Interstate Shellfish Sanitation Conference

Task Force I

Proposals for Consideration

At the 2017 Biennial Meeting
October 14 – 19, 2017
Sheraton Hotel & Convention Center

Sheraton
Myrtle Beach
CONVENTION CENTER HOTEL

Myrtle Beach

South Carolina
THE PALMETTO STATE

Proposal Number	Submitter / Proposal Subject	Page
05-111	Jellett Rapid Testing Ltd. (Joanne Jellett) Rapid Extraction Method for PSP and ASP	1
11-103	Spinney Creek Shellfish, Inc. (Tom Howell) Alternative Male-Specific Coliphage Meat Standard for Restricted Classification of Growing Areas Impacted by wastewater treatment plant outfall.	11
13-107	East Coast Shellfish Growers' Association (Bob Rheault) Sources of Seed for Aquaculture	13
13-109	Interstate Shellfish Sanitation Conference Expanding the Use of the Abraxis Shipboard ELISA for the Determination of Paralytic Shellfish Poisoning (PSP) Toxins	17
13-110	Beacon Analytical Systems, Inc. (Byungchul Kim) Immunoassay Method for Detection of Saxitoxin (PSP) from Shellfish	19
13-111	Abraxis, LLC (Dave Deardorff) DSP PPIA Kit for Determination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish	67
13-113	Neogen Corporation (Jennifer Rice) Reveal 2.0 DSP	126
13-114	Resource Access International (Darcie Couture) Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination	177
13-116	Florida Deparment of Agriculture and Consumer Affairs (Kim Norgren) Shellfish Quarantine Guidance Document	236
15-102	ISSC Growing Area Classification Committee Using Male-Specific Coliphage as a Tool to Redefine Determination of the Size of the Areas to be Classifiec as Prohibited Adjacent to Each Outfall	241
15-109	Maine Department of Marine Resources & Alaska State Environmental Health Laboratory PSP HPLC-PCOX Species Expansion	253
15-110	ISSC Executive Board (Developer William Glover) *V.p.* Enumeration and Detection through MPN & Real-Time PCR	255
15-112	ISSC Executive Board (Developer Jessica Jones) Direct Plating Method for trh	316
15-114	ISSC Executive Board (Developer Kevin Calci) MSC Enumeration in Wastewater by Direct Double-Agar Overlay	336
17-100	Massachusetts Division of Marine Fisheries (Mike Hickey) Marina Definition	371
17-101	New York Department of Environmental Control (Debra Barnes) Parking Lot Mooring/Anchoring Areas in EPA-Approved Vessel No Discharge zones	372
17-102	US Food & Drug Administration (Melissa Abbott) Update Definition of Replicate	373
17-103	US Food & Drug Administration (Melissa Abbott) LC MS MS for Monitoring DSP Toxins	374
17-104	US Food & Drug Administration (Melissa Abbott) Growing Areas 20 Quantitative Analytical Method Verification	402
17-105	Washington Department of Health (Blaine Rhodes) HPLC Method for Domoic Acid	406
17-106	PAC RIM (Michael Jamros) RBA PSP Geoduck	408
17-107	Florida Fish & Wildlife (Leanne Flewelling) ELISA NSP	466

Proposal Number	Submitter / Proposal Subject	Page
17-108	Beacon Analytical Systems, Inc. Detection of ASP biotoxins in *Mytilus edulis* (Blue Mussel) shellfish by ELISA for Domoic Acid	620
17-109	US Food & Drug Administration (Melissa Abbott) Domoic Acid ASP HPLC	645
17-110	US Food & Drug Administration (Melissa Abbott) Vibrio Probe Checklist	655
17-111	US Food & Drug Administration (Melissa Abbott) Vibrio PRC Checklist	669
17-112	US Food & Drug Administration (Melissa Abbott) State Shellfish LEO Guidance	683
17-113	ISSC Male-Specific Coliphage Committee Classification of Shellfish Growing Areas Adjacent to Waste Water Treatment Plants	689
17-114	US Food & Drug Administration (Melissa Abbott) Quality Systems Checklist	765
17-115	Massachusetts Division of Marine Fisheries, Pacific Coast Shellfish Growers Association, NWIFC Treaty Tribes Reconditioning of Recalled Shellfish Implicated in a Norovirus Outbreak	781
17-116	US Food & Drug Administration Aquaculture in Federal Waters	782
17-117	ISSC Male-Specific Coliphage Committee Utilizing Male-Specific Coliphage in Growing Area	784
17-118	Surfside Foods, LLC (Thomas Dameron and BK Rastogo) and Atlantic Capes Fisheries (Chris Shriver) Marine Biotoxin Control/Memorandums of Understanding	789
17-119	US Food & Drug Administration (Melissa Abbott) Marine Biotoxin Control in Federal Waters	791
17-120	PAC RIM (Michael Jamros) Monitoring and Control of Surveillance	799
17-121	US Food & Drug Administration Disposal of Human Sewage and Bodily Fluids	801
17-122	ISSC Executive Office Marine Biotoxin Control	803
17-123	ISSC Executive Office Marine Biotoxin Control Guidance	811

Proposal No. **05-111**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Joanne Jellett
Affiliation	Jellett Rapid Testing Ltd.
Address Line 1	4654 Route 3
Address Line 2	Chester Basin
City, State, Zip	Nova Scotia, Canada B0J 1K0
Phone	902-275-5104
Fax	902-275-2242
Email	jjellett@ns.sympatico.ca
Proposal Subject	Rapid Extraction Method for PSP and ASP
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter III Laboratory @.02 Methods ISSC Constitution, Bylaws, and Procedures Procedure XVI.
Text of Proposal/ Requested Action	Procedure for Acceptance and Approval of Analytical Methods for the NSSP Marine Biotoxins affect farmed and wild fish and shellfish, as well as having a deleterious effect on humans. Jellett Rapid Testing has designed and developed rugged tests for the presence of Paralytic Shellfish Poison, Amnesic Shellfish Poison and Diarrhetic Shellfish Poison (under development at the time of this submittal). To facilitate the use of these tests in the field (for aquaculturists, campers, regulatory officials, etc.), Jellett Rapid Testing has developed a "low-tech" rugged alternative to the standard AOAC method designed to extract the toxins in the field as well as the laboratory. The AOAC method requires the sample to be boiled in acid at low pH and the pH adjusted with strong acids. This requires a fully equipped laboratory and significant safety precautions. The JRT Rapid Extraction Method was designed for use in remote areas, with little sophisticated backup support, by average individuals with little training and education. It is faster, less labor-intensive and less expensive than the other available method. The rapid extraction method requires vinegar and rubbing alcohol to extract the toxins. A simple, rapid, safe method such as this would make rapid tests for marine Biotoxins available in remote areas, to fishermen, aquaculturists, and regulatory officials on an instant basis. The method developed by Jellett Rapid Testing Ltd has been presented to regulatory bodies over the past several years. In cooperation with individuals, governments and those organizations, the analytical method has been refined and improved. The Rapid Extraction Method is being tested in several states and foreign countries. Publications will be forthcoming. The CONSTITUTION BY-LAWS and PROCEDURES of the INTERSTATE SHELLFISH SANITATION CONFERENCE allows the ISSC, through the Laboratory Methods Review Committee, to accept analytical methods that are sufficiently validated but are not AOAC or APHA methods. This is defined in the Constitution, PROCEDURE XVI. PROCEDURE FOR ACCEPTANCE AND APPROVAL OF ANALYTICAL METHODS FOR THE NSSP. Two possible reasons for considering a method are found in Subdivisions i and ii. Subdivision i. Meets immediate or continuing need;

	Subdivision ii. Improves analytical capability under the NSSP as an alternative to other approved or accepted method(s) Currently, only the AOAC extraction for PSP and ASP are accepted. The need for a simple safe extraction method has been expressed by regulatory agencies, governmental organizations and industry for many years. The Jellett Rapid Extraction Method is being validated over a wide geographic area to demonstrate its simplicity, reliability, precision and accuracy. As a result of demonstrations of efficacy and the need that has been expressed by industry and state agencies, the Jellett Rapid Extraction Method is presented as an alternative extraction method for PSP and ASP for the NSSP as a Type III or Type IV method. Please see attached additional information. Suggested wording: Section II, Chapter III Laboratory @.02 Methods C. Biotoxin. Methods for the analyses of shellfish and shellfish harvest waters shall be: (1) The current AOAC and APHA methods used in bioassay for paralytic shellfish poisoning toxins; and (2) The current APHA method used in bioassay for *Karemia breve* toxins. (3) The Jellett Rapid Extraction Method may be used for extracting PSP and ASP toxins from Shellfish by regulatory and industry laboratories.
Public Health Significance	Currently, only the AOAC extraction for PSP and ASP analyses are accepted. Because of many significant constraints, in practical terms, this means that analyses can be conducted only in laboratories, and then under dangerous conditions. Acceptance of the Jellett Rapid Extraction Method for PSP and ASP would allow harvesters, processors, and regulatory agencies to screen for PSP and ASP with an accepted standardized method that provides valid useable data. The Jellett Rapid Extraction Method for PSP and ASP was developed over several years in answer to the oft-stated need for a rapid, reliable, rugged, simple and safe sample preparation method. The Jellett Rapid Extraction Method for PSP and ASP is not meant to be a definitive “Standard Method”, but rather to provide a supplementary extraction method that can be used in the field as well as in the lab. Possible applications for The Jellett Rapid Extraction Method for PSP and ASP include: • as a supplement to analytical methods of screening out negative samples in shellfish regulatory labs; • as a harvest management tool at aquaculture facilities or in wild shellfish harvest areas (especially near shore areas) to supplement available methods to determine if shellfish are free of PSP or ASP and safe to harvest; • as a supplement to quality control methods for shellfish processing plants, distributors and wholesalers to ensure incoming shellfish are free of PSP and ASP toxins before processing or further distribution (this test could become part of the plant's HACCP program); • as a supplement to analytical methods for water classification for

	Biotoxins; and • as a supplement to analytical methods for broad scale ecological monitoring. The rationale for using the Jellett Rapid Extraction Method for PSP and ASP is that the method provides a rapid, reliable, rugged, simple, safe and cost-effective extraction method (especially in low-volume laboratories) for PSP and ASP that can supplement accepted tests and substantially reduce the cost of analyses. Used in conjunction with other rapid methods, the Jellett Rapid Extraction Method for PSP and ASP will supplement regulatory agency efforts and help prevent the harvest of contaminated product. Having the ability to conduct tests using an accepted rapid extraction method will allow those processors who choose to use this test to demonstrate that they are truly controlling for PSP and ASP hazards in the harvested shellfish. The Jellett Rapid Extraction Method for PSP and ASP could contribute to building long-term databases on broader scales than a regulatory lab can afford and, by using an accepted standardized method, will provide consistent results. These databases could be supplemented with industry testing in areas where there is no testing currently. This would extend, augment and strengthen the current food safety system broadening and refining the food safety net by increasing the number of testing sites and generating long term data in more areas. A simple, rapid, rugged, effective, reliable, safe and cost-effective extraction method, available to all harvesters, regulators, and processors, would increase the monitoring and reduce the chance that shellfish containing ASP toxins above the regulatory limit would be harvested or marketed
Cost Information	It is difficult to determine exact costs because many government cost models do not consider capital costs. Both extraction methods are the same through puree step, the chemicals used in both cases are minimal, as is the cost of incidental equipment (blender, pipettes, etc.). However, a comparison of time required using the Rapid Extraction Method (Add rapid liquid; Filter) with the time required using the AOAC Extraction (Add HCL; Boil; Wait; Filter; Pour in tube; Check PH) shows a significant difference. Our experience shows that it takes about 22 minutes for this portion of the AOAC extraction while it takes less than 2 minutes to complete the Jellett Rapid Extraction Method. At a salary of $33 / hour, that is a savings of $11.00 per sample extract.
Action by 2005 Laboratory Methods Review Committee	Recommended referral of Proposal 05-111 to the appropriate committee as determined by the Conference Chairman.
Action by 2005 Task Force I	Recommended adoption of the Laboratory Methods Review Committee recommendation of Proposal 05-111.
Action by 2005 General Assembly	Adopted recommendation of 2005 Task Force I.
Action by USFDA	Concurred with Conference action.
Action by 2007 Laboratory Methods Review Committee	Recommended no action on Proposal 05-111. Rationale – Alternative extraction method for JRT PSP should be adopted to expand utility of the test; however there are insufficient data for acceptance at this time. The submitter will send data to the Executive Office for Conference approval.
Action by 2007 Task Force I	Recommended referral of Proposal 05-111 to an appropriate committee as determined by the Conference Chairman.
Action by 2007	Adopted recommendation of 2007 Task Force I.

General Assembly	
Action by USFDA	December 20, 2007 Concurred with Conference action with the following comments and recommendations for ISSC consideration. The Conference has made considerable progress in its efforts to recognize new and developing analytical methods for the detection of indicators, pathogens, and marine toxins. Much credit goes to the Laboratory Methods Review Committee and its leadership for ensuring a scientifically defensible process for adopting analytical methods under the NSSP. At the 2007 meeting numerous analytical methods were proposed for ISSC adoption. However, many of these methods were lacking the validation and associated data needed by the Laboratory Methods Review Committee to make a final determination regarding their efficacy for use in the NSSP. As a result the General Assembly voted "No Action" on analytical method Proposals 05-107, 05-108, 05-109, 05-111, 05-113, and 05-114. It is FDA's understanding that the intent of the "No Action" vote was not to remove these Proposals from ISSC deliberation as "No Action" normally suggests, but rather to maintain them before the Conference pending submission of additional data for further consideration. The Voting Delegates, by requesting the Proposal submitters provide additional data to the Executive Office for methods approval consistent with Procedure XVI, clearly recognized the importance and utility of these methods and intended to maintain them before the Conference for possible adoption following additional data submission. FDA requests that the ISSC Executive Board confirm FDA's understanding of this outcome. FDA fully supports such a Conference action and encourages the Executive Office to pursue submission of additional data as necessary to move forward with acceptance of these methods.
Action by 2009 Laboratory Methods Review Committee	Recommended no action on Proposal 05-111. Rationale: Requested additional information has not been submitted.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation of Proposal 05-111.
Action by 2009 General Assembly	Referred Proposal 05-111 to the Laboratory Methods Review Committee.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 05-111.
Action by 2011 Laboratory Methods Review Committee	Recommended acceptance of the rapid extraction method in Proposal 05-111, specifically 70% isopropanol: 5% acetic acid 2.5:1, only for use with the Abraxis shipboard ELISA for PSP as an Emerging Method solely for use in the onboard screening dockside testing protocol in the Northeast region, including George's Bank. The Laboratory Methods Review Committee further recommends: 1. The data collected during the dockside testing study be submitted to the LMRC in the SLV Method Application Protocol within 6 months of the concurrence by FDA in the Summary of Actions. 2. The validation study conducted by the State of Maine of the Abraxis laboratory ELISA with the extraction method in Proposal 05-111 be submitted to the LMRC in the SLV Method Application Protocol within 6 months of the concurrence by FDA in the Summary of Actions.

	3. No action on the requested language change in Proposal 05-111 for the Model Ordinance Section II, Chapter III Laboratory @.02 Methods. Section II, Chapter III Laboratory @.02 Methods C. Biotoxin. Methods for the analyses of shellfish and shellfish harvest waters shall be: (1) The current AOAC and APHA methods used in bioassay for paralytic shellfish poisoning toxins; and (2) The current APHA method used in bioassay for *Karenia breve* toxins. (3) ~~The Jellett Rapid Extraction Method may be used for extracting PSP and ASP toxins from Shellfish by regulatory and industry laboratories.~~
Action by 2011 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendations on Proposal 05-111.
Action by 2011 General Assembly	Adopted recommendation of 2011 Task Force I on Proposal 05-111.
Action by FDA February 26, 2012	Concurred with Conference action on Proposal 05-111.
Action by 2013 Laboratory Methods Review and Quality Assurance Committee	Recommended no action on Proposal 05-111 Rationale - Proposal 05-111 is resolved by action on Proposal 13-109.
Action by 2013 Task Force I	Recommended adoption of Laboratory Methods Review and Quality Assurance Committee recommendation on Proposal 05-111.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 05-111.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 05-111.
Action by 2015 Laboratory Methods Review Committee	Recommended the following: 1) Change the name of the Jellett Rapid Test to Scotia Rapid Test and the Jellett Rapid Extraction to Scotia Rapid Extraction in the next revision of the NSSP Guide for the Control of Molluscan Shellfish (Section IV. Guidance Documents Chapter II Growing Areas 4. Approved Limited Use Methods for Marine Biotoxin Testing). 2) Refer Proposal 05-111 for PSP to an appropriate committee as determined by the Conference Chair and further recommended to direct the Executive Office to send a letter to the method submitter requesting additional information as detailed by the LMRC. 3) No action on the Scotia Rapid Extraction Method for ASP as there is no data nor did the submitter indicate that data would be submitted for ASP.
Action by 2015 Task Force I	Recommended adoption of the Laboratory Methods Review Committee on Proposal 05-111 with the following amendments: 1. Remove "and ASP" and change "toxins" to "toxin" throughout the proposal and adopt the Laboratory Method Review Committee recommendation 1 2. Refer Proposal 05-111 to appropriate committee as determined by Conference Chair. 3. No action on recommendation 3 as this is covered by the proposal as amended by the Task Force.
Action by 2015 General Assembly	Adopted recommendations 2. And 3. of Task Force I on Proposal 05-111. Recommendation 1. Was ruled out of order and the General Assembly did not take any action on this recommendation.
Action by FDA	Concurred with Conference action on Proposal 05-111.

January 11, 2016	

Lab #	CFIA Sample #	CFIA Result HPLC (µg/g)	Jellett Result Approx. (µg/g)
04-01847	1	24.1	16-24
04-02156	2	1.4	0-4
04-01784	3	70.0	72-80
04-01968	4	71.9	72-92
04-01647	5	8.9	12-16
04-02328	6	9.3	6.4-11.2
04-02467	7	4.2	6.0-7.2
04-01646	8	31.2	40-64
04-02351	9	9.4	9.6-12
04-02238	10	4.7	4-5.6
04-01862	11	96.7	60-80
04-02240	12	10.3	12-20
04-01750	13	30.7	24-32
04-02231	14	2.5	0-4
04-01969	15	40.1	64-72

Jellett Rapid Testing Ltd.: NOAA Study - JREM Trial
Sample Record Sheet – Homogenate
State of Alaska - Department of Environmental Conservation

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	Date	Species	Field / Site / Lab Name	Date	Size of Sample (mL)	Field / Site / Lab Name	Date	Batch # - Test	Batch # - Buffer	Result (1=Pos, 0=Neg)	Intensity of C Line as % of T	Lab Name	Date	Toxin Standard Used	# of Mice Dead	Result (µg/100g)	# of Mice Sick
20053168-C	3/06/05	Geoduck Viscera	ADEC-EHL	3/14/05	66[2]	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	71	0
20053169-C	3/06/05	Geoduck Viscera	ADEC-EHL	3/14/05	495	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	<10%	ADEC-EHL	03/15/05	FDA	3	39	0
20053170-C	3/06/05		ADEC-EHL	3/14/05	650	ADEC-EHL	3/14/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	71	0
20053183-C	3/13/05	Geoduck	ADEC-EHL	3/15/05	416	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	>0%, <25%	ADEC-EHL	03/15/05	FDA	3	70	0
20053184-C	3/13/05	Geoduck	ADEC-EHL	3/15/05	632	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	54	0
20053185-C	3/14/05	Geoduck	ADEC-EHL	3/15/05	561	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	72	0
20053186-C	3/15/05	Geoduck	ADEC-EHL	3/15/05	301	ADEC-EHL	3/15/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/15/05	FDA	3	90	0
20053137	03/06/05	Oyster	ADEC-EHL	03/08/05	150	ADEC-EHL	03/08/05	40000-13Aug04	40005-05Nov04	INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053136	03/06/05	Oyster	ADEC-EHL	03/08/05	500	ADEC-EHL	03/08/05	40000-13Aug04	40005-05Nov04	N/A INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053138	03/05/05	Oyster	ADEC-EHL	03/08/05	500	ADEC-EHL	03/09/05	40000-13Aug04	40005-05Nov04	INV	C <25% T	ADEC-EHL	03/08/05	FDA	0	NDT	0
20053142	03/06/05	Oyster	ADEC-EHL	03/09/05	50	ADEC-EHL	03/09/05	40000-13Aug04	40005-05Nov04	INV	C <50% T	ADEC-EHL	03/09/05	FDA	0	NDT	0
20053124-C	3/5/05	Geoduck	ADEC-EHL	3/7/05	495	ADEC-EHL	3/7/05	40000-13Aug04	40005-05Nov04	1	0%	ADEC-EHL	03/07/05	FDA	3	117	0
20053125-C	3/5/05	Geoduck	ADEC-EHL	3/7/05	404	ADEC-EHL	3/7/05	40000-13Aug04	40005-05Nov04	1	75%	ADEC-EHL	03/07/05	FDA	3	58	0
20053006	2/29/05	Oyster	ADEC-EHL	3/3/05	125	ADEC-EHL	3/3/05	40000-13Aug04	40005-05Nov04			ADEC-EHL	3/3/05	FDA	0	NDT	0
20053040-C	03/01/05	Geoduck Viscera	ADEC-EHL	03/02/05	545	ADEC-EHL	03/02/05	40000-13Aug04	40009-06Oct04	1	50%	ADEC-EHL	03/02/05	FDA	3	86	0
20053039-C	03/01/05	Geoduck Viscera	ADEC-EHL	03/02/05	340	ADEC-EHL	03/02/05	40000-13Aug04	40009-06Oct04	1	10%	ADEC-EHL	03/02/05	FDA	3	175	0
20053007-C	02/26/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	25%	ADEC-EHL	02/28/05	FDA	3	59	0
20053010-C	02/26/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	<25%	ADEC-EHL	02/28/05	FDA	3	65	0
2005301-C	02/27/05	Geoduck Viscera	ADEC-EHL	02/28/05	750	ADEC-EHL	03/01/05	40000-13Aug04	40009-06Oct04	1	0%	ADEC-EHL	02/28/05	FDA	3	151	0

Jellett Rapid Testing Ltd.: NOAA Study
JREM Trial Sample Record Sheet - Homogenate
California - Microbial Disease Lab

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	**Collection Date**	**Species**	**Field / Site / Lab Name**	**Date**	**Size of Sample (mL)**	**Field / Site / Lab Name**	**Date**	**Batch # - Test**	**Batch # - Buffer**	**Result (1=Pos, 0=Neg)**	**Intensity of C Line as % of T**	**Lab Name**	**Date**	**Toxin Standard Used**	**# of Mice Dead**	**Result µg/100g**	**# of Mice Sick**
05E-00110	02/05/05	LBMU	CA-DHS-EMDS	02/09/05	>130	CA-DHS-EMDS	02/09/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	2/09/05	FDA	0	<36	0
05W-00099	02/01/05	SSMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<34	0
05E-00096	02/28/05	CBMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<36	0
05W-00093	02/01/05	SBMU	CA-DHS-EMDS	02/02/05	>130	CA-DHS-EMDS	02/02/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	02/02/05	FDA	0	<36	0
05W-00079	01/25/05	SSMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/26/05	FDA	0	<35	0
05W-00076	01/22/05	CBMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	1	50%	CA-DHS-EMDS	01/26/05	FDA	3	39	0
05W-00069	01/24/05	SBMU	CA-DHS-EMDS	01/26/05	>130	CA-DHS-EMDS	01/26/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	01/26/05	FDA	0	<36	3
05W-00059	01/18/05	SSMU	CA-DHS-EMDS	01/19/05	>130	CA-DHS-EMDS	01/19/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/19/05	FDA	0	<35	3
05W-00055	01/14/05	CBMU	CA-DHS-EMDS	01/18/005	>130	CA-DHS-EMDS	01/18/05	40000-8/13/04	40005-9/7/04	1	25%	CA-DHS-EMDS	01/18/05	FDA	3	37	
05W-00052	01/17/05	SBMU	CA-DHS-EMDS	01/18/05	>130	CA-DHS-EMDS	01/18/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	01/18/05	FDA	0	<36	0
05W-00025	1/10/05	SBMU	CA-DHS-EMDS	1/12/05	>130	CA-DHS-EMDS	1/12/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/12/05	FDA	0	<35	0
05W-00023	1/11/05	SSMU	CA-DHS-EMDS	1/12/05	>130	CA-DHS-EMDS	1/12/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/12/05	FDA	0	<36	0
05W-00020	1/7/05	CBMU	CA-DHS-EMDS	01/11/05	>130	CA-DHS-EMDS	01/11/05	40000-8/13/04	40005-9/7/04	1	25%	CA-DHS-EMDS	1/11/05	FDA	3	44	0

Jellett Rapid Testing Ltd.: NOAA Study
JREM Trial Sample Record Sheet - Homogenate
California - Microbial Disease Lab (CONTINUED)

	Collection		Homogenization			Jellett Test						MBA Test					
Sample ID	**Collection Date**	**Species**	**Field / Site / Lab Name**	**Date**	**Size of Sample (mL)**	**Field / Site / Lab Name**	**Date**	**Batch # - Test**	**Batch # - Buffer**	**Result (1=Pos, 0=Neg)**	**Intensity of C Line as % of T**	**Lab Name**	**Date**	**Toxin Standard Used**	**# of Mice Dead**	**Result µg/100g**	**# of Mice Sick**
05W-00011	1/3/05	SBMU	CA-DHS-EMDS	1/5/05	>130	CA-DHS-EMDS	1/5/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/5/05	FDA	0	<34	0
05W-00007	1/4/05	SSMU	CA-DHS-EMDS	1/5/05	>130	CA-DHS-EMDS	1/5/05	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	1/5/05	FDA	0	<34	0
05W-00002	12/30/04	CBMU	CA-DHS-EMDS	1/04/05	>130	CA-DHS-EMDS	1/04/05	40000-8/13/04	40005-9/7/04	0	75%	CA-DHS-EMDS	1/04/05	FDA	2	36	1
04W-01458	12/28/04	SSMU	CA-DHS-EMDS	12/29/04	>130	CA-DHS-EMDS	12/29/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/29/04	FDA	0	<36	0
04W-01454	12/27/04	SBMU	CA-DHS-EMDS	12/29/04	>130	CA-DHS-EMDS	12/29/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/29/04	FDA	0	<36	0
04W-01457	12/24/04	CBMU	CA-DHS-EMDS	12/28/04	>130	CA-DHS-EMDS	12/28/04	40000-8/13/04	40005-9/7/04	1	<25%	CA-DHS-EMDS	12/28/04	FDA	3	42	0
04W-1446	12/21/04	SSMU	CA-DHS-EMDS	12/22/04	>130	CA-DHS-EMDS	12/22/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/22/04	FDA	0	<34	0
04W-01436	12/20/04	SBMU	CA-DHS-EMDS	12/21/04	>130	CA-DHS-EMDS	12/21/04	40000-8/13/04	40005-9/7/04	0	75%	CA-DHS-EMDS	12/21/04	FDA	0	<34	3
04W-01399	12/13/04	SBMU	CA-DHS-EMDS	12/14/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	1	50%	CA-DHS-EMDS	12/15/04	FDA	2	35	0
04W-01421	12/11/04	CBMU	CA-DHS-EMDS	12/15/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	1	0%	CA-DHS-EMDS	12/15/04	FDA	3	48	0
04W-01424	12/14/04	SSMU	CA-DHS-EMDS	12/15/04	>130	CA-DHS-EMDS	12/15/04	40000-8/13/04	40005-9/7/04	0	100%	CA-DHS-EMDS	12/15/04	FDA	0	<35	0

Proposal No. **11-103**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Thomas L. Howell
Affiliation	Spinney Creek Shellfish, Inc.
Address Line 1	PO Box 310
Address Line 2	
City, State, Zip	Eliot, ME 03903
Phone	207-439-2719
Fax	207-439-7643
Email	tlhowell@spineycreek.com
Proposal Subject	Alternative Male-specific Coliphage Meat Standard for Restricted Classification of Growing Areas Impacted by wastewater treatment plant outfall.
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Area @ .02 Bacteriological Standards
Text of Proposal/ Requested Action	G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration. (4) Exception. If the Male-specific Coliphage indicator is used for supplemental process verification using an end-point meat standard of < 50PFU/100gm and existing fecal coliform testing requirements in Chapter XV .03 J. are used, then FC water quality monitoring is not required for the restricted classification of growing areas affected by point sources such as wastewater treatment plant outfall.
Public Health Significance	Under shellfish relay, water quality requirements are not needed for the restricted classification when a contaminant reduction study is conducted and a minimum time period of two weeks is used. For depuration, the restricted classification requires water quality monitoring and standards. The reason for these upper FC limits is that FC meat indicator does not adequately reflect the viral risk and/or viral depuration kinetics. Male-specific coliphage is a viral indicator organism to be used in growing areas impacted by point source sewage contamination. MSC demonstrates significant advantages over FC alone for both the assessment of viral contamination and assessment of viral depuration kinetics. Upper FC limits were put into the NSSP to prevent shellfish with higher levels of viruses from being depurated. Several studies clearly show that conventional depuration using FC for process validation is not adequate to protect public health with respect to virus contamination in growing areas with significant wastewater treatment plant and sewage impact. Studies have also shown that viral levels in shellfish impacted by sewage and partially treated sewage detected using MSC and molecular techniques are much lower in the summer months than the winter months. Additionally, the viral depuration rate is higher in the summer with process waters >18°C. Recent studies have also shown that MSC is an appropriate viral indicator to assess viral depuration. Therefore, seasonal viral depuration using male-specific coliphage as well as FC for process verification is a superior approach to taking water samples using FC in a growing area adjacent to wastewater treatment plant outfall. Combining the bacterial indicator of FC and the viral indicator MSC for mitigation strategies that use meat scores is far more direct and effective than water quality sampling in this context.
Cost Information	The Male-specific Coliphage (MSC) method is an inexpensive double-agar pour plate method that can be run in any state-certified microbiological laboratory. A

	refrigerated centrifuge capable of 9,000G is required which costs $10K to $12K (USD). Significant cost savings and a higher level of public health protection may be realized using strategies such as seasonal coliphage depuration process validated using MSC and seasonal coliphage relay using MSC in contaminant reduction studies than requiring water quality limits using FC.
Action by 2011 Task Force I	Recommend referral of Proposal 11-103 to the appropriate committee as determined by the Conference Chairman.
Action by 2011 General Assembly	Adopted recommendation of 2011 Task Force I on Proposal 11-103.
Action by FDA February 26, 2012	Concurred with Conference action on Proposal 11-103.
Action by 2013 Growing Area Classification Committee	Recommend referral of Proposal 11-103 to the appropriate committee as determined by the Conference Chairman. It was additionally recommended that a workgroup be formed to look at current MSC data and the science behind its potential use and applicability for use in the NSSP. The workgroup will organize a summit of outside experts, academia, and scientists to present current information and science on MSC. The group will meet at least quarterly and respond back to the Growing Area Classification Committee on its findings and recommendations. Recommended that the ISSC pursue funding to facilitate scheduling a summit to bring together experts to present the current science in the use of MSC.
Action by 2013 Task Force I	Recommended adoption of Growing Area Classification Committee action on Proposal 11-103.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 11-103.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 11-103.
Action by 2015 Growing Area Classification Committee	Recommended referral of Proposal 11-103 to appropriate committee as determined by the Conference Chair.
Action by 2015 Task Force I	Recommended adoption of Growing Area Classification Committee recommendation on Proposal 11-103.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 11-103.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 11-103.

Proposal No. **13-107**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Robert Rheault
Affiliation	East Coast Shellfish Growers Association
Address Line 1	1623 Whitesville Road
Address Line 2	
City, State, Zip	Toms River, NJ 08755
Phone	401-783-3360
Fax	
Email	bob@ecsga.org
Proposal Subject	Sources of Seed for Aquaculture
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VI. Shellfish Aquaculture
Text of Proposal/ Requested Action	.03 Seed Shellstock Seed may come from any growing area, or from any growing area in any classification, provided that: A. The source of the seed is sanctioned by the Authority B. Seed from growing areas ~~or growing areas~~ in the restricted or prohibited classification have acceptable levels of poisonous or deleterious substances; and C. Seed from growing areas ~~or growing areas~~ in the prohibited classification are cultured for a minimum of ~~six (6) months~~ one month while average daily water temperatures are above 50 degrees F.
Public Health Significance	Shellfish seed collected or cultured in certain growing areas that are in the prohibited classification have been shown through repeated sampling to be free of deleterious substances (John Mullen RI DOH, unpub. data, Rheault unpubl. data, Rice unpub. data, Leavitt unpub. data). A period of one month is typically adequate to purge viral and bacterial contaminants provided water temperatures are high enough to maintain active metabolic activity (above 60 degrees F or 15 degrees C) (Richards 1988). Once the Authority is satisfied that adequate sampling has demonstrated that the seed have "acceptable levels of deleterious substances", then a 30 day period of culture in open waters should be adequate to allow purging of bacterial and viral contaminants to ensure that public health is protected. The Authority retains the right to deny seed collection and culture in any area, or to require additional testing for deleterious substances, or to require longer periods to purge contaminants as necessary. The original intent of this section was to provide for purging of viral and bacterial contamination prior to harvest for consumption on the assumption that deleterious substances were at acceptable levels prior to moving the seed to grow out areas The six-month requirement was implemented as a short-hand way to ensure that seed were grown for at least one month when water temperatures exceeded 60 degrees F. It makes little sense to require relay times in excess of one month for seed that are typically more than six months from harvest size when shellstock relay times as short as two weeks are common.

	References Cited: Richards, G. (1988), Microbial Purification of Shellfish: A Review of Depuration and Relaying, J. Food Protection 51(3)218-251. Supporting Information: RI DOH metals data (oyster seed grown in Billington Cove Marina) Unpublished data from Rd. Dale Leavitt (clam seed grown in Warwick Cove Marina)
Cost Information	This change should facilitate record keeping and documentation efforts required to ensure that seed from prohibited waters do not get harvested until bacterial and viral contamination has been purged.
Action by 2013 Task Force I	Recommended referral of Proposal 13-107 to an appropriate committee as determined by the Conference Chairman.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-107.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-107.
Action by 2015 Aquaculture Facility Inspection Committee	Recommended the following: (1) Referral of Proposal 13-107 back to Committee as appointed by the Conference Chair. (2) The charge of the Committee be expanded to include updating and revising the Aquaculture Chapter of the Model Ordinance to reflect current practices and methods and submit proposals for the next Annual Meeting.
Action by 2015 Task Force I	Recommended adoption of Aquaculture Facility Inspection Committee recommendations on Proposal 13-107.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 13-107.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-107.

ODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB #

SAMPLE COLLECTION FORM

Anal BY

DATE OF COLLECTION		DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
8/4/98	NLS	8/13/98	8/17/98	PCE

CONDITION: HOT, FROZEN, COLD, OTHER — SEALED: YES (circled), NO

ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
Oysters			61

EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Billington Cove Aquafarm	01

ADDRESS — FOLLOW UP SAMPLE #1 — SOURCE: 10

SHIPPER/PACKER & ADDRESS — ANALYSIS: 13

COMPLAINT* — ORIGINAL CONTAINER YES / NO — DATE PURCHASED

NAME & LOCATION OF STORE WHERE PURCHASED — PRODUCT USED YES / NO

HOW STORED: FROZEN / AMBIENT / COLD	IMPORT PRODUCT YES / NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE, TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM	SIGNED TO	DATE	TIME	HOW STORED
[signature]	[signature]	8/4/98	1:45	Refrigerated
AGENT'S PRINT NAME: 164 John Mullen	PRINT NAME: MCALLISTER SILVA			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. MFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %

9. SORBATES ppm	10. TBA mg malo	11. FFA %	12. WT/VOL	13. $NaNO_3$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g

17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID µg/ml	23. TMA mgN/100g	24. VITAMIN A IU/Gt

25. pH	26. BRIX %	27. INSECT	28. ACCENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE µg/100g	32. VITAMIN D IU/Gt

33. PEG INT	34. AFLATOXIN ppb	35. Pb 0.47 ppm	36. Cd 0.45 ppm	37. Cu 127 ppm	38. Cr <0.15 ppm	39. Zn 798 ppm	40. A_w

41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %

49. FRUCTOSE %	50. SUCROSE %	51. LACTOSE %	52. MALTOSE %	53. YEAST	54. MOLD	55. PSF	56. OTHER

56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO

64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC

COLLECTOR'S NOTES:
Moosetom Oyster nursery stock. appt. 36 mm

LABORATORY NOTES:
Fe: 133 ppm

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY, SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION, PROCESSING & DISTRIBUTION OF FOOD*

* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

Data Collected by Dr. Dale Leavitt, Roger William University

Hard Clam Seed from Warwick Cove Marina

Warwick Cove Upweller

Quahog Seed 30-Oct-08 Note: µg/kg = ppb

Group (n=15)	avg Length (mm)	stdev	avg Live Weight (g)	stdev	avg Soft Tissue Wet Weight (g)	stdev			avg Hg/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cr/Soft Tissue Wet Weight (µg/kg)	stdev	avg Fe/Soft Tissue Wet Weight* (µg/kg)	stdev	avg Ni/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cu/Soft Tissue Wet Weight (µg/kg)	stdev
1	15.1	2.9	0.967	0.730	0.267	0.238			7.81		0.20		35.57		0.20		8.18	
2	12.6	1.6	0.545	0.202	0.139	0.056			9.41		0.28		34.00		0.22		11.80	
3	13.9	1.2	0.685	0.201	0.182	0.058			8.24		0.26		33.33		0.20		9.30	
Total	13.9	2.2	0.732	0.476	0.196	0.152			8.49	0.83	0.25	0.04	34.30	1.15	0.21	0.01	9.76	1.85
Group (n=15)					avg Soft Tissue Dry Weight (g)	stdev	avg % Dry Weight	stdev	avg Hg/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cr/Soft Tissue Dry Weight (µg/kg)	stdev	avg Fe/Soft Tissue Dry Weight* (µg/kg)	stdev	avg Ni/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cu/Soft Tissue Dry Weight (µg/kg)	stdev
1					0.041	0.041	14.8%	2.4%	52.75		1.38		240.70		1.37		55.33	
2					0.022	0.008	16.1%	1.5%	58.45		1.73		210.60		1.35		73.09	
3					0.027	0.008	15.2%	1.7%	54.22		1.70		219.20		1.31		61.16	
Total					0.030	0.025	15.4%	2.0%	55.14	2.96	1.60	0.19	223.50	15.50	1.34	0.03	63.19	9.05

Group (n=15)	avg Zn/Soft Tissue Wet Weight* (µg/kg)	stdev	avg As/Soft Tissue Wet Weight (µg/kg)	stdev	avg Se/Soft Tissue Wet Weight (µg/kg)	stdev	avg Sn/Soft Tissue Wet Weight (µg/kg)	stdev	avg Ag/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cd/Soft Tissue Wet Weight (µg/kg)	stdev	avg Pb/Soft Tissue Wet Weight (µg/kg)	stdev
1	55.10		1.77		0.87		15.28		0.03		0.06		0.31	
2	66.07		1.99		1.29		14.07		0.03		0.05		0.28	
3	55.07		1.55		0.53		11.94		0.03		0.11		0.26	
Total	58.75	6.34	1.77	0.22	0.89	0.38	13.76	1.69	0.03	0.00	0.07	0.03	0.28	0.03
Group (n=15)	avg Zn/Soft Tissue Dry Weight* (µg/kg)	stdev	avg As/Soft Tissue Dry Weight (µg/kg)	stdev	avg Se/Soft Tissue Dry Weight (µg/kg)	stdev	avg Sn/Soft Tissue Dry Weight (µg/kg)	stdev	avg Ag/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cd/Soft Tissue Dry Weight (µg/kg)	stdev	avg Pb/Soft Tissue Dry Weight (µg/kg)	stdev
1	372.90		12.01		5.89		103.40		0.22		0.43		2.09	
2	409.30		12.32		7.97		87.14		0.16		0.29		1.76	
3	362.20		10.17		3.47		78.55		0.22		0.69		1.68	
Total	381.47	24.69	11.50	1.16	5.78	2.26	89.70	12.62	0.20	0.04	0.47	0.20	1.84	0.22

Proposal No. **13-109**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Executive Office
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Expanding the use of the Abraxis Shipboard ELISA for the determination of paralytic shellfish poisoning (PSP) toxins
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	4. Approved Limited Use Methods for Marine Biotoxin Testing This submission presents the Abraxis Shipboard ELISA for paralytic shellfish poisoning (PSP) toxins as a screening method for consideration as an NSSP Approved Limited Use Method. Currently the Abraxis Shipboard ELISA is approved for limited use in conjunction with the Jellett Rapid Extraction (mixture of rubbing alcohol and vinegar) and specifically for the onboard testing protocol. This proposal presents more data on the Abraxis test using the rapid extraction and also provides new data and comparisons of the test when AOAC extractions (boiling with hydrochloric acid) are performed. The data presented supports expanding the use of the Abraxis Shipboard ELISA to (1) allow for the rapid extraction OR the AOAC extraction method and (2) allow the kit to be used as a screening method beyond the onboard screening protocol
Public Health Significance	Paralytic shellfish poisoning intoxications result from the consumption of seafood (primarily bivalve molluscs) contaminated with neurotoxins known as paralytic shellfish toxins (PSTs). To protect public health, harvesting closures are implemented when toxicity exceeds the guidance level of 80 micrograms saxitoxin equivalents per 100 grams of shellfish tissue. As such, accurate screening and analytical methods are needed to monitor shellfish toxicity for making decisions regarding opening and closing shellfish growing areas accordingly. While the Abraxis Shipboard ELISA is already an NSSP Approved Limited Use Method for PSP toxicity determination, being able to use AOAC extractions with this kit would allow for the same extraction to be used with this method during screening and with the MBA as necessary for confirmation (without requiring a second extraction). Further expanding the use of the method beyond the onboard screening protocol would be beneficial as it would make the Abraxis Shipboard ELISA available for use by monitoring laboratories.
Cost Information	Each 96 well plate costs ~$500.
Action by 2013 Laboratory Method and Quality Assurance Review Committee	Recommended referral of Proposal 13-109 to an appropriate committee as determined by the Conference Chairman.
Action by 2013 Task Force I	Recommended adoption of Laboratory Method and Quality Assurance Review Committee recommendation on Proposal 13-109.

Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-109.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-109.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-109 to an appropriate committee as determined by the Conference Chair until data that supports the use of the Abraxis ELISA beyond the use of the onboard procedure is made available.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-109.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 13-109.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-109.

Proposal No. **13-110**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Byungchul Kim
Affiliation	Beacon Analytical Systems, Inc.
Address Line 1	82 Industrial Park Rd.
Address Line 2	
City, State, Zip	Saco, ME, 04072
Phone	207-571-4302
Fax	207-602-6502
Email	bkim@beaconkits.com
Proposal Subject	Immunoassay Method for Detection of Saxitoxin (PSP) from Shellfish
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	2. Approved Methods for Marine Biotoxin Testing and 4. Approved Limited Use Methods for Marine Biotoxin Testing. Review the validation for Saxitoxin (PSP) Microtiter Plate Test Kit by the Proposal Review Committee. Single Laboratory Validation Protocol for Method Approval attached.
Public Health Significance	Rapid screening method can handle numerous samples and screen out negative samples so that it reduces the size of sample to be confirmed with regulatory methods such as mouse bioassay (MBA) or liquid chromatography with post-column oxidation (PCOX). This results in saving resources of the laboratories, and makes the laboratories able to provide rapid warning. References attached.
Cost Information	Approximate cost for the basic set up of the method is $3600.
Action by 2013 Laboratory Methods and Quality Assurance Review Committee	Recommended referral of Proposal 13-110 to an appropriate committee as determined by the Conference Chairman and directs the Executive Office send a letter to the submitter requesting additional information as requested by the Laboratory Methods Review and Quality Assurance Committee.
Action by 2013 Task Force I	Recommended adoption of Laboratory Method Review and Quality Assurance Committee recommendation on Proposal 13-110.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-110.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-110.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-110 to the appropriate committee as determined by the Conference Chair until additional data are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-110.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 13-110.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-110.

Single Laboratory Validation (SLV) Protocol For Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Justification for New Method

- Name of the New Method.
  Saxitoxin (PSP) Microtiter Plate Test Kit.

- Specify the Type of Method.
  Enzyme linked immunosorbent assay (ELISA) using anti-saxitoxin polyclonal antibody.

- Name of Method Developer.
  Drs. Titan Fan and Byungchul Kim

- Developer Contact Information.
  Beacon Analytical Systems, INC.
  82 Industrial Park Rd. Saco, 04072
  Phone: 207-571-4302
  Email: titan@beaconkits.com or bkim@beaconkits.com

- Date of Submission.
  June 26, 2013

- Purpose and Intended Use of the Method.
  Rapid analysis of saxitoxin (PSP) from shellfish such as blue mussels, steamers and mahogany clams. This method can be used for screening purpose that screens out negative samples (below 30 µg/100g). Suspicious samples with PSP levels between 30 and 100 µg/100g will need confirmation with NSSP Approved Method, mouse bioassay (MBA). PSP levels higher than 100 µg/100g would be considered as positive, and may not need further confirmation.

- Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods.
  The regulatory methods for PSP toxins are mouse bioassay (MBA) as NSSP Approved Method, and high performance liquid chromatography (HPLC) and liquid chromatography with post-column oxidation (PCOX) as NSSP Approved Limited Use Method. These methods are laborious, time consuming and expensive. Using these regulatory methods, it is difficult to process large amount of shellfish samples with limited resources. Therefore, there is a need of screening technique prior to the regulatory method that can screen out negative shellfish samples containing low levels of PSP (below 30 µg/100g). Only suspicious samples with PSP levels between 30 µg/100g and 100 µg/100g need further confirmation test with mouse bioassay. Therefore, this screening procedure will dramatically reduce the volume of samples to be confirmed with MBA, and save time and resources for the private, certified or state laboratories.

- Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable to Specific Matrix Types.

Due to extremely high sensitivity of the method, sample can be easily diluted with buffer solution (total 15000 folds). This high degree of sample dilution results in reduction in sample preparation time and elimination of any potential matrix effects either positive or negative from shellfish samples. Therefore, it is possible that this method could be used for any shellfish species for the determination of PSP level.

- Other Comments.

Method Documentation

- Method Title.
  Saxitoxin (PSP) Microtiter Plate Test Kit.

- Method Scope.
  The method is a competitive enzyme linked immunosorbent assay (ELISA) for the detection of saxitoxins in blue mussels, steamers and mahogany clams from North Atlantic Ocean as an Approved Limited Use Method.

- References.
  1. B. J. Yakes, S. M. Prezioso, S. L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta 2012, 99, 668-676.
  2. G. B. Inami, C. Crandall, D. Csuti, M. Oshiro, R. Brenden. Presence/Absence Screening for Saxitoxin in Frozen Acidified Mussel and Oyster Extracts from the Coast of California with In Vitro Methods, J AOAC. Int. 2004, 87 (5), 1133-1142.
  3. E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer. Immunoassay Methods for Paralytic Shellfish Poisoning Toxins. J. AOAC. Int. 2001, 84 (5), 1649-1656.
  4. Anderson, D.M., P. Andersen, V. M. Bricelj, J. J. Cullen, and J. EE. Rensel, 2001. Monitoring and management strategies for harmful algal blooms in coastal waters, APEC #201-MR-01.1, Asia Pacific Economic Program and Intergovernmental Oceanographic Commission Technical Series No. 59, Paris.
  5. Fun S. Chu and Titan S. L. Fan. Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. Journal-Association of Official Analytical Chemists 1985, 68 (1):13-16.

- Principle.
  The Beacon Saxitoxin (PSP) Microtiter Plate Kit is a competitive enzyme-labeled immunoassay. The Saxitoxin HRP conjugate, sample extract and calibrators are pipetted into the test wells followed by Saxitoxin antibody into the test wells to initiate the reaction. During the 30 minute incubation period, PSP toxins from the sample and Saxitoxin HRP conjugate compete for binding to Saxitoxin antibody. The Saxitoxin antibody is captured on the walls of the test well. Following this 30 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound PSP toxins, Saxitoxin HRP conjugate and free Saxitoxin antibody. After wash, a clear substrate is then added to the wells and any bound enzyme conjugate causes the conversion to a blue color. Following a 30 minute incubation, the reaction is stopped and the amount of color in each well is read. The color of the unknown samples is compared to the color of the calibrators and the Saxitoxin concentration of the samples is derived.

- Analytes/Measurands.
  Paralytic shellfish poisoning toxins (Saxitoxin, Neo-saxitoxin, Decarbamoylsaxitoxin, Gonyautoxin-2 and -3)

- Proprietary Aspects.
  Beacon Analytical Systems developed the kit including antibody and enzyme conjugate.

- Reagents provided.
  Antibody coated microplate
  Calibrators
  Enzyme conjugate
  Anti-saxitoxin rabbit polyclonal antibody
  Wash solution concentrate
  Substrate
  Stop solution

- Materials required but not provided.
  Laboratory quality distilled or deionized water
  20 mM Phosphate buffered saline
  Pipet with disposable tips capable of dispensing 50 µL
  Multi-channel pipet; 8-channel capable of dispensing 50 and 100 µL
  Paper towels or equivalent absorbent material
  Microwell plate or strip reader with 450nm filter
  Timer
  Wash bottle

- Media.
  A mixture of 70% Isopropanol and 5% Acetic acid in a ratio of 2 to 1 is used to extract PSP toxins from shellfish homogenate. To dilute the extract, 10% Methanol in 20 mM PBS buffer (10 mL of Methanol + 90 mL of 20 mM PBS) is used.
  Phosphate buffered saline with tween 20 (PBST) is used for washing.

- Matrices of Interest.
  Blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*).

- Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
  Preparation: Shellfish (12 animals) are shucked, rinsed and homogenized with a kitchen blender. Five grams (5 g) of homogenate is weighed in a 50 mL centrifuge tube, and 10 mL of extraction solvent (70% Isopropanol + 5% acetic acid solution, 2 + 1) is added. The tube is vortexed for 3 min. Approximately 1 mL of the extract is transferred into a microcentrifuge tube, and centrifuged for 5 min at 12,000 rpm. The supernatant is diluted with 10% Methanol in 20 mM PBS buffer to 5000 folds (e.g. 0.1 mL of supernatant + 9.9 mL of buffer and 0.1 mL of the diluted solution + 4.9 mL of buffer), and ready for the analysis.

- Safety Requirements.
  Personal safety items such as safety glasses, gloves and lab coat must be required. Calibrators and enzyme conjugate are toxic materials, and must be kept in the original vials when they are

not used. Since stop solution is a strong acidic solution (1 N hydrochloric acid), skin or eye contact must be avoided. Lab items with contact of toxins (sample extract and calibrator) such as pipette tips and lab wares must be soaked in 50% dilution of house bleach at least 1 hour before washing or disposal.

- Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]).
    - Cost of the method: Saxitoxin (PSP) Microtiter Plate Test Kit costs $325 and can test up to 44 samples in duplicate (approximately $7 per sample)
    - Special technical skills: Experience in ELISA assay would be preferable but not necessary. Basic lab experience is recommended such as pipetting and safety training.
    - Special equipment and cost:

Single channel micropipette (200 and 1000 µL)	$560
Multi channel micropipette (either 200 or 300 µL)	$680
Microplate reader (capable of reading at 450 nm, StatFax 303+)	$2,100

    - Abbreviations and acronyms:
      PSP – Paralytic Shellfish Poisoning
      ELISA – Enzyme Linked ImmunoSorbent Assay
      HRP – Horse Radish Peroxidase
      PBS – Phosphate Buffered Saline
      MBA – Mouse BioAssay
      HPLC-PCOX – High Performance Liquid Chromatography with Post-Column OXidation
    - Turnaround time: More than 200 samples can be tested in a day (8 hours). MBA and HPLC-PCOX may test up to 60 and 15 samples, respectively. (Reference 4, Page 33)

- Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.). Please refer to the instructional booklet (Appendix).
    1. Prepare the 1X wash solution by adding the contents of the 10X wash concentrate bottle to 450 mL Lab grade water in a wash bottle.
    2. Allow reagents and sample extracts to reach room temperature prior to running the test. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
    3. Using a pipet with disposable tips, add 50 µL enzyme conjugate to the appropriate test wells. Be sure to use a clean pipet tip for each.
    4. Add 50 µL of Calibrators or Sample extract to each well. Dispense 50 µL of Antibody Solution into each test well. Shake the plate gently for 30 seconds and incubate the test wells for 30 minutes.
    5. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.
    6. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the wash solution. Dispense 100 µL of Substrate, and incubate for 30 minutes.
    7. Dispense 100 µL of Stop Solution into each test well. Read and record the absorbance of the wells at 450nm using a strip or plate reader.
    8. The concentration of PSP toxins in the sample is calculated based on the calibration curve (4-parameter fit). The dilution factor of 15000 must be applied to the calculated concentration (e.g., 1 ppb as calculated concentration X 15000 = 15000 ppb as real

concentration of PSP in sample). Then, the value in ppb (µg/1000 g) can be converted to µg/100 g by dividing by 10. For people who don't have 4-parameter fit in their readers, the Microsoft spreadsheet for the calculation would be provided upon request (Attached separately).

- Quality Control (Provide Specific Steps).
  Coefficient of variation (CV, %) of the results from duplicate wells for each test should be below 15%. If this CV (%) is not calculated by the reader, it can be manually calculated as standard deviation divided by average, and then multiply by 100. Coefficient of determination (R^2) from the calibration curve must be higher than 0.990, which indicates the assay performs accurately. Absorbance of zero calibrator should not be higher than 2.5. Customized QC protocol can be developed with assistance from Beacon Analytical Systems.

- Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

  Accuracy/Trueness
  The range chosen is 5 to 160 µg/100 g because any results higher than 100 µg/100 g are considered as positive. There is no reason to test the accuracy and trueness at such high levels.
  Sample type is shellfish tissue. Shellfish samples were purchased from a local fish market (Portland, Maine) and tested prior to the study to see if any considerable levels of PSP toxins are found. Only negligible levels of PSP were found (less than 3 µg/100 g). Samples used for spiking are blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*). Since the regulatory limit of PSP toxin is 80 µg/100 g, a broad range of saxitoxin levels were spiked between 5 and 160 µg/100 g. The standard saxitoxin used for the spike was purchased from NIST (RM 8642, FDA Saxitoxin Dihydrocholoride Solution, National Institute of Standards and Technology). Detailed procedures are as below.

  1. Twelve animals (mussel, steamer or mahogany clam) were shucked and homogenized in a kitchen blender and stored in -20 °C freezer.
  2. Five grams of the homogenate was weighed in 50 mL centrifuge tube and saxitoxin was added into the tube so that the final concentration in 5 g is 5, 10, 20, 30, 40, 50, 55, 60, 65, 70, 75, 80, 90, 100, 110, 120, 130, 140, 150 or 160 µg/100 g.
  3. Extraction solvent of 10 mL was added into the tube, and vortexed for 3 minutes.
     (Extraction solvent is a mixture of 70% Isopropanol and 5% Acetic acid in a ratio of 2 to 1)
  4. Transfer 1 mL of extract into a microcentrifuge tube to spin at 12,000 rpm for 5 minutes.
  5. Mix 0.1 mL of the supernatant with 9.9 mL of 10% Methanol/20 mM PBS buffer for 100 times dilution, and transfer 0.1 mL of the diluted extract into a 4.9 mL of buffer solution. to make the final dilution of 5000 times. This diluted extract was used for the assay. As directed in the instructional booklet, multiply the assay result by 15000 in order to compensate the dilution. This results in saxitoxin concentration in the shellfish tissue (ppb). Then, the value in ppb (µg/1000 g) can be converted to µg/100 g by dividing by 10.

  Ruggedness

Sample type is shellfish tissue. Organisms used for spiking are blue mussel (*Mytilus edulis*), steamer (*Mya arenaria*) and mahogany clam (*Arctica islandica*). Spike and extraction were conducted same as Accuracy/Trueness. Spike levels chosen were 10, 20, 40, 50, 60, 70, 80, 90, 100 and 120 µg/100 g. However, two aliquots from each sample were tested with two different lots of the kit.

Precision

Working range, sample type and organism used for spiking are same as Accuracy/Trueness. Spike and extraction were conducted same as Accuracy/Trueness. However, each sample was spiked with three different levels. Spike levels chosen were 20, 80 and 160 µg/100 g. Each level of spiked sample was analyzed twice.

Recovery

Same sample procedure as Precision test.

Specificity

The cross reactivity was evaluated by US FDA. Betsy Jean Yakes, Samantha M. Prezioso, Stacey L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta (2012), 99, 668-676.

Linearity/Limit of Detection/Limit of Quantitation/Sensitivity

Sample type is shellfish tissue. Working range is 10 to 240 µg/100 g. Range of interest is 20 to 80 µg/100 g. Range in spiking levels used is 5 to 240 µg/100 g. For the linearity, STX standards were used (0, 0.01, 0.08 and 0.32ppb), and this range of calibrators covers the saxitoxin levels of 15 through 480 µg/100 g in sample when the dilution was performed as directed in the instructional booklet. For the LOD, LOQ and sensitivity, blue mussel, steamer and mahogany clam were used for spiking. Spike and extraction were conducted same as Accuracy/Trueness. However, each sample was spiked with six different levels including zero. Ten samples were prepared. Spike levels chosen for each sample were 0, 5, 20, 80, 160 and 240 µg/100 g. Each spike level of the samples except zero was analyzed in triplicate.

Table 1. Accuracy/Trueness for blue mussel.

Mussel	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
A	5	5.86	1.72	4.14	0.86
B	10	11.19	1.64	9.55	0.45
C	20	20.73	1.56	19.17	0.83
D	30	31.85	1.89	29.96	0.04
E	40	41.88	1.71	40.17	-0.16
F	50	56.23	2.16	54.07	-4.07
G	55	63.23	2.16	61.07	-6.07
H	60	63.65	2.08	61.57	-1.57
I	65	70.6	2.18	68.42	-3.42
J	70	75.28	2.3	72.98	-2.98
K	75	83.61	2.12	81.49	-6.49
L	80	85.27	2.02	83.25	-3.25
M	90	90.82	2.05	88.77	1.23
N	100	122.34	2.27	120.07	-20.07
O	110	124.91	2.18	122.73	-12.73
P	120	138.26	2.35	135.91	-15.91
Q	130	151.76	2.31	149.45	-19.45
R	140	157.85	2.38	155.47	-15.47
S	150	180.79	2.64	178.15	-28.15
T	160	191.12	2.52	188.60	-28.60
Average	78	88.36	2.11	86.25	-8.25
Standard dev.					9.78
Accuracy/Trueness (%)	111				
Measurement uncertainty (µg/100 g)	4.29				

Table 2. Accuracy/Trueness for steamer.

Steamer	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
A	5	5.56	1.01	4.55	0.45
B	10	10.61	0.89	9.72	0.28
C	20	19.74	0.67	19.07	0.93
D	30	29.68	0.81	28.87	1.13
E	40	41.02	0.91	40.11	-0.11
F	50	53.74	1.21	52.53	-2.53
G	55	59.27	0.86	58.41	-3.41
H	60	58.31	1.16	57.15	2.85
I	65	67.78	1.17	66.61	-1.61
J	70	73.17	1.17	72.00	-2.00
K	75	76.69	1.07	75.62	-0.62
L	80	70.02	1.29	68.73	11.27
M	90	93.12	1.11	92.01	-2.01
N	100	102.03	1.24	100.79	-0.79
O	110	115.1	1.69	113.41	-3.41
P	120	127.62	1.61	126.01	-6.01
Q	130	131.82	1.78	130.04	-0.04
R	140	146.65	1.73	144.92	-4.92
S	150	161.68	1.79	159.89	-9.89
T	160	179.26	1.79	177.47	-17.47
Average	78	81.14	1.25	79.90	-1.90
Standard dev.					5.48
Accuracy/Trueness (%)	102				
Measurement uncertainty (µg/100 g)	2.40				

Table 3. Accuracy/Trueness for mahogany clam.

Mahogany clam	Saxitoxin (µg/100 g)				
Sample	Spike level	Determined	Blank	Blank subtracted	Difference
1	5	7.00	1.62	5.38	-0.38
2	10	11.72	1.62	10.10	-0.1
3	20	19.59	1.62	17.97	2.03
4	30	30.33	1.95	28.38	1.62
5	40	41.32	2.01	39.31	0.69
6	50	54.72	1.86	52.86	-2.86
7	55	60.24	1.84	58.40	-3.40
8	60	62.42	2.26	60.16	-0.16
9	65	64.70	2.27	62.43	2.57
10	70	70.83	2.38	68.45	1.55
11	75	76.86	2.55	74.31	0.69
12	80	81.45	2.29	79.16	0.84
13	90	92.70	2.64	90.06	-0.06
14	100	103.11	2.61	100.50	-0.50
15	110	113.75	2.29	111.46	-1.46
16	120	132.55	2.58	129.97	-9.97
17	130	137.53	2.56	134.97	-4.97
18	140	138.86	2.39	136.47	3.53
19	150	150.25	2.54	147.71	2.29
20	160	170.89	2.63	168.26	-8.26
Average	78	81.04	2.23	78.82	-0.82
Standard dev.					3.44
Accuracy/Trueness (%)	101				
Measurement uncertainty (µg/100 g)	1.55				

Table 4. Ruggedness analysis.

	Mussel (µg/100 g)		Steamer (µg/100 g)		Mahogany (µg/100 g)	
Sample	Lot #1	Lot #2	Lot #1	Lot #2	Lot #1	Lot #2
1	9.75	9.84	8.58	8.61	10.89	12.71
2	17.66	18.12	14.09	15.73	21.74	21.14
3	33.46	33.97	32.92	32.95	39.91	39.69
4	46.52	45.17	42.39	41.12	49.29	55.45
5	57.9	56.55	48.16	50.02	62.33	69.42
6	65.32	66.89	61.67	64.72	79.93	70.44
7	77.52	74.98	69.05	67.32	84.86	87.97
8	79.31	78.15	72.38	76.77	88.01	97.9
9	90.31	98.38	82.33	87.42	101.3	103.77
10	112.45	116.24	102.51	103.14	130.54	124.97
Skewness	-0.04	0.10	-0.04	-0.05	0.06	-0.12
Variance	1064.05	1157.37	893.83	942.89	1378.60	1337.38
Ratio of variances	1.09		1.05		1.03	
P-value (Paired t-test)	0.43		0.09		0.45	
Significant difference	NO		NO		NO	

Table 5. Precision for blue mussel.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L*	1	20.32	6	L	1	15.54
1	L	2	24.59	6	L	2	21.30
1	M*	1	81.33	6	M	1	83.87
1	M	2	79.36	6	M	2	86.25
1	H*	1	162.73	6	H	1	175.71
1	H	2	152.45	6	H	2	168.75
2	L	1	19.77	7	L	1	16.99
2	L	2	14.40	7	L	2	19.75
2	M	1	78.70	7	M	1	86.41
2	M	2	79.60	7	M	2	85.06
2	H	1	149.92	7	H	1	172.50
2	H	2	158.63	7	H	2	167.14
3	L	1	25.15	8	L	1	21.48
3	L	2	18.34	8	L	2	21.10
3	M	1	72.79	8	M	1	85.97
3	M	2	76.45	8	M	2	86.22
3	H	1	152.89	8	H	1	166.83
3	H	2	152.19	8	H	2	170.77
4	L	1	23.05	9	L	1	23.24
4	L	2	18.48	9	L	2	24.06
4	M	1	73.24	9	M	1	84.01
4	M	2	74.61	9	M	2	92.05
4	H	1	149.37	9	H	1	170.87
4	H	2	141.79	9	H	2	171.03
5	L	1	20.60	10	L	1	20.16
5	L	2	16.54	10	L	2	17.87
5	M	1	75.65	10	M	1	84.29
5	M	2	78.27	10	M	2	86.37
5	H	1	152.04	10	H	1	175.94
5	H	2	158.34	10	H	2	170.48

*L; Low level spike (20 µg/100 g), M; Medium level spike (80 µg/100 g), H; High level spike (160 µg/100 g)

Table 6. Nested ANOVA for blue mussel sample.

Source of variation	DF	SS	MS	F value
Samples	9	1384.16	153.80	0.02
Concentrations in samples	20	203507	10175	894.24
Determinations within concentrations	30	341.36	11.38	
Total	59	205233	3478.52	

Table 7. Precision for steamer.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L	1	18.68	6	L	1	16.87
1	L	2	23.16	6	L	2	21.63
1	M	1	77.46	6	M	1	81.76
1	M	2	76.23	6	M	2	82.96
1	H	1	154.04	6	H	1	167.80
1	H	2	154.51	6	H	2	163.75
2	L	1	20.14	7	L	1	18.73
2	L	2	21.66	7	L	2	24.59
2	M	1	85.17	7	M	1	83.62
2	M	2	79.32	7	M	2	80.45
2	H	1	160.30	7	H	1	155.48
2	H	2	165.08	7	H	2	165.04
3	L	1	23.13	8	L	1	23.69
3	L	2	19.95	8	L	2	20.61
3	M	1	79.99	8	M	1	87.19
3	M	2	80.85	8	M	2	87.25
3	H	1	153.02	8	H	1	169.35
3	H	2	151.95	8	H	2	166.76
4	L	1	26.14	9	L	1	23.20
4	L	2	23.63	9	L	2	26.76
4	M	1	82.59	9	M	1	85.59
4	M	2	84.22	9	M	2	92.55
4	H	1	163.35	9	H	1	174.61
4	H	2	155.64	9	H	2	167.74
5	L	1	21.51	10	L	1	20.71
5	L	2	23.76	10	L	2	22.71
5	M	1	78.95	10	M	1	88.30
5	M	2	81.70	10	M	2	88.46
5	H	1	168.83	10	H	1	171.01
5	H	2	160.60	10	H	2	169.67

Table 8. Nested ANOVA for steamer sample.

Source of variation	DF	SS	MS	F value
Samples	9	666.39	74.04	0.01
Concentrations in samples	20	199960	9997.98	1093.56
Determinations within concentrations	30	274.28	9.14	
Total	59	200900	3405.09	

Table 9. Precision for mahogany clam.

Sample	Concentration	Determination	STX Score	Sample	Concentration	Determination	STX Score
1	L	1	16.52	6	L	1	22.28
1	L	2	23.15	6	L	2	22.54
1	M	1	81.58	6	M	1	78.12
1	M	2	78.26	6	M	2	79.69
1	H	1	155.89	6	H	1	171.77
1	H	2	158.16	6	H	2	162.53
2	L	1	20.78	7	L	1	21.89
2	L	2	19.63	7	L	2	27.09
2	M	1	80.07	7	M	1	89.17
2	M	2	74.56	7	M	2	89.24
2	H	1	161.16	7	H	1	168.43
2	H	2	152.61	7	H	2	161.55
3	L	1	26.17	8	L	1	29.98
3	L	2	21.59	8	L	2	26.59
3	M	1	76.65	8	M	1	96.99
3	M	2	77.17	8	M	2	83.19
3	H	1	152.00	8	H	1	164.97
3	H	2	159.93	8	H	2	165.68
4	L	1	22.83	9	L	1	24.67
4	L	2	23.56	9	L	2	25.65
4	M	1	80.57	9	M	1	83.07
4	M	2	80.61	9	M	2	86.46
4	H	1	162.48	9	H	1	169.98
4	H	2	155.49	9	H	2	156.82
5	L	1	21.18	10	L	1	22.74
5	L	2	26.06	10	L	2	28.91
5	M	1	82.48	10	M	1	88.31
5	M	2	85.91	10	M	2	89.45
5	H	1	165.33	10	H	1	165.92
5	H	2	156.10	10	H	2	162.24

Table 10. Nested ANOVA for mahogany clam sample.

Source of variation	DF	SS	MS	F value
Samples	9	646.57	71.84	0.01
Concentrations in samples	20	191207	9560.33	561.00
Determinations within concentrations	30	511.25	17.04	
Total	59	192364	3260.41	

Table 11. Recovery for blue mussel.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	20.32	24.59	22.46	-2.46
L (20)	19.77	14.40	17.09	2.92
L (20)	25.15	18.34	21.75	-1.75
L (20)	23.05	18.48	20.77	-0.77
L (20)	20.60	16.54	18.57	1.43
L (20)	15.54	21.30	18.42	1.58
L (20)	16.99	19.75	18.37	1.63
L (20)	21.48	21.10	21.29	-1.29
L (20)	23.24	24.06	23.65	-3.65
L (20)	20.16	17.87	19.02	0.99
M (80)	81.33	79.36	80.35	-0.35
M (80)	78.70	79.60	79.15	0.85
M (80)	72.79	76.45	74.62	5.38
M (80)	73.24	74.61	73.93	6.08
M (80)	75.65	78.27	76.96	3.04
M (80)	83.87	86.25	85.06	-5.06
M (80)	86.41	85.06	85.74	-5.74
M (80)	85.97	86.22	86.10	-6.10
M (80)	84.01	92.05	88.03	-8.03
M (80)	84.29	86.37	85.33	-5.33
H (160)	162.73	152.45	157.59	2.41
H (160)	149.92	158.63	154.28	5.73
H (160)	152.89	152.19	152.54	7.46
H (160)	149.37	141.79	145.58	14.42
H (160)	152.04	158.34	155.19	4.81
H (160)	175.71	168.75	172.23	-12.23
H (160)	172.50	167.14	169.82	-9.82
H (160)	166.83	170.77	168.80	-8.80
H (160)	170.87	171.03	170.95	-10.95
H (160)	175.94	170.48	173.21	-13.21

Table 12. One-way ANOVA for blue mussel sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	19.045	9.52	0.22	0.81	3.35
Error	27	1184.75	43.88			
Total	29	1203.79				

Table 13. Recovery for steamer.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	18.68	23.16	20.92	-0.92
L (20)	20.14	21.66	20.90	-0.90
L (20)	23.13	19.95	21.54	-1.54
L (20)	24.14	23.63	24.89	-4.89
L (20)	21.51	23.76	22.64	-2.64
L (20)	16.87	21.63	19.25	0.76
L (20)	18.73	24.59	21.66	-1.66
L (20)	23.69	20.61	22.15	-2.15
L (20)	23.20	26.76	24.98	-4.98
L (20)	20.71	22.71	21.71	-1.71
M (80)	77.46	76.23	76.85	3.16
M (80)	85.17	79.32	82.24	-2.24
M (80)	79.99	80.85	80.42	-0.42
M (80)	82.59	84.22	83.41	-3.41
M (80)	78.95	81.70	80.33	-0.33
M (80)	81.76	82.96	82.36	-2.36
M (80)	83.62	80.45	82.04	-2.04
M (80)	87.19	87.25	87.22	-7.22
M (80)	85.59	92.55	89.07	-9.07
M (80)	88.30	88.46	88.38	-8.38
H (160)	154.04	154.51	154.28	5.73
H (160)	160.30	165.08	162.69	-2.69
H (160)	153.02	151.95	152.49	7.52
H (160)	163.35	155.64	159.50	0.51
H (160)	168.83	160.60	164.72	-4.72
H (160)	167.80	163.75	165.77	-5.77
H (160)	155.48	165.04	160.26	-0.26
H (160)	169.35	166.76	168.05	-8.05
H (160)	174.61	167.74	171.18	-11.18
H (160)	171.01	169.67	170.34	-10.34

Table 14. One-way ANOVA for steamer sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	7.34	3.67	0.19	0.83	3.35
Error	27	528.18	19.56			
Total	29	535.51				

Table 15. Recovery for mahogany clam.

Spike (µg/100 g)	Replicate 1	Replicate 2	Average	Spike - Average
L (20)	16.52	23.15	19.84	0.17
L (20)	20.78	19.63	20.21	-0.21
L (20)	26.17	21.59	23.88	-3.88
L (20)	22.83	23.56	23.20	-3.20
L (20)	21.18	26.06	23.62	-3.62
L (20)	22.28	22.54	22.41	-2.41
L (20)	21.89	27.09	24.49	-4.49
L (20)	29.98	26.59	28.29	-8.29
L (20)	24.67	25.65	25.16	-5.16
L (20)	22.74	28.91	25.83	-5.83
M (80)	81.58	78.26	78.26	1.74
M (80)	80.07	74.56	74.56	5.44
M (80)	76.65	77.17	77.17	2.83
M (80)	80.57	80.61	80.61	-0.61
M (80)	82.48	85.91	85.91	-5.91
M (80)	78.12	79.69	78.91	1.10
M (80)	89.17	89.24	89.21	-9.21
M (80)	96.99	83.19	90.09	-10.09
M (80)	83.07	86.46	84.77	-4.77
M (80)	88.31	89.45	88.88	-8.88
H (160)	155.89	158.16	157.03	2.98
H (160)	161.16	152.61	156.89	3.12
H (160)	152.00	159.93	155.97	4.04
H (160)	162.48	155.49	158.99	1.02
H (160)	165.33	156.10	160.72	-0.72
H (160)	171.77	162.53	167.15	-7.15
H (160)	168.43	161.55	164.99	-4.99
H (160)	164.97	165.68	165.33	-5.33
H (160)	169.98	156.82	163.40	-3.40
H (160)	165.92	162.24	164.08	-4.08

Table 16. One-way ANOVA for steamer sample.

Source of variation	DF	SS	MS	F	P-value	F critcal
Concentration	2	25.52	12.76	0.70	0.50	3.35
Error	27	490.11	18.15			
Total	29	515.63				

Table 17. Percent recovery of the method for each tissue type.

		Blue Mussel		Steamer		Mahogany Clam	
	Spike (µg/100 g)	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
	20	20.32	24.59	18.68	23.16	16.52	23.15
	20	19.77	14.40	20.14	21.66	20.78	19.63
	20	25.15	18.34	23.13	19.95	26.17	21.59
	20	23.05	18.48	24.14	23.63	22.83	23.56
	20	20.60	16.54	21.51	23.76	21.18	26.06
	20	15.54	21.30	16.87	21.63	22.28	22.54
	20	16.99	19.75	18.73	24.59	21.89	27.09
	20	21.48	21.10	23.69	20.61	29.98	26.59
	20	23.24	24.06	23.20	26.76	24.67	25.65
	20	20.16	17.87	20.71	22.71	22.74	28.91
	80	81.33	79.36	77.46	76.23	81.58	78.26
	80	78.70	79.60	85.17	79.32	80.07	74.56
	80	72.79	76.45	79.99	80.85	76.65	77.17
	80	73.24	74.61	82.59	84.22	80.57	80.61
	80	75.65	78.27	78.95	81.70	82.48	85.91
	80	83.87	86.25	81.76	82.96	78.12	79.69
	80	86.41	85.06	83.62	80.45	89.17	89.24
	80	85.97	86.22	87.19	87.25	96.99	83.19
	80	84.01	92.05	85.59	92.55	83.07	86.46
	80	84.29	86.37	88.30	88.46	88.31	89.45
	160	162.73	152.45	154.04	154.51	155.89	158.16
	160	149.92	158.63	160.30	165.08	161.16	152.61
	160	152.89	152.19	153.02	151.95	152.00	159.93
	160	149.37	141.79	163.35	155.64	162.48	155.49
	160	152.04	158.34	168.83	160.60	165.33	156.10
	160	175.71	168.75	167.80	163.75	171.77	162.53
	160	172.50	167.14	155.48	165.04	168.43	161.55
	160	166.83	170.77	169.35	166.76	164.97	165.68
	160	170.87	171.03	174.61	167.74	169.98	156.82
	160	175.94	170.48	171.01	169.67	165.92	162.24
Average	86.67		87.89		89.37		89.41
Recovery (%)			101.42		103.12		103.16

Table 18. Cross reactivity of antibody to saxitoxin-related congeners.

Compound	Cross reactivity (%)
Saxitoxin dihydrochloride	100.00
Neosaxitoxin	2.28
Decarbamoyl STX	42.30
GTX2 & 3	50.00
GTX1 & 4	0.48
Decarbamoyl GTX2 & 3	2.36
Decarbamoyl NeoSTX	1.50
B1	16.95
C1& 2	12.10

*The cross reactivity was evaluated by US FDA. Betsy Jean Yakes, Samantha M. Prezioso, Stacey L. DeGrasse. Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta (2012), 99, 668-676.

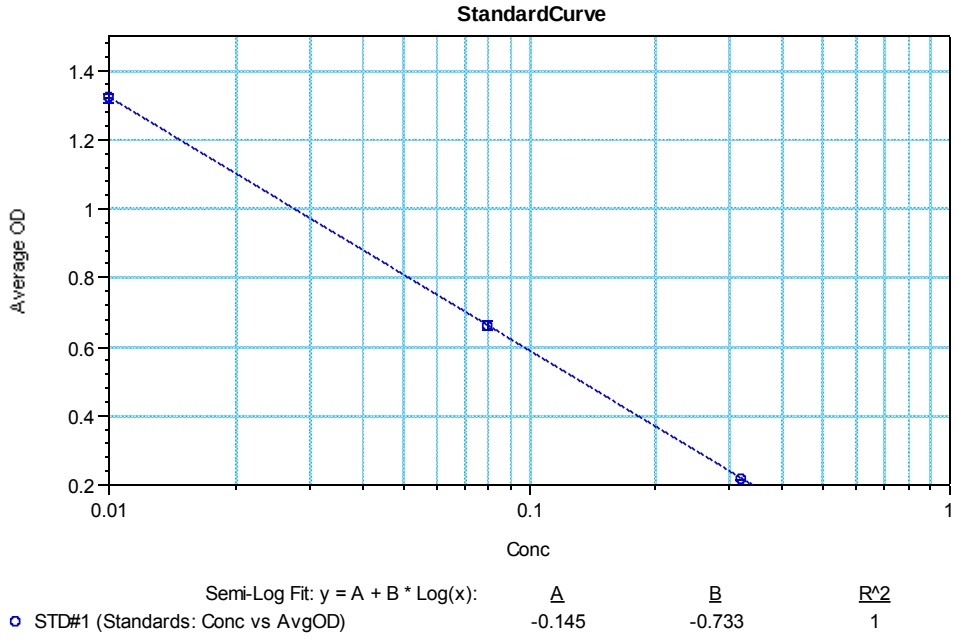


Figure 1. Calibration curve.

Table 19. LOD and LOQ for blue mussel.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	8.00	19.44	81.56	154.58	218.59
	2	5.23	22.13	79.71	151.94	222.88
	3	6.70	20.32	76.53	150.97	227.44
2	1	8.98	20.01	77.37	158.08	222.52
	2	6.22	21.09	79.20	153.75	225.14
	3	8.17	19.67	76.63	153.63	229.74
3	1	5.51	19.89	79.46	165.71	220.13
	2	7.55	21.71	80.02	167.96	231.76
	3	6.21	22.31	77.98	174.00	232.58
4	1	6.21	19.33	78.04	155.51	232.76
	2	6.91	22.19	83.81	156.42	237.66
	3	7.01	22.34	85.43	162.98	229.39
5	1	7.87	22.06	86.31	163.41	234.75
	2	10.65	24.62	80.05	160.72	231.98
	3	9.46	22.04	79.33	161.27	228.36
6	1	3.17	21.17	81.48	158.63	234.11
	2	4.90	23.31	81.72	161.60	237.80
	3	10.09	22.82	84.49	166.57	241.66
7	1	7.54	22.04	83.74	167.89	242.64
	2	7.25	21.40	80.08	160.91	238.06
	3	6.04	20.19	80.64	158.23	235.43
8	1	4.43	20.31	69.33	155.01	228.79
	2	6.31	22.70	75.59	161.16	243.85
	3	6.86	23.74	77.60	162.91	244.77
9	1	4.88	20.13	68.81	154.66	228.89
	2	5.83	22.57	74.57	160.32	243.81
	3	8.13	23.92	77.61	163.21	246.43
10	1	6.30	24.06	76.86	161.50	246.37
	2	8.02	22.43	81.28	168.06	244.21
	3	8.23	24.54	82.96	170.67	243.64
Average		6.89	22.16	79.47	162.47	236.66
Standard Deviation		1.76	1.47	4.40	4.98	7.03
CV (%)		25.48	6.63	5.54	3.06	2.97

Limit of quantitation (LOQ) is 13 µg/100 g and limit of detection (LOD) is 3.9 µg/100 g.

Table 20. LOD and LOQ for steamer.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	5.89	19.23	74.16	154.92	228.85
	2	4.93	20.26	75.06	155.50	235.79
	3	5.84	21.23	76.36	154.92	233.99
2	1	4.92	18.49	72.48	153.03	228.16
	2	4.24	18.74	73.04	153.62	233.76
	3	5.25	20.56	75.21	154.07	235.08
3	1	5.26	15.70	70.34	142.34	225.09
	2	5.68	19.10	75.49	147.96	221.74
	3	5.94	18.87	76.12	150.07	228.19
4	1	4.46	19.77	75.80	147.91	234.36
	2	2.53	21.12	77.35	152.37	229.56
	3	3.16	22.37	79.89	156.60	238.98
5	1	4.91	20.53	82.73	154.60	237.12
	2	4.33	21.12	84.16	165.37	244.30
	3	6.82	21.95	85.43	161.95	244.16
6	1	5.93	22.43	84.55	160.50	240.98
	2	2.43	20.55	82.53	162.05	239.87
	3	3.93	19.76	81.05	153.58	233.75
7	1	6.80	22.04	78.88	161.82	249.64
	2	4.74	19.42	87.73	169.54	258.67
	3	7.70	21.53	84.61	170.70	250.27
8	1	5.37	21.95	82.42	167.55	246.71
	2	3.99	17.22	82.09	164.19	247.66
	3	5.13	18.27	78.32	161.26	243.13
9	1	8.23	20.57	76.89	150.28	240.76
	2	7.65	19.04	74.81	148.37	235.24
	3	9.36	19.46	76.15	156.55	243.71
10	1	6.86	22.15	77.17	152.94	242.10
	2	7.05	23.59	76.91	155.44	244.50
	3	8.62	22.69	83.93	157.17	249.33
Average		5.60	20.32	78.72	156.57	238.85
Standard Deviation		1.70	1.76	4.42	6.77	8.45
CV (%)		30.39	8.67	5.61	4.32	3.54

Limit of quantitation (LOQ) is 17 µg/100 g and limit of detection (LOD) is 5.2 µg/100 g.

Table 21. LOD and LOQ for Mahogany clam.

Sample	Replicate	Spike (µg/100 g)				
		5	20	80	160	240
1	1	4.79	20.72	74.76	156.09	235.92
	2	3.66	20.58	73.55	152.94	238.18
	3	5.52	22.54	78.00	146.95	241.35
2	1	5.30	21.52	76.18	151.08	240.92
	2	2.32	20.46	71.91	150.13	239.19
	3	4.50	19.50	75.17	154.73	233.91
3	1	5.14	20.74	75.36	150.57	239.47
	2	7.62	19.13	70.52	148.60	237.14
	3	3.80	18.70	74.66	154.31	233.02
4	1	4.75	20.38	75.15	150.26	240.21
	2	3.13	18.20	74.55	153.86	233.42
	3	6.36	18.37	69.73	147.80	237.09
5	1	5.67	18.83	75.84	149.32	229.98
	2	6.38	21.11	77.56	155.13	242.33
	3	6.64	20.18	81.71	160.98	246.92
6	1	6.09	18.92	82.98	161.94	248.69
	2	5.09	19.40	76.25	155.23	243.44
	3	4.92	17.48	75.18	148.89	231.89
7	1	3.37	18.46	76.29	154.20	240.17
	2	6.03	18.95	83.97	161.17	246.89
	3	4.46	17.25	75.97	149.58	231.00
8	1	9.50	22.67	83.28	153.81	231.91
	2	8.39	23.87	82.56	158.22	231.64
	3	6.29	22.53	86.47	149.23	232.41
9	1	7.62	19.18	73.82	158.28	237.57
	2	7.13	22.08	75.80	156.14	223.80
	3	7.71	21.37	77.12	153.62	223.58
10	1	5.36	19.00	73.77	157.04	235.65
	2	2.84	21.28	74.66	155.53	221.84
	3	5.85	21.49	78.05	156.17	224.65
Average		5.54	20.16	76.69	153.73	235.81
Standard Deviation		1.69	1.66	3.98	4.08	6.93
CV (%)		30.45	8.24	5.19	2.66	2.94

Limit of quantitation (LOQ) is 16 µg/100 g and limit of detection (LOD) is 4.9 µg/100 g.

Table 22. Comparison between ELISA and MBA for samples collected from same location over the seasons.

Date of collection	ELISA (µg/100g)	MBA (µg/100g)	Result from ELISA	Result from MBA
6/7/2010	35	45	Suspicious	Below limit
6/9/2010	5	0	Negative	Negative
6/14/2010	31	45	Suspicious	Below limit
6/16/2010	18	0	Negative	Negative
6/21/2010	66	59	Suspicious	Below limit
6/23/2010	31	48	Suspicious	Below limit
6/28/2010	133	274	Positive	Positive
6/30/2010	433	567	Positive	Positive
7/5/2010	1006	1204	Positive	Positive
7/7/2010	115	163	Positive	Positive
7/12/2010	276	264	Positive	Positive
7/14/2010	11	0	Negative	Negative
7/19/2010	10	0	Negative	Negative
7/21/2010	6	0	Negative	Negative
7/26/2010	0	0	Negative	Negative
7/28/2010	1	0	Negative	Negative
8/2/2010	0	0	Negative	Negative
8/9/2010	0	0	Negative	Negative
8/11/2010	12	0	Negative	Negative
8/16/2010	8	0	Negative	Negative
8/18/2010	8	0	Negative	Negative
5/9/2011	42	54	Suspicious	Below limit
5/16/2011	34	42	Suspicious	Below limit
5/30/2011	25	0	Negative	Negative
6/6/2011	184	124	Positive	Positive
6/13/2011	288	382	Positive	Positive

Table 23. Comparison between ELISA and MBA for samples collected from different locations.

Location of collection	ELISA (µg/100g)	MBA (µg/100g)	Result from ELISA	Result from MBA
Black Rock	101	121	Positive	Positive
Lumbos Hole	124	156	Positive	Positive
Ogunquit R.	24	48	Negative	Below limit
Hermit Island East	120	136	Positive	Positive
Long Point	72	67	Suspicious	Below limit
Gurnet	264	156	Positive	Positive
Head Beach	32	39	Suspicious	Below limit
Little johns Bridge	52	52	Suspicious	Below limit
Black Rock	32	50	Suspicious	Below limit
Bangs Island	40	54	Suspicious	Below limit
Ash Point	99	74	Suspicious	Below limit
Basin Point	44	44	Suspicious	Below limit
Ash Point	44	48	Suspicious	Below limit
Lumbos Hole	36	49	Suspicious	Below limit
CB 004 Youngs Point	64	55	Suspicious	Below limit
Matthews Island	56	47	Suspicious	Below limit

Discussion

The accuracy/trueness of the method for mussel, steamer and mahogany clam were 111%, 102% and 101%, respectively. The measurement uncertainty for mussel, steamer and mahogany clam were 4.29, 2.40 and 1.55 µg/100 g, respectively. Based on these results, mahogany clam sample gives better accuracy/trueness and measurement uncertainty for the method (Table 1 – 3).

The skewness (within -2 to +2) and the ratio of variances (close to 1) indicate that this is a symmetric distribution and the variance is homogeneous, respectively. Therefore, the paired t-test was chosen for the analysis instead of Welch's t-test in order to see the significant difference between two lots of each tissue type test. P-value below 0.05 is considered statistically significant at a significance level of 0.05, while one of 0.05 or greater indicates no significant difference between the groups. Therefore, all three tissue types had no significant differences between lots when tested with the method over the tested range of 10 – 120 µg/100 g. This indicates that the method is sufficiently rugged to be used routinely (Table 4).

For mussel sample, we obtained an F value of 0.02 for sample which is less than the critical value of 2.39 for 9 and 20 degrees of freedom at the 0.05 significance level (Table 6). This indicates that the mean values from the samples are not significantly different. The F value for concentration in sample is greater than the critical value of 1.93 for 20 and 30 degrees of freedom a the 0.05 significance level. This indicates that the mean values of each concentration within samples are significantly different. From this nested ANOVA table, we can conclude that the precision of the method is consistent over the samples. Each sample has three different levels of saxitoxin contamination. However, the precision of the method is not consistent for each spiked concentration when repeated with different samples. The other two samples, steamer and mahogany had the same results as mussel sample (Table 5 – 10).

From one-way ANOVA for recovery, since F value for each shellfish type is smaller than F critical (tabulated F value at 95% confidence level), there is no significance between groups (20, 80 and 160). Therefore, the recoveries for mussel, steamer and mahogany samples are consistent over the range tested by the method (20 through 160 µg/100 g) (Table 11 – 17).

Cross reactivity of the antibody used for the kit was evaluated by the third party (Table 18). The detailed information regarding the cross reactivity can be achieved from the reference 1 (Yakes et al., 2012). However, it is common that the results may vary depending on the assay formats used to evaluate the cross reactivity.

For the linearity of the method, calibrators were run by the method. OD (optical density) values at 450 nm were graphed on the y-axis, and log concentrations of calibrators (0.01 – 0.32 µg/L) were graphed on the x-axis. A linear curve fit was applied to the data. The curve in Figure 1 is very good with coefficient of determination of 1.0. This indicates the method can detect the levels of saxitoxin between 15 and 480 µg/100 g in sample with good linearity.

To determine the LOQ for each tissue type, coefficient of variation (%) was plotted on the y-axis and saxitoxin spike level was plotted on the x-axis. From this graph, LOQ was determined by finding the saxitoxin concentration where the CV (%) is 10%. LOQs for mussel, steamer and mahogany samples are 13, 17 and 16 µg/100 g, respectively. LOD was determined by dividing the LOQ by 3.3. LODs for mussel, steamer and mahogany samples are 3.9, 5.2 and 4.9 µg/100 g, respectively (Table 19 – 21).

ELISA and MBA methods were compared for the determination of PSP from naturally contaminated blue mussels (Table 22 – 23). All the suspicious results from ELISA (between 30 µg/100 g and 100 µg/100 g) agreed with the results from MBA (Detectable but below limit, < 80 µg/100 g). Therefore, these suspicious results from ELISA testing can be retested by MBA to confirm the PSP level. All the results either negative or positive from ELISA well matched with the results from MBA except one negative result from ELISA (Ogunquit R.). However, the result from MBA for the sample is quite below the legal limit (48 µg/100 g) as well. Therefore, all the naturally contaminated samples were correctly screened by the ELISA indicating this ELISA method can be used as a screening tool for PSP analysis in the laboratory.

Summary

Rapid screening method for saxitoxin (PSP) in blue mussels, steamers and mahogany clams was developed and evaluated. This method can be used in the private, certified or state laboratories for the determination of PSP toxins in order to screen out negative samples (below 30 µg/100g of saxitoxin equivalent) prior to mouse bioassay (MBA) as a confirmation method. Suspicious samples with PSP levels between 30 µg/100g and 100 µg/100 g will need confirmation with MBA. However, PSP levels higher than 100 µg/100g would be considered as positive, and may not need further confirmation. Therefore, this screening method will dramatically reduce the volume of samples to be confirmed with MBA, and save time and resources for the laboratories.

CALCULATE RESULTS

1. Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbance of the calibrator wells. Samples containing less color than a calibrator will have a concentration of Saxitoxin greater than the concentration of the calibrator. Samples containing more color than a calibrator well have a concentration less than the concentration of the calibrator.
2. Quantitative interpretation requires graphing the absorbances of the calibrators (Y axis) versus the log of the calibrator concentration (X axis) on semi-log graph paper. A straight line is drawn through the calibrator points and the sample absorbances are located on the line. The corresponding point on the X axis is the concentration of the sample Alternatively, Beacon can supply a spreadsheet template which can be used for data reduction. Please contact Beacon for further details.

SAMPLE CALCULATIONS

Well Contents	OD	Average OD ± SD*	%RSD	%Bo**	STX conc. (ppb)
Negative Control	1.438 1.461	1.449 ± 0.016	1.1	100	N/A
0.01 ppb Calibrator	1.312 1.330	1.321 ± 0.012	0.9	91.2	N/A
0.08 ppb Calibrator	0.669 0.653	0.661± 0.011	1.7	45.6	N/A
0.32 ppb Calibrator	0.217 0.216	0.217 ± 0.001	0.4	15.0	N/A
Sample	0.491 0.511	0.501 ± 0.014	2.8	34.6	0.119

Actual values may vary; this data is for example purposes only.

* standard deviation

** %Bo equals average sample absorbance divided by average negative control absorbance times 100%.

TECHNICAL ASSISTANCE

For questions regarding this kit or for additional information about Beacon products, call (207) 571-4302.

SAFETY

To receive complete safety information on this product, contact Beacon Analytical Systems, Inc. and request Material Safety Data Sheets. Stop Solution is 1N hydrochloric acid. Handle with care.

General Limited Warranty

Beacon Analytical Systems, Inc. ("Beacon") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond a product's printed expiration date. BEACON MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The warranty provided herein and the data, specifications and descriptions of Beacon products appearing in published catalogues and product literature may not be altered except by express written agreement signed by an officer of Beacon. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and, if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Beacon's sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Beacon promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as Beacon is willing and able to repair or replace any nonconforming Beacon product or part. Beacon shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by a customer from the use of its products. However, in some states the purchaser may have rights under state law in addition to those provided by this warranty.

BEACON ANALYTICAL SYSTEMS, INC.
82 Industrial Park Road
Saco, ME 04072
Tel. (207) 571-4302
Fax (207) 602-6502
www.beaconkits.com

Beacon Analytical Systems Inc.

Saxitoxin (PSP) Microtiter Plate Test Kit

Instructional Booklet

READ COMPLETELY BEFORE USE.

INTENDED USE

The Beacon Saxitoxin Plate Kit is a competitive ELISA for the quantitative analysis of Saxitoxin in contaminated samples

USE PRINCIPLES

The Beacon Saxitoxin (PSP) Microtiter Plate Test Kit is a competitive enzyme-labeled immunoassay. The Saxitoxin HRP conjugate, sample extract and calibrators are pipetted into the test wells followed by Saxitoxin antibody into the test wells to initiate the reaction. During the 30 minute incubation period, PSP toxins from the sample and Saxitoxin HRP conjugate compete for binding to Saxitoxin antibody. The Saxitoxin antibody is captured on the walls of the test well. Following this 30 minute incubation, the contents of the well are removed and the wells are washed to remove any unbound PSP toxins, Saxitoxin HRP conjugate and free Saxitoxin antibody. After wash, a clear substrate is then added to the wells and any bound enzyme conjugate causes the conversion to a blue color. Following a 30 minute incubation, the reaction is stopped and the amount of color in each well is read. The color of the unknown samples is compared to the color of the calibrators and the Saxitoxin concentration of the samples is derived.

MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2 – 8ºC.

1 plate containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.

1 vial of Negative control (Zero ppb Saxitoxin)

3 vials each containing 2 mL of Saxitoxin calibrators corresponding to 0.01, 0.08 and 0.32 µg/L (ppb) of Saxitoxin.

1 vial containing 7 mL Saxitoxin HRP Enzyme Conjugate.

1 vial containing 7 mL of Polyclonal anti-Saxitoxin antibody.

1 bottle containing 50 mL 10X Wash solution concentrate.

1 vial containing 14 mL of Substrate.

1 vial containing 14 mL of Stop Solution. (Caution! 1N HCl. Handle with care.)

1 Instructional Booklet

MATERIALS REQUIRED BUT NOT PROVIDED

- Laboratory quality distilled or deionized water.
- 20 mM PBS (phosphate buffered saline).
- Pipet with disposable tips capable of dispensing 50 µL.
- Multi-channel pipet; 8-channel capable of dispensing 50 and 100 µL.
- Paper towels or equivalent absorbent material.
- Microwell plate or strip reader with 450nm filter.
- Timer
- Wash bottle

PERFORMANCE CHARACTERISTICS

SPECIFICITY

The following table shows the % cross reactivity of Saxitoxin.

Compound	% CR
Saxitoxin dihydrochloride	**100.00 %**
Neosaxitoxin	**2.28 %**
Decarbamoyl STX	**42.30 %**
GTX 2& 3	**50.00 %**
GTX 1 & 4	**0.48 %**
Decarbamoyl GTX 2& 3	**2.36 %**
Decarbamoyl NeoSTX	**1.50 %**
B1	**16.95 %**
C1 and 2	**12.10 %**

PRECAUTIONS

- Store all kit components at 4°C to 8°C (39°F to 46°F) when not in use.
- Each reagent is optimized for use in the Beacon Saxitoxin (PSP) Microtiter Plate Test Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Beacon Saxitoxin Plate Kits with different Lot numbers.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Do not use reagents after expiration date.
- Reagents should be brought to room temperature, 20 – 28ºC (62 – 82ºF) prior to use. Avoid prolonged (> 24 hours) storage at room temperature.
- Saxitoxin is a toxin and should be treated with care.
- The Stop Solution is 1N hydrochloric acid. Avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.
- Transfer of samples and reagents by pipette requires constant monitoring of technique. Pipetting errors are the major source of error in immunoassay methodology.

EXTRACTION SOLUTION AND DILUTION BUFFER

1. Prepare 70% Isopropanol by mixing 70 mL of Isopropanol with 30 mL of lab grade water.
2. Prepare 5% Acetic acid solution by mixing 5 mL of Acetic acid with 95 mL of lab grade water.
3. Mix 2 parts (100 mL) of 70% Isopropanol with 1 part (50 mL) of 5% Acetic acid. Use for sample extraction.
4. For sample dilution buffer, mix 10 mL of Methanol with 90 mL of 20 mM PBS buffer.

SAMPLE PREPARATION (MUSSELS)

1. Shellfish are shucked and rinsed with lab grade water.
2. Transfer 12 mussels to a sieve and gently shake the sieve to drain the excess liquid.
3. Put the drained tissue into a kitchen blender jar and homogenize to a soupy texture.
4. Tare a 50 mL conical tube and weigh 5 grams of homogenized tissue.
5. Add 10 mL of extraction solvent and vortex for 3 minutes.
6. Transfer approximately 1 mL of the extract into a microcentrifuge tube, and centrifuge for 5 minutes at 12,000 rpm.
7. Dilute the supernatant with 10% MeOH/20 mM PBS to 5000 folds before running in assay.

   (e.g. 0.1 mL of the supernatant + 9.9 mL of buffer, and 0.1 mL of the diluted supernatant + 4.9 mL of buffrer)

ASSAY PROCEDURE

(Note: Running calibrators and samples in duplicate will improve assay precision and accuracy.)

1. Prepare the 1X wash solution by adding the contents of the 10X wash concentrate bottle to 450 mL Lab grade water in a wash bottle.
2. Allow reagents and sample extracts to reach room temperature prior to running the test.
3. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
4. Using a pipet with disposable tips, add **50 µL enzyme conjugate** to the appropriate test wells. Be sure to use a clean pipet tip for each. Add **50 µL of Calibrators or Sample** extract to each well.
5. Dispense **50 µL of Antibody Solution** into each test well.
6. Shake the plate gently for 30 seconds and incubate the test wells for **30 minutes**.
7. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.
8. Following the last wash tap the inverted wells onto absorbent paper to remove the last of the wash solution.
9. Dispense **100 µL of Substrate** into each well.
10. Incubate the wells for **30 minutes**.
11. Dispense **100 µL of Stop Solution** into each test well.
12. Read and record the absorbance of the wells at 450nm using a strip or plate reader.

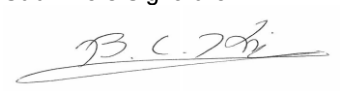
ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**Saxitoxin (PSP) Microtiter Plate Test Kit**
Name of the Method Developer	**Titan Fan and Byungchul Kim**
Developer Contact Information	**Beacon Analytical Systems, Inc.** **82 Industrial Park Road** **Saco, ME 04072** **Phone: 207-571-4302** **Fax: 207-602-6502** **titan@beaconkits.com** **bkim@beaconkits.com**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Rapid and inexpensive saxitoxin (PSP) screening analysis
2. What is the intended purpose of the method?	Y	For monitoring saxitoxin levels (PSP) from shellfish (mussels, clams, steamers) as an Approved Limited Use Method.
3. Is there an acknowledged need for this method in the NSSP?	Y	Rapid screening tool prior to confirmation analysis with regulatory method
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Enzyme-Linked Immunosorbent Assay (ELISA)
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Saxitoxin (PSP) Microtiter Plate Test Kit
Method Scope	Y	For the analysis of PSP toxins in blue mussels, steamers and mahogany clams from North Atlantic Ocean as an Approved Limited Use Method.
References	Y	See Reference 1 (Page 673). See Reference 2 (Page1133). See Reference 3 (Page1649). See Reference 4 (Page 33). See Reference 5 (Page 13). Reference 1: B. J. Yakes, S. M. Prezioso, S. L. DeGrasse. (2012) Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies. Talanta, 99, 668-676.

		Reference 2: G. B. Inami, C. Crandall, D. Csuti, M. Oshiro, R. Brenden. (2004) Presence/Absence Screening for Saxitoxin in Frozen Acidified Mussel and Oyster Extracts from the Coast of California with In Vitro Methods, J AOAC. Int. 87 (5), 1133-1142. Reference 3: E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer. (2001) Immunoassay Methods for Paralytic Shellfish Poisoning Toxins. J. AOAC. Int. 84 (5), 1649-1656. Reference 4: Anderson, D.M., P. Andersen, V. M. Bricelj, J. J. Cullen, and J. EE. Rensel, 2001. Monitoring and management strategies for harmful algal blooms in coastal waters, APEC #201-MR-01.1, Asia Pacific Economic Program and Intergovernmental Oceanographic Commission Technical Series No. 59, Paris. Reference 5: F. S. Chu, T. S. L. Fan. (1985) Indirect Enzyme-Linked Immunosorbent Assay for Saxitoxin in Shellfish. J. AOAC. 68 (1), 13-16.
Principle	Y	Competitive ELISA
Any Proprietary Aspects	Y	Antibody and STX-HRP conjugate
Equipment Required	Y	Listed in the protocol (Page 3)
Reagents Required	Y	Listed in the protocol (Page 3)
Sample Collection, Preservation and Storage Requirements	Y	Described in the protocol (Page 3)
Safety Requirements	Y	Personal safety items such as safety glasses, gloves and lab coat must be required. Calibrators and enzyme conjugate are toxic materials, and must be kept in the original vials when they are not used. Since stop solution is a strong acidic solution (1 N hydrochloric acid), skin or eye contact must be avoided. Lab items with contact of toxins (sample extract and calibrator) such as pipette tips and lab wares must be soaked in 50% of house bleach solution at least 1 hour before washing or disposal.
Clear and Easy to Follow Step-by-Step Procedure	Y	Described in the protocol (Page 4)
Quality Control Steps Specific for this Method	Y	Described in the protocol (Page 5)
C. Validation Criteria		
1. Accuracy / Trueness	Y	Described in the protocol (Page 5, Table 1 - 3)
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	The precision of the method is consistent for the three sample matrices. Each sample was tested with three different levels of saxitoxin. See the details on Page 23 and Table 5 - 10.
4. Recovery	Y	Described in the protocol (Table 11 - 17)
5. Specificity	Y	Described in the protocol (Page 6, Table 18)
6. Working and Linear Ranges	Y	Described in the protocol (Page 6, Figure 1)
7. Limit of Detection	Y	Described in the protocol (Page 6, Table 19 - 21)
8. Limit of Quantitation / Sensitivity	Y	Described in the protocol (page 6, Table 19 - 21)
9. Ruggedness	Y	Described in the protocol (Page 5, Table 4)
10. Matrix Effects	Y	There is no matrix effect observed due to the high degree of dilution (15000) of sample before the assay. The potential matrix effect from any shellfish species

		could be eliminated by this high dilution.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		
D. Other Information		
1. Cost of the Method	Y	$325 per kit to test up to 44 samples (≈$7 per sample)
2. Special Technical Skills Required to Perform the Method	Y	Some ELISA experience or basic training required
3. Special Equipment Required and Associated Cost	Y	Described in the protocol (Page 3)
4. Abbreviations and Acronyms Defined	Y	Described in the protocol (Page 4)
5. Details of Turn Around Times (time involved to complete the method)	Y	One person may test 50 – 100 samples in a day including sample preparation time (shucking, grinding and extracting). Single assay can be done in 90 minutes testing up to 44 samples with duplicate.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The manufacture of the kit was conducted in compliance with GMP (Good Manufacturing Practices) regulations.
Submitters Signature 	Date: June 26, 2013	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Talanta 99 (2012) 668–676

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Developing improved immunoassays for paralytic shellfish toxins: The need for multiple, superior antibodies

Betsy Jean Yakes [a,*], Samantha M. Prezioso [b,1], Stacey L. DeGrasse [a]

[a] U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5100 Paint Branch Parkway, College Park, MD 20740, USA
[b] Joint Institute for Food Safety and Applied Nutrition (JIFSAN), University of Maryland, College Park, MD 20742, USA

ARTICLE INFO

Article history:
Received 17 April 2012
Accepted 25 June 2012
Available online 25 July 2012

Keywords:
Surface plasmon resonance
Paralytic shellfish poisoning
Saxitoxin
Lateral flow immuno-chromatography
Immunoassay
Cross-reactivity

ABSTRACT

Paralytic shellfish toxins (PSTs) are a risk to humans upon consumption of contaminated seafood. The PST family is comprised of more than twenty congeners, with each form having a different potency. In order to adequately protect consumers yet reduce unnecessary closures of non-contaminated harvesting areas, a rapid method that allows for analysis of sample toxicity is needed. While a number of PST immunoassays exist, the outstanding challenge is linking quantitative response to sample toxicity, as no single antibody reacts to the PST congeners in a manner that correlates with potency. A novel approach, then, is to combine multiple antibodies of varying reactivity to create a screening assay. This research details our investigation of three currently available antibodies for their reactivity profiles determined using a surface plasmon resonance biosensor assay. While our study shows challenges with detection of the R1-hydroxylated PSTs, results indicate that using multiple antibodies may provide more confidence in determining overall toxicity and the toxin profile. A multiplexed approach would not only improve biosensor assays but could also be applied to lateral flow immuno-chromatographic platforms, and such a theoretical device incorporating the three antibodies is presented. These improved assays could reduce the number of animal bioassays and confirmatory analyses (e.g., LC/MS), thereby improving food safety and economic use of shellfish resources.

Published by Elsevier B.V.

1. Introduction

Paralytic shellfish poisoning (PSP) is caused by a suite of toxins, known collectively as paralytic shellfish toxins (PSTs) [1]. Saxitoxin (STX) and its congeners originate from certain dinoflagellates and some cyanobacteria [2]. Filter feeding bivalves (e.g., mussels, clams, cockles, scallops and oysters), as well as other seafood species, can accumulate and metabolize these toxins which can then lead to potentially dangerous seafood [3,4]. Human consumption of toxic seafood can result in tingling, numbness, respiratory paralysis and potentially death [5], as the PSTs bind to site 1 and block the opening of voltage gated sodium channels [6]. These small molecule toxins are also quite robust, and typical preventative food safety measures (i.e., use of heat or acid during cooking) do not destroy the PSTs [1].

Proper monitoring and implementation of harvesting bans when toxin concentrations exceed safe levels (typically 80 μg STX equivalents per 100 g tissue) have minimized PSP illnesses [1]. However, outbreaks still occur, especially in developing countries [7] and with an estimated worldwide mortality of 6% [8]. For example, a major PSP epidemic occurred in Guatemala in 1987 that claimed the lives of 26 people out of the 187 affected [7,9]. A review of PSP cases and outbreaks has been compiled by FAO, which reports PSP prevalence along coastal European nations, parts of Africa, the West Coast and Northeast region of North America, South America, and parts of Asia [10]. Within the US, the majority of illnesses and outbreaks are reported from recreational harvests among fishermen and tribal communities. For example, during May and June of 2011, 21 cases of PSP illness were reported in Southeast Alaska due to unprecedented high levels of PSTs in surrounding waters [11].

Recent reviews on PST detection have focused on improved analysis of both coastal waters and seafood [1,2,12,13]. The mouse bioassay (MBA) is one of the AOAC approved and most commonly used testing methods for PSTs [14]. While simple, this bioassay suffers performance related challenges (e.g., poor quantitation and low dynamic range, interferences to detection, low sample throughput, and lack of determination of the specific toxin associated with death) as well as ethical concerns.

A second AOAC approved method for determining PSTs is high performance liquid chromatography (HPLC) with fluorescence detection (FD) [15,16]. This method is quite effective at identifying and quantifying the toxins in a seafood sample. However, it requires a lengthy sample clean-up and pre-column oxidation procedure to

* Corresponding author. Tel.: +1 240 402 2720, fax: +1 301 436 2624.
E-mail address: betsy.yakes@fda.hhs.gov (B.J. Yakes).
[1] Present address: Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333, USA.

0039-9140/$ - see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.talanta.2012.06.073

create fluorescent derivatives of the toxins for detection as well as multiple analytical runs for complete PST determination. The post-column HPLC-FD method created by Oshima [17] was refined [18] and is also now AOAC approved [19]. This post-column oxidation method has a simpler sample preparation procedure than pre-column HPLC-FD; however, multiple analytical runs under different chromatographic conditions must be conducted in order to analyze all potential PST congeners. Furthermore, both HPLC-FD approaches can be hindered by sample materials that have native fluorescence, requiring additional steps to ensure the presence of toxins [20].

Other analytical techniques that are advancing include liquid chromatography (LC) coupled with mass spectrometry (MS) [21,22], some in tandem with biosensors [23]. The major limitation of this analytical approach is matrix interference and ionization suppression, which restricts its ability to serve as a reliable, quantitative monitoring tool. Limited availability of internal reference standards (e.g., isotopically labeled toxins) currently hinders wider-spread implementation of monitoring by LC/MS.

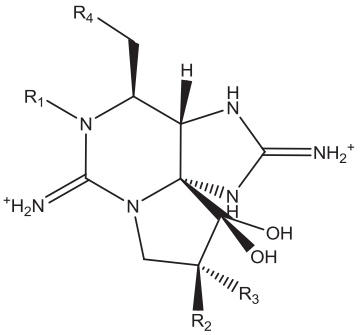
In order to overcome the challenges associated with MBA and LC methods, rapid screening techniques have been explored. These methods can be simple, cost-effective, sensitive, and accurate for high-throughput detection needs. Such methods include receptor binding assays (RBA) [24–27], lateral flow immuno-chromatography [28,29], enzyme-linked immunosorbent assays (ELISA) [30–32], and cell bioassays [33,34]. While these methods allow for high throughput and ease of use, they suffer from the use of difficult to procure radiolabeled materials for RBA, high probability of false-positive and potential for false-negative results with current immuno-chromatographic PSP tests, large amounts of manual labor and limited antibody cross-reactivity for ELISA, and nonspecific toxin recognition for the cell bioassays.

An immunological technique that has been shown to provide high throughput detection of PSTs is surface plasmon resonance (SPR) biosensors [35–38], though this method faces the same challenges with respect to antibody reactivity. SPR immunoassays are based on specific biosensor platforms that bind the molecule of interest at the surface. The change in mass due to binding is detected as a change in refractive index (RI) at the dielectric interface (i.e., gold immunoassay substrate and solution in the flow cell). This RI change causes a shift in the SPR band position that can be tracked in real-time using standard spectroscopy optics [39]. This automated technique allows for real-time analysis of PST-containing samples, requires minimal sample cleanup, no labeling of the analytes, and yields sub-ppb limits of detection in less than ten min [40,41].

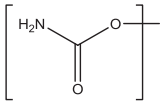
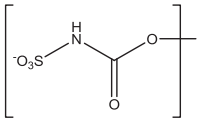
The SPR assay for the determination of PSTs currently implemented in our laboratory is robust and shows good repeatability and reproducibility; however, quantitative results do not always correlate with overall sample toxicity due to the many PST congeners having widely varying potency. The toxicities for common PSTs are shown in Table 1, and the inability to correlate results with sample toxicity when using immunological assays could lead to unsafe seafood harvested for consumers (false-negative) or destruction of safe seafood and closure of non-contaminated harvesting areas (false-positive). Clearly, there is a need for improved assays to not only protect the public but also to improve the economic viability of the industry and utilization of seafood resources. Unfortunately, a single antibody that reacts to the congeners with respect to their potency has yet to be produced. An advantage to the SPR assay is that while the response may not always correlate with toxicity, the cross-reactivity of individual congeners with an antibody can be calculated. A novel approach, then, would be to combine multiple antibodies of varying reactivity to the congeners, as screened via the SPR assay, to create a multiplexed immunoassay.

One disadvantage to SPR biosensors is the size of instrumentation and cost of materials which could prohibit routine testing in the field or dockside. Lateral flow immuno-chromatographic tests (LFIs) have been used for PST testing and could fulfill the requirements of an easy-to-use and cost-effective technique for monitoring potential toxicity of seafood when the quantitation and automation of the SPR instrumentation is not necessary.

Table 1
PST structure, congener forms, and relative toxicities [17]. Toxins used in this study are listed in bold.



Toxin	Relative Toxicity
STX	1.00
dcSTX	0.51
GTX2,3	0.36, 0.64
B1 (GTX5)	0.06
C1,2	0.01, 0.10
dcGTX2,3	0.15, 0.38
NEO	0.92
dcNEO	–
GTX1,4	0.99, 0.73

R1	R2	R3	Carbamate	Decarbamoyl	*N*-sulfocarbamoyl
H	H	H	**STX**	**dcSTX**	**B1 (GTX 5)**
OH	H	H	**NEO**	**dcNEO**	B2 (GTX 6)
OH	H	OSO_3^-	**GTX1**	dcGTX1	C3
H	H	OSO_3^-	**GTX2**	**dcGTX2**	**C1**
H	OSO_3^-	H	**GTX3**	**dcGTX3**	**C2**
OH	OSO_3^-	H	**GTX4**	dcGTX4	C4
		R4:			

The challenge with these rapid tests also lies in the inability to accurately measure sample toxicity, and the performance of such devices has been extensively studied [28,29,42]. To potentially enhance the reliability of the LFIs, multiple antibodies that have distinct reactivity patterns as determined in the SPR assay could be employed.

This manuscript details our work that evaluated three antibodies for their reactivity to nine commonly occurring PSTs. The data indicate that a multiplexed approach may not only improve SPR biosensor assays but could also be incorporated into LFI platforms for more reliable, rapid, inexpensive screening options. Such approaches could then allow for more successful assessment of overall sample toxicity and better use of confirmatory (e.g., LC-MS or MBA) techniques. The research introduced herein sets the stage for these multi-antibody devices and discusses the potential challenges when using the antibodies profiled in this study.

2. Materials and methods

2.1. Reagents

Saxitoxin (STX) dihydrochloride used in this research is the FDA reference standard, now available from the National Institute of Standards and Technology (81.0 μg/mL free base in 20% ethanol/80% water). *N*-sulfocarbamoyl-gonyautoxin-2 and -3 (C1,2, 70.8 μg/mL), decarbamoylgonyautoxin-2 and -3 (dcGTX2,3, 51.4 μg/mL), decarbamoylneosaxitoxin dihydrochloride (dcNEO, 7.93 μg/mL free base), decarbamoylsaxitoxin dihydrochloride (dcSTX, 16.0 μg/mL free base), gonyautoxin-1 and -4 (GTX1,4, 58.0 μg/mL), gonyautoxin-2 and -3 (GTX2,3, 62.1 μg/mL), gonyautoxin-5 (B1, 24.7 μg/mL), and neosaxitoxin dihydrochloride (NEO, 20.6 μg/mL free base) were purchased from NRC Certified Reference Materials Program, Institute for Marine Biosciences (Halifax, Nova Scotia, Canada).

Standard laboratory reagents were procured from Sigma-Aldrich (St. Louis, MO), Pharmaco AAPER (Shelbyville, KY), and J.T. Baker (Phillipsburg, NJ). Millipore Milli-Q 18.2 MΩ cm water (Billerica, MA) was used to prepare buffers. Sensor chips, amine coupling kit (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine), and buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) surfactant P20 buffer (HBS-EP+)) were obtained from GE Healthcare, Biacore (Piscataway, NJ).

2.2. Clam matrix

Control clam extract was prepared following a standard shellfish extraction procedure for PSTs (see [15] for procedure). A total of 100 g of clam was homogenized, from which a 5.0 g ($\pm$0.1 g) aliquot was thoroughly mixed with 3.0 mL of 1% acetic acid in water. This was heated at 95 °C for 5 min. The sample was then removed and placed on ice until cool enough to handle. The sample was vortexed and then centrifuged at 3600*g* for 10 min. The supernatant was collected into a 15 mL glass centrifuge tube. Another 3.0 mL, 1% acetic acid aliquot was added to the homogenate; the solution was vortexed and centrifuged at 3600*g* for 10 min. Following collection of the supernatant in the same 15 mL centrifuge tube, the extract volume was brought up to 10 mL with 0.1 N HCl with a final pH of 4.0. The clam extract was then filtered through a Supelco Supelclean LC-18, 3 mL solid phase extraction (SPE) cartridge (Sigma-Aldrich). The cartridge was conditioned with 6 mL methanol followed by 6 mL of water. Clam extract (1 mL) was added to the cartridge followed by 2 mL of water, and the cartridge was run dry. This extraction procedure produced 5 g of clam tissue per 40 mL and at the action level (i.e., 80 μg STX equivalents per 100 g tissue), equates to 100 ng STX eq./mL in the SPR biosensor assay. Standards were prepared by spiking the control clam matrix with the PSTs followed by serial dilution of these stock concentrations using the control clam matrix. Blank solutions containing no PSTs (0 ng/mL) were performed with control clam matrix for each PST calibration curve.

2.3. Mixed PST standards

Stock solutions of 90% STX with 10% NEO, 77% B1 with 23% STX, and 80% GTX1,4 with 20% STX were prepared and then serially diluted in clam matrix. The stock solutions were designed to have overall toxicity of 160 μg STX eq./100 g tissue for those standards containing NEO and GTX1,4, while the B1 standard had a stock concentration of 80 μg STX eq./100 g.

2.4. Antibodies

Two antibodies used in this research are commercially available in ELISA kits. The first antibody (Ab1) was used as received from the kit (Ridascreen Fast PSP SC, R-Biopharm AG, Darmstadt, Germany). Standard antibody dilutions were run on the STX chip; the 1:5 dilution in HBS-EP+ used throughout these studies had a response similar to that obtained from the 8 μg/mL burro anti-STX also used in this study as described below (see [41]).

The second antibody (polyclonal anti-STX, Ab2) was generously supplied in a purified form (ammonium sulfate precipitation followed by dialysis against 10 mM PBS, pH 7.3) from Beacon Analytical Systems (Saco, ME). The concentration that gave the same signal ($\sim$150 RU) as 8 μg/mL burro anti-STX was 25 μg/mL, and this concentration was used for all PST immunoassays.

The third antibody (polyclonal, protein G purified burro anti-STX, Ab3) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD). Antibody dilutions were run to determine the appropriate concentration for immunoassay, and 8 μg/mL was used for all studies herein.

2.5. Instrumentation

A Biacore T100 (GE Healthcare) surface plasmon resonance biosensor was used for all SPR immunoassays. The instrument was run via the Biacore T100 Controller Software v. 2.0, and data evaluation was performed with the Biacore T100 Evaluation Software v. 2.0. The instrument and sensor chips were normalized following the manufacturer procedures prior to performing the PST immunoassays. The SPR response (Resonance Unit, RU) is a measure of the angle of minimum reflected intensity that occurs upon changes in refractive index where 1 RU corresponds to a 10^{-6} change in refractive index ($\sim 10^{-4}$ degree angle shift).

2.6. Sensor chip

The STX biosensor surface was prepared on a Series S CM5 sensor chip and has been previously described [41]. Briefly, all flow cells were activated to succinimidyl esters using the instrument amine immobilization wizard and EDC/NHS from the amine coupling kit. Flow cell one was then deactivated with ethanolamine to create a reference surface, while flow cells two through four were activated with jeffamine, and unreacted sites were blocked with ethanolamine. The chip was removed from the instrument, and STX was conjugated to the chip surface via 15 h, 37 °C reaction with formaldehyde in 100 mM phosphate buffer. The chip was then rinsed with water, dried with $N_{2(g)}$, and docked into the SPR instrument. The fluidics and sensor chip were primed with HBS-EP+, and three startup cycles with 50 mM NaOH were performed prior to running the standards.

2.7. Immunoassay

Immunoassays were performed using the Biacore T100 optical biosensor with HBS-EP+ as the running buffer. The sample compartment temperature was set at 10 °C while the analysis temperature was held constant at 25 °C. The antibodies were diluted as noted above and then mixed in the instrument autosampler (90% Ab to 10% standard, 600 s mix time) prior to injection. This mixture was injected over the STX sensor chip at a flow rate of 20 μL/min for 120 s followed by a 60 s dissociation period and then a 240 s regeneration with 50 mM NaOH. STX controls (0.3, 3, 30, and 300 ng/mL in HBS-EP+ with 8 μg/mL Ab3) were run with every cycle to ensure chip stability.

2.8. Data processing

The response (RU_{PST}) for each sample was obtained by subtracting the baseline (10 s prior to injection) from the stability point (15 s after sample injection completion). These values were then normalized to the blank (0 ng/mL PST for associated antibody, RU_0), and the results multiplied by 100 to achieve percent binding for each antibody/PST combination: % binding = $(RU_{PST}/RU_0) \times 100$.

Each flow cell was normalized separately, and the data from flow cells 2, 3, and 4 were averaged together. Due to nonspecific binding and bulk effects for Ab1, the background from the blank measurement (0.45 normalized response) was subtracted from each data point for this antibody. The average response was then plotted versus the original solution concentration for each PST in ng/mL. Curve fitting was performed using a variable slope, four parameter model for log(inhibitor) vs. response in GraphPad Prism (v. 5.02, La Jolla, CA). GraphPad Prism was then used to calculate the values at inhibitory concentrations (IC): IC_{20} (80% binding), IC_{50} (50% binding), and IC_{80} (20% binding). Additionally, the response at 100 ng/mL for each PST based on the generated curves was determined. For concentrations where full inhibition was not seen (e.g., C1,2 with Ab3 where the highest standard gave 49.8% binding without flattening of the curve), the software extrapolates the curve fit to determine the 20% binding point.

Cross-reactivities (CR) for each PST were calculated from the IC_{50} values of each toxin with respect to the IC_{50} of STX: % CR = $(IC_{50,\ STX}/IC_{50,\ PST}) \times 100$. The 100 ng/mL level is defined as the response at the action level (RAL) and is equivalent to a sample containing only STX at 80 μg/100 g tissue based on extraction dilution.

For the theoretical lateral flow immuno-chromatography, the reactivity for each antibody-PST congener was translated to a band in the device. For Fig. 3, the RALs (100 ng/mL from Table 3) were used, while in Fig. 4 the response at 80 μg STX eq./100 g tissue were translated to band patterns. To account for differing sensor chips and nonspecific binding in Fig. 3, the values were corrected for each antibody by subtracting the background (Ab1 = 12.45, Ab2 = 8.60, Ab3 = 3.43) from each response. Using PowerPoint, the color of the band was defined by the RGB parameters 128 red, 0 green, 0 blue with the transparency of the band equal to (100—Response).

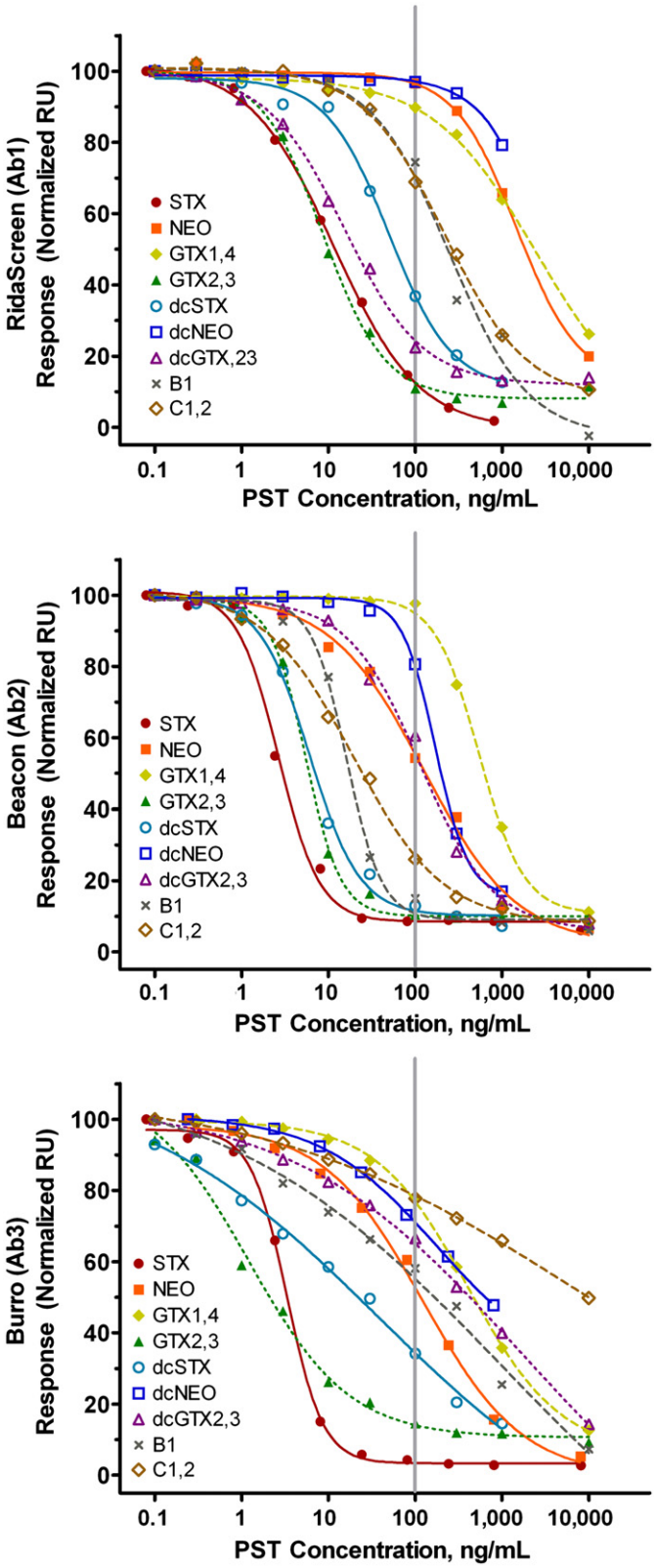


Fig. 1. Antibody reactivity with nine common PSTs. $N = 3$ with error bars smaller than the data points. The vertical grey line at 100 ng/mL represents the action level for a sample containing only STX (80 μg STX/100 g tissue).

3. Results and discussion

3.1. Cross-reactivity (CR) of antibodies

Previous research has focused on developing rapid, sensitive SPR assays that are capable of detecting PSTs in buffer [41] and common shellfish matrices [36]. The challenge to correctly estimating sample potency lies in the current inability of antibodies to react with the congeners according to their toxicities (Table 1). The PSTs can be classified into three major categories with their toxicities generally following the R4 substituent groupings: carbamate > decarbamoyl > *N*-sulfocarbamoyl. It has been shown that

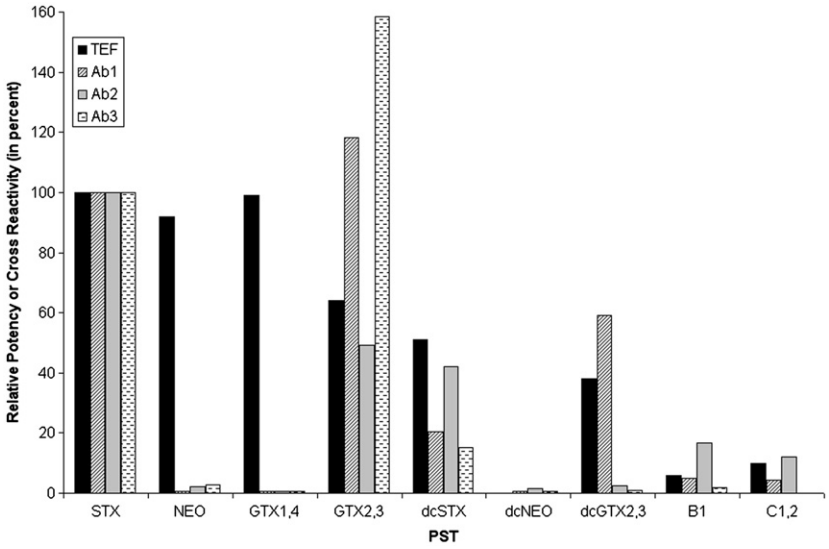


Fig. 2. Cross-reactivity for each antibody (from Table 3) in comparison to the toxin equivalency factors (from [17]) for each PST. For PST congeners where epimers are reported in pairs (e.g., GTX1,4) the value of the epimer with the higher TEF is used. No TEF value for dcNEO is reported by Oshima.

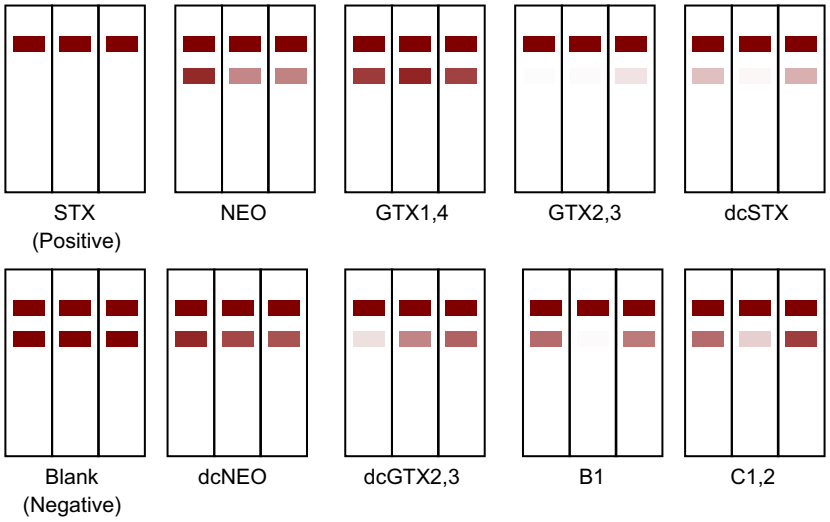


Fig. 3. Conceptual lateral flow immunochromatographic devices for samples containing a single PST at 100 ng/mL. Each strip corresponds to an individual antibody (left to right: Ab1, Ab2, Ab3) in which a competition assay is performed with the analyte.

Table 2
IC_{50} and dynamic range (DR = IC_{20} to IC_{80}) for each antibody. All values in ng/mL.

PST	Ab1: RidaScreen		Ab2: Beacon		Ab3: Burro	
	IC_{50}	DR	IC_{50}	DR	IC_{50}	DR
STX	11.9	2.7–54.3	3.0	1.4–7.7	3.4	1.6–6.9
NEO	1834.9	530.5–9986.0	131.7	23.1–793.1	116.0	17.5–726.4
GTX1,4	2346.7	324.9–16197.1	630.3	260.9–1955.7	470.3	79.2–3438.4
GTX2,3	10.1	3.1–41.4	6.0	3.0–14.3	2.1	0.4–24.6
dcSTX	58.6	16.2–290.7	7.1	2.6–24.7	22.0	0.8–442.4
dcNEO	2369.6	964.5–3724.2	199.3	100.5–593.0	642.5	43.2–39711.6
dcGTX2,3	20.1	4.2–165.9	126.9	30.7–606.1	426.6	15.9–5851.5
B1	236.4	58.1–920.7	17.7	8.8–40.8	167.4	5.5–2673.5
C1,2	266.4	56.1–1716.3	24.8	4.6–190.7	9983.5	75.1–2091542

different antibodies can have different cross-reactivities to the PST congeners [43]. To take advantage of this characteristic, the cross-reactivity of three available antibodies are examined herein using SPR biosensor evaluation to determine the feasibility and merits of using a multi-antibody approach for both SPR biosensors and LFIs.

Fig. 1 shows the cross-reactivity curves of the binding of each antibody with STX, NEO, GTX1,4, GTX2,3, dcSTX, dcNEO, dcGTX2,3, B1 and C1,2. These assays were designed to have nearly-complete inhibition with STX at the action level (indicated by a vertical grey line in the graphs). Qualitatively, it is clear that

Table 3
SPR-determined antibody cross-reactivities (CR in %), reported ELISA cross-reactivities, and responses at the action level (RAL in normalized RU, at 100 ng STX/mL).

PST	Ab1			Ab2			Ab3	
	CR	ELISA[a]	RAL	CR	ELISA[a]	RAL	CR	RAL
STX	100.0	100	12.5	100.0	100.0	8.6	100.0	3.4
NEO	0.7	12	96.5	2.3	0.8	55.4	2.9	52.8
GTX1,4	0.5		89.6	0.5	< 0.1	94.9	0.7	76.9
GTX2,3	118.3	70	12.5	49.4	12.0	10.2	158.5	14.1
dcSTX	20.4	20	37.0	42.0	18.0	11.5	15.2	34.4
dcNEO	0.5		97.1	1.5	0.7	80.2	0.5	71.1
dcGTX2,3	59.2		24.3	2.3	0.4	55.7	0.8	65.0
B1	5.0		70.1	16.8		10.8	2.0	55.3
C1,2	4.5		70.2	12.0		26.9	0.03	78.5

[a] Values for the ELISA cross-reactivities obtained from pamphlet information contained in the kits.

the antibodies have distinct reactivity with the nine PST congeners, as the calibration curve patterns for each antibody are dissimilar. This can be further seen in the individual congener plots (Supplementary materials A) where it is visually easy to compare the similarities (e.g., the three antibody curves for STX are similar with Ab2 and Ab3 responses nearly overlapping) and differences (e.g., the curve shapes for the three antibodies reacting with B1 are quite dissimilar). The data can also be quantitatively evaluated via IC_{50} and dynamic range (Table 2) as well as percent cross-reactivities and responses at the action level (RAL) (Table 3). When these results are considered together, it is clear that no antibody profile accurately reflects potency; however, each antibody has a distinct profile with advantages and disadvantages as discussed below.

For RidaScreen (Ab1), high reactivity (> 100% CR) with STX and GTX2,3 is seen while low reactivity (< 5% CR) with many congeners is observed. When used in an assay alone, Ab1 would be expected to yield false-negative results when the highly toxic NEO and GTX1,4 are present. Beacon (Ab2) has high reactivity with STX but low reactivity with NEO, GTX1,4, dcNEO, and dcGTX2,3. In this case, samples high in GTX1,4 and/or NEO may also result in false-negatives. Additionally, the moderate reactivity (~ 15% CR) with low potency B1 and C1,2 may result in false-positive results if these toxins dominated the profile. Finally, the Burro (Ab3) has high reactivity with STX and GTX2,3 and to a lesser extent with all congeners tested. In this scenario, a sample with a toxin profile dominated by GTX1,4, and/or NEO may be screened as negative when toxin levels may be above the action level, whereas a false-positive may occur if GTX2,3 dominated the sample.

When comparing the profiles based on substitution groups (R1 to R4 as shown in Table 1, Supplementary materials B1–B3), all antibodies show limited reactivity with OH modification at the R1 group potentially due to steric hindrance, charge, or hydrophilicity imparted by this group. Indeed, weak reactivity with the R1-hydroxylated PSTs is frequently found with antibodies [43]. Antibody cross-reactivity for the non-hydroxylated compounds is mainly driven by the R4 functionality with highest reactivity seen in the carbamate modified PSTs followed by decarbamoyl PST forms and *N*-sulfocarbamoyl conjugations.

The two antibodies used for this work that were obtained from commercial ELISA kits have been previously evaluated for cross-reactivities (Table 3). For Ab1, the published cross-reactivity order (STX > GTX2,3 > dcSTX > NEO) is similar to the SPR results (GTX2,3 > STX > dcGTX2,3 > dcSTX > B1 > C1,2 > NEO > GTX1,4 = dcNEO) except for the exchange of STX with GTX2,3. This discrepancy may not be significant due to the very similar reactivity of the antibody to these PSTs as seen in the SPR curves in Fig. 1. For Ab2, the published results are STX > dcSTX > GTX2,3 > NEO > dcNEO > dcGTX2,3 > GTX1,4; however, the SPR analysis showed STX > GTX2,3 > dcSTX > B1 > C1,2 > NEO = dcGTX2,3 > dcNEO > GTX1,4. The order differences of GTX2,3/dcSTX as well as NEO/dcNEO/dcGT2,3 could be expected due to the very similar curve shapes and, with the error in measurements, these values may not be significantly different.

One further and important consideration is how cross-reactivity corresponds to toxin-equivalency factors (TEFs) for each PST (Fig. 2). The values for STX are set at 100%, based on the definitions for TEF and CR, and therefore show no differences between potency and cross-reactivity with each antibody. Ideally, the best antibody profile would have CRs to each congener that match their TEFs. However, it is clear that antibodies perform poorly at matching the TEF values, especially for R1-hydroxylated toxins (e.g., NEO and GTX1,4) and with varied success for the non-hydroxylated congeners. For example, Ab2 cross-reactivities correspond reasonably well with the TEF for GTX2,3, dcSTX and C1,2, while Ab1 and Ab3 cross-reactivities correlate closely with the TEF for B1. These distinctions in reactivity between the antibodies could be exploited for use in a multiplexed format to create an assay that would yield more information regarding the toxin profile and thus more confidence in sample potency.

3.2. Theoretical lateral flow immuno-chromatography

In general, the format of the LFI used to detect PSTs is a competitive displacement assay. In this assay, sample extract is added to the sample well of the pad and is drawn up through the membrane. The toxin first interacts with the conjugate pad containing antitoxin-coated gold particles. The antibody and toxin interact and remaining antitoxin-coated gold particles bind to the test line coated with toxin conjugated protein. The higher the toxin concentration in solution the fainter the red line at the test line position. A control line is also present and should always yield a strong red response. In this way, two red bands indicate that a sample contains little to no toxin, whereas a single band at the control position indicates the sample contains toxin.

By using the antibodies screened via SPR, combinations of antibodies that create a unique pattern could be incorporated into an LFI for higher confidence in sample toxicity. Prior to undertaking extensive studies in incorporating multiple antibodies into an LFI, a theoretical model was designed and is shown in Fig. 3 with each strip in the three-strip system containing a single antibody-gold colloid. In envisioning the LFI functionality, only single toxin solutions at 100 ng/mL (e.g., equivalent to the action level for a toxin containing only STX, per extraction procedure used) are applied to each three-strip system. As can be seen in the STX LFI and Blank LFI, a positive sample would have only the three control bands while a negative result would show control bands as well as three strong bands at the test line position below each control band.

The theoretical LFIs for the other PST congeners show that, indeed, more confidence in sample toxicity could be gained by using multiple antibodies. For example, the pattern for B1 (which has low toxicity) with only Ab2 indicates an unsafe sample (a false-positive result), but when used in combination with the information from the Ab1 and Ab3 strips the pattern and strength of the bands could allow a user to realize the sample is safe for harvest and consumption. Unfortunately, false-negative results were still not eliminated. This is demonstrated in the NEO and GTX1,4 conceptual tests in which these two LFIs demonstrate very little discernible difference from the blank (negative), yet have concentrations equivalent to toxicity near the action level.

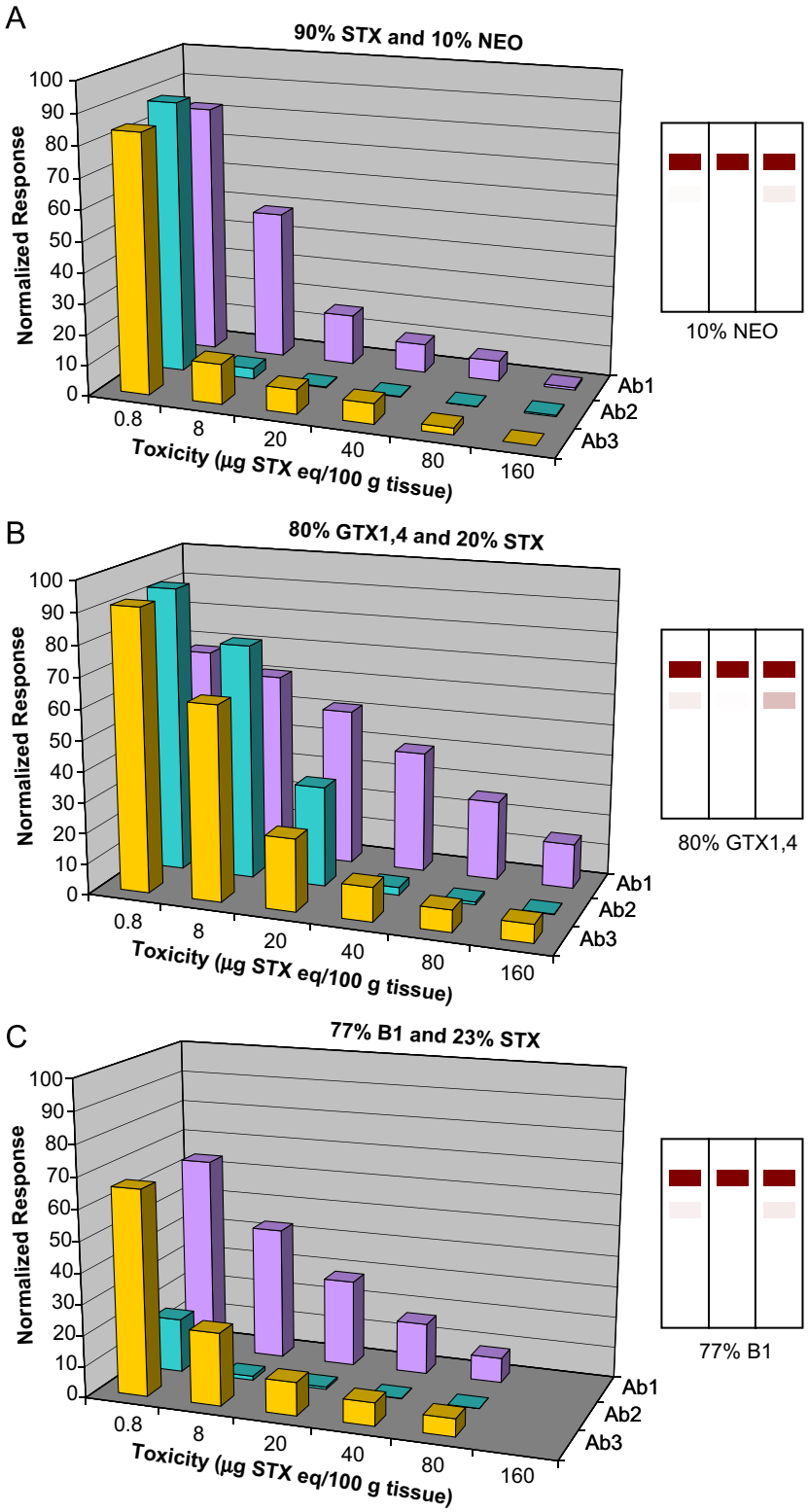


Fig. 4. Reactivity of antibodies to PST mixtures using the SPR biosensor and corresponding theoretical LFI device for mixtures containing 80 µg STX eq. per 100 g tissue for (A) 90% STX and 10% NEO, (B) 80% GTX1,4 and 20% STX, and (C) 77% B1 and 23% STX.

3.3. *PST mixtures*

PST congeners commonly exist as mixtures in naturally contaminated shellfish which could complicate such a simplistic "pattern matching" approach of the LFIs. To investigate this, experiments were performed with the antibodies and mixtures of PST compounds. Differing toxicity PST mixtures were tested to determine if the antibodies would perform well in situations that challenged high and low cross-reactors that do not correlate with toxicity (i.e., the potential for false-positive or false-negative results, respectively). The selected mixtures represented examples encountered in natural waters: 90% STX with 10% NEO

(e.g., *Alexandrium* sp. and shellfish in Alaska [anticipated accurate test performance]), 80% GTX1,4 with 20% STX (e.g., shellfish in Scotland [anticipated potential false-negative]), and 77% B1 with 23% STX (e.g., *Pyrodinium* sp. in Florida [anticipated potential false-positive]). Results from these SPR assays, and corresponding theoretical LFIs, are shown in Fig. 4 where the toxicity at 80 μg STX eq. per 100 g tissue is the action level in the United States.

For all mixtures, the antibodies respond well for samples in which there is high toxicity, with all SPR biosensor data showing the desired inhibition at the 80 μg STX eq./100 g tissue action level. If these were naturally contaminated samples, the SPR screening technique would indicate that further, confirmatory testing is required. With the mixture of 90% STX with 10% NEO, the curves nearly follow that predicted from a sample of STX alone, thus yielding a representative, accurate test. From this, an arbitrary cut-off level of all antibodies having an SPR response below 50 could be set for the level at which further confirmatory testing would be required. For the 90% STX with 10% NEO, this level corresponds to samples of approx. 8 μg STX eq./100 g tissue and would allow for adequate screening without over burdening confirmatory testing techniques. As expected, the potential false-negative system (80% GTX1,4 with 20% STX) would only have further testing indicated for samples containing more than 20 μg STX eq./100 g tissue. While this is below the action level, any potential systematic errors could lead to a false-negative result. Finally, for the potential false-positive mixture of 77% B1 with 23% STX, samples very low in toxicity (<8 μg STX eq./100 g tissue) would be indicated for further testing. In this case, false-positive results could still be common.

With regard to the theoretical LFI devices for these mixtures, all conceptual LFIs with the three antibody system show patterns which indicate further testing would be required based on the faint-red response of the test lines for all three antibodies. In the case of the 80% GTX1,4 with 20% STX, the red line from Ab3 is clearly visible and a faint line from Ab1 can be seen. If a pattern matching approach was used, the pattern for this toxic sample could mimic that of the nontoxic B1 sample in Fig. 3. Thus, while the pattern matching and intensity approach could enhance reliability, the current antibody combination does not fully alleviate the challenges associated with false-negative and false-positive results.

While this three antibody system could increase the knowledge of a sample composition and potential toxicity, the antibodies and LFI format used herein do not allow for full resolution of the false-negative and false-positive challenges that currently plague PST immunoassays. Furthermore, the SPR results will also suffer from similar challenges but could allow for more confidence in results due to the quantitative nature of the immunoassay versus reliance on visual readout of minor hue variations from the LFI device. Our research studies continue to focus on the generation and screening of antibodies with differing cross-reactivities, especially with improved reactivity to the hydroxylated toxins, and to determine if a mixture of multiple antibodies [44] or spatially separate antibodies (as shown in the conceptual LFIs) will be best for rapid tests.

4. Conclusions

Rapidly screening seafood samples for potential contamination by PSTs remains an analytical challenge. Sensitive, real-time SPR assays for PSTs have been developed and were used to evaluate the reactivity of three antibodies. The results show that each antibody has a unique reactivity for the PST congeners. This highlights the potential for developing antibodies that could have a higher correlation of response with sample potency. While the production of such antibodies continues, techniques employing the current antibodies for higher-confidence screening were evaluated and a conceptual model was created. This model indicated that while false-positive and false-negative results were not completely eliminated, there is potential to improve immunoassays and reduce the use of MBA and confirmatory analytical tests. Current research is focusing on the development of PST binders (i.e., antibodies, aptamers, receptors) that have better cross-reactivity with the congeners. Once candidate binders are evaluated by the SPR biosensor, they will be incorporated into a multi-binder, rapid test to fully realize the potential of a potency-based screening technique.

Acknowledgments

The authors express their gratitude to Mark Poli and his colleagues at USAMRIID for providing the burro anti-STX used in this work. We also thank Titan Fan from Beacon Analytical for providing purified anti-STX. Thank you to Katrina Campbell, Christopher Elliott, and Simon Haughey for valuable discussions and their contributions to the field of PST biosensors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.06.073.

References

[1] S.M. Etheridge, Toxicon 56 (2010) 108–122.
[2] A.R. Humpage, V.F. Magalhaes, S.M. Froscio, Anal. Bioanal. Chem. 397 (2010) 1655–1671.
[3] M. Kodama, S. Sato, in: L.M. Botana (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, CRC Press, Taylor and Francis Group, Boca Raton, FL, 2008, pp. 165–175.
[4] S.E. Shumway, Rev. Fish. Sci. 3 (1995) 1–31.
[5] C.Y. Kao, in: I.R. Falconer (Ed.), Algal Toxins in Seafood and Drinking Water, Academic Press, San Diego, CA, 1993, pp. 75–86.
[6] G.M. Lipkind, H.A. Fozzard, Biophys. J. 66 (1994) 1–13.
[7] J.A. Kalaitzis, R. Chau, G.S. Kohli, S.A. Murray, B.A. Neilan, Toxicon 56 (2009) 244–258.
[8] G.K. Isbister, M.C. Kiernan, Lancet Neurol. 4 (2005) 219–228.
[9] D.C. Rodrigue, R.A. Etzel, S. Hall, E. Deporras, O.H. Velasquez, R.V. Tauxe, E.M. Kilbourne, P.A. Blake, Am. J. Trop. Med. Hyg. 42 (1990) 267–271.
[10] FAO, in, Food and Agriculture Organization of the United Nations (FAO) Food and Nutrition Paper 80, Rome, 2004.
[11] Bulletin, in, June 23, 2011.
[12] N. Vilarino, E.S. Fonfria, M.C. Louzao, L.M. Botana, Sensors 9 (2009) 9414–9443.
[13] B. Ben-Gigirey, A. Villar-Gonzalez, in: L.M. Botana (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection, CRC Press, Taylor and Francis Group, Boca Raton, FL, 2008, pp. 177–196.
[14] AOAC, in: K. Helrich (Ed.), Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 1990, pp. 881–882.
[15] AOAC, Paralytic shellfish poisoning toxins in shellfish: Prechromatographic oxidation and liquid chromatography with fluorescence detection. Method 2005.06, in: Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 2005.
[16] J.F. Lawrence, B. Niedzwiadek, C. Menard, J. AOAC Int. 88 (2005) 1714–1732.
[17] Y. Oshima, J. AOAC Int. 78 (1995) 528–532.
[18] J.M. van de Riet, R.S. Gibbs, F.W. Chou, P.M. Muggah, W.A. Rourke, G. Burns, K. Thomas, M.A. Quilliam, J. AOAC Int. 92 (2009) 1690–1704.
[19] AOAC, Paralytic shellfish toxins in mussels, clams, oysters, and scallops post-column oxidation (PCOX). First Action, Method 2011.02, in: Official Methods of Analysis, Association of Official Analytical Chemists (AOAC), Arlington, VA, 2011.
[20] S. Etheridge, J. Deeds, S. Hall, K. White, L. Flewelling, J. Abbott, J. Landsberg, S. Conrad, D. Bodager, G. Jackow, Afr. J. Mar. Sci. 28 (2006) 383–387.
[21] C. Dell'Aversano, P. Hess, M.A. Quilliam, J. Chromatogr. A 1081 (2005) 190–201.
[22] E. Turrell, L. Stobo, J.P. Lacaze, S. Piletsky, E. Piletska, J. AOAC Int. 91 (2008) 1372–1386.

[23] G.R. Marchesini, H. Hooijerink, W. Haasnoot, J. Buijs, K. Campbell, C.T. Elliott, M.W.F. Nielen, TrAC, Trends Anal. Chem. 28 (2009) 792–803.
[24] G.J. Doucette, M.M. Logan, J.S. Ramsdell, F.M. VanDolah, Toxicon 35 (1997) 625–636.
[25] C.L. Powell, G.J. Doucette, Nat. Toxins 7 (1999) 393–400.
[26] G. Usup, C.P. Leaw, M.Y. Cheah, A. Ahmad, B.K. Ng, Toxicon 44 (2004) 37–43.
[27] F.M. van Dolah, T.A. Leighfield, G.J. Doucette, L. Bean, B. Niedzwiadek, D.F.K. Rawn, J. AOAC Int. 92 (2009) 1705–1713.
[28] M.V. Laycock, M.A. Donovan, D.J. Easy, Toxicon 55 (2010) 597–605.
[29] C.-K. Wong, P. Hung, E.A.L. Ng, K.L.H. Lee, G.T.C. Wong, K.-M. Kam, Harmful Algae 9 (2010) 636–646.
[30] E. Garet, A. Gonzalez-Fernandez, J. Lago, J.M. Vieites, A.G. Cabado, J. Agric. Food Chem. 58 (2010) 1410–1415.
[31] F. Kasuga, Y. HaraKudo, K. Machii, J. Food Hyg. Soc. Jpn. 37 (1996) 407–410.
[32] E. Usleber, R. Dietrich, C. Burk, E. Schneider, E. Martlbauer, J. AOAC Int. 84 (2001) 1649–1656.
[33] R.L. Manger, L.S. Leja, S.Y. Lee, J.M. Hungerford, M.A. Kirkpatrick, T. Yasumoto, M.M. Wekell, J. AOAC Int. 86 (2003) 540–543.
[34] M. Okumura, H. Tsuzuki, B. Tomita, Toxicon 46 (2005) 93–98.
[35] H.X. Chen, Y.S. Kim, S.R. Keum, S.H. Kim, H.J. Choi, J.B. Lee, W.G. An, K. Koh, Sensors 7 (2007) 1216–1223.
[36] K. Campbell, S.A. Haughey, H. van den Top, H. van Egmond, N. Vilarino, L.M. Botana, C.T. Elliott, Anal. Chem. 82 (2010) 2977–2988.
[37] E.S. Fonfria, N. Vilarino, K. Campbell, C. Elliott, S.A. Haughey, B. Ben-Gigirey, J.M. Vieites, K. Kawatsu, L.M. Botana, Anal. Chem. 79 (2007) 6303–6311.
[38] S.A. Haughey, K. Campbell, B.J. Yakes, S.M. Prezioso, S.L. DeGrasse, K. Kawatsu, C.T. Elliott, Talanta 85 (2011) 519–526.
[39] B.J. Yakes, M.M. Mossoba, in: S. Carnazza (Ed.), New Research Trends in Protein Chip Technology, Transworld Research Network, Kerala, India, 2010, pp. 55–73.
[40] H.J. van den Top, C.T. Elliott, S.A. Haughey, N. Vilarinì *f* o, H.P. van Egmond, L.M. Botana, K. Campbell, Anal. Chem. 83 (2011) 4206–4213.
[41] B.J. Yakes, S. Prezioso, S.A. Haughey, K. Campbell, C.T. Elliott, S.L. DeGrasse, Sens. Actuators, B 156 (2011) 805–811.
[42] A. Komano, H. Maruko, H. Sekiguchi, Y. Seto, Forensic Toxicol. 29 (2011) 38–43.
[43] K. Campbell, L.D. Stewart, G.J. Doucette, T.L. Fodey, S.A. Haughey, N. Vilarino, K. Kawatsu, C.T. Elliott, Anal. Chem. 79 (2007) 5906–5914.
[44] K. Campbell, D.F.K. Rawn, B. Niedzwiadek, C. Elliott, Food Addit. Contam., Part A 28 (2011) 711–725.

SEAFOOD TOXINS

Indirect Enzyme-Linked Immunosorbent Assay for Saxitoxin in Shellfish

FUN S. CHU and TITAN S. L. FAN
University of Wisconsin, Food Research Institute and Department of Food Microbiology and Toxicology, Madison WI 53706

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for the detection of saxitoxin (STX). Antibodies against STX were demonstrated in rabbits 5 weeks after immunizing with STX-bovine serum albumin (STX-HCHO-BSA). In the ELISA, STX-HCHO-BSA or polylysine-STX was coated onto the microtiter plate, followed by incubation with standard toxin and anti-STX antibody. The amount of antibody bound to the solid phase was determined by incubation with goat anti-rabbit IgG peroxidase conjugate and a reaction with chromogenic substrate. Competitive indirect ELISA revealed that the antiserum did not cross-react with either carbamoyl-neo-STX-sulfate or tetrodotoxin. The antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX about 56% and 16% as much as they did with STX, respectively. The lower detection limits for STX, decarbamoyl-STX, and neo-STX in this sytem were about 25, 45, and 156 pg per assay, respectively. When STX added to clams or mussels was assayed, the detection limit for STX was about 50–100 ppb, and recoveries were in the range of 86.8–107%.

Saxitoxin is one of the major and most potent in a group of toxins involved in paralytic shellfish poisoning (PSP) (1–3). The toxin is produced predominantly by the dinoflagellate *Gonyaulax catenalla* (4) and is primarily isolated from toxic mussels, clams (2), and other marine animals in waters inhabited by *Gonyaulax*. The poison has no adverse effect on the shellfish using the dinoflagellate as a food source. Human ingestion of toxin-contaminated shellfish has resulted, however, in paralytic poisoning and occasionally death (2). The amount of PSP poison that causes death in humans is estimated to be about 0.5–4 mg (2). The Food and Drug Administration has set the maximum acceptable level for paralytic poison in fresh, frozen, or canned shellfish at no more than 400 mouse units (MU) or about 80 μg/100 g edible portion (2).

Because of the potential health hazard, a quick, sensitive, and specific method is needed to determine the presence of toxins in shellfish. Due to its simplicity, the mouse bioassay (5) has been adopted as an official method for monitoring the poison; however, this method is neither specific nor sensitive, and also requires a continuous supply of mice. About 0.18 μg STX is required to kill a 20 g mouse in 15 min. Other methods, including fluorometric assay (6) and colorimetric techniques (7), also have sensitivity and specificity problems. More recently, a liquid chromatographic method was developed by Sullivan and Iwaoka (8). Although this method can detect low levels of PSP (0.5–25 ng/assay), it requires expensive equipment, and samples must be analyzed one at a time (8, 9).

Because of the highly specific antigen-antibody interaction, several laboratories have attempted to develop an immunoassay for PSP. As early as 1964, Johnson et al. (10) reported an immunoassay involving a hemoagglutination reaction; however, the sensitivity was not high enough for practical application of the immunoassay. Most recently, Carlson and Guire (11) demonstrated a radioimmunoassay system that permitted detection of less than 1 ng STX, but the antiserum did not react with neo-STX, and, therefore, the antiserum has only limited use. In our laboratory, we have attempted to produce specific antibodies against STX. Among several approaches tested, we found that the antibody produced in rabbits after immunizing with STX conjugated to bovine serum albumin was useful for STX assay. Subsequently, we developed an indirect enzyme-linked immunosorbent assay (ELISA), and details for production, characterization, and the ELISA protocols for determination of STX in clams and mussels are described here.

Experimental

Materials

Purified STX was provided by E. J. Schantz and R. W. Wannemacher, Jr. Decarbamoyl STX was prepared according to the method of Ghazarossian et al. (12, 13). Neo-STX and tetrodotoxin were supplied by R. E. Carlson, and carbamoyl-neo-STX sulfate was provided by H. Schnoes. Bovine serum albumin (BSA, RIA grade), polylysine (mol. wt. 60,000), Tween 20, 2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), 30% hydrogen peroxide, and goat anti-rabbit IgG peroxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freunds's adjuvant were obtained from Difco Laboratories (Detroit, MI). Gelatin was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Polystyrene microwell plates (NUNC product, Denmark; No. 2-69620) were obtained from Vangard International Inc. (Neptune, NJ). CF-1 mice were purchased from Harlan/Sprague-Dawley (Madison, WI). Albino rabbits, female, 5 lb size, were purchased from Klubertanz Rabbit Farm (Edgerton, WI) and were tested to be *Pasteurella*-negative before use. All chemicals and organic solvents were reagent grade or better.

The cultivated mussels *(Mytilus recurvus)* and cherrystone clams *(Mercenaria mercenaria),* harvested from the Chesapeake Bay, MD, were purchased from a local seafood store. Clams were kept refrigerated and mussels were kept in crushed ice. Both extracts were tested by mouse bioassay which showed no toxicity. The naturally contaminated scallop (Pecten grandis) samples, supplied by E. J. Schantz who collected them from the Bay of Fundy in 1980, were shucked and kept frozen at −20°C before extraction.

Preparation of Saxitoxin Antigen

The antigen (STX-HCHO-BSA) was prepared according to Johnson et al. (10) with slight modifications. In a typical experiment, 14.25 mg bovine serum albumin in 6 mL 0.1M sodium acetate buffer (pH 4.2) was reacted with 1.5 mg saxitoxin in the presence of 0.081 mL 37% formaldehyde (w/w). The reaction was carried out at room temperature for 72 h and then at 5°C for another 12 h. The reaction mixture was dialyzed against 2 L acetic acid (0.001M) at 5°C for 72 h with 2 changes of the acetic solution during this period to remove residual-free saxitoxin. The molar ratio of STX:BSA was estimated to be about 16:1 by measuring the free amino groups

Received May 24, 1984. Accepted August 20, 1984.
Presented at the 97th Annual International Meeting of the AOAC, Oct. 3–6, 1983, at Washington, DC.

before and after reaction (14). The same method was used to prepare the STX-polylysine. Succinyl-STX-BSA and decarbamoyl-STX BSA were prepared according to the procedure of Ghazarossian (15).

Immunization Schedule

Three rabbits were immunized with STX antigen, using a multiple site injection method (16). The fur along the back and the proximal limbs of the rabbits was shaved before injection. Two mL of emulsion was made by mixing 500 μg STX-HCHO-BSA in 0.5 mL sterilized saline and 1.5 mL complete Freund's adjuvant. About 30–50 μL emulsion was injected intradermally at each site over the shaved area, and 0.2–0.25 mL emulsion was injected subcutaneously on each shoulder. Each animal received about 30–40 injections. Three or four weeks after the initial immunization, the rabbits were bled on a weekly basis, and the titers were determined. Six to eight weeks after the initial injection, the animals were boosted intramuscularly with another 500 μg antigen in incomplete Freund's adjuvant emulsion prepared by emulsifying 1 volume of antigen with 1 volume of adjuvant. The same schedule (5–6 weeks interval) was used for subsequent booster injections. The antiserum collected was precipitated with ammonium sulfate to a final 33% saturation. The precipitate was redissolved in water to the same original serum volume, dialyzed against 2 L 0.1M PBS overnight, and then lyophilized. The lyophilized, purified antibody was stored at −4°C.

Enzyme-linked Immunosorbent Assay

Titration of antibody titers.—The ELISA protocols are essentially the same as those we described for the analysis of aflatoxin B_1 (17). The optimal dilution of antigen necessary for precoating microtiter plates was determined by the checkerboard test. Fifty μL antigen (STX-HCHO-BSA) at a concentration of 1.6 μg/mL in 0.5M bicarbonate buffer at pH 9.6, was added in each well and incubated overnight at 4°C. The plate was washed with various amounts of washing solution (0.1M sodium phosphate buffered saline containing 0.1% Tween 20, pH 7.5) in the following sequence: twice with 0.1 mL, twice with 0.2 mL, and 3 times with 0.32 mL each well. Three hundred μL 0.1% gelatin in PBS was added to each well to eliminate nonspecific binding by blocking the plastic surface where protein was not bound. After 30 min to 1 h incubation at 37°C, the well was washed 4 times each with 0.32 mL washing solution. Next, 50 μL of various dilutions of antibody, diluted in PBS containing 0.1% BSA, was added to each well. Following 1 h incubation at 37°C, the plate was washed again to remove the free antibody. Fifty μL goat antirabbit IgG peroxidase conjugate (1:500 dilution in PBS containing 0.1% BSA) was added to each well, and the wells were incubated another hour at 37°C. The plate was washed again. The peroxidase substrate (ABTS in citrate buffer, pH 4.0) of 0.1 mL was finally added to each well. After developing the color 20 min at 37°C, the reaction was terminated by adding 0.1 mL hydrofluoric acid–ethylenediamine tetracetic acid stopping reagent, and absorbance at 410 nm was determined by a Dynatech minireader. The antibody titer was defined as the reciprocal of the antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum.

Competitive indirect ELISA.—Protocols used for titration of antibody titers were slightly modified in this assay. One hundred μL of an appropriate dilution of antigen in 0.05M carbonate buffer, pH 9.6, was coated to the plate. In addition, 50 μL purified STX, other PSP toxins, or unknown samples were incubated together with 50 μL of appropriate dilution of antibody in each well. The amount of bound antibody was determined by adding 0.1 mL goat antirabbit IgG peroxidase conjugate.

Preparation of Samples

The AOAC acid extraction method (5) was used throughout the experiment for extraction of STX from clam and mussel before ELISA. The whole tissue of clam was used for extraction whereas only the dark gland of mussel was used. Mussels were shucked and the dark glands (hepatopancreas) were removed. About 15 mussels gave a total of 5 g dark glands. The samples (50–100 g clam or 5 g dark gland) were homogenized with an equal volume of 0.1N HCl and then boiled 5 min. After cooling to room temperature, the solution was adjusted to pH 4.0–4.5 and then centrifuged to remove the tissue. The acidic extract was adjusted to pH 7.0 and immediately used in the ELISA.

In the recovery experiment, different concentrations of STX diluted in PBS were injected into 6 groups (5 g each group) of dark gland samples. After 30 min, 5 mL 0.1N HCl was added, and the sample was extracted by the procedures described. For clam samples, toxin was added to 50 g of sample during the homogenization step.

Analysis of Samples by Competitive Indirect ELISA

In a preliminary study, the effect of the acid extracts of blank samples on the indirect ELISA was tested. However, we observed significant interference in the ELISA where more than 50 mg sample was used. The interference was minimal when 5 mg/mL of mussel dark gland or 50 mg/mL of clam meat was used. Subsequently, a phosphate-buffered saline solution (0.1M, pH 7.4) containing either blank mussel dark gland or blank clam meat acid extracts, at concentrations of 5 and 50 mg/mL, respectively, was used for preparation of STX standard and sample solutions for ELISA. A naturally contaminated scallop sample was also analyzed for STX by ELISA. A high level of STX was present in this sample, so high dilution was necessary (1 to 10,000 dilution of the acid extract). ELISA was carried out in regular phosphate buffer instead of buffer containing blank samples for this naturally contaminated sample.

Results and Discussion

In the initial studies, we tested several different STX-BSA conjugates for their ability to produce antibodies against STX, using indirect ELISA to monitor the antibody titer. We found that decarbamoyl-STX BSA, succinyl-STX BSA, and STX-HCHO-BSA all elicited antibody against STX. Because the best antibody titers were obtained from rabbits that had been immunized with STX-HCHO-BSA, our efforts were concentrated on this immunogen. Figure 1 shows a typical titration curve for the antibody titer for a rabbit immunized with this antigen. Figure 2 shows results for the average antibody titers of 3 rabbits over a period of 10 weeks. Good antibody titers were obtained from rabbits as early as the 5th week after immunization. Antiserum titer increased considerably after the first booster.

Antibody specificity was determined by a competitive indirect ELISA in which different STX derivatives were present in the assay system to compete the binding of STX coated to the solid phase with the antibody. In this assay, the STX-polylysine conjugate was coated to the plate because this conjugate gave less aggregation after repeated thawing. After incubation with the antibody and different STX derivatives, the rabbit antibody bound to the STX-polylysine solid phase

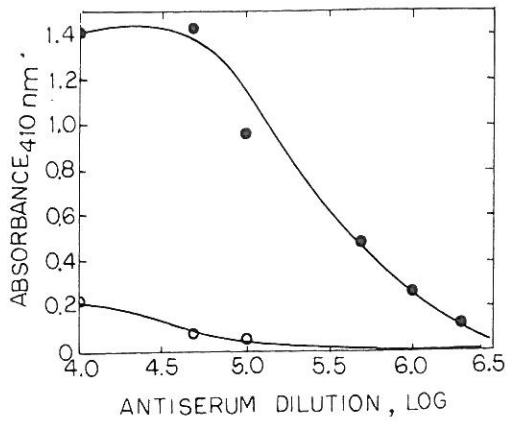


Figure 1. Titration of antibody titer by indirect ELISA. See text for description of procedure. Titer is defined as reciprocal of antiserum dilution that gives an absorbance at 410 nm that is 0.1 unit greater than that of the pre-immune serum. Antisera from rabbits immunized with STX-HCHO-BAS (●); pre-immune serum (○).

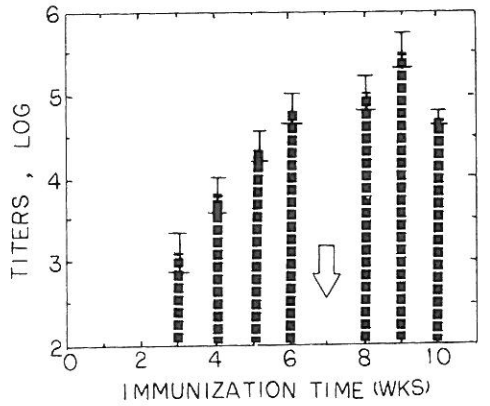


Figure 2. Average antibody titers for 3 rabbits immunized with STX-HCHO-BSA. Range of antibody titer for bleeding of 3 rabbits is represented by an error bar. Arrow indicates time of booster injection.

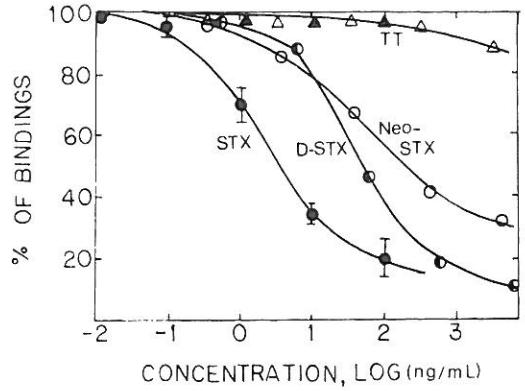


Figure 3. Competitive indirect ELISA for STX. See text for description of procedure. Saxitoxin (●), neo-saxitoxin (○), decarbamoyl-saxitoxin (◐), carbamoyl-neo-saxitoxin sulfate (△), and tetrodotoxin (▲).

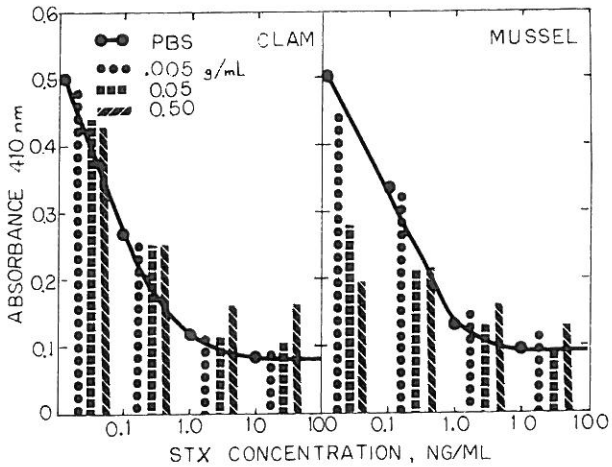


Figure 4. Effect of clam and mussel extracts on indirect ELISA of STX. Curves represent data obtained from experiments using STX in PBS buffer. Data from experiments using clam and mussel extracts are shown as bars. STX concentrations for spiking experiments are indicated to left of bars on X-axis. All data represent average of 3 measurements.

was determined by reaction with goat-antirabbit peroxidase complex and enzyme substrate. Results (Figure 3) indicate that the antibody has high affinity for STX. The concentrations that cause 50% inhibition of binding of the antibody to the solid phase antigen (i.e., STX-HCHO-polylysine) by STX, decarbamoyl-STX and neo-STX are 3.2, 50, and 180 ng per assay, respectively. Thus, the antibodies for STX cross-reacted with decarbamoyl-STX and neo-STX only about 56.2% and 16% as much as they did with STX. Carbamoyl neosaxitoxin sulfate and tetrodotoxin showed no cross-reaction with the antibody at the maximal concentration tested.

Carbamoyl neo-STX sulfate did not cross-react with the antibodies whereas neo-STX did, so modification of the structure of the C-11 position may greatly affect the conformation of STX and thus prevent these derivatives from reacting with the antibody. Because the decarbamoyl-STX cross-reacts with the antibodies somewhat, the role of the terminal amide bond in the STX in determining the antibody specificity may not be as important as the side chain in the C-11 position. Additional experiments using other derivatives such as 11-hydroxysaxitoxin sulfate, which is produced by *Gonyaulax tamarensis* and is also a major PSP along the Alantic Coast (3), are needed to v

Competitive ELI
system can detect

150 pg neo-STX (3 ng/mL) in each assay where 3 standard deviations of the blank response were 5% of binding (Figure 3). It has been reported that the reactivity of neo-STX with anti-saxitoxinol antibody, which was obtained from rabbits immunized with saxitoxinol-BSA conjugate, is less than 1% of those for STX (11). Therefore, the anti-BSA-HCHO-STX antiserum has some advantage over the anti-saxitoxinol antibody, and can be used for monitoring the neo-STX, one of the major toxins in PSP.

Although the indirect ELISA could detect as little as 2 pg STX in each assay when the analysis was carried out with pure STX in buffered solution, high interference was observed when the food samples were analyzed. The problem was particularly serious when whole mussel meat extract was used. Results (Figure 4) indicate only a slight effect on the standard curve when an extract equivalent to 50 mg/mL (or 2.5 mg/assay) of clam meat was used; however, high interference was observed when an extract equivalent to as little as 5 mg/mL (0.25 mg/assay) of mussel meat was used. Therefore, subsequent recovery experiments, including the standards, were carried out in a buffer that contained either blank extract of clam meat (50 mg/mL) or blank mussel dark gland

sing dark gland in the
ne observation (1) that
lated in this gland, and

Table 1. Recovery (%) of saxitoxin from clam and mussel by ELISA[a]

Toxin added, ppb	Clam			Mussel		
	Rec., % (N)	SD[b]	CV[b]	Rec., % (N)	SD[b]	CV[b]
50	207 (12)	27.3	13.2	95.2 (12)	17.4	18.3
100	103 (20)	15.8	15.3	97.5 (20)	15.8	16.2
200	107 (24)	9.5	8.9	106.5 (12)	9.6	9.0
250	—[c]	—[c]	—[c]	89.6 (8)	8.8	9.8
500	90.8 (24)	11.8	13.0	86.8 (12)	11.4	13.1
1000	105.2 (24)	9.3	8.8	95.4 (14)	19.0	19.9

[a]Saxitoxin was added to whole clam tissue or mussel dark gland. In the assay, either whole tissue (clam) or whole dark gland (mussel) extract was used. In the final calculation, weight of whole mussel was used. In general, dark gland of mussel accounted for about 10% of whole mussel weight.
[b]SD and CV represent standard deviation (%) and coefficient of variation (%), respectively.
[c]Not determined.

that this gland is relatively easy to separate from the meat tissue. Several cleanup protocols were tested, but we felt that adding the blank sample extracts to the assay was the simplest approach in solving the interference problem. Table 1 gives results for the recovery of STX spiked in mussel dark gland and clam. Recoveries were between 86.8 and 107% in the range 50–1000 ppb (on the basis of total tissue weight) and 90.8–105% in the range of 100–1000 ppb STX added to the mussel dark gland and clam samples, respectively. Coefficients of variation for these assays are in the range 9–20%, which is in the range for most ELISAs. The detection limit appears to be in the range of 50–100 ppb where the blank response at 95% confidence was 3 standard deviations.

A naturally contaminated scallop sample was tested by mouse bioassay according to the AOAC method (5) and by the present ELISA. The sample contained 97.8 ± 7.8 μg STX/g sample by ELISA and 112.53 ± 6.53 μg/g by mouse assay. In the bioassay, a series of standard solutions were injected into the same strain of mice under the same experimental conditions where 0.363 μg STX was determined to be 1 mouse unit. Present results indicate that 86.9% of the STX determined by mouse bioassay was detected by ELISA. The lower recovery by ELISA may result from the presence of other toxins in the sample or from problems existing in both assays. Further comparative analyses for additional naturally contaminated samples by both methods as well as testing the cross-reactivity with other PSP toxins, are needed to establish the validity of the assay.

Conclusions

Results obtained from the recent study indicate that antibodies obtained from rabbits immunized with STX-HCHO-BSA are adequate for the analysis of STX in foods, according to ELISA protocols described. Although the antibodies also recognize neo-STX, the ELISA for this toxin is not as sensitive as that for STX. The antibodies, however, could not recognize STX when the OH-group at the C-11 position was esterified by sulfate, i.e., carbamoyl-neo-STX-sulfate. Additional experiments are needed to prepare other STX-protein conjugates for eliciting antibodies that will recognize other PSP toxins.

Acknowledgments

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin-Madison, and by contract No. DAMD-80-C-2021 from the U.S. Army Medical Research and Development Command of the Department of Defense.

The authors thank E. J. Schantz and R. W. Wannemacher for supplying the saxitoxin and R. E. Carlson for providing the neosaxitoxin and tetrodotoxin in the present study; S. W. Li for his help in the preparation of saxitoxin-protein conjugate; and E. J. Schantz and N. Higley for their suggestions and discussion throughout the present study.

References

(1) Baden, D. G. (1983) *Int. Rev. Cytol.* **82**, 99–150
(2) Schantz, E. J. (1979) *Pure Appl. Chem.* **52**, 183–188
(3) Boyer, G. L., Wichmann, C. F., Mosser, J., Schantz, E. J., & Schnoes, H. K. (1979) in *Toxic Dinoflagellate Blooms*, D. L. Taylor & H. H. Saliger (Eds), Elsevier North Holland, Inc., New York, NY, pp. 373–376
(4) Sommer, H., Whedon, W. F., Kofoid, C. A., & Stohler, R. (1937) *Arch. Pathol.* **24**, 537–559
(5) *Official Methods of Analysis* (1984) 14th Ed., AOAC, Arlington, VA, secs **18.086–18.092**
(6) Bates, H. A., Kostriken, R., & Rapport, H. (1978) *J. Agric. Food Chem.* **26**, 252–254
(7) Gershey, R. M., Nevé, R. A., Musgrave, D. L., & Reichardt, F. B. (1977) *J. Fish. Res. Board Can.* **34**, 559–563
(8) Sullivan, J. J., & Iwaoka, W. T. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 297–303
(9) Sullivan, J. J., Simon, W. G., & Iwaoka, W. T. (1983) *J. Food Sci.* **48**, 1312–1314
(10) Johnson, H. M., Frey, P. A., Angelotti, R., Campbell, J. E., & Lewis, K. H. (1964) *Proc. Soc. Exp. Biol. Med.* **117**, 425–430
(11) Carlson, R. E., & Guire, P. E. (1983) in *Symposium on Seafood Toxins*, Sept. 1983, American Chemical Society, Washington, DC
(12) Ghazarossian, V. Z., Schantz, E. J., Schnoes, H. K., & Strong, F. M. (1976) *Biochem. Biophys. Res. Commun.* **68**, 776–780
(13) Koehn, F. E., Ghazarossian, V. E., Schantz, E. J., Schnoes, H. K., & Strong, F. M. (1981) *Bioorg. Chem.* **10**, 412–428
(14) Habeeb, A. F. S. A. (1966) *Anal. Biochem.* **14**, 328–336
(15) Ghazarossian, B. E. (1977) Ph.D. thesis, The University of Wisconsin-Madison, Madison, WI
(16) Nieschlag, E., Kley, H. K., & Usadel, K. H. (1975) in *Steroid Immunoassay*, E. H. D. Cameron, S. G. Hillier, & K. Griffiths (Eds), Alpha Omega Publications, Ltd, Wales, pp. 87–96
(17) Fan, T. S. L., & Chu, F. S. (1984) *J. Food Prot.* **47**, 263–266

Reprinted from the *Journal of the Association of Official Analytical Chemists*, Vol. 68 #1

Beacon Analytical Systems, Inc.

4 parameter ELISA Data Reduction Worksheet

Operator:

Date:

Assay ID:

Kit Lot#:

Section I)

Standards Data

#	ppb	ABS	% Bo
	0.00	1.449	100.0
	0.01	1.321	91.2
	0.08	0.661	45.6
	0.32	0.217	15.0

Section II)

Curve Fitting

Starting/Final values for Parameters A - D

A	B	C	D
100.00	1.22	0.07	2.61

Squares of differences between actual and calculated curves

X	0.00	0.01	0.08	0.32
Y Predicted	100.00	91.17	45.62	14.98
(Y-Y Pred.)^2	0.00	0.00	0.00	0.00

Minimize sum of square of differences

Min. (Y-Y Pred.)^2 =	**0.0**

Correlation Coeff.

R^2 =	1.000

Section III)

Sample Calculations

#	Sample	ID	ABS	% Bo	ppb
			0.501	34.6	0.119
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!
				0.0	#NUM!

Reviewed & Accepted:

Date:

Checklist for Saxitoxin (PSP) Microtiter Plate Test Kit

Operator:		
Date:		
Sample:		
Assay ID:		
Kit name:		
Kit Lot#:		
Kit expiration date:		
Laboratory equipment		
The kitchen blender and its accessories are clean.	☐ YES	☐ NO
The balance provides a sensitivity of at least 0.1 g.	☐ YES	☐ NO
The balance calibration is checked monthly.	☐ YES	☐ NO
Microcentrifuge has a maximum speed of ≥ 12,000 rpm.	☐ YES	☐ NO
Single-channel (200 and 1000 µL) and multi-channel micropipette (200 µL) are calibrated every six months.	☐ YES	☐ NO
Microwell plate or strip reader has a filter for 450 nm wavelength.	☐ YES	☐ NO
Reagents and chemicals		
Kit components are stored at 4 – 8 °C and equilibrated to room temperature for 1 hr before use.	☐ YES	☐ NO
Make up water is distilled or deionized.	☐ YES	☐ NO
Isopropanol (2-Propanol) and Methanol are laboratory grade.	☐ YES	☐ NO
Acetic acid, Sodium Phosphate Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$) and Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4) are ACS grade.	☐ YES	☐ NO
<u>Extraction solution</u> 1. 70% Isopropanol – 70 mL of Isopropyl alcohol + 30 mL of water 2. 5% Acetic acid – 5 mL of Acetic acid + 95 mL of water 3. A mixture of 70% Isopropanol and 5% Acetic acid – 100 mL of 70% Isopropyl alcohol + 50 mL of 5% Acetic acid	☐ YES	☐ NO
<u>Dilution buffer solution</u> 1. 20 mM PBS (pH 7.2) – Sodium Phosphate Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$): 0.77g – Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4): 2.04g – Sodium Chloride (NaCl): 8.5g – Distilled or deionized water: 1L 2. 10% Methanol/20mM PBS – 10 mL of Methanol + 90 mL of 20 mM PBS	☐ YES	☐ NO

Sample preparation		
1. At least 12 shellfish are used per sample.	☐ YES	☐ NO
2. The inside of each shell is rinsed with laboratory grade water.		
3. Shellfish are shucked and strained through a #10 mesh sieve (or equivalent).		
4. Strained meats are blended at high speed until homogenous.		
5. 5 g of sample and 10 mL of extract solution are accurately measured in a 50 mL centrifuge tube and vortexed for 3 minutes.		
6. Approximately 1 mL of the extract is transferred to a microcentrifuge tube and centrifuged for 5 minutes at 12,000 rpm.		
7. The supernatant is diluted with dilution buffer 5000 fold. e.g. 0.1 mL of supernatant + 9.9 mL of dilution buffer -> 0.1 mL of the diluted supernatant + 4.9 mL of dilution buffer		
Assay		
1. Prepare the 1X wash solution	☐ YES	☐ NO
2. Allow reagents and sample extracts to reach room temperature prior to running the test.		
3. Dispense 50 µL enzyme conjugate to the appropriate test wells. Be sure to use a clean pipet tip for each.		
4. Add 50 µL of Calibrators or sample extract to each well.		
5. Dispense 50 µL of antibody solution into each test well.		
6. Shake the plate gently for 30 seconds and incubate the test wells for 30 minutes.		
7. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and dump. Repeat 3X for a total of four washes.		
8. Tap the inverted wells onto absorbent paper to remove the last of the wash solution.		
9. Dispense 100 µL of substrate, and incubate for 30 minutes.		
10. Dispense 100 µL of stop solution into each test well.		
11. Read and record the absorbance of the wells at 450nm.		
Calculation		
1. Use a 4-parameter curve fit for calculation.	☐ YES	☐ NO
2. When the data reduction spreadsheet is used for calculation, type in absorbance values for both calibrator and sample. Follow the instruction in the spreadsheet to calculate the concentration in ppb.		
3. Multiply the results in ppb by dilution factor (15000).		
4. The unit (ppb) of the result can be converted to µg/100 g by dividing the result by 10.		

5. Any samples below 30 µg/100 g are considered negative, and no further testing is needed. Samples falling between 30 and 100 µg/100 g are considered as suspicious, and confirmation test (MBA) is followed. Samples above 100 µg/100 g are considered positive, and may not require further tests to confirm.	

Operator Signature:_____ Date:_____

Supervisor Signature:_____ Date:_____

Proposal No. **13-111**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	David C. Deardorff
Affiliation	Abraxis LLC
Address Line 1	54 Steamwhistle Drive
Address Line 2	
City, State, Zip	Warminster, PA 18974
Phone	215-357-3911
Fax	215-357-5232
Email	ddeardorff@abraxiskits.com
Proposal Subject	DSP PPIA Kit for Determination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests Marine Biotoxin Testing
Text of Proposal/ Requested Action	The DSP PPIA kit be approved as a Marine Biotoxin Laboratory Test Method.
Public Health Significance	Okadaic acid (OA) and its analogues, DTX1, DTX2, together with their ester forms are known as the group of OA-toxins. These toxins, lipophilic and heat stable, are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter feeding bivalve molluscs. The OA-toxins group causes Diarrheic Shellfish Poisoning (DSP), which is characterized by symptoms such as diarrhea, nausea, vomiting and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs such as mussels, clams, scallops or oysters. Inhibition of serine/threonine phosphoprotein phosphatases is assumed to be responsible for these toxic effects. Recently in the Pacific Northwest harvest areas, outbreaks of DSP have occurred.
Cost Information	Refer to Para D.1. of the Checklist
Action by 2013 Laboratory Methods Review and Quality Assurance Committee	Recommended referral of Proposal 13-111 to an appropriate committee as determined by the Conference Chairman and directed the Executive Office send a letter to the submitter requesting additional information as provided by the Laboratory Methods Review and Quality Assurance Committee.
Action by 2013 Task Force I	Recommended adoption of Laboratory Methods Review and Quality Assurance Committee recommendation on Proposal 13-111.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-111.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-111.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-111 to an appropriate committee as determined by the Conference Chair until additional data are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-111.
Action by 2015 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-111.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-111.

OKATEST

ZE/OA48C
ZE/OA96C

Test for detection of Okadaic Acid-toxins group

Test para la detección de las toxinas del grupo del Ácido Okadaico

ZEULAB, S.L.
C/ Bari, 25 dpdo. • 50197 Zaragoza (SPAIN)
Tel.: +34 976 731 533 • Fax: +34 976 524 078
info@zeulab.com • www.zeulab.com

SCOPE

This protocol specifies a method for the quantitative determination of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, cockle, scallops, etc.

PRINCIPLE

Test based on the phophatase activity inhibition by OA-toxins group, responsible for diarrheic shell-fish poisoning (DSP).
Phosphatase enzyme PP2A is able to hydrolyse a specific substrate, yielding a product that can be detected at 405 nm. Samples containing toxins from the okadaic acid group will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample can be calculated using a standard curve.

KIT CONTENTS

	48 Tests Kit	96 Tests Kit
Microtiter plate strips (8 wells per strip)	6	12
Vials of Phosphatase *(Phosphatase)*	2	4
Set of Okadaic Acid Standards (*Okadaic acid 0.5, 0.8, 1.2, 1.8 and 2.8 nM)*	1	1
Chromogenic Substrate *(Chromogenic Substrate)*	1	1
Phosphatase Dilution Buffer *(Phosphatase Dilution Buffer)*	1	1
Stock Buffer Solution *(Stock Buffer Solution)*	1	1
Stop Solution *(Stop Solution)*	1	1
Adhesive film	1	2
Kit instructions	1	1

ADDITIONAL MATERIAL AND REAGENTS NEEDED

- Micropipettes
- Blender (Ultraturax) or mortar and pestle
- Heater at 30ºC ± 2 ºC (i.e. FX Incubator, Ref ZE/FX, from ZEULAB)
- Microplate reader (wavelength at 405 nm)
- Water bath for 76 ± 2 ºC
- Methanol (analytical grade)
- NaOH 2.5 N made by titration, (NaOH of analytical grade)
- HCl 2.5 N made by titration, (HCl of analytical grade)
- Deionised water (grade 2, ISO3696)
- Graded 50 mL centrifuge tubes with screw caps
- Tube shaker
- Centrifuge

SOLUTIONS

1.- Okadaic Acid Standards: to make sure these solutions are homogeneous, it is very important to mix well using a vortex, before applying to the plate.

2.- Chromogenic Substrate solution: The solution contains stabilization resin. Make sure this resin is not added to the microwells. To assure that, it is recommended to transfer the volume needed into a transparent labware (i.e.: test tube or eppendorf) and take the solution from that container to add into the wells. *Note:* Do not use this solution if the absorbance of 90 µL of this solution at 405 nm is over 0.6.

3.- Phophatase solution: Add 2.0 mL of phosphatase dilution buffer *(Phosphatase Dilution Buffer)* to one of the phosphatase vials *(Phosphatase)* and dissolve by mixing gently for 1 hour ± 5 minutes at room temperature (22 ± 2 ºC) to ensure that the enzyme is fully hydrated. Do not use the tube shaker at any moment. This solution must be stored under refrigeration if not in use immediately after preparation. Do not use the phosphatase solution for following days. Each enzyme vial contains enough volume for 24 wells. If more than one vial is used in the assay, dissolve each vial as described above, make a pool with the content of the vials and mix gently, by inversion, before use.
*Attention: this reagent is blue and becomes brownish when dissolved. If brownish colour is noticed before hydratation, discard this reagent as it could be damaged.

4.- Buffer solution x1: dilute the *Stock Buffer Solution* included in the kit by mixing 1 volume with 9 volumes of deionised water. Use buffer solution x1 only freshly made, and store under refrigeration if not in use immediately.

5.- 2.5 N NaOH: weigh 100 g of NaOH and add 500 mL of water and dissolve. Transfer to a volumetric flask and add deionised water up to a final volume of 1000 mL.

6.- 2.5 N HCl: add 205 mL of HCl (37 %) to 400 mL of deionised water already contained in a volumetric flask. Make the volume up to 1000 mL with deionised water.

SAMPLES EXTRACTION

The method described below includes a hydrolysis step to detect all toxins forms of okadaic acid (okadaic acid and dinophisistoxins).

1.- Clean the shell thoroughly using water
2.- Open the shellfish by cutting the adductor muscles.
3.- Wash inside the shell thoroughly to remove any dirt.
4.- Remove the tissue inside the shell by cutting all the muscles attached to the shell.
5.- Place the shellfish tissue in a filter paper for few minutes to remove water in excess.

It is recommended to use graded 50 mL centrifuge tubes with screw caps during the following steps of hydrolysis in order to prevent loses due to labware changes.

6.- Mash the shellfish tissue to obtain a representative sample and weigh 5 g. Add 25 mL of Methanol and homogenise the mixture for 2 minutes using a tube shaker.
7.- Centrifuge at 2000 g for 10 min at 4 ºC. The supernatant (*methanolic extract*) is poured into a centrifuge tube.
8.- Take 640 µL of *methanolic extract* and pour into another centrifuge tube.
9.- Add 100 µL of 2.5 N NaOH.
10.- Seal and heat at 76 ± 2 ºC for 40 minutes.

11.- Add 80 µL of 2.5 N HCl (the sample does not need to be cooled down previously).
12.- Add up to 20 mL of Buffer solution x1.

TEST PROCEDURE

Warning:

The volume of some reagents used in this assay is small and special attention must be paid when added to the wells:

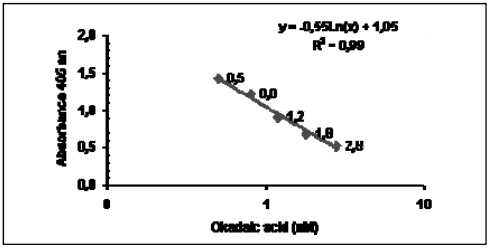
- Make sure the pipettes are calibrated before running the assay.
- Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 or 200 µL.
- Be sure that the incubator's temperature is stabilized before use.

It is recommended to run samples and standards in duplicate.

1.- Add 50 µL of samples or standards.
2.- Add 70 µL of the Phosphatase Solution to each well. Mix well by gentle tapping on the side of the plate.
3.- Cover the plate with the adhesive film provided and incubate for 20 ± 0.5 minutes at 30 ± 2 ºC.
4.- Remove the adhesive film and add 90 µL of Chromogenic Substrate to each well. Mix well by gently tapping on the side of the plate.
5.- Cover the plate with the adhesive film and incubate 30 ± 0.5 minutes at 30 ± 2 ºC.
6.- Remove the adhesive film and add 70 µL of Stop Solution to each well.
7.- Read absorbance of samples and standards at 405 nm.

GRAPHIC REPRESENTATION AND CALCULATIONS OF RESULTS

1.- Obtain a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of okadaic acid in a logarithmic *x axis* and use a logarithmic fitting as shown in the graphic next page. R^2 has to be greater than or equal to 0.96.



2.- The OA concentration contained in the sample (Cs) is calculated by interpolation into the calibration curve or using the following equation:

$$x = \text{EXP}\,(y - b/a)$$

Where x is the OA concentration in the sample (Cs) and y the absorbance of the sample.

Note: An Excel worksheet to calculate results is available upon request.

3.- Calculate the diarrheic shellfish toxins concentration in tissue (Ct) as follows:

$$\text{Ct } (\mu g/kg) = \frac{(\text{Cs (nM)} \times \text{FD} \times \text{MW (g/mol)} \times \text{Ve (L)})}{\text{Mt (g)}}$$

Ct: toxins concentration in tissue, expressed as equivalents of OA; Cs: toxins concentration in sample; FD: Methanolic extract dilution factor (i.e. 640 μL/20 mL → x 31.25); MW: Okadaic acid molecular weight = 805; Ve: Methanolic extract volume (0.025L); Mt: Tissue weight (5g).

Example: for OA concentration of 1.5 nM: 1.5 nM x 31.25 x 805 g/mol x 0.025L / 5g = 189 μg OA eq/kg.

NOTE: For samples with OA concentration falling outside the working range (< 0.5 nM or > 2.8 nM), results will be reported as < 0.5 nM (or < 63 μg/Kg) or > 2.8 nM (or > 352 μg/kg), respectively. When sample absorbance is below the value obtained for 2.8 nM the methanolic extract could be diluted up to 1:4 and samples re-tested

STABILITY AND STORAGE

The kit contents must be stored at 4 - 12 ºC and protected from light. This kit has a shelf life of 8 months when stored under optimal conditions. See the expiry date on the kit package.

SAFETY

Safety clothing should be worn and skin contact with the reagents avoided. Do not ingest.
A SAFETY DATA SHEET is available from your local distributor on request.

*Warning: Okadaic Acid is toxic. Gloves, mask and other protective clothing must be worn when handling okadaic acid solutions.

REFERENCES

1. Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. *Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin*. *FEBS Lett*. 1987, 21781-21784.

2. Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins.* *Toxins,* 2012, *5*, 339-352.

3. Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study.* *Journal AOAC*, 2013. 96, 1, 77-85.

OkaTest complies with the requirements established under chapter III A (4) a, b and c from Appendix III of the European Regulation (EC) 2074/2005 and can be used as complementary method.
For further information, please visit the European Reference Laboratory website:
http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

OBJETIVO

Test para la determinación cuantitativa de Ácido Okadaico (OA) y otras toxinas del grupo del OA, incluyendo DTX1, DTX2 y DTX3. Consiste en un ensayo colorimétrico de inhibición de la actividad enzimática de una fosfatasa. Este método es aplicable a especies como mejillones, almejas, berberechos, vieiras, etc.

PRINCIPIO

Okatest es un test basado en la inhibición de la actividad enzimática de una fosfatasa (PP2A) por toxinas del grupo del ácido okadaico. En condiciones normales, la fosfatasa es capaz de hidrolizar un sustrato específico obteniéndose un producto que puede ser detectado a 405 nm. En presencia de toxina diarreica se producirá una inhibición de la actividad enzimática proporcional a la cantidad de toxina diarreica presente en la muestra. Mediante la utilización de una curva de calibrado se pueden obtener los valores de concentración de toxina presentes en la muestra analizada.

COMPONENTES DEL KIT

	Kit de 48 Tests	Kit de 96 Tests
Tiras de 8 pocillos de placa microtiter	6	12
Fosfatasa *(Phosphatase)*	2	4
Set de patrones de ácido okadaico *(Okadaic acid 0.5, 0.8, 1.2, 1.8 y 2.8 nM)*	1	1
Sustrato Cromogénico *(Chromogenic Substrate)*	1	1
Solución de Dilución de la Fosfatasa *(Phosphatase Dilution Buffer)*	1	1
Solución Tamponante *(Stock Buffer Solution)*	1	1
Solución Stop *(Stop Solution)*	1	1
Lámina adhesiva	1	2
Guión de instrucciones	1	1

MATERIAL Y REACTIVOS ADICIONALES NECESARIOS

- Micropipetas
- Homogeneizador (e.j. Ultraturax) o mortero
- Incubador a 30 ± 2ºC. (Ej. FX Incubator Ref ZE/FX, de ZEULAB)
- Lector de placas microtiter con filtro a 405 nm.
- Baño termostático 76 ± 2ºC
- Metanol (grado analítico)
- NaOH (grado analítico)
- HCl (grado analítico)
- Agua desionizada (al menos de grado 2, ISO 3696)
- Tubos de centrifuga de 50 mL
- Centrífuga
- Agitador para tubos (tipo vortex)

SOLUCIONES

1.- Estándares de Ácido Okadaico: Es muy importante agitar bien estas disoluciones justo antes de su utilización (p.e.: en vortex), para asegurar su homogeneidad
2.- Sustrato Cromogénico: esta solución contiene una resina estabilizante que no debe añadirse a los pocillos. Con este fin, se recomienda transvasar el volumen a utilizar a un vial transparente (p.e.: eppendorf o tubo de ensayo), asegurándose de no coger resina, y de ahí pipetear a los pocillos. *Nota*: no usar esta solución si la absorbancia de 90 μL es superior a 0.6.
3.- Preparación de la Fosfatasa: reconstituir el liofilizado de Fosfatasa (*Phosphatase*) en 2.0 mL de Solución de Dilución de la Fosfatasa (*Phosphatase Dilution Buffer*). Mantener la solución a temperatura ambiente (22 ± 2ºC) y con agitación suave durante 1 hora para asegurar así la correcta hidratación del liofilizado. No usar el agitador de tubos en ningún momento. Una vez reconstituido el enzima, mantenerlo en condiciones de refrigeración. No conservar la solución de Fosfatasa para su uso en días posteriores.
Cada vial de Fostatasa contiene la cantidad necesaria para 24 pocillos. Si se va a utilizar más de uno, disolver cada vial como se ha explicado anteriormente y mezclar el contenido de todos en uno único antes de usar. Agitar suavemente antes de su utilización.
Atención: el liofilizado posee una coloración azulada y al reconstituirlo se convierte en marrón. Si observa que este reactivo posee una coloración marrón antes de reconstituirlo, no usarlo, ya que podría estar dañado.
4.- Solución Tamponante x1: diluir la *Stock Buffer Solution* incluida en el kit, mezclando 1 volumen de esta solución con 9 volúmenes de agua desionizada. Preparar sólo la que se vaya a utilizar en el momento y mantener en refrigeración hasta entonces.
5.- NaOH 2.5 N: pesar 100 g de NaOH y disolver en 500 mL de agua desionizada. Seguidamente, enrasar hasta un volumen final de 1000 mL usando un matraz aforado.
6.- HCl 2.5 N: Añadir 205 mL de HCl (37 %) a 400 mL de agua desionizada. Mezclar y enrasar hasta 1000 mL con agua desionizada usando un matraz aforado.

EXTRACCIÓN DE LAS MUESTRAS

El método de preparación de muestras que se describe a continuación incluye una etapa de hidrólisis que permite la detección de todas las formas tóxicas de ácido okadaico (ácido okadaico y dinofisistoxinas).

1.- Limpiar la superficie externa del molusco con agua.
2.- Abrir los moluscos seccionando los músculos aductores.
3.- Lavar el contenido de las conchas con agua hasta conseguir eliminar todas las sustancias extrañas que puedan contener.
4.- Separar la carne de las conchas, retirando todos los músculos o tejidos que estén en contacto con ellas.
5.- Colocarlos en un papel de filtro y dejarlos secar durante unos minutos.
Se recomienda el uso de tubos calibrados para centrífuga de 50 mL durante las siguientes etapas de hidrólisis para evitar pérdidas por transvase de líquidos.
6.- Triturar el tejido hasta obtener una muestra homogénea, tomar 5 g (peso húmedo) y extraer con 25 mL de Metanol durante 2 minutos, usando un agitador para tubos.
7.- Centrifugar el homogeneizado a 2000 g durante 10 minutos a 4 ºC. Al sobrenadante lo llamaremos *extracto metanólico* y lo pasaremos a otro tubo de centrífuga por decantación.

8.- Tomar 640 µL del *extracto metanólico* y transvasarlo a un tubo para centrífuga nuevo.
9.- Añadir 100 µL de NaOH 2.5 N.
10.- Cerrar y calentar la muestra a 76 ± 2 ºC durante 40 minutos.
11.- Sin dejar enfriar, añadir 80 µL de HCl 2.5 N
12.- Añadir Solución Tamponante x1 hasta un volumen final de 20 mL.

PROCEDIMIENTO DE ENSAYO

Atención:

En este ensayo se usan reactivos en volúmenes pequeños y se debe tener especial cuidado cuando se añaden a la placa:

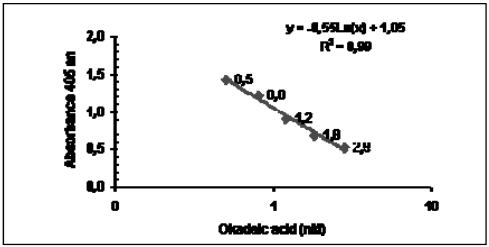
- Asegurarse de que las pipetas están calibradas antes de realizar el ensayo.
- Usar pipetas de 100 ó 200 µL de volumen máximo.
- Comprobar que la temperatura del incubador está estabilizada antes de su uso.

Es aconsejable aplicar las muestras y patrones por duplicado.

1.- Aplicar 50 µL de cada estándar o muestra.
2.- Aplicar en cada pocillo 70 µL de la Solución de Fosfatasa. Mezclar bien golpeando suavemente en el lateral de la placa.
3.- Tapar la placa con la lámina adhesiva incluida en el kit e incubar a 30 ± 2 ºC durante 20 ± 0.5 minutos.
4.- Aplicar 90 µL en cada pocillo de Sustrato Cromogénico y tapar la placa con la lámina adhesiva.
5.- Incubar a 30 ± 2 ºC durante 30 ± 0.5 minutos.
6.- Retirar la lámina adhesiva y añadir en cada pocillo 70 µL de Solución Stop.
7.– Leer la absorbancia a 405 nm en un lector de placas microtiter.

REPRESENTACIÓN Y CÁLCULO DE LOS RESULTADOS

1.- Obtener una curva de calibrado representando las absorbancias en el eje de ordenadas frente a las concentraciones de ácido okadaico en el eje de abscisas (este último en escala logarítmica). A continuación se muestra un ejemplo de curva patrón. R^2 deberá ser mayor o igual a 0.96.



2.- A partir de la curva de calibrado obtener los valores de ácido okadaico de las muestras (Cs) por interpolación o aplicando la ecuación correspondiente:

$$x = EXP\ (y - b/a)$$

x: concentración de ácido okadaico en la muestra
y: absorbancia de la muestra

*ZEULAB puede proporcionar una plantilla Excel para calcular los resultados. Para más información contacte con nosotros.

3.- Calcular la concentración de toxinas diarreicas en el tejido (Ct) a partir de la siguiente fórmula:

$$Ct\ (\mu g/kg) = \frac{Cs\ (nM) \times FD \times PM\ (g/mol) \times Ve\ (L)}{Mt\ (g)}$$

Ct: Concentración de toxinas en tejido; Cs: Concentración de toxinas de cada muestra aplicada en el pocillo; FD: Factor de dilución del extracto metanólico en la preparación de la muestra (p.e. 640 µL/20 mL → x 31.25); PM: Peso molecular ácido okadaico = 805; Ve: Volumen de extracto metanólico obtenido (0.025L); Mt: Masa de tejido pesada inicialmente (5 g).

Ej.: Para una muestra 1.5 nM de OA: 1.5 nM x 31.25 x 805 g/mol x 0.025 L / 5 g = 189 µg eq OA/kg

NOTA: Aquellas muestras cuya concentración (Cs) esté fuera del rango de trabajo (< 0.5 nM ó > 2.8 nM), los resultados se expresarán como < 0.5 nM (ó < 63 µg/Kg) ó > 2.8 nM (ó > 352 µg/kg) respectivamente.
Muestras con absorbancias inferiores a las obtenidas para el patrón 2.8 nM pueden ser analizadas de nuevo haciendo una dilución máxima de 1/4 del extracto metanólico.

ESTABILIDAD Y ALMACENAMIENTO

Conservar los componentes del kit de 4 -12 ºC y en oscuridad. El kit tiene una estabilidad de 8 meses en las condiciones de conservación anteriormente indicadas.

SEGURIDAD

Se recomienda seguir unas prácticas correctas de laboratorio, así como el empleo de ropa y material de seguridad adecuados para el desarrollo del test. Evitar el contacto directo con la piel. No ingerir.

*Atención: El ácido okadaico es un producto tóxico, para su manejo es imprescindible el uso de guantes y trabajar con precaución.
Puede solicitar la hoja de seguridad del producto contactando con su distribuidor habitual o fabricante.

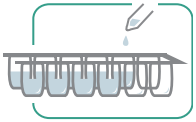
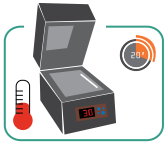
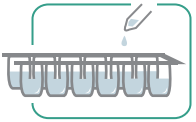
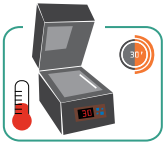
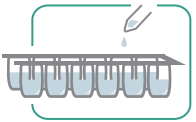
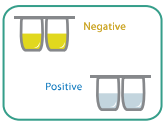
BIBLIOGRAFÍA

1.- Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. *Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin*. *FEBS Lett*. 1987, 21781-21784.

2.- .Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins,* 2012, *5*, 339-352.

3.- Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal AOAC*, 2013. 96, 1, 77-85.

OkaTest cumple con los requisitos del capítulo III A (4) a, b y c del Anexo III de la Regulación Europea (EC) 2074/2005 y puede ser usado como método complementario tal y como indica el Laboratorio de Referencia Europeo en su página web: http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

FLOWCHART PROCEDURE	*ESQUEMA DEL PROCEDIMIENTO*
	
1. Add 50 µL samples/standars	*1. Añadir 50 µL muestras/estándares*
2. Add 70 µL Phosphatase Solution	*2. Aplicar 70 µL Solución de Fosfatasa*
	
3. Incubate 20 min at 30ºC	*3. Incubar 20 min a 30ºC*
	
4. Add 90 µL Cromogenic Substrate	*4. Añadir 90 µL Sustrato Cromogénico*
	
5. Incubate 30 min at 30ºC	*5. Incubar 30 min a 30ºC*
	
6. Add 70 µL Stop Solution	*6. Añadir 70 µL Solución Stop*
	
7. Read absorbance at 405 nm	*7. Leer absorbancia a 405 nm*

DSP PPIA kit-OkaTest

Single Laboratory Validation Report

1- EXECUTIVE SUMMARY 2

2- METHOD PRINCIPLE AND SCOPE 3

3- VALIDATION 3

3.1 Accuracy/Truness 3

3.2. Measure of Uncertainty 4

3.3. Precision 5

3.4. Recovery 7

3.5. Specificity 8

3.6. Working Range and Linear Ranges 8

3.7. Limit of detection and Limit of quantification 11

3.8. Ruggedness 12

3.8.1- Assay temperature 12

3.8.2- Assay incubation times 12

3.8.3- Influence of pipetting volumes 13

3.8.4- Influence of phosphatase solubility 14

3.8.5- Ruggedness between batches in samples 15

3.9- Matrix Effects: 17

3.10. Method comparison Comparability 18

4- LITERATURE 23

1- EXECUTIVE SUMMARY

The **DSP PPIA (**commercial name **OkaTest)** is a test for detection of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. It is a rapid and simple method suitable for quantitative determination of the OA- toxins group from 63 to 352 µg of OA equivalents per Kg, including the maximum limit established as 160 µg of OA equivalents /Kg in the Commission Regulation of 29 April 2004 (Regulation (EC) 853/2004). Test applicable to shellfish species such as mussels, clams, oysters and scallops.

The **OkaTest** kit was developed by ZEULAB (previous name ZEU- INMUNOTEC) based on the research work carried out by Vieytes et al. The method uses the inhibitory activity of OA and DTXs against the enzyme phosphate, which is responsible for their toxic effect, for the detection of OA-toxins group in molluscs. **OkaTest** uses a colorimetric detection system (Takai and Mieskes, 1991), while the original method (Vieytes et al., 1997) was based on fluorimetric detection.

A single laboratory validation was carried out at ZEULAB, followed by a collaborative study with 16 laboratories from 11 different countries. Both validations have been published in scientific journals; Toxins in 2012 by Smienk et al. and Journal of AOAC in 2013 by Smienk et al., respectively. Besides, OkaTest participates annually in intenational proficency exercises (Quasimeme, The Neatherlands).

OkaTest complies with the requirements established by the European Regulation (EC) 2074/2005 as complementary to the reference method.

http://www.aecosan.msssi.gob.es/en/CRLMB/web/public_documents/seccion/other_crlmb_standard_operating_procedures.htm.

Furthermore, **OkaTest** has been compared with other methodologies and using samples from the USA, UK and Argentina (Bich-Thuy et al., 2013, Turner & Goya, 2016 and Johnson et al., 2016).

This report shows the data obtained in the initial single laboratory validation (Smienk et al, 2012) that has been completed with additional information requested by the ISSC. Following a summary of the validation parameters:

Parameter	Results
Accuracy/Truness	98,00%
Measurement Uncertainty	14.92 - 31.08 µg equivalentes OA /kg
Precision	
Repeatibility:	1,4%- 3,9 % (Mean= 2,65%)
Reproducibility	0,8 %-17,7% (Mean= 6,45%)
Recovery	Okadaic acid: 78-114%
	DTX-1: 79-102%
	DTX-2: 83-94%
Working Range	63 - 352 µg equivalents OA /kg
Limit of Detection (LOD)	44 µg equivalets OA /kg
Limit of Quantification (LOQ)	56 µg equivalents OA/kg

2- METHOD PRINCIPLE AND SCOPE

DSP PPIA (OkaTest) is a protein phosphatase inhibition assay (PPIA), where the phophatase activity is ihnibited by the OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). The PPIAs have been identified for a long time as an alternative for the detection of the OA-toxins, as Ser/Thr phosphatases are known to be their natural target (Bialojan & Takai, 1988). Under normal circumstances, a phosphatase enzyme is able to hydrolyse a specific substrate producing a reagent that can be detected by absorbance measurement (405 nm). Samples containing OA toxins will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample.

OkaTest is applicable to shellfish species such as mussels, clams, oysters and scallops. It is a quantitative method for determination of the OA- toxins group, where concentration of toxins present in the sample is calculated using a standard curve.

OkaTest includes five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), phosphatase enzyme and substrate reagents ready to use.

The test procedure is extensively described in the user manual G-COM-OA.06.

3- VALIDATION

To evaluate the performance of the OkaTest kit, accuracy, uncertainty, precision, limit of detection and quantification were calculated. The assay temperature, incubation times and other variables affecting rugedness, together with specificity and matrix effects were also evaluated. Finally, a method comparison was carried out.

3.1 Accuracy/Trueness

To estimate the accuracy of the method 20 blank mussel samples (*Mytilus edulis*) were spiked with OA at 80, 120, 160, 240 and 300 µg/kg. Percentage of recoveries were calculated and are shown in Table 1.

Table 1. Recovery values from 20 different mussels samples spiked with OA at different levels along the working range. Mean (M), standard deviation (SD) and relative standard deviation (RSD). ND < 63 µg/kg

	µg OA equivalents/kg					
Theoretical Spike	**Before spiked**	**After spiked**	**Recovery**	**Mean**	**SD**	**RDS**
80	ND	73	91.3%			
80	ND	91	113.8%			
80	ND	87	108.8%	**112.5%**	**0.18**	**16.68%**
80	ND	112	140.0%			
80	ND	87	108.8%			
120	ND	133	110.8%	**106.7%**	**0.06**	**5.52%**
120	ND	123	102.5%			
160	ND	128	80.0%			
160	ND	169	105.6%	**98.8%**	**0.13**	**12.98%**
160	ND	173	108.1%			
160	ND	162	101.3%			
200	ND	186	93.0%			
200	ND	185	92.5%	**91.3%**	**0.30**	**0.27%**
200	ND	177	88.5			
240	ND	219	91.3%			
240	ND	205	85.4%	**96.1%**	**0.21**	**21.59%**
240	ND	195	81.3%			
240	66	304	126.7%			
300	ND	250	83.3%	**82.7%**	**0.01**	**1.14%**
300	ND	246	82.0%			

3.2. Measure of Uncertainty

Measurement of uncertainty was calculated using the results obtained in the accuracy experiment considering a confidence interval of 95%. Mean and standard deviation of the difference between the concentration of the spiked sample and the spiked amount were calculated. The coefficient of confidence (Z) and maximum error (E. max) were then determined (Table 2) according to the following equation:

$$E.\ max = Z_{\alpha/2} * SD/\sqrt{n},\ \text{where}$$

E. max: maximum error, **Z:** confidence coefficient; α 95% confidence interval**, SD**: standard deviation, **n**: number of samples.

Table 2. Estimation of uncertainty based on recovery data from 20 different mussels. ABS: absolute value of differences between OA concentration in spiked samples and spike concentration. Z= coefficient of confidence. SD= standard deviation. ABS E. max=absolute value of maximum error. ND < 63 µg/kg

Sample	Spike (OA µg/kg)	Blank Sample µg OA equiv. /kg	Spiked Sample	Recovery	ABS differences	Mean	SD	ABS E. Max
1	80	ND	73	91.3%	7	13	10.87	9.53
2	80	ND	91	113.8%	11			
3	80	ND	87	108.8%	7			
4	80	ND	112	140.0%	32			
5	80	ND	87	108.8%	7			
6	120	ND	133	110.8%	13	8	7.07	6.20
7	120	ND	123	102.5%	3			
8	160	ND	128	80.0%	32			
9	160	ND	169	105.6%	9			
10	160	ND	173	108.1%	13			
11	160	ND	162	101.3%	2	14	12.83	11.25
12	200	ND	186	93.0%	14			
13	200	ND	185	92.5%	15			
14	200	ND	177	88.5%	23	17	4.95	4.34
15	240	ND	219	91.3%	21			
16	240	ND	205	85.4%	35			
17	240	ND	195	81.3%	45			
18	240	66	304	126.7%	64	41	18.08	15.85
19	300	ND	250	83.3%	50			
20	300	ND	246	82.0%	54	52	2.83	2.48
				Mean	**23**			
				SD	**18.44**			
				ABS E. Max	**8.08**			

3.3. Precision

To determine the precision of the method, relative standards devidation (RSD) for repetibility and reproducibility were calculated.

To calculate repeatability eight replicates of two mussel samples at two levels of concentration were analysed on the same day. Mean, standard deviation and relative standard deviation were calculated. The RSD obtained for the samples tested were, 1.4 and 3.9%, respectively. These values are far below the reference value of 15% (Horwitz W., 2002).

Table 3. Repeatability of 2 different mussel samples. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	Sample 1 (µg OA equiv./kg)	Sample 2 (µg OA equiv./kg)
1	269	124
2	276	125
3	276	131
4	273	129
5	280	121
6	278	117
7	281	127
8	275	118
Mean	**276**	**124**
SD	**3.9**	**4.8**
RSD	**1.4%**	**3.9%**

Intermediate precision/Reproducibility

Intermediate precision was estimated by testing 13 different samples (10 mussel samples and 3 from other species) at different levels of concentration on 3 different days by the same analyst (Table 4).

Mean values, standard deviation and relative standard deviation were calculated. An average of 6.45% of RSD was calculated for all the samples with different levels of concentration. Only sample 3, at a concentration below the regulatory limit showed a RSD above 15%, which is the variability expected for this concentration range (Horwitz, 2002).

Table 4. Reproducibility of thirteen different mussel (*Mytilus edulis*), king scallop (*Pecten maximus*) and clam (*Venerupis pullastra* and *V. vomboides*) samples. Mean, standard deviation (SD), relative standard deviation (RSD) were calculated.

Sample	Matrix	Day 1	Day 2	Day 3	Mean	SD	RSD
		µ OA equivalents /kg					
1	Mussel	82	94	90	**88**	6.17	**7.0%**
2	Mussel	106	95	90	**97**	8.05	**8.3%**
3	Mussel	98	101	72	**90**	15.95	**17.7%**
4	Mussel	109	106	95	**101**	7.80	**7.8%**
5	King Scallop	125	108	117	**117**	8.20	**7.0%**
6	Mussel	122	132	113	**122**	9.57	**7.8%**
7	Mussel	196	196	215	**202**	10.57	**5.2%**
8	Mussel	211	227	187	**208**	19.84	**9.5%**
9	Clam	261	251	260	**257**	5.51	**2.1%**
10	Mussel	257	250	258	**255**	4.36	**1.7%**
11	Mussel	250	253	281	**261**	16.90	**6.5%**
12	Mussel	277	279	289	**282**	6.62	**2.4%**
13	Clam	285	285	281	**284**	2.31	**0.8%**

The intermediate precision was also further evaluated in a collaborative study with 5 samples analysed by 16 different laboratories. Values of 11.2% and 13.2% were determined as the

highest relative standard deviation for repeatability and reproducibility, respectively (Smienk et al 2013).

3.4. Recovery

Recovery was calculated by spiking mussel and scallop samples (*Mytilus edulis* and *Pecten maximus*, respectively) with okadaic acid (OA) at 0.5, 1 and 1.5 times the regulatory limit. Samples were also spiked with 80, 160 and 240 µg/kg of DTX-1 and 80 and 160 µg/kg of DTX-2. Three to five repetitions of each concentration were analysed on different days. Results are shown in Tables 5 and 6.

Table 5. Results (µg OA equivalents/kg) from recovery of OA in mussel and scallop samples at 80, 160 and 240 µg/kg. Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	Mussel				King Scallop			
	spiked OA (µg/kg)							
Repetition	**0**	**80**	**160**	**240**	**0**	**80**	**160**	**240**
1	86	158	230	271	ND	82	162	252
2	87	134	211	282	ND	84	142	218
3	87	178	216	257	ND	89	150	268
4	95	193	253	298	ND	102	177	268
5	95	191	257	280	ND	99	158	271
Mean	**90**	**171**	**233**	**277**	**-**	**91**	**157**	**255**
SD	4.8	25.0	20.9	15.1	-	9.0	13.3	22.2
RSD	5.4%	14.6%	8.9%	5.4%	-	9.9%	8.4%	8.7%
Recovery	**-**	**101%**	**90%**	**78%**	**-**	**114%**	**98%**	**106%**

Table 6. Results (µg OA equivalents/kg) from recovery of DTX-1 and DTX-2 in mussel and scallop samples spiked at 80, 160 and 240 µg/kg. Mean, Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	King scallop				Mussel					
	spiked DTX - 1 (µg/kg)				spiked DTX2 (µg/kg)					
Repetition	**0**	**80**	**160**	**240**	**0**	**160**	**0**	**80**	**0**	**160**
1	ND	63	101	211	ND	145	86	157	ND	128
2	ND	91	127	179	ND	156	101	163	ND	130
3	ND	81	132	175	ND	151	-	-	ND	124
4	ND	82	132	261	-	-	-	-	-	-
5	ND	93	140	228	-	-	-	-	-	-
Mean	**ND**	**82**	**126**	**211**	**ND**	**151**	**93.5**	**160**	**ND**	**127**
SD	-	11.9	14.8	35.6	-	5.5	-	4.2	-	2.7
RSDr	-	14.5%	11.7%	16.9%	-	3.7%	-	2.7%	-	2.1%
Recovery	**-**	**102%**	**79%**	**88%**	**-**	**94%**	**-**	**83%**	**-**	**80%**

The mean of recoveries obtained for the different concentrations tested and toxins were acceptable and ranged from 78 to 114%.

3.5. Specificity

Specificity was studied by determining the possible interferences caused by other lipophilic toxins such as Azaspirazides (AZA), Yessotoxins (YTX) and Pectenotoxins (PTX).

A mussel sample naturally contaminated was spiked, on two different days, with 160 µg/kg of AZA-1 (NRC, Institute for Marine Biosciences, Canada), 160 µg/kg PTX-2 (Cifga laboratories, Spain) and 1000 µg/kg of YTX (NRC, Institute for Marine Biosciences, Canada) and concentration of OA determined following the kits´ instructions. Results obtained for spiked and non-spiked samples were very similar and within the method variability, showing no interferences by the toxins tested.

Table 6. Results obtained from spiking a mussel sample with 160 µg/kg of azaspirazides (AZA), 160 µg/kg of pectenotoxins (PTX) and 1000 µg/kg of yessotoxins (YTX).

Spiked Mussel	Day 1	Day 2
	µg equiv. OA /kg	
0	82	82
160 (µg/kg) **PTX-2**	83	79
160 (µg/kg) **AZA-1**	82	73
1000 (µg/kg) **YTX**	82	82

3.6. Working Range and Linear Ranges

The working range is understood as the range of OA concentrations that do correctly adapt to the fitting procedure. The working range of the assay depends on the quantity and quality of the phosphatase present. Therefore, assays were performed with at least 3 different phosphatase batches and the "goodness of fit" was evaluated according to the kits´ specifications ($R^2 > 0.96$) with standard concentrations rising from 0.25 to 3.5 nM OA.

Figure 1 shows the results of three assays covering the range from 0.25 to 3.0 nM OA as this was the range that always fitted correctly (R^2>0.96). This covers sufficiently the actual range of the standards in the kit (0.5 to 2.8 nM OA).

The linearity of an assay was tested to find out whether the response of this assay is a function of the concentration of the analyte. The OkaTest assay uses a logarithmic fitting procedure.

As such the linearity of the assays' response was tested by 'backcalculation' of the standard concentration. For 'backcalculation' the equation of the standard curves used to calculate the concentration of these standards from their absorbances (Table 7).

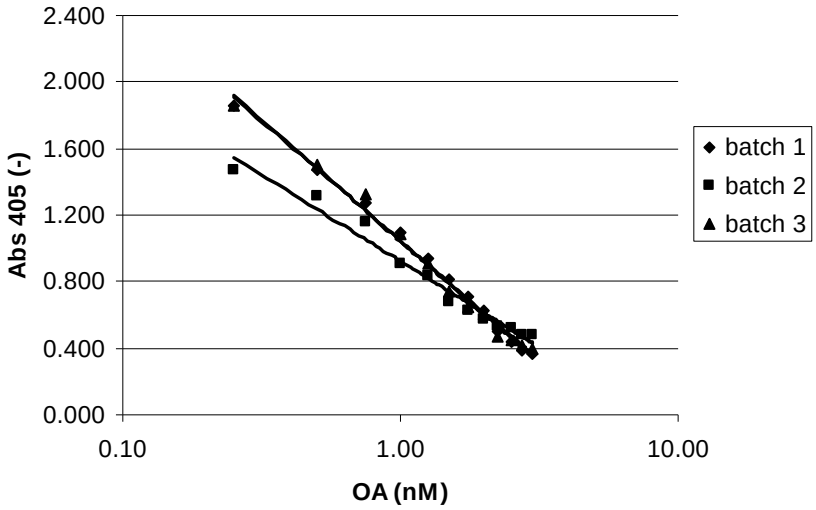


Fig 1. Working range of the assay for 3 different phosphatase batches. R^2: 0.99, 0.98 and 0.99 for batch 1, batch 2 and batch 3, respectively. Working range from 0.25 to 3.0 nM OA.

Table 7. Linearity of the assay. OA (nM) was calculated by using the standard curve of batch 1.

Standards OA (nM)	Batch 1 OA (nM)	Batch 2 OA (nM)
0.5	0.6	0.5
0.8	0.7	0.7
1.2	1.1	1.2
1.8	1.9	1.9
2.8	2.9	2.8

To check the linearity of the response, the theoretical concentration was compared to the calculated concentration for both batches (see figure 2 for the results shown in Table 1) and a linear fit was performed. The Pearson correlation coefficient (R^2) for batch 1 was 0.99 and 1.00 for batch 2.

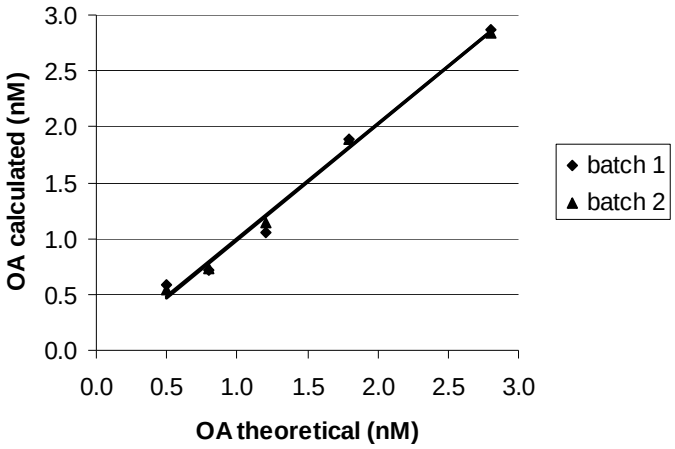


Fig 2. Comparison of the theoretical and calculated standard concentration. Concentration were `backcalculated´ by using the standard curve obtained with batch 1. R^2: 0.99 and 1.00 for batches 1 and 2, respectively.

The linearity was also determined by testing 10 blank mussel samples spiked at 80, 160, 200, 240 and 300 µg/kg (Table 8). OA concentrations obtained were divided by the spiked concentration (relative recovery). Mean of relative recovery per concentration was plot against the spiked concentration and curve equation to observe the relative response (Figure 3).

Table 8. Assay linerarity. Results obtained from 10 blank samples spiked with 80, 120, 160, 200 and 240 µg/kg to determine linearity of the assay.

Spike OA µg/kg	After spiked µg OA equiv. /kg	Relative recovery	Mean Relative Recovery
80	91	1,14	1,11
80	87	1,09	
160	169	1,06	0,96
160	162	1,01	
200	186	0,93	0,91
200	177	0,88	
240	219	0,91	0,88
240	205	0,85	
300	250	0,83	0,83
300	246	0,82	

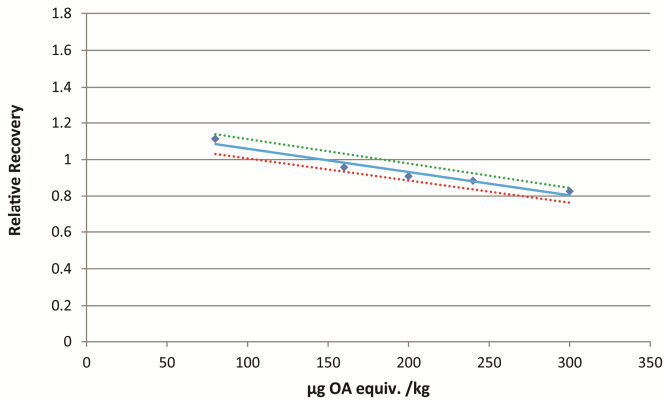


Figure 3. Assay linearity. Relative recovery data plot against spiked OA concentration µg/kg in solid blue line. Green and red dotted lines were obtained by multiplying the OA concentration by 0.95 and 1.05.

3.7. Limit of detection and Limit of quantification

To estimate the LOD and LOQ a blank mussel material was extracted ten times and analyzed according the kits' instructions. The mean and standard deviation were calculated and the limit of detection was estimated by the equation below:

$$LOD_{99\%} = X + 3SD$$

The LOQ (the lowest concentration that can be determined with an acceptable level of repeatability precision and trueness) was estimated using the same data and equation, but applying a higher factor:

$$LOQ_{99\%} = X + 10SD$$

The mean result obtained for the blank sample was 38 µg/kg. The estimated LOD and LOQ were 44 µg/kg and 56 µg/kg, respectively (Table 11).

Table 9. Quantification of the standard solvent (10 repetitions) as OA concentration equivalents (µg/kg) to estimate the LOD and LOQ. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	µg OA equivalents /kg
1	36
2	38
3	36
4	37
5	41
6	37
7	40
8	38
9	40
10	38
mean	38
SD	1.8
RSD	4.6%
LOD	**44**
LOQ	**56**

3.8. Ruggedness

The influence of different experimental conditions critical for the kits' performance such as assay temperature, incubation times or reaction component volumes were evaluated. The ruggedness between batches with spiked mussel samples was also evaluated.

3.8.1- Assay temperature

The hydrolysis of the substrate by the phosphatase is temperature dependent and shows the typical behaviour of an enzymatic reaction with higher reaction rates close to the optimum temperature (37°C). However, a lower assay temperature was chosen to guarantee enzyme stability during the assay and to get stable reaction rates. The assay was tested at temperatures varying from 20 to 40 °C. 30 °C was chosen as the optimum temperature. At this temperature a 2 °C variation can be expected in any incubator. So, to show the influence of this temperature variation, 3 samples were quantified performing a complete assay (standard curve and samples) at each of these temperatures (Table 10).

Table 10. Influence of the assay temperature on the results of the test. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	28 ºC	30 ºC	32 ºC	mean	SD	RSD
1	104	100	97	100	3.4	3.4%
2	176	173	176	175	1.7	1.0%
3	302	303	298	301	2.6	0.9%

Mean and relative standard deviation were calculated. For all three samples RSD were below the 15%, variation that can be expected at this concentration (Horwitz, 2002).

3.8.2- Assay incubation times

The assay consists of two different incubation steps that could affect the outcome of the test. During the first incubation the sample and the phosphatase are mixed, and the inhibition reaction should reach its endpoint. Following, the substrate is added and the plates are incubated for the second time. The main risk of this incubation step is phosphatase activity loss.

To determine the influence of time on the first incubation of the assay (normally 20 minutes), this step was varied between 18 and 24 min, while maintaining the rest of the assays' conditions according the kits' instructions. Three control samples were quantified and the variation in the relative standard deviation was evaluated. For each of the incubation conditions an independent assay was performed (Table 11).

Table 11. Influence of time on the first incubation of the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 2 min	0	+ 2 min	+ 4 min	mean	SD	RSD
1	85	87	87	90	88	2.1	2.4%
2	152	155	161	164	158	5.7	3.6%
3	311	291	317	320	310	12.9	4.2%

In all cases the assay complied with the criterion (R^2>0.96). The relative standard deviations were comparable to those obtained when performing the test under standard conditions (highest 4.2%).

The second incubation was evaluated similarly. A 10% error from normal incubation time (30 minutes) was applied, adding some extra time (up to 20% or 6 minutes). The assay was performed as described; although no stopping solution was added to permit reading the same assay. The RSD was 2.9% at highest, a bit lower than the ones obtained for the first incubation time (Table 11).

Table 12. Influence of the incubation time (2nd incubation with the substrate) on the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 3 min	0	+ 3 min	+ 6 min	mean	SD	RSD
1	89	90	89	91	90	1.0	1.1%
2	143	152	145	149	147	4.3	2.9%
3	309	321	315	313	315	5.2	1.7%

3.8.3- Influence of pipetting volumes

The OkaTest assay consists of three pipetting steps of relatively small volumes. First, 50 µL samples of standards are applied in duplicate and 70 µl of phosphatase is added. Then, after the first incubation, 80 µL of substrate and finally 70 µL of stopping solution are added. The influence of pipetting error was evaluated by introducing a 2 µL systematic error in each of the pipetting steps, e.g. a -2 µL error means pipetting 48, 68, 78 and 68 µL for samples/standard, phosphatase, substrate and stopping solution, respectively. This relatively big error (4% of the sample volume) is quite above the systematic error that can be expected in correctly calibrated pipettes (2%), but it was chosen in order to get clear results for obvious interpretation. The RSD and error were evaluated (Table 13).

Table 13. Effect of the sistematic pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Sample	- 2 µL	0	+ 2 µL	mean	SD	RSD	E. Max*
1	83	85	93	87	4.9	5.6%	8.0 (9.4%)
2	161	148	156	155	6.7	4.3%	13 (8.8%)
3	303	289	304	299	8.5	2.8%	15 (5.1%)

*E. max = maximum difference from standard (0) conditions in µg/kg and percentage.

The RSD was at highest 5.6% and in accordance with the values normally obtained with OkaTest. The error introduced changed from 9.4 to 5.1% of the standard conditions.

The effect of a single pipetting error was evaluated by introducing a 5 µL error in one of the pipetting steps. In this case, the standard curve was performed according the kits' instructions and the error was introduced in the samples that were quantified. For example, a -5 µL error in the phosphatase means that 65 µL phosphatase was added to 50 µL sample (in duplicate) after which the assay was performed as usual. Also in this case, a relatively big error was chosen (10-6.3 % error, depending on the assay volume) (Table 14).

Table 14. Effect of a single pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Variable	-5 µL	0	+ 5 µL	mean	SD	RSD	E Max*
Sample	132	148	173	151	20.5	13.6%	25 (17%)
Phosphatase	180	148	130	153	25.2	16.5%	32 (22%)
Substrate	167	148	159	158	9.6	6.1%	19 (13%)
Stop solution	170	148	153	157	11.6	7.4%	22 (15%)

*E. max = maximum difference from standard (0) conditions in µg/kg and percentage.

Table 14 shows that pipetting errors in sample and phosphatase volume have the biggest effect and special care have to be taken when applying these. Also the logical tendencies can be seen; when applying less samples underestimation can be expected, while with the phosphatase occurs the contrary. This is to be expected, less phosphatase means more inhibitor per amount of phosphatase and so higher estimates of the toxin concentration. Table 14 also shows that high RSDr values (above 10%, ZEULAB in-house 5%) are a good indication for pipetting error. Substrate and stop solution pipetting errors seem to be much less important RSDr < 10%.

3.8.4- Influence of phosphatase solubility

In the previous paragraph was shown that the amount of phosphatase added to each well is important for correct quantification. The phosphatase is the only component of the kit that is not ready to use. It has to be dissolved previously and insufficient solubilisation could lead to

overestimation of the toxin concentration. Therefore the solubilisation time was evaluated by dissolving three phosphatase vials of the same batch for 30, 60 and 90 minutes (normal resuspension time use is 60 minutes), and always under agitation. Three control samples were quantified and the RSD was evaluated (table 15).

Table 15. Test results after dissolving the phosphatase for 30, the normal 60 and 90 minutes. The remaining part of the assay was performed according to the kits instructions. Mean, standard deviation (SD), relative standard deviation (RSD) were calculated.

Sample	30 min	60 min	90 min	Mean	SD	RSD
1	100	95	99	98	2.5	2.5%
2	167	151	157	158	8.0	5.0%
3	317	304	318	313	8.1	2.6%

The RSD values obtained were at highest 5.0% and comparable to those obtained for within batch variability (see table 15).

3.8.5- Ruggedness between batches in samples

The ruggedness of the assay with molluscs samples was also determined. Ten blank mussel samples were spiked at 80, 120, 160, 200 and 240 µg/kg of okadaic acid and tested following the kits´ instructions in two different days and using two different batches. Differences between concentrations obtained in each batch for the different samples were calculated. Mean and standard deviation of the differences together with the experimental t-score and critical t values were also determined (Table 16).

$$t\,exp = \frac{|Mean|}{\frac{s}{\sqrt{n}}}$$

Mean ≡ mean of the difference of skewness

s ≡ Standard deviation; n ≡ number of samples

The critical value was calculated for a significance of α = 0.05 (95% confidence) for n-1 degrees of freedom. If the calculated value of experimental-t is less than the critical-t, we can affirm that the hypothesis is true, so that there is an equivalence between both methods.

The experimental t-score was smaller than the critical t-value (t exp< t crit; 1.42<2.26); and so the range of skewness was acceptable. There is not significant difference between batch 1 samples and batch 2 concentrations.

Table 16. Results from testing 10 different mussel samples spiked at different concentrations and tested with two different batches in two different days. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Batch No. 1	Batch No. 2	Differences between batches
	OA equivalents µg/kg		
1	91	74	-17
2	87	79	-8
3	133	102	-31
4	123	150	27
5	169	145	-24
6	162	177	15
7	186	177	-9
8	185	168	-17
9	219	174	-45
10	159	169	10
		Mean	**-9.9**
		SD	**22.01**
		Experimental t-score	**1.42**
		Critical t-value	**2.26**

The data was also analyzed using a Welch's test or unequal variances t-test, which is a two-sample location test used to check the hypothesis that two populations have equal means (H_0).

Therefore, considering that the null hypothesis (H_0) refers to the fact that the two batches do not show differences in the analysis of samples spiked with a known concentration of okadaic acid. Mean, variance and p-value were calculated (Table 17).

P-value (0.603) was higher than 0.05 (0.603>0.05), therefore we do not reject the null hypothesis. The observed difference between the sample´s means is not convincing enough to say that the average value between both batches differing significantly.

Table 17. Mean, variance and p-value calculated for results obtained from 10 spiked mussel samples tested with bath 1 and batch 2 of OkaTest (results from Table 16).

	Batch 1	Batch 2
Mean	151.40	141.50
Variance	1812.93	1682.50
p value	0.603	

Residual values analysis evaluates the goodness of the test. A linear relationship is confirmed when the residues have symmetry around zero and a homogeneous random dispersion. Graphical representation is the most common methodology, being a very visual and simple method to evaluate symmetry. Residual standard values were also calculated (table 18) and

the distribution plotted. The adjustment is adequate since the residual values have a random and homogeneous distribution around 0, being between ± 2 (Figure 4).

Table 18. Residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest.

Sample	Residual standard values
1	5.51E-05
2	-0.39
3	0.79
4	-1.67
5	0.67
6	-0.98
7	0.13
8	0.46
9	1.78
10	-0.79

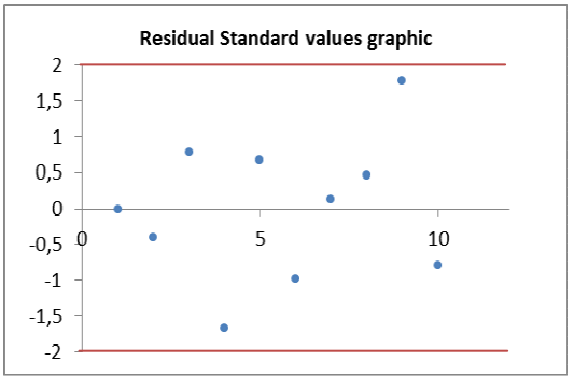


Figure 4. Distribution of residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest

3.9- Matrix Effects:

To determine the matrix effect 10 different molluscs' samples were tested according to the kit instructions and further diluted; where the final concentration of diluted samples was calculated multiplying by the appropriate dilution factor. Mean and SD of the differences between concentrations for diluted samples were calculated.

To evaluate if the concentrations obtained for diluted samples were within the assay variability and not due to matrix effect the experimental t-score and t-critical values were calculated (Table 19):

As the experimental t-score is smaller than the critical t-value (0.93<2.26) the skewness obtained is acceptable and does not indicate matrix effect.

Table 19. OA equivalents µg/kg for 10 mussel samples tested a two different dilutions. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Dilution 1	Dilution 2	Diferences
	OA equiv. µg/kg		
1	40	37	-3
2	980	974	-6
3	29	30	1
4	620	628	8
5	595	560	-35
6	138	104	-34
7	1192	1287	95
8	1149	1318	169
9	118	108	-10
10	85	87	2
		mean	**18.7**
		SD	**63.84**
		Experimental- t score	**0.93**
		Critical-t value	**2.26**

3.10. Method comparison

A method comparison was performed with the mouse bioassay (MBA), reference method in Europe until 2011 and LC-MS/MS (current official reference method in Europe).

To compare results from OkaTest and MBA, values obtained by OkaTest with a concentration ≥ 160 µg/kg were regarded as positive while samples with a concentration < 160 µg/kg were reported negative.

Twenty-three out of thirty-one samples tested positive for both methods and five samples were negative for both methods. However, three samples were positive for MBA and negative for OkaTest (Table 20). In all three samples OA toxins were detected, but below the regulatory limit of 160 µg/kg (144, 135 and 124 µg/kg OA toxins, respectively). Those samples were also tested by LC-MS/MS where two out of three results were above the regulatory limit as well by MBA, showing slightly higher quantifications compared to OkaTest (185, 152 and 177 OA toxins µg/kg, respectively).

OkaTest was compared with LC-MS/MS for a total of 69 samples, where results from the reference method came from two different laboratories (Tables 20 and 21).

Table 20. Results from MBA, OkaTest and LC-MS/MS. Positive results (+): ≥160 µk/kg. Negative result (-): <160 µg/kg. *HPLC-MS results were not with toxicity factors. However only 4 samples contained DTX-2. LC-MS/MS carried out at the Reference Laboratory in Vigo.

Sample	Matrix	MBA	OkaTest	OkaTest µg OA equiv. /kg	LC-MS/MS µg OA /kg
1	**Mussel**	-	-	122	ND
2	**Scallop**	-	-	ND	ND
3	**Mussel**	-	-	ND	ND
4	**Donax**	-	-	97	82
5	**Cockle**	-	-	ND	ND
6	**Mussel**	+	+	196	158
7	**Mussel**	+	+	232	502
8	**Mussel**	+	+	268	ND
9	**Scallop**	+	+	264	184
10	**Mussel**	+	+	250	177
11	**Mussel**	+	+	265	288
12	**Mussel**	+	+	196	318
13	**Mussel**	+	+	>377	604
14	**Mussel**	+	+	>377	894
15	**Mussel**	+	+	277	390
16	**Mussel**	+	+	305	658
17	**Mussel**	+	+	306	414
18	**Mussel**	+	+	310	392
19	**Mussel**	+	+	>377	444
20	**Mussel**	+	+	315	329
21	**Mussel**	+	+	270	232
22	**Mussel**	+	+	277	235
23	**Mussel**	+	-	135	152
24	**Mussel**	+	+	164	98
25	**Mussel**	+	+	211	168
26	**Mussel**	+	+	251	209
27	**Mussel**	+	+	191	113
28	**Mussel**	+	-	124	177
29	**Cockle**	+	+	252	193
30	**Mussel**	+	+	216	247
31	**Mussel**	+	-	144	185
32	**Mussel**		-	ND	ND
33	**Mussel**		+	>377	357
34	**Mussel**		-	ND	292
35	**Mussel**		-	ND	ND
36	**Mussel**		-	ND	ND
37	**Mussel**		+	304	316

A comparison of OkaTest and the reference method LC-MS/MS was made for those samples which showed a quantitative value with both methods. The samples were analyzed by paired t-test to determine the equivalence of the two analytical methods, comparing both means to determine if the difference between the expected means surpasses the one produced randomly.

The hypothetical difference of Means should be zero (Null hypothesis H_0), which means that both methods are considered equivalents.

Table 21. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 20.

	OkaTest	LC-MS
Mean	240.33	281.71
t-statistic	1.74	
P(T≤t) value (probability value) for the t-statistic (one-tailed)	0.048	
Critical value of a t-distribution (one-tailed)	1.72	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.097	
Critical value of a t-distribution (two-tailed)	2.09	

The null hypothesis was accepted because critical- t two-tail < t Stat < t Critical two-tail (-2.09 < -1.74 < 2.09) and p (0.097)>0.05. The observed difference between the sample means (240.33 and 281.71) was not convincing enough to say that the average value between LC-MS and Okatest differ significantly.

Besides, the test t was applied manually to the difference of values obtained for each sample. For this application, the value of the experimental t-score statistic was calculated, as well as the critical t- value:

We could affirm that the hypothesis is true because the calculated experimental-t value was smaller than the critical-t value (1.65<2.08). The skewness is acceptable and the methods Okatest and LC-MS/MS are considered to be similar (Table 22).

Table 22. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	37.77
SD	107.56
Number of samples	22
Experimental t-score	1.65
Critical-t value	2.08

Table 23. Results from OkaTest and LC-MS/MS (EU harmonized SOP, v2, 2010)
Assays carried out by Jonathan Deeds from the FDA, US

Shellfish/Location	OkaTest µg equiv. OA/Kg	LC-MS/MS µg equiv. OA/Kg	OA	DTX1	DTX2
Softshell Clams (*Mya arenaria*) State: New York US East Coast Atlantic Ocean	336	373	255	118	ND
	315	325	202	105	ND
	295	307	217	108	ND
	285	260	136	69	ND
	240	205	171	89	ND
	190	155	102	53	ND
	118	75	49	26	ND
	<63	39	26	13	ND
	<63	ND	ND	ND	ND
Oysters (*Crassostrea virginica*) State: Texas US Gulf Coast Gulf of Mexico	322	563	563	ND	ND
	300	519	519	ND	ND
	245	202	202	ND	ND
	240	194	194	ND	ND
	239	221	221	ND	ND
	235	189	189	ND	ND
	198	189	189	ND	ND
	155	88	88	ND	ND
	154	97	97	ND	ND
	88	38	38	ND	ND
	<63	16	16	ND	ND
Mussels (*Mytilus edulis*) State: Washington US West Coast Pacific Ocean	>352	525	ND	525	ND
	266	272	ND	272	ND
	256	263	ND	263	ND
	171	165	ND	165	ND
	157	164	ND	164	ND
	141	131	ND	131	ND
	134	128	ND	128	ND
	127	121	ND	121	ND
	90	76	ND	76	ND
	<63	76	ND	76	ND
	<63	33	ND	33	ND
	<63	ND	ND	ND	ND

Mussels' results were statistically analyzed by applying a t-Student match pairs test to the results above the limit of quantification for each method.

The null hypothesis was accepted because the critical-t two-tail < t Stat < Critical-t two-tail (-2.37 < 0.94 < 2.37) and p (0.8) > 0.05. Therefore, we do not reject the null hypothesis. The observed difference between the sample means (167.75 and 165.00) is not convincing enough to say that the average value between LC-MS and Okatest differ significantly (Table 24).

Table 24. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 22:

	OkaTest	LC-MS
Mean	167.75	165.00
t-statistic	0.94	
P(T≤t) value (probability value) for thet-statistic (one-tailed)	0.19	
Critical value of a t-distribution (one-tailed)	1.89	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.38	
Critical value of a t-distribution (two-tailed)	2.36	

We applied the test t manually to the difference of values obtained for each sample. For this application the value of the experimental t-score statistic was calculated, as well as the critical-t value (Table 25).

Table 25. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	-2.75
SD	8.26
Number of samples	8
Experimental t-score	0.94
Critical-t value	2.36

We could affirm that the hypothesis is true because the calculated value of experimental-t is smaller than the critical-t (0.94<2.36). The skewness is acceptable and the values obtaines by Okatest and LC-MS/MS are considered similars (Table 23).

LITERATURE

1. Bialojan, C.; Takai, *A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases.* Biochem. J. **1988**, 256, 283-290.
2. Eberthart B.-T. L., Moore L. K., Harrington N., Adams N. G., Borchert J. and Trainer V.L. *Screening tests for the rapid detection of diarrhetic shellfish toxins in Washington State.* Marine Drugs, 11, 3718-3734. **2013**.
3. *EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS*, version 2, July **2010.**
4. Horwitz W., *Protocol for the design, conduct and interpretation of method-performance studies.* Pure & Appl. Chem., Vol 67, No. 2, pp 331-343, **1995**.
5. Horwitz W. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*. **2002**
6. Jonhson S., Harrison K. And Turner A.D. *Application of rapid test kits for the determination of Diarrhetic Shellfish Poisoning (DSP) toxin in bivalve molluscs from Great Britain*. Toxincon. 111, 121-129. **2016.**
7. Smienk H., Calvo D., Razquin P., Domínguez E. y Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins.* Toxins*, 5*, 339-352; **2012**
8. Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal AOAC*, 96, 1, 77-85, **2013**.
9. Takai A and Mieskes. *Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases.*, Biochem. J. 275, pp 233-239, **1991**.
10. Turner A. D. & Goya A. B. *Comparison of four rapid test kits for the detection of okadaic acid groups toxins in bivalve shellfish from Argentina. Food Control*, 59, 829-840. **2016.**
11. Vieytes M.R., Fontal O.I., Leira F., Baptista de Sousa J.M.V., and Botana L.M. *A Fluorescente microplate assay for diarrheic shellfish toxins.* Analytical Biochemistry, 248, pp 258-264, **1997**.

Toxins **2012**, *5*, 339-352; doi:10.3390/toxins4050339

OPEN ACCESS

toxins

ISSN 2072-6651

www.mdpi.com/journal/toxins

Article

Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins

Henry G. F. Smienk, Dolores Calvo, Pedro Razquin, Elena Domínguez * and Luis Mata

Zeu-Inmunotec, Polígono PLAZA, C/Bari 25 Dpdo, 50197, Zaragoza, Spain;
E-Mails: hsmienk@zeulab.com (H.G.F.S.); dcalvo@zeulab.com (D.C.);
prazquin@zeulab.com (P.R.); lmata@zeulab.com (L.M.)

* Author to whom correspondence should be addressed; E-Mail: edominguez@zeulab.com;
Tel.: +34-976731533; Fax: +34976524078.

Received: 31 January 2012; in revised form: 4 April 2012 / Accepted: 18 April 2012 / Published: 30 April 2012

Abstract: A phosphatase inhibition assay for detection of okadaic acid (OA) toxins in shellfish, OkaTest, was single laboratory validated according to international recognized guidelines (AOAC, EURACHEM). Special emphasis was placed on the ruggedness of the method and stability of the components. All reagents were stable for more than 6 months and the method was highly robust under normal laboratory conditions. The limit of detection and quantification were 44 and 56 µg/kg, respectively; both below the European legal limit of 160 µg/kg. The repeatability was evaluated with 2 naturally contaminated samples. The relative standard deviation (RSD) calculated was 1.4% at a level of 276 µg/kg and 3.9% at 124 µg/kg. Intermediate precision was estimated by testing 10 different samples (mussel and scallop) on three different days and ranged between 2.4 and 9.5%. The IC_{50} values of the phosphatase used in this assay were determined for OA (1.2 nM), DTX-1 (1.6 nM) and DTX-2 (1.2 nM). The accuracy of the method was estimated by recovery testing for OA (mussel, 78–101%; king scallop, 98–114%), DTX-1 (king scallop, 79–102%) and DTX-2 (king scallop, 93%). Finally, the method was qualitatively compared to the mouse bioassay and LC-MS/MS.

Keywords: protein phosphatase inhibition assay (PPIA); protein phosphatase 2A (PP2A); validation; okadaic acid (OA); diarrheic shellfish poisoning (DSP)

1. Introduction

Diarrheic shellfish poisoning (DSP) is a consequence of the ingestion of a series of lipophilic toxins produced by dinoflagellates that can be present in shellfish for human consumption. These lipophilic toxins can be subdivided into four groups: the okadaic acid group (OA-toxins) including the dinophysistoxins (DTX), the pectenotoxin group (PTX), the yessotoxin group (YTX) and finally the azaspiracids (AZA). Only the OA-toxins and AZA are known to cause gastrointestinal problems [1,2]. For many years the mouse bioassay (MBA) has been the official method of detection for lipophilic toxins in the European Union [3], but with the publication of Commission Regulation (EU) No. 15/2011 [4], LC-MS/MS has become the reference method for their determination. This regulation also states that alternative or complementary methods can be used as long as an equivalent level of public health protection is provided, and the method performance criteria stipulated by the European Union Reference Laboratory on Marine Biotoxins (EU-RLMB) are fulfilled. Such methods should be intra-laboratory validated and successfully tested under a recognized proficiency test scheme.

Protein phosphatase inhibition assays (PPIA) have been identified for a long time as an alternative for the detection of OA-toxins, as ser/thr phosphatases are known to be their natural target [5,6]. As such, a validated phosphatase inhibition assay can be very useful in lipophilic toxin detection, complementary to the more complex, expensive and time consuming LC-MS/MS; or as an alternative when only OA-toxins are present in the samples. Different laboratories have developed in-house PPIA with good qualifications, using colorimetric or fluorimetric substrates to monitor enzyme inhibition. [7–12]. A collaborative study was also performed with a fluorimetric assay [13]. However, specific equipment, not often available in routine testing laboratories, makes difficult the use of fluorimetric assays for monitoring purposes. Besides, fluorimetric substrates are less stable than colorimetric ones and therefore less appropriate for ready-to-use kits. A standardized commercial test based on PPIA has not been available until recently. In this paper, we present a single laboratory validation of a commercial colorimetric PP2A assay (OkaTest) for the determination of OA-toxins in bivalve mollusks.

2. Materials and Methods

2.1. Reagents and Equipment

OkaTest kit (formerly Toxiline-DSP): The kit includes a 96-well microtiter plate, four vials of lyophilized protein phosphatase 2A (PP2A), purified from human red blood cells, five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM) prepared from the OA Certified Reference Material (NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), a liquid chromogenic substrate (p-Nitrophenyl phosphate), phosphatase dilution buffer and buffer solution.

Other reagents not included in the OkaTest kit: Methanol (Reagent grade, Carlo Erba), HCl (Reagent grade, 37% v/v, Carlo Erba), NaOH (Reagent grade, Scharlau), de-ionized water (type II, ISO 3696), certified Reference Materials (NRC CRM-DSP-MUS-b, NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), DTX-1 (042-28661, Wako) and DTX2 (00-DTX2, Cifga).

Equipment: Ultra homogenizer (IKA werken), a water bath at 76 ± 2 °C (Raypa), a FX-incubator at 30 °C ± 2 °C (ZEU-INMUNOTEC), a microplate absorbance reader (405 nm ± 10 nm wavelength

filter, Multiskan RC, Thermo-Labsystems), roller mixer, centrifuge, micropipettes, graduated 50 mL centrifuge tubes and laboratory glassware.

2.2. Sample Preparation

Market samples were thoroughly washed, the whole mollusk tissue recovered from the shell, and then blended. Portions of 5 ± 0.1 g were prepared and used for fresh testing, or stored frozen (below −15 °C) for future analysis. The portions were extracted by adding 25 mL of methanol (100% v/v) and mixing with a vortex for 2 min. The methanolic extract was separated by centrifugation for 10 min. at 2000 × g. To perform the hydrolysis, 640 µL of the methanolic extract and 100 µL of 3 N NaOH were mixed and incubated for 40 ± 1 min. at 76 ± 1 °C. To stop the reaction, 80 µL of HCl were added and sample preparation buffer used to make up a final volume of 20 mL. For non-hydrolyzed samples, 640 µL of methanolic extract were diluted up to 20 mL with sample preparation buffer. Hydrolysis was carried out in most samples unless otherwise specified.

2.3. Assay Procedure

The phosphatase solution was prepared by adding 2 mL of dilution buffer to each vial of lyophilized PP2A. To assure full hydration of the lyophilized enzyme, it was mixed gently for 1 h ± 5 min. at room temperature (22 °C ± 2 °C) on a roller mixer. Then, 50 µL of samples or ready-to-use OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), and 70 µL of the prepared phosphatase solution were added in duplicate to a microwell plate. This mixture was equilibrated in an incubator for 20 ± 2 min. at 30 °C. Finally, 90 µL of the chromogenic substrate were added to each well and incubated for 30 ± 2 min. at 30 °C. The absorbance was read at 405 nm.

2.4. Calculations

The results were calculated from a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of OA in a logarithmic *x axis*, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient r^2 had to be greater than or equal to 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = \mathrm{EXP}\ (y - \mathrm{b})/\mathrm{a}$$

where x is the OA concentration in the sample (C_s) and y the absorbance of the sample.

The OA-toxin concentration in shellfish tissue was calculated as follows:

$$C_t\ (\mu g/kg) = (C_s\ (nM) \times FD \times MW\ (g/mol) \times V_e\ (L))/M_t\ (g)$$

where C_t is the toxin concentration in tissue, expressed as equivalents of OA, *FD* is the methanolic extract dilution factor (31.25), *MW* is the OA molecular weight = 805, V_e is the methanolic extract volume (0.025 L), M_t is the tissue weight (5 g).

Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

2.5. Ruggedness Testing

The ruggedness testing was performed by introducing changes in the procedure and determining the effects on the sample quantification [14]. The variations used were chosen according to the values expected under normal laboratory conditions.

2.6. Spiking Procedure

Samples were spiked with OA Certified Reference Calibration Solution (NRC CRM-OA-c). The reference solution was prediluted to 2 µM in sample buffer and added accordingly. No Certified Reference Materials were available for DTX-1 and DTX-2 at the time of the performance testing. These toxins were first dissolved in methanol and diluted to 2 µM in sample buffer before adding to the samples.

A Certified Reference Material (NRC CRM-DSP-MUS-b) was also tested. However, the certified concentration of this material is far above the working range of the assay and the sample had to be diluted with blank mussel or king scallop. To do this, an amount of reference material was added as precisely as possible to 50 mL tubes, and weighed. The blank material was added on top and the mixture weighed again. Then, the amount of the mussel reference material per sample was calculated. This value was used as the theoretical spiked amount. The samples were analyzed with and without hydrolysis, as the reference material was only certified for OA and DTX-1, but ester derivates of the OA-toxins could also be present as indicated in the CRM certificate. The total recovery was calculated according to the AOAC Official methods of analysis [15].

2.7. Method Comparison

A method comparison was also carried out with OkaTest, the mouse bioassay (MBA) and LC-MS/MS, using EU harmonized protocols for the last two methods [16,17].

Shellfish samples were previously tested by a third party laboratory using mouse bioassay (MBA) and LC-MS/MS, and kindly donated to do the method comparison.

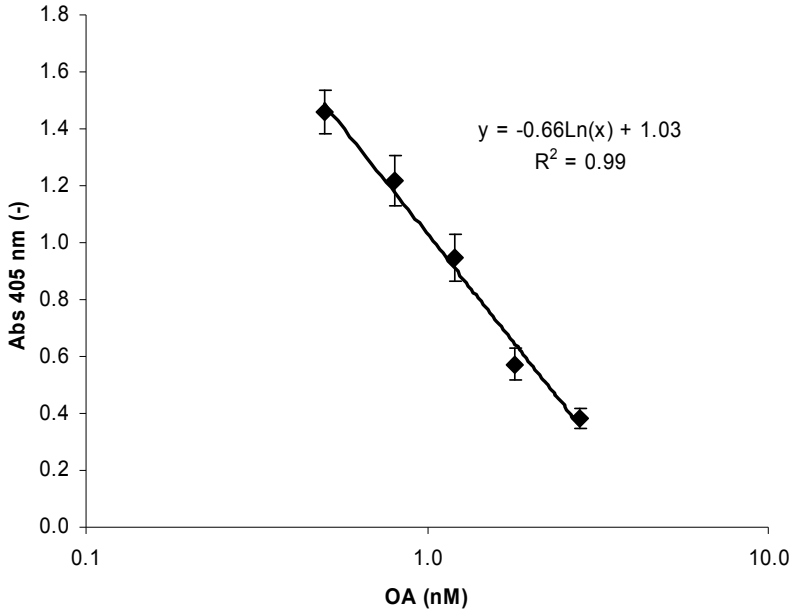
As MBA is a qualitative method, results obtained by OkaTest and LC-MS/MS were interpreted qualitatively for comparison purposes. Therefore, samples with a concentration ≥160 µg/kg were regarded as positive, while samples with a concentration <160 µg/kg were reported negative.

3. Results and Discussion

3.1. Calibration of the Assay

The assay is calibrated by five OA standards prepared by dilution from the NRC CRM-OA-c with a concentration between 0.5 and 2.8 nM OA. Following the kits sample preparation (see material and methods), this will result in a working range between 63 and 352 µg/kg. Figure 1 shows a typical calibration curve from 5 different assays using different phosphatase batches. All calibration curves were evaluated according to the Pearson correlation coefficient obtained after a logarithmic fitting procedure ($r^2 > 0.96$).

Figure 1. Typical calibration curve of OkaTest produced as the mean of 5 phosphatase batches. The Pearson correlation coefficient (r^2) of the logarithmic fit was >0.96 for each batch. The figure shows the equation and r^2 of the mean. The error bars were calculated as ±1 SD.



The bias introduced by the logarithmic fitting procedure on the calibration curve of the kit was estimated by recalculating the concentration of the OA dilutions using its own standard curve. The relative absolute difference was then calculated as the absolute difference between the theoretical and calculated OA concentration divided by the theoretical OA concentration and multiplied by 100 (Table 1). The best accuracy was found at levels around the regulatory limit (0.8% at 1.2 nM OA standards equals 151 µg OA equivalents/kg mollusk), while below that level (0.5 nM of OA), a 9.0% overestimation was calculated. Only minor deviations were calculated over the legal limit.

Table 1. Bias introduced due to the fitting procedure. Relative absolute difference was calculated from mean of 5 standard curves by relating the absolute difference to the theoretical OA concentration.

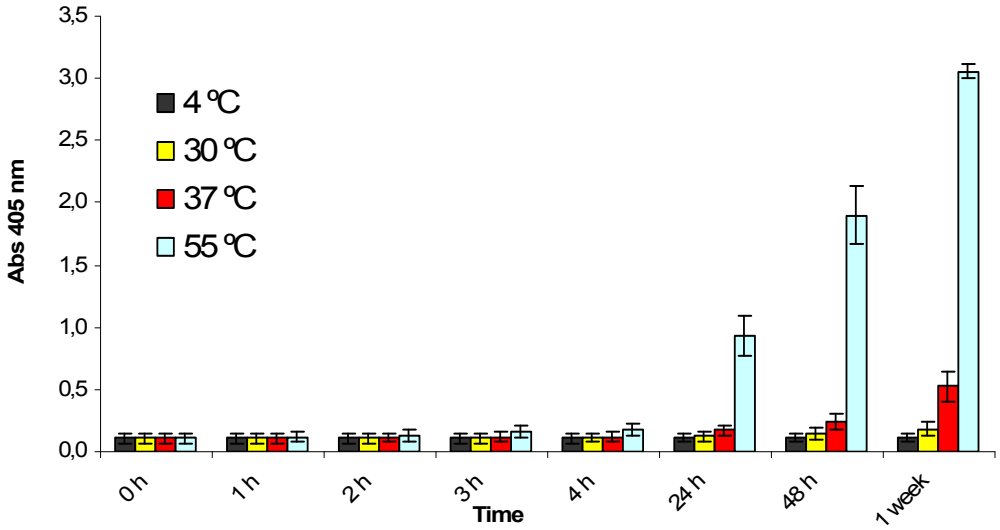
OA theoretical (nM)	OA calculated (nM)	Relative Absolute Difference
0.50	0.55	9.0%
0.80	0.83	3.8%
1.20	1.21	0.8%
1.80	1.78	1.1%
2.80	2.73	2.5%

3.2. Stability and Homogeneity of the Components

The stability and homogeneity of the critical components of the kit were studied by combining a real time and accelerated study design. Water soluble buffers such as the phosphatase dilution solution and the sample buffer were considered less critical, as sufficient internal know-how was available for these components and no stability problems were expected. Other components, such as the

ready-to-use chromogenic substrate, the PP2A or the OA standards, were specially developed for the phosphatase inhibition assay and were more extensively tested. Reagents were normally analyzed within the assay system or by performing specific tests depending on their particular characteristics. The ready-to-use substrate performed correctly in the OkaTest assay when stored for a year at temperatures between 2 and 15 °C (results not shown), as the background absorbance remained acceptable (below 0.3 absorbance units). However, accelerated studies showed that the substrate is sensitive to higher temperatures (Figure 2). After 24 h at 55 °C, the substrate was strongly hydrolyzed and after 1 week at 37 °C the absorbance of the substrate was above 0.6. Nevertheless, these results indicate that although the hydrolysis rate increases with temperature, it is very stable at temperatures below 15 °C and no problems should be expected under normal conditions of usage and storage.

Figure 2. Study of the temperature stability for the ready-to-use chromogenic substrate (p-Nitrophenyl phosphate). Absorbance at 405 nm was measured at different times and temperatures. Assays were performed in triplicate. The error bars were calculated as ±1 SD.



The OA standards and the PP2A were estimated the most critical components, as their quantity and quality establish the working range and, to a great extent, the ruggedness of the assay. The enzyme quantity determines the amount of analyte that is needed for inhibition, while the enzyme quality assures the amount of product formed per time unit [18]. Likewise, the lack of stability or impurities of the OA standards directly affect the quantification, either overestimating, in the case of degradation of the OA, or underestimating, when impurities that can inhibit the PP2A are present. Therefore, greater emphasis was put on these components and the 'between batch homogeneity' was evaluated besides the stability of the components. The between batch homogeneity was studied by taking 1 set of standards or 1 vial of phosphatase from 5 different batches. These batches were chosen along the estimated shelf life of the compounds and tested in one single assay together with internal control samples. All batches performed according to the assays' specifications ($r^2 > 0.96$) and the relative standard deviation was far below 15%, the expected value for samples assayed under repeatability conditions [19]. These results proved the stability of the enzyme for over 12 months at 4 °C and the homogeneity of between all batches tested (Table 2).

Table 2. Phosphatase stability and homogeneity. Five different phosphatase batches were tested at different stages of shelf life. Mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated. Three internal control samples were used to verify correct quantification.

PP2A batch (shelf life)	Sample 1 (µg/kg)	Sample 2 (µg/kg)	Sample 3 (µg/kg)
1 (2 months)	95	160	310
2 (4 months)	100	169	304
3 (8 months)	88	162	323
4 (10 months)	94	156	300
5 (12 months)	90	144	341
mean	93	158	316
SD	5	9	17
RSDR	4.8%	6.0%	5.2%

For the OA standards, the same strategy was used. Five batches, covering 90% of the shelf life of the component (6 months), were tested in one assay to be able to single out the variation due to the standards' stability and homogeneity (Table 3). A sample shown to be blank (0 nM) was included to be able to calculate the effect of variables other than OA. The RSDr calculated from the absorbance values were all <3%, proving the stability and homogeneity of the standards over 6 months.

Table 3. OA standards stability and homogeneity. Five different batches of OA standards were tested at different stages of shelf life. The absorbances (405 nm) obtained for each of the standards are shown. Mean, standard deviation (SD) and relative standard deviation (RSDr) of these absorbances were calculated.

	Absorbance 405 nm							
Standards OA (nM)	batch 1 5 months	batch 2 4 months	batch 3 3 months	batch 4 2 months	batch 5 1 week	mean	SD	RSDr
0.0	2.042	2.100	2.064	2.073	2.120	2.079	0.031	1.5%
0.5	1.622	1.614	1.649	1.625	1.678	1.637	0.026	1.6%
0.8	1.462	1.390	1.386	1.375	1.372	1.397	0.037	2.7%
1.2	1.124	1.116	1.101	1.092	1.134	1.113	0.017	1.5%
1.8	0.772	0.792	0.769	0.822	0.809	0.793	0.023	2.9%
2.8	0.619	0.646	0.606	0.637	0.613	0.624	0.017	2.7%

3.3. Ruggedness

Enzymatic assays, such as OkaTest, can be sensitive to environmental factors, such as temperature, incubation time or reagent volume. To determine the impact of these factors, samples with concentrations around the regulatory limit were quantified at normal and suboptimal conditions (Table 4). The effect of temperature was tested by performing the OkaTest assay at three different temperatures 28, 30 and 32 °C, obtaining a RSD of 1.0%. These results showed that temperature variations of 2 °C did not affect the performance as RSDr values were lower than 10% usually obtained in the assay (Table 5).

Duration and pipetting volumes were evaluated alike and none of the variables affected the results of the test, with the exception of large pipetting errors. Pipetting errors of 5 µL in samples or phosphatase addition (errors of 10% and 7.1%, respectively) gave RSDr values of 14% and 17%, respectively. Precision in substrate addition was less critical. Pipetting samples and phosphatase are, however, the main sources of variability affecting PPIA and therefore care should be taken when adding these components.

Table 4. Ruggedness testing. The effects of variations of the normal assay conditions on sample quantification are shown.

Variable	Normal value	Variation	Mean value (µg/kg)	RSDr
Temperature	30 °C	±2 °C	175	1.0%
Pre-incubation	20 min	18, 20, 22, 24 min	158	3.6%
Incubation	30 min	27, 30, 33, 36 min	147	2.9%
Syst. pipetting error	50, 70, 90 µL	±2 µL	155	4.3%
Random pipetting error				
Sample	50 µL	±5 µL	151	14%
PP2A	70 µL	±5 µL	153	17%
Substrate	90 µL	±5 µL	158	6.1%
Phosphatase solubility time	60 ± 5 min	±30 min	158	5.0%

Table 5. Intermediate precision of ten different mussel and scallops samples. Mean, standard deviation (SD), relative standard deviation (RSDr) were calculated. < 63: below the working range of the assay (63–352 µg/kg).

Sample	Origin	Day 1 (µg/kg)	Day 2 (µg/kg)	Day 3 (µg/kg)	Mean	SD	RSDr
1	Mussel	211	227	187	208	20	9.5%
2	Mussel	122	132	113	122	10	7.8%
3	Scallop	<63	<63	<63	-	-	-
4	Mussel	82	94	90	88	6	7.0%
5	Mussel	196	196	215	202	11	5.2%
6	Scallop	<63	<63	<63	-	-	-
7	Mussel	<63	<63	<63	-	-	-
8	Scallop	125	108	117	117	8	7.0%
9	Mussel	250	253	281	261	17	6.5%
10	Mussel	277	279	289	282	7	2.4%

3.4. Applicability

There are numerous descriptions of the application of protein phosphatase inhibition assays for determination of OA and its derivatives [7–13]. However, the inhibition pattern of OA, DTX1 and DTX2 is different and is supposed to correspond to their toxicity. One way to evaluate the inhibition capacity of toxins on an enzyme is by determining the IC_{50}, the concentration of toxin able to inhibit 50% of the maximum enzyme activity. This concentration depends, among others, on the amount of enzyme and the substrate concentration present in the assay [20] and therefore the IC_{50} values published for these toxins are difficult to compare [7,8,12,18,21,22]. The IC_{50} values found in our study were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1 (Figure 3) and are in accordance

with the ones obtained recently by Huhn *et al*., 2009 [21]. However, these do not exactly correspond to the toxicity factors (TEF) that are used in analytical methods such as LC-MS/MS; as OA and DTX-1 have a TEF of 1, while DTX-2 has a TEF of 0.6, indicating equal toxicity for DTX-1 and OA and less toxicity for DTX-2 [2]. According to these values, our results would lead to an overestimation of the amount of DTX-2 and an underestimation of the amount of DTX-1 when compared with methods such as LC-MS/MS. However, the recovery data obtained for both DTX-1 and DTX-2 were similar to the ones obtained for OA (Table 6) suggesting that difference has a low impact in the determination of the level of toxins in shellfish samples.

Figure 3. Phosphatase inhibition curve obtained with okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2). Each point is the mean obtained from three different phosphatase batches. The standard deviation is not shown to maintain the figure legible. The IC_{50} values were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1.

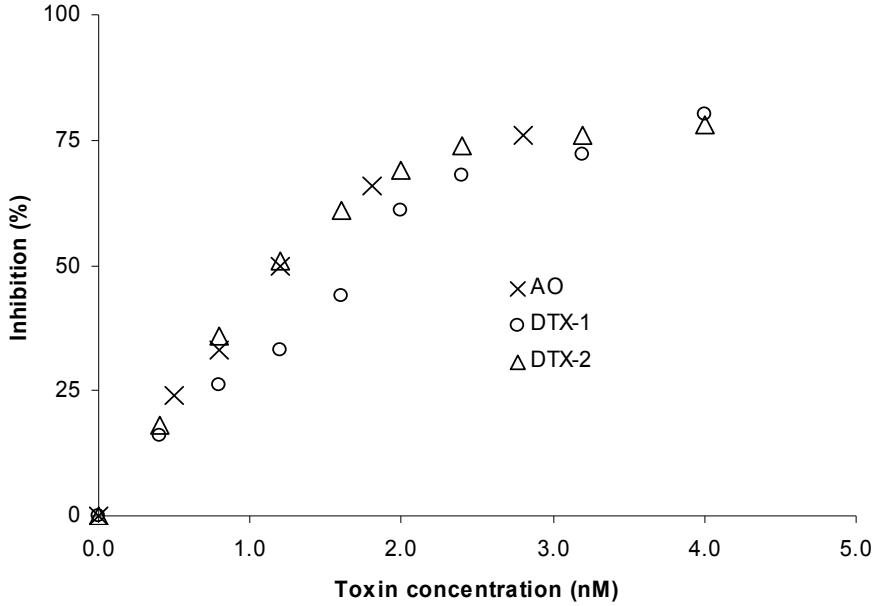


Table 6. Recovery of the different toxins was calculated testing 5 samples at 0.5, 1 and 1.5 times the regulatory limit on 3 different days. OA Certified Reference Material (NRC CRM-OA-c) was spiked on mussel and king scallop. DTX-1 and DTX-2 were spiked on king scallop. ND: not determined.

Toxin	Matrix	Recovery (RSDr)		
		80 µg/Kg	160 µg/Kg	240 µg/Kg
OA	Mussel	101% (15%)	90% (8.9%)	78% (5.4%)
	King scallop	114% (9.9%)	98% (8.4%)	106% (8.7%)
DTX-1	King scallop	102% (15%)	79% (12%)	88% (17%)
DTX-2	King scallop	93% (2.3%)	ND	ND

3.5. Limit of Detection, Limit of Quantification, Repeatability and Reproducibility

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using a blank +3 SD or blank +10 SD approach [14]. For blank mussel material, the LOD and LOQ were 44 and

56 µg/kg, respectively. These values are both below the working range of the test and sufficiently below the current European legal limit of 160 µg/kg.

To estimate the precision, the assay was tested both under repeatability and intermediate precision conditions. The repeatability characteristics were estimated by analyzing 8 fractions of two naturally contaminated mussel samples and RSDr of 1.4% with a mean of 276 µg/kg, and 3.9% with a mean of 124 µg/kg were obtained (results not shown). The intermediate precision of the test was estimated by analyzing 7 samples with OA-toxin levels covering the working range of the assay on three different days by the same analyst. For all samples, the RSDr was well below the 15% RSDr limit as calculated by Horwitz [19]. Three samples tested as negative by LC-MS/MS were included to evaluate the consistency of the negative results (Table 5).

3.6. Accuracy

The accuracy of the method was estimated by calculating recoveries for OA, DTX-1 and DTX-2 and by testing a Certified Reference Material (NRC-CNRC). Five portions containing 5 grams of mussel or king scallop were spiked with one of the three toxins at 0.5, 1 and 1.5 times the regulatory limit (80, 160 and 240 µg/kg), except for DTX-2 that was only added up to a concentration of 80 µg/kg. The five portions were analysed on three different days to determine the intermediate precision characteristics of the test. OA recoveries between 78 and 101% in mussel and 98 and 114% in king scallop were obtained. RSDr values for this toxin were below or equal to 15%. Similar recoveries were obtained for the other two toxins (Table 6). These recoveries are in agreement with the 75 to 120% range that is expected for this concentration range [19]. The RSDr results in this study were higher than the ones obtained in the precision experiments (Table 4), specially for DTX-1. This might be a consequence of the spiking. As mentioned before, the higher IC_{50} for DTX-1 compared to OA and DTX-2 had a low impact on the recovery.

Finally, four aliquotes of blank samples were spiked with the Certified Reference Material. The methanolic extract obtained was analysed with and without hydrolysis, and the recovery was estimated using the DTX-1 and OA content reported for the certified material. The recovery for the non-hydrolysed samples ranged from 71% to 98%, with a mean of 87% for mussle and 91% for king scallop (Table 7). These are acceptable recoveries and in accordance with the results showed in Table 6. However, the mean recovery of the hydrolysed samples was a 146% and 163% for mussle and king scallop, respectively. These percentages were far above the expected content of OA-toxins indicated in the reference material [23]. This could be due to the fact that the material is only certified for OA and DTX-1. Other esters of OA and DTX are reported in the certificate of anlaysis for this material.

Table 7. Recovery experiment with Certified Reference Material (NRC CRM-DSP-MUS-b). Samples were analysed with and without hydrolysis.

Matrix	Spiked level (µg/kg) (*n*)	Without hydrolysis		With hydrolysis	
		Recovery	RSDr	Recovery	RSDr
mussel	219 (4)	87%	14%	146%	12%
king scallop	180 (4)	91%	5.0%	163%	2.8%

3.7. Method Comparison

A method comparison among MBA, LC-MS/MS and OkaTest was performed with a total of 37 samples. Results were compared qualitatively for all three methods and quantitatively between OkaTest and LC-MS/MS. The 160 µg/kg regulatory limit was used to decide whether the samples were positive or negative (Table 8).

Table 8. Methods comparison. Results from OkaTest, MBA and LC-MS/MS. 31 of the 37 samples were tested by MBA. Positive results (+): ≥160 µk/kg. Negative results (−): <160 µg/kg. LOQ. Limit of quantification. NA: not available.

ID	M	MBA	LC-MS/MS	OKATEST	LC-MS/MS	OKATEST
1	**Cockle**	-	-	-	<LOQ	<LOQ
2	**Cockle**	+	+	+	193	252
3	**Donax**	-	-	-	82	97
4	**Mussel**	+	+	+	502	232
5	**Mussel**	+	-	+	<LOQ	268
6	**Mussel**	+	+	+	604	>352
7	**Mussel**	+	+	+	894	>352
8	**Mussel**	+	+	+	414	306
9	**Mussel**	+	+	+	444	>352
10	**Mussel**	NA	-	-	<LOQ	<LOQ
11	**Mussel**	NA	+	+	357	>352
12	**Mussel**	NA	-	-	<LOQ	<LOQ
13	**Mussel**	NA	-	-	<LOQ	<LOQ
14	**Mussel**	-	-	-	<LOQ	122
15	**Mussel**	+	-	+	158	196
16	**Mussel**	+	+	+	177	250
17	**Mussel**	+	+	+	288	265
18	**Mussel**	+	+	+	202	196
19	**Mussel**	+	+	+	390	277
20	**Mussel**	+	+	+	658	305
21	**Mussel**	+	+	+	392	310
22	**Mussel**	+	+	+	329	315
23	**Mussel**	+	+	+	232	270
24	**Mussel**	+	+	+	235	277
25	**Mussel**	+	-	-	152	135
26	**Mussel**	+	-	+	98	164
27	**Mussel**	+	+	+	168	211
28	**Mussel**	+	+	+	209	251
29	**Mussel**	+	-	+	113	191
30	**Mussel**	NA	+	-	292	<LOQ
31	**Mussel**	NA	+	+	316	304
32	**Mussel**	-	-	-	<LOQ	<LOQ
33	**Mussel**	+	+	-	177	124
34	**Mussel**	+	+	+	247	216
35	**Mussel**	+	+	-	185	144
36	**Scallop**	+	+	+	184	264
37	**Scallop**	-	-	-	<LOQ	<LOQ

In general, the qualitative interpretation of the results indicates that the three methods obtained equivalent results, especially taking into account that these are conceptually different methods. The OkaTest disagreed with both MBA and LC-MS/MS on two occasions (samples 33 and 35). OkaTest detected levels of OA-toxins in those two samples, but below the EU regulatory limit (124 and 144 µg/kg), while the samples were positive according to the other two methods). A third sample (25) was also identified as negative by OkaTest and positive by MBA. LC-MS/MS also gave a negative result for sample 25. The concentration of this sample determined by both methods was just below the EU regulatory limit.

The LC-MS/MS differed on four occasions: all four negative according to LC-MS/MS, but positive by the other two methods. Three of the samples (15, 26 and 29) contained OA-toxins below the EU refulatory limit, but sample 5 was quantified under the method's LOQ. Finally, one sample (30) was positive by LC-MS/MS, but under the LOQ by OkaTest. Sample 30 was not tested by MBA due to lack of material.

Quantitative results obtained by LC-MS/MS and Okatest showed some differencies. About two thirds of the samples gave similar results (±25%) with both methods, but the rest of the samples did not show a clear tendency. There is no evident explanation for this and further investigation would be required.

4. Conclusions

A colorimetric phosphatase inhibition assay for determination of OA-toxins, OkaTest, was single laboratory validated according to international methods validation guidelines. The limit of quantification of the method is well below the EU regulatory limit and the method permitted the easy quantification of up to 43 samples within one hour, excluding sample preparation. The method is robust, with very good precision characteristics, adequate specificity and accuracy.

This colorimetric phosphatase inhibition assay could be used as a complementary assay to the reference method for determination of lipophilic toxins, once a collaborative study has been completed and it has been successfully tested under recognized proficiency tests. This assay could be applied for monitoring purposes when OA-toxins are identified to be responsible for a bloom.

Acknowledgements

We thank María Rodríguez Velasco, from the EURLMB (European Reference Laboratory for Marine Biotoxins), for revising the data, providing the mollusk material and coordinating the method comparison. We also want to thank Adriano Rodriguez from the EURLMB for performing the LC-MS/MS determination and Anfaco-Cecopesca for the MBA and mollusk material.

Note: Collaborative efforts between the EURLMB and ZEU-INMUNOTEC do not amount to an endorsement of the firm's products.

References

1. Gerssen, A.; Pol-Hofstad, I.E.; Poelman, M.; Mulder, P.P.J.; van Den Top, H.J.; de Boer, J. Marine Toxins: Chemistry, toxicity, occurrence and detection with special reference to the Dutch situation. *Toxins* **2010**, *2*, 878–904.

2. EFSA. Marine biotoxins in shellfish-okadaic acid and analogues—Scientific Opinion of the Panel on Contaminants in the Food Chain. *EFSA J*. **2008**, *589*, 1–62.
3. European Commission. EC Commission Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No. 854/2004. *Off. J. Eur. Comm.* **2005**, *338*, 27–59.
4. European Commission. EC Commission Regulation (EC) No. 15/2011 of 10 January 2011 amending Regulation (EC) No. 274/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Comm.* **2011**, *6*, 3–6.
5. Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. *FEBS Lett*. **1987**, 21781–21784.
6. Bialojan, C.; Takai, A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J*. **1988**, *256*, 283–290.
7. Tubaro, A.; Florio, C.; Luxich, E.; Sosa, S.; Della Loggia, R.; Yasumoto, T. A protein phasphatase 2A inbhition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. *Toxicon* **1996**, *34*, 743–752.
8. Honkanen, R.E.; Stapleton, J.D.; Bryan, D.E.; Abercrombie, J. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. *Toxicon* **1996**, *34*, 1385–1392.
9. Mountfort, D.O.; Kennedy, G.; Garthwaite, I.; Quilliam, M.; Truman, P.; Hannah, D.J. Evaluation of the fluorimetric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. *Toxicon* **1999**, *37*, 909–922.
10. Ramstad, H.; Shen, J.L.; Larsen, S.; Aune, T. The validity of two HPLC methods and a colorimetric PP2A assay related to the mouse bioassay in quantification of diarrheic toxins in blue mussels (*mytilus edulis*). *Toxicon* **2001**, *39*, 1387–1391.
11. Simon, J.F.; Vemoux, J.P. Highly sensitive assay of okadaic acid using protein phosphatase and paranitrophenyl phosphate. *Nat. Toxins* **1994**, *2*, 293–301.
12. Vieytes, M.R.; Fontal, O.I.; Leira, F.; Baptista de Sousa, J.M.V.; Botana, L.M. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal. Biochem*. **1997**, *248*, 258–264.
13. Gonzalez, J.C.; Leira, F.; fontal, O.I.; Vieytes, M.R.; Arévalo, F.F.; Vieites, J.M.; Bermúdez-Puente, M.; Muñiz, S.; Salgado, C.; Yasumoto, T.; *et al*. Inter-laboratory validation of the fluorescent protein phosphatase inhibitions assay to determine diarrheic shellfish toxins: Intercomparison with liquid chromatography and mouse bioassay. *Anal. Chim. Acta* **2002**, *466*, 233–246.
14. EURACHEM. *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; EURACHEM: Prague, Czech Republic, 1998.
15. AOAC. *Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*; AOAC International: Gaithersburg, MD, USA, 2002.

16. EURLMB. EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. In *EU Harmonised SOP LIPO LCMSM Version 1*; EURLMB: Vigo, Spain, 2006;
17. EURLMB. EU Harmonised Standard Operating Procedure for detection of lipophilic toxins by mouse bioassay. In *EU Harmonised SOP MBA Lipophilic Version 4*; EURLMB: Vigo, Spain, 2007;
18. Takai, A.; Murata, M.; Torigoe, K.; Isobe, M.; Mieskes, G.; Yasumoto, T. Inhibitory effect of okadaic acid derivatives on protein phosphatases. *Biochem. J.* **1992**, *284*, 539–544.
19. Horwitz, W. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*; AOAC International: Gaithersburg, MD, USA, 2002; pp. 12–19.
20. Engel, P.C. *Enzymology Labfax*; Academic Press: San Diego, CA, USA, 1996.
21. Huhn, J.; Jeffrey, P.D.; Larsen, K.; Rundberget, T.; Rise, F.; Cox, N.R.; Arcus, V.; Shi, Y.; Miles, C.O. A structural basis for the reduced toxicity of dinophysistoxin-2. *Chem. Res. Toxicol.* **2009**, *22*, 1782–1786.
22. Aune, T.; Larsen, S.; Aasen, J.A.B.; Rehman, N.; Satake, M.; Hess, P. Relative toxicity of dinphysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* **2007**, *49*, 1–7.
23. Perez, R.A.; Crain, S.M.; Walter, J.A.; Quilliam, M.A.; Melanson; J.E. *NRC CRM-OA-c*, *Certified Calibration Solution for Okadaic Acid*; Technical Report for CRMP: Halifax, Canada, 2008.

FOOD CHEMICAL CONTAMINANTS

Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study

HENRY SMIENK and ELENA DOMÍNGUEZ[1]
ZEU-INMUNOTEC, C/Bari 25 Dpdo. 50197, Zaragoza, Spain
MARÍA L. RODRÍGUEZ-VELASCO
EURLMB-AESAN, Estación Marítima S/N, Muelle de Trasatlánticos, 36200 Vigo, Spain
DAVID CLARKE
Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
KATRIN KAPP
BFR—Federal Institute for Risk Assessment, Thielallee 88-92, 14195, Berlin, Germany
PANAGIOTA KATIKOU
NRL Marine Biotoxins, Institute of Food Hygiene, 3A Limnou St, 54627 Thessaloniki, Greece
ANA G. CABADO, ALBERTO OTERO, and JUAN M. VIEITES
ANFACO CECOPESCA, Ctra. Colexio Universitario, 16, Lagoas, 36310, Vigo, Spain
PEDRO RAZQUIN and LUIS MATA
ZEU-INMUNOTEC, C/Bari 25 Dpdo., 50197, Zaragoza, Spain

An interlaboratory collaborative study to validate a colorimetric phosphatase inhibition assay for quantitative determination of the okadaic acid (OA) toxins group in molluscs, OkaTest, was conducted. Eight test materials, including mussels, scallops, clams, and cockles, were analyzed as blind duplicates. Blank samples and materials containing different OA toxin levels ranging from 98 to 275 µg/kg OA equivalents were included. The study was carried out by a total of 16 laboratories from 11 different countries. Values obtained for repeatability relative standard deviations (RSD_r) ranged from 5.4 to 11.2% (mean 7.5%). Reproducibility RSD (RSD_R) values were between 7.6 and 13.2% (mean 9.9%). The Horwitz ratio (HorRat) values ranged between 0.4 and 0.6. A recovery assay was also carried out using a sample spiked with OA. A mean recovery of 98.0% and an RSD of 14.5% were obtained. The results obtained in this validation study indicate that the colorimetric phosphatase inhibition assay, OkaTest, is suitable for quantitative determination of the OA toxins group. OkaTest could be used as a test that is complementary to the reference method for monitoring the OA toxins group.

Okadaic acid (OA) and its analogs dinophysistoxin-1 and -2 (DTX1, DTX2), together with their ester forms, are known as the OA toxins group. These lipophilic and heat stable toxins are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter-feeding bivalve molluscs.

OA toxins causes diarrheic shellfish poisoning, which is characterized by symptoms, such as diarrhea, nausea, vomiting, and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs, such as mussels, clams, scallops, or oysters. Inhibition of serine/threonine phosphoprotein phosphatases (PPs) is assumed to be responsible for these toxic effects. These compounds are also involved in tumor promotion (1). Therefore, these toxins are regulated by European Union law.

Regulation (EC) No. 853/2004 (2) states that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed 160 µg of OA equivalents/kg for OA, dinophysistoxins, and pectenotoxins together.

Commission Regulation (EC) No. 15/2011 (3) indicates that in the case of lipophilic toxins including OA toxins, LC/MS/MS is the reference method for routine testing of official controls or any checks done by food operators. This regulation has recently amended the Commission Regulation (EC) No. 2074/2005 (4), in which biological methods (mouse and rat bioassay) were considered the reference. From now on, they will only be used for a transitional period of time (until the end of 2014) or in special circumstances.

Both regulations (No. 2074/2005 and No. 15/2011) contemplate other methods for routine testing of lipophilic toxins, providing they are intralaboratory-validated and successfully tested under a recognized proficiency test scheme. Those methods should detect, either alone or in combination with others, all of the lipophilic toxin analogs (OA, pectenotoxins, yesotoxins, and azaspiracids group toxins). The protein phosphatase inhibition assay (PPIA) is specifically mentioned in these regulations as an alternative or complementary method, considering that the PPs are known to be OA-toxins natural targets (5, 6). In-house PPIAs using different phosphatase sources and colorimetric or fluorometric substrates have been previously developed (7–12). Later improvements to detect all OA derivatives by hydrolysis of samples were also suggested

Received October 28, 2011. Accepted by AP May 23, 2012.
[1] Corresponding author's e-mail: edominguez@zeulab.com
DOI: 10.5740/jaoacint.11-465

Table 1. Details of matrixes and species origin of test materials used in this study

Code	Matrix/Species	Origin
A	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
D	Clam (*V. pullastra*)	Food & Agricultural Organization, 37 Mediterranean Sea
E	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
F	Scallop (*P. maximus*)	FAO 27 NE Atlantic
G	Clam (*V. decussatus*)	Galicia (NW Spain)
K	Clam (*V. romboides*)	Galicia (NW Spain)
L	Cockle (*C. edulis*)	Portugal and Galicia (NW Spain)
N	Mussel (*M. edulis*)	Ireland
BM	Scallop (*P. maximus*)	Scotland

(13), and a collaborative study was also performed with a fluorometric PPIA (14). However, none of those assays was commercially available for routine analysis, nor were they demonstrated to comply with the legislation requirements.

ZEU-INMUNOTEC (Zaragoza, Spain) has developed a commercial kit (OkaTest, formerly Toxiline-DSP) based on a colorimetric PP2A inhibition assay for quantification of the OA toxins group in molluscs (15).

The PPIA described in this study uses a human PP2A purified by ZEU-INMUNOTEC that has showed higher sensitivity than other commercial and genetic engineering produced enzymes (16). PP2A was stabilized by freeze-drying to obtain a standardized assay with shelf life of up to 12 months at 4°C (15). Colorimetric substrate was chosen over a fluorometric one as the latter is less stable and, therefore, less appropriate for ready-to-use kits. Besides, fluorometric assays require specific equipment not often available in routine testing laboratories; therefore, they are difficult to use for monitoring purposes.

The robustness and performance of OkaTest were evaluated by the manufacturer in a single-laboratory validation according to AOAC and Eurachem guidelines (15). All of the results obtained showed that the OkaTest kit is robust and accurate, and, therefore, suitable for an interlaboratory study.

Interlaboratory Study

A colorimetric PPIA, OkaTest, was interlaboratory-validated for quantification of the OA toxins group. The main purpose of this study was to determine repeatability and between-laboratory reproducibility. A recovery assay was also carried out, and accuracy of the method confirmed.

A validation management team (David Clarke, Elena Domínguez, Katrin Kapp, Panagiota Katikou, and María Luisa Rodríguez) was appointed to supervise, advise on the accomplishment of the study, and ensure its independence. A total of 16 laboratories from 11 different countries in Europe and South America participated in the study.

The study plan including details of the test method, experimental design, preparation of test materials, instructions for participants, key personnel, schedule, and data analysis was prepared and agreed to by the validation management team. Participants were fully informed of the study design prior to distribution of testing materials.

Eight different test materials, as blind duplicates, were analyzed by each laboratory on 2 different days. Five materials contained different OA toxin levels, all naturally contaminated except for one that was partially spiked. Three of the test materials were blank samples. An additional blank material (BM) was used in the recovery study. The test materials comprised four different genera of molluscs (*Mytilus spp, Pecten spp., Venerupis spp.,* and *Cerastoderma spp*.) and seven different species. Details of the materials used are shown in Table 1. The materials were prepared by the Spanish Association of Seafood Products Manufacturers (ANFACO-CECOPESCA; Vigo, Spain) as explained below.

All participants sent back an electronic copy of a tailor-made Excel reporting sheet for each day of analysis with raw data and final results for each test material. The reporting sheets were checked upon receipt for obvious errors in sample codes and calculations.

Participants also completed a questionnaire with details of the equipment used and preparation of reagents and samples, as well as feedback on the assay.

Preparation of Test Materials

Materials A and E (mussel) and D and K (clam) were purchased from the retail market fresh and alive. They were thoroughly cleaned outside and inside with fresh water to remove sand and any other foreign materials. Tissues were removed from the shell, transferred to strainers, and drained for 5 min before homogenization (blender and Ultraturrax®; IKA, Staufen, Germany). The homogenate (at least 450 g) was then distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until analysis or the day of shipment.

Materials F (scallop) and G (clam) were purchased frozen

Table 2. Total concentration of OA toxins group (µg/kg) determined by OkaTest, and toxins profile by LC/MS/MS

Test material[a]	Matrix/species	Total OA equivalents, µg/kg[b]	OA toxins content[c]
BM	Scallop (*P. maximus*)	<LOD	—
A	Mussel (*M. galloprovincialis*)	<LOD	—
F	Scallop (*P. maximus*)	<LOD	—
G	Clam (*V. decussatus*)	<LOD	—
E	Mussel (*M. galloprovincialis*)	79 ± 5	OA
L	Cockle[d] (*C. edulis*)	168 ± 11	OA, DTX1, and DTX2
D	Clam (*V. pullastra*)	240 ± 9	OA
K	Clam (*V. romboides*)	250 ± 6	OA
N	Mussel[e] (*M. edulis*)	276 ± 6	OA and DTX2

[a] Samples presented in increasing order of concentration.

[b] Determined by OkaTest; LOD = 44 OA equivalents µg/kg.

[c] Determined by LC/MS/MS.

[d] Artificially contaminated with DTX1 and mixed with blank material.

[e] Mixed with blank material.

Table 3. Results from homogeneity study for test materials for the determination of OA (µg OA total equivalents/kg)

Test material	Variance of sums, Vs	Analytical variance, s_{an}^2	Allowable sampling variance, σ_{all} ^2	Sampling variance, S_{sam}^2	Critical value, c	Test for homogeneity result
D	166	90.7	36.8	116	310	S_{sam}^2 < c
E	84.7	8.09	19.8	11.1	29.1	S_{sam}^2 < c
K	139	19.6	32.5	126	257	S_{sam}^2 < c
L	356	46.9	85.7	55.6	152	S_{sam}^2 < c
N	124	24.2	28.4	154	314	S_{sam}^2 < c

from the retail market. They were thawed at room temperature, cleaned, and prepared as described above.

Material L (cockle) was provided cleaned, blended, and frozen by the European Reference Laboratory for Marine Biotoxins (EURLMB, Vigo, Spain). The sample contained OA, DTX2, and traces of DTX1. In order to achieve a suitable toxin profile, the sample was mixed with fresh cockle from the same species (*C. edulis*) without toxin prior to being spiked with DTX1 (Wako Chemicals, Neuss, Germany). The sample was thawed at room temperature, mixed with the cockle blank material (purchased in Porto, Portugal), and spiked. Then, it was distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until the day of shipment.

Material N (mussel) was provided cleaned, blended, and frozen by the National Reference Laboratory of Ireland, Galway, Ireland. The sample contained a high level of OA toxins, so it was mixed with mussel (*M. edulis*) without toxin (purchased in a retail market in Ireland) to achieve a suitable toxin concentration. The sample was thawed at room temperature, mixed, and distributed into plastic containers (5.0 ± 0.1 g). The material was then frozen and stored at –20 ± 2°C until the day of shipment.

The BM (scallop) was provided blended and homogenized by Integrin Advanced Bioscience (Oban, Scotland) and stored frozen at approximately –20 ± 2°C until the day of shipment.

Homogeneity and stability of test materials were studied according to the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories (17). Ten containers of 5 g were randomly selected for each material. The content of each container was homogenized and extracted, and two test portions (from the sample extract) were analyzed to estimate the analytical variance. A total of 20 portions/material were tested under repeatability conditions and in a random order using the OkaTest kit.

To ensure the stability of the materials during shipment to participants and the study duration, aliquots of each material were taken randomly and split into two subsets, each of them containing five samples. One subset was used as control and stored at –18 ± 1°C. The second was stored under experimental conditions of 9.0 ± 1°C for 5 days. Samples of both subsets were randomized before testing and analysis simultaneously using the OkaTest kit under repeatability conditions. The test materials were also analyzed by LC/MS/MS (18, 19) to determine the OA toxin profile.

The test materials were blind coded by EURLMB and distributed by ANFACO-CECOPESCA to the participants. The codes were securely kept by EURLMB until statistical analysis was carried out.

The materials were shipped in isothermal boxes with dry ice and were received within the following 2 days by most participants. Materials sent to South American countries were delivered more than a week after the dispatch date, as they have long customs check up procedures. Samples were, however, reported to have been kept frozen while stored at customs. Two laboratories informed that the box containing the samples did not arrive in good conditions, and six reported that samples were cold, but defrosted.

PPIA

Principle

OkaTest is an enzymatic test based on a colorimetric PPIA for quantitative determination of OA and other toxins of the OA group, including DTX1, DTX2, and their ester forms.

Table 4. Results obtained for the stability assays conducted for materials D, E, K, L, and N

	Storage conditions						
	–18 ± 1°C	9.0 ± 1°C					
	Mean						
Test material	Total OA equivalents, µg/kg		Absolute difference D	Variance *F*-test	*t*-test	Test criterion C	D < C
D	265 ± 10	262 ± 15	3.02	0.54	0.71	34.5	Pass
E	84.0 ± 4	85.1 ± 3	–1.19	0.45	0.62	10.9	Pass
K	255 ± 8	257 ± 7	–1.57	0.87	0.75	33.2	Pass
L	171 ± 7	169 ± 8	1.63	0.79	0.73	22.2	Pass
N	343 ± 24	355 ± 32	–13.0	0.58	0.49	44.6	Pass

Table 5. Calibration curve parameters obtained by each laboratory every day of the study

	R^2		Slope		Absorbance 405 nm, lowest standard 0.5 nM		Absorbance 405 nm, highest standard 2.8 nm	
Lab	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
A	0.99	0.98	–0.12	–0.45	0.734	1.287	0.524	0.505
B	0.99	0.99	–0.50	–0.65	1.157	1.425	0.334	0.339
C	0.98	0.98	–0.64	–0.44	1.530	1.177	0.496	0.468
D	0.98	0.98	–0.67	–0.58	1.537	1.402	0.430	0.459
E	0.97	0.98	–0.51	–0.48	1.222	1.221	0.409	0.436
F	1.00	0.99	–0.72	–0.74	1.684	1.726	0.482	0.491
G	0.98	1.00	–0.79	–0.58	1.781	1.411	0.462	0.423
H	0.99	0.99	–0.78	–0.73	1.644	1.609	0.366	0.414
I	0.99	0.99	–0.76	–0.68	1.661	1.486	0.409	0.357
J	0.97	0.98	–0.41	–0.45	1.164	1.204	0.498	0.458
K	0.99	0.98	–0.77	–0.74	1.712	1.690	0,438	0,485
L[a]	0.93	0.96	–0.63	–1.13	1.488	2.588	0.425	0.709
M	0.99	0.99	–0.78	–0.65	1.697	1,464	0.419	0.390
N	0.99	0.98	–0.54	–0.65	1.273	1,497	0.384	0.444
O	0.97	0.98	–0.49	–0.32	1.188	0,992	0,396	0.470
P	0.97	0.99	–0.27	–0.58	1.015	1.474	0.549	0.520

[a] Standard curve obtained by Laboratory L on Day 1 was rejected as R^2 criterion was not met. Assay could not be repeated due to time issues.

This method is applicable to shellfish species, such as mussels, clams, cockles, and scallops.

The toxicity of the OA toxins group is directly related to its inhibitory activity against a family of structurally related PPs, in particular PP1 and PP2A. OkaTest uses this strong inhibitory activity to determine the OA content in shellfish using the PP2A with a chromogenic substrate for this enzyme. After the substrate's hydrolysis by the enzyme, the product can be measured at 405 nm by a microplate reader. As the ability of the PPs to hydrolyze the substrate depends on the amount of OA and analogs in the samples, the toxin concentration can be calculated by using a standard curve.

Apparatus

(**a**) *Micropipets*.—Adjustable 100, 200, and 1000 µL (Thermo Labsystems, Helsinki, Finland).

(**b**) *Ultra homogenizer*.

(**c**) *Block heater or incubator*.—For 30 ± 2°C (ZEU-INMUNOTEC, Zaragoza, Spain).

(**d**) *Microwell absorbance reader*.—405 ± 10 nm wavelength filter (Thermo Labsystems).

(**e**) *Water bath*.—Set at 76 ± 2°C (Raypa, Barcelona, Spain).

(**f**) *Centrifuge tubes*.—Graduated 50 mL.

(**g**) *Laboratory glassware*.

Reagents

(**a**) *Extraction solvent*.—Methanol, reagent grade, 100% (v/v; Sharlab, Barcelona, Spain).

(**b**) *HCl*.—Reagent grade, 37% (v/v; Sharlab).

(**c**) *NaOH*.—Reagent grade (Sharlab).

(**d**) *Deionized water*.—Type II, ISO 3696 (Ellix 5; Millipore, Germany).

(**e**) *OkaTest kit*.—From ZEU-INMUNOTEC containing:

(*1*) 96-well microtiter plate and plate adhesive film.

(*2*) Lyophilized PP2A purified from human blood cells.

(*3*) Ready-to-use OA Standards of 0.5, 0.8, 1.2, 1.8, and 2.8 nM, prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences, Halifax, Canada).

(*4*) Chromogenic substrate.

(*5*) Phosphatase dilution buffer.

(*6*) Stock buffer solution.

(*7*) OA Spiking solution (2 µM) prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences).

Spiking Procedure

Due to the limited experience on the homogeneity and stability of spiked samples with OA toxins, each participant prepared a spiked sample on the day of the assay. A BM and an OA solution of known concentration (2 µM, to prepare a final concentration of 161 µg/kg) were provided to each participant.

A blank sample was spiked with OA solution for the recovery study as follows:

(**a**) Mix 500 µL OA spiking solution (2 µM) with 5.0 ± 0.1 g homogenous blank sample.

(**b**) Add 25 mL extraction solvent [methanol, 100% (v/v)] to the mixture and shake for 2 min by vortexing. Proceed with the extraction procedure described below under point (**b**).

Sample Extraction

(**a**) Thaw each aliquot with 5.0 ± 0.1 g homogenized mollusc at room temperature (22 ± 2°C). Add 25 mL extraction solvent [methanol, 100% (v/v)]; then mix for 2 min using an ultra homogenizer.

(**b**) Centrifuge at 2000 g for 10 min at 4°C. The supernatant is called "methanolic extract."

(**c**) Pipet 640 µL methanolic extract into a 50 mL graduated centrifuge tube and add 100 µL 2.5 M NaOH.

(**d**) Seal the test tube and heat at 76 ± 2°C for 40 min in a water bath.

(**e**) Do not cool the sample; add 80 µL 2.5 M HCl immediately.

(**f**) Add 19.18 mL buffer solution with a glass pipet up to a total volume of 20 mL.

Assay Procedure

(**a**) Rehydrate the lyophilized phosphatase (PP2A) by adding 2.0 mL phosphatase dilution buffer to the vial and mix gently for 60 ± 5 min at room temperature (22 ± 2°C) on a roller mixer or a shaker (maximum 60 rpm) (both from JP Selecta, Barcelona, Spain).

(**b**) Add 50 µL each sample extract or standard to wells. Samples and standards have to be analyzed in duplicate.

(**c**) Add 70 µL phosphatase solution to each well. Cover the plate with the adhesive film provided in the kit, and mix by gentle tapping on the side.

(**d**) Incubate at 30 ± 2°C for 20 ± 0.5 min.

(**e**) Remove the adhesive film and add 90 µL chromogenic substrate to each well and mix by tapping gently on the side. Incubate at 30 ± 2°C for 30 ± 0.5 min.

(**f**) Read the absorbance of samples and standards at 405 ± 10 nm.

Calculations

The results were calculated from a standard curve by plotting the absorbance values on a linear y axis and the concentration of OA on a logarithmic x axis, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient R^2 had to be equal to or greater than 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = EXP\ (y - b)/a$$

where x is the OA concentration in the sample (Cs), y the absorbance of the sample, a is the slope, and b is the y-intercept.

The OA toxin concentration in shellfish tissue was calculated as follows:

$$Ct,\ \mu g/kg = [Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L)]/\ Mt\ (g)$$

where Ct is the toxin concentration in tissue expressed as equivalents of OA, FD is the methanolic extract dilution factor, MW of OA = 805, Ve is the methanolic extract volume (0.025 L), and Mt is the tissue weight (5 g).

Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

Results were recorded by each participant in a tailor-made Excel spreadsheet with which the results were automatically calculated when the absorbance values were entered. All participants sent back an electronic copy of the reporting sheet for each day of analysis.

Table 6. Individual results (µg OA total equivalents/kg) reported from laboratories A to P for Materials A, D, E, F, G, K, L, and N on Days 1 and 2. Invalid or incorrect results are those in bold type.

	µg OA total equivalents/kg															
	Material															
	A		D		E		F		G		K		L		N	
	Day															
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	<63	<63	186	239	97	102	<63	<63	<63	<63	248	281	167	174	210	247
B	<63	<63	251	266	100	101	<63	<63	<63	<63	302	299	177	190	273	277
C	<63	<63	244	233	96	87	<63	<63	<63	<63	279	246	174	160	256	251
D	<63	<63	264	253	125	100	<63	<63	<63	<63	282	277	189	223	269	295
E	<63	<63	210	233	101	120	<63	<63	<63	<63	239	244	156	181	226	219
F	<63	<63	252	250	113	116	<63	<63	<63	<63	287	286	166	165	271	275
G	<63	<63	246	252	89	100	<63	<63	<63	<63	356[a]	269[a]	192	192	274	236
H	<63	<63	253	250	90	99	<63	<63	<63	<63	291	301	175	179	271	270
I	<63	<63	252	254	95	87	<63	<63	<63	<63	284	283	169	161	265	253
J	70[a]	98[a]	238	239	163[a]	102[a]	<63	<63	78[a]	67[a]	248	268	239	184	246	235
K	<63	<63	253	264	81	81	<63	<63	<63	<63	295	300	152	160	247	266
L	**—**	**<63**	**—**	**242**	**—**	**145**	**—**	**<63**	**—**	**—**	**—**	**266**	**—**	**202**	**—**	**182**
M	<63	<63	257	255	101	104	<63	<63	<63	<63	292	274	177	176	271	272
N	<63	<63	261	251	98	101	<63	<63	<63	<63	285	285	161	181	257	250
O	<63	<63	221	223	91	94	<63	<63	<63	<63	270	249	179	184	259	244
P	<63	<63	192	241	69[a]	153[a]	<63	<63	<63	<63	226	278	97	173	206	259

[a] Outlier.

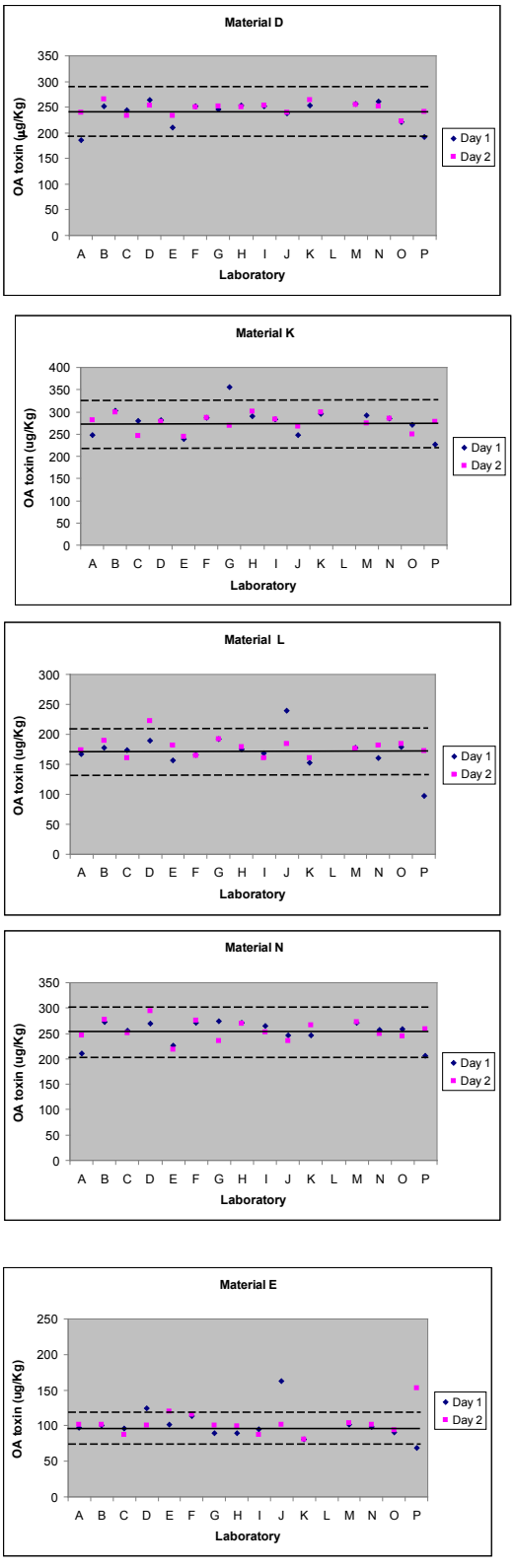


Figure 1. Individual results for each test material obtained per lab and per day of analysis (including outliers). The solid line shows the assigned mean value calculated in this study for each material. The dashed lines indicate the theoretical reproducibility SD determined for each material in this study ($PRSD_R$).

Statistics

Analysis of Valid Data and Outliers

Statistical data analysis was carried out following the approach described in the AOAC/IUPAC guidelines (17, 20). Submitted results were initially reviewed to remove invalid data. Results from assays with calibration curves with a $R^2 < 0.96$ and results outside the working range or showing deviations from the Standard Operating Procedure were considered invalid.

The valid data were first analyzed for possible outliers applying the Cochran and Grubbs tests. Then, precision parameters, HorRat values, and recovery were calculated.

The Cochran test was applied to remove laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material. A 1-tail test at a probability value of 2.5% was applied (17, 20).

The Grubbs test was used to remove results from laboratories with extreme averages (17, 20). This test was applied to the remaining values from the Cochran test. A single value test (two-tail, $P = 2.5\%$) was first applied, followed by a pair value test (two values at the highest end, two at the lowest end, and one at each end, at an overall $P = 2.5\%$).

Precision

To estimate the precision of the method, the within-laboratory repeatability and between-laboratory reproducibility were determined by calculating s_r (repeatability SD), s_R (reproducibility SD), RSDs (RSD_r and RSD_R), repeatability and reproducibility limits (r and R), and HorRat values. These parameters were calculated following the AOAC guidelines (20).

Recovery

For recovery calculations, the marginal recovery was calculated as follows:

$$\text{Recovery, \%} = 100\,(C_f - C_u)/C_A),$$

where C_f is the amount found for the spiked concentration, C_u is the amount present originally for the unspiked concentration, and C_A is the amount added.

Results and Discussion

Test Material Results

The test materials were first analyzed by OkaTest and LC/MS/MS to determine the content and profile of OA toxins. Results obtained by both methods for samples A, F, and G showed concentration for OA toxins below their LOD (44 and 40 µg/kg, respectively). The BM was tested by LC/MS/MS (19) at EURLMB, and no peaks were detected for this group of toxins (LOD for this method is 15 µg/kg). Therefore, materials A, F, G, and BM were considered blank; therefore, no homogeneity or stability studies were carried out.

Analyses by LC/MS/MS were used to identify the toxin profile and to ensure that all toxins belonging to the OA group were present in the materials. Table 2 shows concentration

Table 7. Details of the test materials, number of results submitted, and results after removing outliers, together with performance values of precision (repeatability and reproducibility) obtained for the colorimetric OkaTest[a]

							Repeatability[c]			Reproducibility[c]			
							µg total equiv.OA/kg						
Test material	Matrix	Runs/lab	No. labs submitting results	No. labs after invalid/incorrect results	No. of labs after outliers[b]	Mean (µg total equivalent OA/kg)[c]	S_r	r	RSD_r, %	S_R	R	RSD_R, %	HorRat
A	Mussel *M. galloprovincials*	2	16	14	—	<63	—	—	—	—	—	—	—
D	Clam *V. pullastra*	2	16	15	15 (0)	242	14.7	41.2	6.1	19.4	54.4	8.0	0.4
E	Mussel *M. galloprovincialis*	2	16	15	13 (2)	98.8 (102)	7.32 (20.8)	20.5 (58.4)	7.4 (20.5)	10.7 (19.6)	30.0 (54.8)	10.7 (19.2)	0.5 (0.8)
F	Scallop *P. maximus*	2	16	15	—	<63	—	—	—	—	—	—	—
G	Clam *V. decussatus*	2	16	14	—	<63	—	—	—	—	—	—	—
K	Clam *V. romboides*	2	16	15	14 (1)	275 (277)	14.9 (21.4)	41.8 60.1)	5.4 (7.7)	21.0 (25.0)	58.7 (70.1)	7.6 (9.0)	0.4 (0.5)
L	Cockle *C. edulis*	2	16	15	15 (0)	175	19.6	55.0	11.2	23.2	64.9	13.2	0.6
N	Mussel *M. edulis*	2	16	15	15 (0)	255	15.6	43.7	6.1	20.7	58.1	8.1	0.4

[a] S_r = Repeatability SD, S_R = reproducibility SD, RSD_r = repeatability RSD, RSD_R = reproducibility RSD, r = repeatability limit, R = reproducibility limit.

[b] Number of laboratories remaining after removal of outliers (number of outliers).

[c] Mean, repeatability, and reproducibility (values obtained including outliers).

in OA equivalents determined by OkaTest and toxins profile of the different materials used. All test materials were found to be stable for the duration of the study and with sufficient homogeneity (Tables 3 and 4).

Interlaboratory Study Results

All participants who received test materials reported results. The sample concentration was calculated by standard curves obtained by each laboratory every day of analysis. Fit parameters of each standard curve are shown Table 5. Although the slopes show differences depending on the laboratory and day, the calculated samples concentration was not affected. The data obtained by each laboratory per test material and day of analysis are shown in Table 6.

All individual values obtained per material, day and laboratory were also plotted. One graph per material is shown in Figure 1. The solid lines represent the assigned mean value obtained for each material in this study (Table 7). The area between the dashed lines demonstrates the range of deviation from the mean value based on the theoretical reproducibility SD ($PRSD_R$).

Two laboratories reported one of the assays with $R^2 < 0.96$; one (Laboratory A) repeated the analysis obtaining R^2 within the required criterion. Laboratory L, however, could not repeat the assay on time, and those results were considered invalid and removed for statistical analysis.

Materials A, F, and G were not statistically analyzed, as they were blank samples. However, Laboratory J reported values within the working range of the test for Materials A and G. These values are considered incorrect according to the AOAC guidelines (20), as they are positive values found for a blank material. All the other laboratories in the study identified the blank materials below the working range of the test.

The valid data from the contaminated test materials (D, E, K, L, and N) were then analyzed for identification of outliers applying Cochran and Grubbs tests (20). Results from Laboratory L could not be included in the statistical analysis, as only one value per material was available.

The Cochran test showed Laboratory G for Material K and Laboratory P for Material E as outliers. This test was applied again after these outliers were removed. Laboratory J for Material E was also excluded in a second round. The Grubbs single and pair values tests were then applied; no further outliers were identified.

The mean values assigned for OA-toxins for the test materials were 98.8, 175.4, 242.8, 255.0, and 275.0 µg total equivalents OA/kg for Materials E, L, D, N, and K, respectively (Table 7).

Values obtained for repeatability SD (S_r) ranged from 7.3 µg/kg for Material E to 19.6 µg/kg for Material L, with repeatability RSDs (RSD_r) from 5.4% for Material K to 11.2% for Material L (Table 7). The reproducibility SD (S_R) calculated for the five test materials ranged from 10.7 to 23.2 µg/kg, with reproducibility RSD (RSD_R) values from 7.6 to 13.2% for Materials K and L, respectively (Table 7).

The HorRat values obtained were 0.4 for Materials D, K, and N, 0.5 for Material E, and 0.6 for Material L (Table 7), indicating a very good performance of the method. These values are just at the lower limit of the range considered as normally expected for a good reproducibility of a method ($0.5 < HorRat \leq 1.5$), according to the AOAC guidelines (20). HorRat values between 0.64 and 2.61 for OA-toxins group (21), 0.3 and 2.0 for paralytic

Table 8. Results from the recovery experiment carried out during Day 2 of the interlaboratory study

	µg OA total eq./kg			
Lab code	BM[a]	Spiked concn.	BM + OA[b]	Recovery, %
A	—	161	172	107.1
B	—	161	162	100.7
C	—	161	155	96.3
D	—	161	115	71.6
E	—	161	124	77.3
F	—	161	138	85.5
G	—	161	162	100.7
H	—	161	131	81.1
I	—	161	152	94.4
J	—	161	197	122.3
K	—	161	152	94.4
L	—	161	196	121.6
M	—	161	153	95.0
N	—	161	174	108.3
O	—	161	155	96.3
P	—	161	185	114.7
Mean recovery, %				98.0
SD				14.2
RSD, %				14.5

[a] BM = Blank material. No OA toxins were detected; therefore, a concentration of zero was considered for calculation purposes.

[b] BM + OA = Concentration of the samples spiked with 161 µg/kg.

shellfish toxins (22) and 1.1 to 2.4 for domoic acid (23) were previously described for other methods.

The statistical analysis was also carried out including outliers (Table 7). Although there were some differences when including outlier values, repeatability and reproducibility remained satisfactory and within the expected values for this type of interlaboratory study.

Although the main objective of the validation study was to determine the repeatability and between-laboratory reproducibility of the OkaTest kit, a recovery assay was also carried out. A scallop blank sample (BM) was spiked with OA by each laboratory, and the recovery of OkaTest calculated. Recovery values from all participants ranged from 71.6 to 122.3%. The mean and RSD were 98.0 and 14.5%, respectively (Table 8). These recoveries met the criteria set in the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (24).

Comments from Participants

Most participants reported that the SOP for the method provided all the information they needed to perform the assay and that they did not have difficulties understanding any part of it. Some comments were made about the phosphatase preparation. Those led to the conclusion that the use of a nonorbital shaker does not always guarantee full dissolution of this reagent. Manual mixing, longer preparation, and a final visual check of the solution should be included in the SOP. Other minor comments were made, and were answered or resolved by the study director.

Conclusions

The precision and recovery values determined in this study for OkaTest can be considered satisfactory for this type of methodology and the concentration range required. The colorimetric PPIA, OkaTest, could be used as an assay complementary to the reference method for determination of the OA toxins group in molluscs according to the Commission Regulations (EC) No. 2074/2005 and No. 15/2011. Additional methods have to be implemented in a laboratory to analyze all regulated lipophilic marine biotoxins.

Acknowledgments

We thank the following collaborators for their participation, time, effort, and comments towards this study:

Dolores Calvo, ZEU-INMUNOTEC, Spain;

Monica Campàs, IRTA, Spain;

Guntis Cepurnieks, Institute of Food Safety, Animal Health and Environment, Latvia;

David Clarke, Marine Environment and Food Safety Services, Ireland;

Jorge Correa, INTECMAR, Spain;

Lorena A. Delgado, Public Health Institute, Chile;

Alejandra Goya A, SENASA, Argentina;

Katrin Kapp, BfR (Federal Institute for Risk Assesment), Germany;

Panagiota Katikou, Ministry of Rural Development and Food Centre of Veterinary Institutions of Thessaloniki Institute of Food Hygiene, Greece;

Mirslaw Michalski and Kataryna Graziel, National Veterinary Research Institute, Poland;

María José Chapela, ANFACO-CECOPESCA, Spain;

Sonia Piñero, European Reference Laboratory for Marine Toxins, Spain;

Vlad Serafim, The Institute for Diagnosis and Animal Health, Romania;

Ulrich Schwank, Bavaria's policies on health and consumer protection (LGL), Germany;

Andrew Turner and Clothilde Brunet, CEFAS, UK; and

Paulo Vale and Susana Rodrigues, IPIMAR, Portugal.

Note: Collaborative efforts among the European Reference Laboratory for Marine Toxins (EURLMB), NRLs, and ZEU-INMUNOTEC does not amount to an endorsement of the firm's products.

References

(1) Xing, Y., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B., & Shi, Y. (2006) *Cell* **127**, 341–353. http://dx.doi.org/10.1016/j.cell.2006.09.025

(2) Regulation (EC) No. 853/2004 (2004) *Off. J. Eur. Union* **L139**, 55–205

(3) Commission Regulation (EC) No. 15/2011 (2011) *Off. J. Eur. Union* **L6**, 3–6

(4) Commission Regulation (EC) No. 2074/2005 (2005) *Off. J. Eur. Union* **L338**, 27–59

(5) Takai, A., Bialojan, C., Troschka, M., & Rüegg, J.C. (1987)

FEBS Lett. **217**, 81–84. http://dx.doi.org/10.1016/0014-5793(87)81247-4

(6) Bialojan, C., & Takai, A. (1988) *Biochem. J.* **256**, 283–290

(7) Simon, J.F., & Vemoux, J.-P. (1994) *Nat. Toxins* **2**, 293–301. http://dx.doi.org/10.1002/nt.2620020508

(8) Honkanen, R.E., Stapleton, J.D., Bryan, D.E., & Abercrombie, J. (1996) *Toxicon* **34**, 1385–1392. http://dx.doi.org/10.1016/S0041-0101(96)00095-5

(9) Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., & Yasumoto, T. (1996) *Toxicon* **34**, 743–752. http://dx.doi.org/10.1016/0041-0101(96)00027-X

(10) Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M.V., & Botana, L.M. (1997) *Anal. Biochem*. **248**, 258–264. http://dx.doi.org/10.1006/abio.1997.2127

(11) Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M., Truman, P., & Hannah, D.J. (1999) *Toxicon* **37**, 909–922. http://dx.doi.org/10.1016/S0041-0101(98)00222-0

(12) Ramstad, H., Shen, J.L., Larsen, S., & Aune, T. (2001) *Toxicon* **39**, 1387–1391. http://dx.doi.org/10.1016/S0041-0101(01)00097-6

(13) Mountfort, D.O., Suzuki, T., & Truman, P. (2001) *Toxicon* **39**, 383–390. http://dx.doi.org/10.1016/S0041-0101(00)00144-6

(14) Gonzalez, J.C., Leira, F., Fontal, O.I., Vieytes, M.R., Arévalo, F.F., Vieites, J.M., Bermúdez-Puente, M., Muñiz, S., Salgado, C., Yasumoto, T., & Botana, L.M. (2002) *Anal. Chim. Acta* **466**, 233–246. http://dx.doi.org/10.1016/S0003-2670(02)00597-4

(15) Smienk, H.G.F., Calvo, D., Razquin, P., Domínguez, E., & Mata, L. (2012) *Toxins* **5**, 339–352. http://dx.doi.org/10.3390/toxins4050339

(16) Sassolas, A., Catanante, G., Hayat, A., & Marty, J.-L. (2011) *Anal. Chim. Acta* **702**, 262–268. http://dx.doi.org/10.1016/j.aca.2011.07.002

(17) Thompson, M., Ellison, S.L.R., & Wood, R. (2006) *Pure App. Chem.* **78**, 145–196. http://dx.doi.org/10.1351/pac200678010145

(18) Capela, M.J., Reboreda, A., Vieites, J.M., & Cabado, A.G. (2008) *J. Agric. Food Chem*. **56**, 8979–8986. http://dx.doi.org/10.1021/jf801572j

(19) Villar-González, A., Rodriguez-Velasco, M.L., & Gago, A. (2011) *J. AOAC Int*. **94**, 909–922

(20) *AOAC Official Methods of Analysis, Interlaboratory Collaborative Study* (2002) AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D, p. 9

(21) Van den Top, H.J., Gerssen, A., & Van Egmond, H.P. (2011) *Report on Quantitative Determination of Liphophhilic Toxins in Shellfish by LC/MS/MS*, http://www.edepot.wur.nl/180890

(22) Van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., & MacNeil, J.D. (2011) *J. AOAC Int*. **94**, 1154–1176

(23) Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., & Gokoyr, V. (2007) *J. AOAC Int.* **90**, 1011–1027

(24) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

Proposal No. **13-113**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Jennifer Rice
Affiliation	Neogen Corporation
Address Line 1	620 Lesher Place
Address Line 2	
City, State, Zip	Lansing, MI 48912
Phone	517-372-9200
Fax	517-367-0514
Email	jrice@neogen.com
Proposal Subject	Reveal 2.0 DSP
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas
Text of Proposal/ Requested Action	.11 Approved NSSP Laboratory Tests We request review of the validation study submission for the Reveal 2.0 DSP (okadaic acid group) test kit and consideration of the method for approval as a screening method for qualitative determination of okadaic acid group in shellfish. Add Reveal DSP to Section IV. Guidance Documents, Chapter II. Growing Areas, .11 Approved NSSP Laboratory Tests.
Public Health Significance	Toxins that cause diarrhetic shellfish poisoning (DSP) include the okadaic acid (OA) group of toxins [1, 2] OA is produced by marine dinoflagellates such as Dinophysis, and has structural analogues referred to as the dinophysistoxins (DTXs). The U.S. Food and Drug Administration action limits are 160 ppb OA equivalents (OA, DTX1, DTX2, DTX3) in shellfish. LC-MS/MS methods [3] have been accepted as quantitative reference methods in many parts of the world. Assays facilitating more rapid determination of OA toxins with simplified procedures are needed by the shellfish industry and regulatory authorities. [1] J. Sobel and J. Painter (2005), Illness caused by Marine Biotoxins. Clin. Infect. Dis. 4, 1290. [2] Van Dolah, Frances M. (2000), Marine algal toxins: origins, health effects, and their increased occurrence. Environmental health perspectives 108. Suppl 1, 133. [3]Community Reference Laboratory for Marine biotoxins (CRLMB)., Agencia Española de Seguridad Alimentaria y Nutrición (AESAN). (2009). EU Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS. Version1. http://www.aesan.msps.es/en/CRLMB/web/procedimientos_crlmb/crlmb_standard operating_procedures.shtml
Cost Information	Approximately $17.00 per test. Reader based assay – approximate cost of Reader $1995.
Action by 2013 Laboratory Method and Quality Assurance Review Committee	Recommended referrals of Proposal 13-113 to an appropriate committee as determined by the Conference Chairman and await data to determine if the method is fit for purpose within the NSSP.
Action by 2013	Recommended adoption of Laboratory Method Review and Quality Assurance

Task Force I	Committee recommendation on Proposal 13-113.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-113.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-113.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-113 to an appropriate committee as determined by the Conference Chair until additional data are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-113.
Action by 201 General Assembly	Adopted recommendation of Task Force I on Proposal 13-113.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-113.

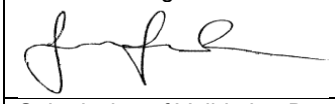
ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Reveal 2.0 DSP
Name of the Method Developer	Neogen Corporation
Developer Contact Information	Jennifer Rice 517-372-9200 Jrice@neogen.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for a simple, rapid screening method for okadaic acid (OA) and dinophysistoxins (DTX) in shellfish, one that can be used in the field as well as in a laboratory setting.
2. What is the intended purpose of the method?		The method is designed for rapid qualitative screening of shellfish for OA group of toxins (OA and DTXs).
3. Is there an acknowledged need for this method in the NSSP?		Simple assays that provide rapid and accurate results are needed.
4. What type of method? i.e. chemical, molecular, culture, etc.		Lateral flow immunoassay in dipstick format.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Reveal 2.0 DSP
Method Scope		Qualitative detection of OA and DTXs in mussels, oysters, clams and scallops.
References		Study report and kit insert included in this submission.
Principle		Competitive lateral flow immunoassay in dipstick format. Solvent extraction of analyte from homogenized shellfish tissue.
Any Proprietary Aspects		Yes, commercial test kit.
Equipment Required		Timer, bag roller, microwell holder, pipettes (1.0, 0.1 mL), heatblock, reader
Reagents Required (consumables)		Reveal DSP test devices, extraction bags with mesh filter, filter syringes, microwells, methanol, screwcap glass vials, sodium hydroxide and hydrochloric acid
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2-8^{o}C before testing.
Safety Requirements		Used test devices, extraction bags, microwells, and pipettes should be treated as if contaminated with OA group toxins and handled accordingly. Gloves and lab coats should be worn while performing the test.
Clear and Easy to Follow Step-by-Step Procedure		Step-by-step procedure in kit insert and study report.
Quality Control Steps Specific for this Method		Test device contains an internal control (control line) that confirms that the device is functioning properly. An okadaic acid solution in buffer at a concentration 5.34

		ng/mL can be used as an external positive control, if desired. This is the equivalent of a shellfish samples containing OA at a level of approx. 320 ug/kg (320 ppb).
C. Validation Criteria		
1. Accuracy / Trueness		No false negatives with incurred samples containing at or above 160 ppb OA eqs (various toxin profiles and species). No false positives with incurred samples containing at or below approx. 80 ppb OA eqs.
2. Measurement Uncertainty		Not applicable.
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable.
5. Specificity (cross-reactivity)		Approx. cross reactivity profiles at cut-off (based on QC): OA: 100% DTX1: 89% DTX2: 47% No impact on test results by potentially interfering compounds – Domoic acid (incurred samples).
6. Working and Linear Ranges		Not applicable.
7. Limit of Detection		Approximate cut off for positive results (based on QC): OA: 125 ppb OA eqs. DTX1: 140 ppb OA eqs DTX2: 160 ppb OA eqs
8. Limit of Quantitation / Sensitivity		Not applicable.
9. Ruggedness		No impacts on performance in results using 3 kit lots, +/- 2 min variation in test incubation time or +/- 10 uL in sample volume.
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		The assay was found comparable to LC-MS/MS reference methods in testing naturally incurred OA/DTX samples
D. Other Information		
1. Cost of the Method		Approximately $17.00 per test (list price)
2. Special Technical Skills Required to Perform the Method		None
3. Special Equipment Required and Associated Cost		Reader (list price approximately $1,995)
4. Abbreviations and Acronyms Defined		ppb = parts per billion, equivalent to ug/kg
5. Details of Turn Around Times (time involved to complete the method)		The test can be fully performed in less than 30 min including sample preparation. If hydrolysis is necessary, this adds approximately 40 min to the procedure.
6. Provide Brief Overview of the Quality Systems Used in the Lab		
Submitters Signature 	Date: June 27, 2013	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Laboratory Evaluation Checklist – Reveal 2.0 DSP

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
SHELLFISH PROGRAM IMPLEMENTATION BRANCH
SHELLFISH SAFETY TEAM
5100 PAINT BRANCH PARKWAY
COLLEGE PARK, MD 20740-3835
TEL. 301-436-2151/2147 FAX 301-436-2672

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**
LABORATORY REPRESENTED BY:	**TITLE:**	
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:** **REGION:**	
OTHER OFFICIALS PRESENT:	**TITLE:**	

Items which do not conform are noted by:

C - Critical **K** - Key **O** - Other **NA** - Not Applicable Conformity is noted by a **"√"**

PART I – QUALITY ASSURANCE		
Code		**Item Description**
		1.1 Quality Assurance (QA) Plan
K		1. Written Plan adequately covers all the following: (check √ those that apply) a. ☐ Organization of the laboratory. b. ☐ Staff training requirements. c. ☐ Standard operating procedures. d. ☐ Internal quality control measures for equipment, calibration, maintenance, repair and performance. e. ☐ Laboratory safety. f. ☐ Quality assessment. g. ☐ Proper animal care.
C		2. QA plan implemented.
		1.2 Work Area
O		1. Adequate for workload and storage.
O		2. Clean and well lighted.
O		3. Adequate temperature control.
O		4. All work surfaces are nonporous and easily cleaned.
C		5. A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.
		1.3 Laboratory Equipment
C		1. The balance provides a sensitivity of at least 0.1g at a load of 150 grams.
K		2. The balance calibration is checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent. Records maintained.
C		3. Refrigerator temperature is maintained between 0 and 4°C.
K		4. Refrigerator temperature is monitored at least once daily. Records maintained.
C		5. Freezer temperature is maintained at -20°C or below.
K		6. Freezer temperature is monitored at least once daily. Record maintained.
C		7. All glassware/plastic used with the high speed blender for homogenization is cleaned with water after each use.
C		8. Accuscan Pro Reader is calibrated before use
C		9. The correct QR code is scanned in the reader for the lot of strips that will be used
C		10. The heat block (or waterbath) is set at 76^{o}C (+/- 2 ^{o}C)
		1.4 Reagent and Reference Solution Preparation and Storage
C		1. Buffers are stored in plastic screw top vials at room temperature.
K		2. Buffers are within expiration date.
C		3. Analytical grade methanol, 2.5M HCL and 2.5M NaOH are used for extracton
		1.5 Collection and Transportation of Samples
K		1. Shellstock are collected in clean, waterproof, puncture resistant containers.
K		2. Samples are appropriately labeled with the collector's name, harvest area and time and date of collection.
K		3. Immediately after collection, shellstock samples are placed in dry storage for transport (e.g. cooler) which is maintained between 0 and 10°C. Upon receipt at the lab, samples are placed under refrigeration.
K		4. The time from collection to completion of the assay does not exceed 48 hours if stored refrigerated. However, if there are significant transportation delays, then shellstock samples are processed immediately as follows *(circle the appropriate choice):* a. Washed, shucked, drained, frozen until extracted; b. Washed, shucked, drained, homogenized and frozen; c. The laboratory has an appropriate contingency plan in place to handle samples which can't be analyzed within 24 hours due to transportation issues.
K		5. Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.

PART II – EXAMINATION OF SHELLFISH FOR DSP TOXIN		
		2.1 Preparation of Sample
C		1. At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.
O		2. The outside of the shell is thoroughly cleaned with fresh water.
O		3. Shellstock are opened by cutting adductor muscles.
O		4. The inside of the shell is rinsed with fresh water to remove sand or other foreign material.
O		5. Shellfish meats are removed from the shell by separating adductor muscles and tissue connecting at the hinge.
K		6. Damage to the body of the mollusk is minimized in the process of opening.
O		7. Shucked shellfish are drained on a #10 mesh sieve (or equivalent) without layering for 5 minutes.
K		8. Pieces of shell and drainage are discarded.
C		9. Drained meats or thawed homogenates are blended at high speed until a homogenous sample is obtained (time required is species dependent).
		2.2 Extraction Procedure – Rapid (to detect OA, DTX1, DTX2)
C		1. The extraction bag is numbered on both sides using a marker, so that one side is labelled "1" and the opposite side labelled "2".
C		2. 2 g (± 0.1 g) of homogenized sample is weighed into the bottom of extraction bag on side "1"
C		3. 8 mL of methanol is added to side "1" of the extraction bag containing the sample.
K		4. The green straw is placed approximately half-way down from the top of the bag and the upper edge of the bag is folded over the green straw ensuring that the sample and methanol remain in the lower half of the bag.
K		5. The white clip is firmly applied to the bag to prevent sample leakage.
C		6. The extraction bag is placed firmly on a surface and the roller is pressed firmly on the sample extraction bag pushing the roller back and forth for 30 seconds.
K		7. The green straw and white clip are removed and the bag contents from side "2" are removed (with pipette or poured) into a clean container.
K		8. The extraction bag is discarded as biohazardous waste.
C		9. The sample extract is poured into the barrel of a filter syringe until the syringe is approximately half full. The plunger is placed on top and approximately 1 mL of the sample is filtered into a collection tube.
C		10. The filtered solution is transparent and not cloudy. If cloudy, then the solution is refiltered through a fresh syringe filter.
C		11. 100 µl of the sample extract is removed using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and added into DSP buffer B vial.
		2.3 Extraction Procedure – Hydrolysis (to detect OA, DTX1, DTX2, DTX3)
C		1. The Heater block is turned on to 76°C
C		2. 800 µL of the sample prepared in step 9 is transferred into a screw cap glass vial.
C		3. 100 µL of NaOH (2.5 M) is added to the vial and the vial is capped tightly.
C		4. The sample is thoroughly mixed using a vortex on high speed for 30 seconds.
C		5. The vial is heated in the heater block at 76°C for 40 mins
K		6. After 40 mins, the vial is removed from the heater block and allowed to cool to room temperature or the sample vial is placed on ice to cool to room temperature.
C		7. 100 µL of HCl (2.5 M) is added to the glass vial and mixed using a vortex on high speed for 30 seconds.
C		8. 100 µl of the sample extract is removed using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and added into a DSP buffer A vial.

		2.4Assay Procedure
K		1. The appropriate number of microwells are removed and place into the microwell holder.
C		2. The DSP buffer vial (containing diluted sample) from either extraction process, is shaken vigorously by hand for 30 seconds.
C		3. 100 µL of diluted sample is transferred into each microwell using a new disposable pipette
K		4. The required number of test strips are removed from the lateral flow device container and the container is immediately closed.
C		5. The DSP test strips with the sample end down (Neogen logo on top) are placed into the microwells.
C		6. The strip is allowed to develop in the microwell for 15 minutes.
C		7. After 15 minute run time, the test strip is immediately removed and read using the AccuScan® Pro reader
		2.5 Reading Test Results
C		1. Test strips are read within 1 minute of completion of the 15 minute incubation.
C		2. The Reveal 2.0 DSP test strip is fully inserted into the black cartridge "R" adapter with the sample end first and results facing out.
K		3. The cartridge with test strip side up is inserted in the AccuScan® Pro. The reader automatically begins analysis of the cartridge. The cartridge is not removed until the reader has completed the analysis.
O		4. Results are displayed on the AccuScan® Pro reader and stored by the reader.
O		5. The reader reports Positive (160ppb or greater OA EQS) or Negative (<160ppb OA EQS).

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Obersvation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DSP COMPONENT: PARTS I and II	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key and Other (O) nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Does Not Conform Status** The DSP component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is $\geq$ 3 **or**
   b. The total # of Key nonconformities is $\geq$ 6 **or**
   c. The total # of Critical, Key and Other is $\geq$ 10

2. **Provisionally Conforms Status**: The PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is $\geq$ 1 but < 3

C. Laboratory Status *(circle appropriate)*
Does Not Conform - Provisionally Conforms – Conforms

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NEOGEN® CORPORATION

June 27, 2013

Laboratory Methods Review & Quality Assurance Committee
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223-1740

Dear Members of the Committee:

Please find enclosed a validation study report and other supporting documentation for the Reveal 2.0 DSP test kit. We respectfully request your review of this submission and consideration of the test for acceptance as an ISSC approved method for qualitative determination of okadaic acid group of toxins (OA, DTX1, DTX2, DTX3) in molluscan shellfish. We believe that the test provides significant advantages in terms of time-to-result and ease of use, and we feel that acceptance of the method by ISSC will be of benefit to the shellfish industry and public health authorities.

I would be pleased to answer any questions or provide any further information that you may require. Thank you very much for your consideration.

Sincerely,

Jennifer Rice, DVM, MSc, Ph.D, MBA
Vice President and Senior Research Director
Neogen Corporation

Enclosures: Validation study report
Reveal 2.0 DSP Kit insert
Hydrolysis Pack Kit insert
Reveal 2.0 DSP Hydrolysis Pack MSDS
Reveal 2.0 DSP MSDS
Single lab validation checklist
2013 ISSC conference proposal
Laboratory Evaluation Checklist - DSP

620 Lesher Place • Lansing, MI 48912-1595
(517) 372-9200 • (800) 234-5333 • Fax: (517) 367-0514
www.neogen.com • e-mail: jrice@neogen.com

Validation Study of the Reveal ® 2.0 DSP Test for the Qualitative Detection of the Okadaic Acid group of toxins in Shellfish

Waqass Jawaid, Karrie Melville, Mark Hooper, Paul Muirhead, Frank Klein and Jennifer Rice*
Neogen Corporation
620 Lesher Place, Lansing, MI 48912 USA
*corresponding author: phone 517-372-9200, email jrice@neogen.com

Submitted June 2013

1. Introduction

Toxins that cause diarrhetic shellfish poisoning (DSP) include the okadaic acid (OA) group of toxins. OA is produced by marine dinoflagellates such as *Dinophysis*, and has structural analogues referred to as the dinophysistoxins (DTXs). The established European Union maximum permitted levels are 160 µg OA equivalents (OA, DTX1, DTX2, DTX3, pectenotoxins) per kg shellfish meat (160 ppb OA eqs). The U.S. Food and Drug Administration action limits are 160 ppb OA equivalents (OA, DTX1, DTX2, DTX3) in shellfish.

LC-MS/MS methods [1] have been accepted as quantitative reference methods in many parts of the world. Assays facilitating more rapid determination of OA toxins with simplified procedures are needed by the shellfish industry and regulatory authorities.

In this report, we describe results of a validation study of the Reveal 2.0 DSP test for qualitative detection of the OA group (OA, DTXs 1 - 3) in shellfish. Reveal 2.0 DSP is a lateral flow immunoassay designed for rapid determination of OA-group toxins at or greater than 160 ppb OA eqs. The test is easy to use and results can be obtained in less than 70 mins, including sample preparation. A preliminary screening result for free toxins (OA, DTX1, DTX2) is also possible to obtain within the first 25 mins, including sample preparation.

2. Principle of the Method

Reveal 2.0 DSP is a single-step, lateral flow device based on a competitive immunoassay format. In summary, the shellfish extract is wicked through a reagent zone, containing antibodies specific for OA-group toxins that have been conjugated to coloured particles. If the toxins are present in the sample, the toxin will be captured by the particle-antibody complex. The complex is then wicked onto a membrane which contains a stationary capture zone of a toxin-protein conjugate. This zone captures any uncomplexed toxin particle-antibody. Therefore, as the concentration of toxins in the sample increases, the test line intensity decreases. The membrane also contains a stationary control zone which always will form regardless of the level of toxins. Results are analyzed qualitatively as either positive or negative using Neogen's AccuScan® Pro Reader.

3. Intended Use

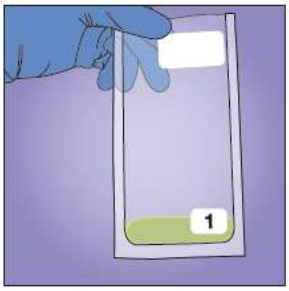
Reveal 2.0 DSP is a simple to use assay intended for the qualitative screening of shellfish for OA-group toxins at levels of 160 ppb or above. The test kit is designed for use by quality control personnel and other personnel who may be involved with handling shellfish possibly contaminated by OA toxins.

4. Reveal 2.0 DSP Method

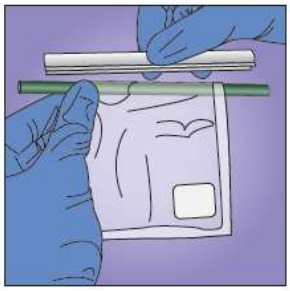
The kit insert is included as Appendix I which describes the rapid extraction method and the hydrolysis method. An overview of the rapid extraction method is shown below.

Rapid Screen Procedure

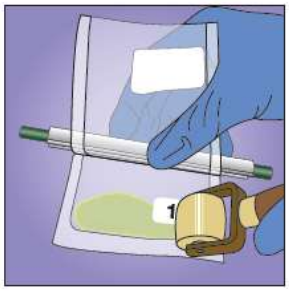
1. Number both sides of the extraction bag, one side is labelled **1** and opposite side labelled **2**



2. Weigh out 2 g (± 0.1 g) of homogenised sample and add to bottom of the extraction bag on side **1**. Add 8ml of analytical grade methanol into side **1** of extraction bag.



3. Fold the upper edge of the bag over the green straw and clip the white clip to prevent leakage of the sample.

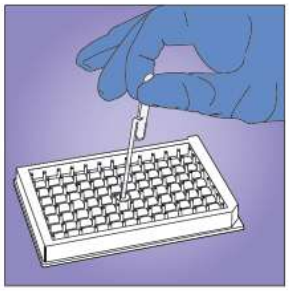


4. Press the roller firmly on the sample extraction bag, pushing the roller back and forth for **30 seconds to aid in obtaining a homogenous sample extract.**

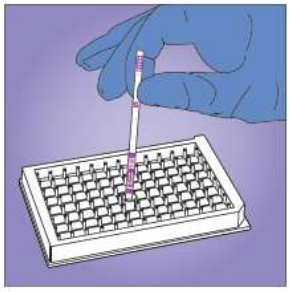
5. Remove straw and clip. Pour all bag contents from side 2 into a suitable container. Discard the used extraction bag.

6. Shake sample cup vigorously by hand for 30 seconds. Filter 1–2 mL of the sample into the collection test tube.

7. Remove 100 uL of the sample extract using a disposable pipettor, add into DSP buffer B vial and mix.



8. Transfer 100 uL of the diluted sample to a microwell.



9. Place the DSP strip into the well. Set a timer for **15 minutes.**

10. Remove promptly at 15 minutes and interpret results using the AccuScan® Pro Reader.

Reveal® 2.0 for DSP, an easy to use, one-step rapid test. Usable in the field as well as in the lab, with consistent interpretation of results using AccuScan® Pro Reader.

4.1. MATERIALS PROVIDED

Reveal 2.0 DSP (Neogen item 9561)

1. 24 Reveal 2.0 DSP lateral flow test strips
2. 24 microwells
3. 24 vials of DSP buffer A (for hydrolysis extraction protocol)
4. 24 vials of DSP buffer B (for rapid extraction protocol)
5. 25 extraction bags
6. 48 disposable exact volume (100 µl) pipettors

4.2. **MATERIALS RECOMMENDED BUT NOT PROVIDED**

1. Marine biotoxins starter kit (Neogen item 9563)
   - Microwell holder
   - 1 roller
   - 1 bag clip (white clip with green straw)
2. Filter syringes (Neogen item 9420)
3. Sample collection cups with lids (Neogen items 9428, 9428B)
4. Blender (Neogen items 9493, 9477 or 9495)
5. Scale capable of weighing 0.5 – 400 g ± 0.1g (Neogen item 9427)
6. Timer (Neogen item 9452)
7. Graduated cylinder, 10 mL
8. AccuScan® Pro reader (Neogen item 9565)

4.3. MATERIALS REQUIRED IF PERFORMING THE HYDROLYSIS EXTRACTION

1. Reveal 2.0 DSP hydrolysis accessory pack (Neogen item 9561), which contains
   - 2.5 M sodium hydroxide (NaOH) solution (5 mL)
   - 2.5 M hydrochloric acid (HCl) solution (5 mL)
2. Methanol (analytical grade recommended)
3. Pipettor, 100-1000 µL and pipette tips
4. Heater block capable of holding 76 ± 2 °C (recommended Stuart item SBH130D)
5. Glass sample vials (recommended Water's item 600000751CV)
6. Vortex (Capable of speeds of approximately 3000 rpm. E.g. Fisher Scientific item 11726477)

4.4. PRECAUTIONS

1. The test strips must remain inside the stay-dry tube before use.
2. Do not use kit contents beyond expiration date.
3. Treat all liquids, including sample extract, and used components as if contaminated with toxin. Gloves and other protective apparel should be worn at all times.
4. To avoid cross-contamination, use clean pipettes, extraction bags and fresh extraction solutions for each sample.
5. A Material Safety Data Sheet (MSDS) is available from Neogen Corp.

4.5. Storage Requirements

Store kit components at controlled room temperature (18-30^{o}C, 64-86^{o}F). Do not freeze. Test strips should remain in their original sample tubes until use to maintain shelf life and ensure optimal performance.

4.6. AccuScan® Pro Reader Set up

1. Enter the lot-specific QR code by selecting the QR code icon on the reader. Place the QR code into the cartridge and insert the cartridge into the reader.
2. Return to the home screen and select the test strip icon. Touch the Marine Biotoxins category, and then select the DSP test type.

4.7. Sample Preparation and Preliminary Extraction

The sample to be tested should be collected according to accepted sampling techniques.

1. Obtain a representative sample. Shell the samples.
2. Thoroughly rinse the samples with distilled or deionized water, and allow any excess water to drain.
3. Homogenize the shellfish in a high-speed blender.
   NOTE: A good homogenate is essential in order to obtain an accurate result.
4. Number both sides of an extraction bag using a marker, so that one side is labelled “1” and the opposite side labelled “2”. Note: The extraction bag contains a mesh filter which allows for partial filtration of the sample. All samples should only ever be added to side “1”
5. Weigh 2 g (± 0.1 g) of homogenized sample in the bottom of the extraction bag on side “1”
6. Add 8 mL of methanol to side “1” of the extraction bag containing the sample.
7. Ensuring that the sample and methanol remain in the lower half of the extraction bag, position and hold the green straw approximately half-way down from te top of the bag. Fold the upper edge of the bag over the green straw. Firmly clip on the white clip to prevent leakage of the sample.
8. Place the extraction bag on a firm surface and press the roller firmly on the sample extraction bag, pushing the roller back and forth for 30 seconds to aid in obtaining a homogenous sample extract.
9. Slide out the green straw and remove the white bag clip
10. Remove the bag contents from side “2” into a suitable container. Discard the used extraction bag.
11. Pour the sample extract into the barrel of a filter syringe until it is approximately half full. Place the plunger on top and filter approximately 1 mL of the sample into a collection tube. IMPORTANT: The filtered solution should be transparent and not cloudy. Should the filter syringe block or should the filtered extract not be clear, pour the unfiltered contents into a fresh syringe filter to ensure a clear solution.

Proceed to the extraction procedure(s) of interest (i.e. Rapid and/or Hydrolysis)

4.8. Extraction Procedure – Rapid (to detect OA, DTX1, DTX2)

1. Remove 100 µl of the sample extract using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and add into a DSP buffer B vial.

4.9. Extraction Procedure – Hydrolysis (to detect OA, DTX1, DTX2, DTX3)

1. Switch on heater block to 76 °C
2. Ensuring that the filtered extract is well-mixed, transfer 800 µL into a glass vial.
3. Add 100 µL of NaOH (2.5 M) and cap tightly. Mix using a vortex on full speed for 30 seconds.
4. Heat the vial in the heater block at 76°C for 40 mins
5. After 40 mins, remove the vial from the heater block and allow it to cool to room temperature. Alternatively, the sample vial can be placed in ice to cool faster,
6. Add 100 µL of HCl (2.5 M) and mix using a vortex on full speed for 30 seconds.
7. Remove 100 µl of the sample extract using a disposable pipettor provided (or alternatively, by use of a standard pipettor), and add into a DSP buffer A vial.

4.10. Test Procedure

1. Remove the appropriate number of microwells and place into the microwell holder.
2. Shake the tube containing sample extract and buffer vigorously by hand or with a vortex on full speed for 30 seconds.
3. Remove 100 µL of the buffered sample extract and add to microwell.
4. Remove the required number of DSP strips from the lateral flow stay dry tube and immediately close the tube
5. Place the DSP strip with the sample end down into the well containing the filtered extract solution. Set a timer for 15 mins to allow the lines on the strip to develop.
6. After 15 mins, remove the test strip and immediately interpret the results using the AccuScan Pro reader.

4.11. Reading Test Results

1. Test strips should be read within 1 min of completion of the 15 min incubation. Refer to AccuScan® Pro Reader Set Up for test selection and set up information.
2. Fully insert the Reveal 2.0 DSP test strip into the black cartridge adapter with the sample end first and results facing out.
3. Insert the cartridge with test strip side up in the AccuScan® Pro. The reader will automatically begin analyzing the cartridge. CAUTION: Removing cartridge prior to completion can result in invalid readings.
4. The AccuScan® Pro reader will analyze the test strip and results will be displayed and stored in the reader. Results will be displayed on the screen of the AccuScan Pro as either positive or negative.

   Notes:

   Ensure device is fully inserted into cartridge.

   The strips must be read using Neogen's AccuScan® Pro reader.

5. Development Aims

The Reveal 2.0 DSP device and extraction methodology has been developed to ensure detection of OA, DTX1, DTX2 and DTX3 toxins. The method for extracting these toxins for the Reveal 2.0 DSP strip is based on the EU Harmonized Standard Operating Procedure [1]. During development, the rationale was to retain key steps from the accepted analytical method, whist aiming to ensure a relatively simple extraction procedure without the need for expensive equipment nor a high degree of expertise.

DTX2 is less toxic than OA and DTX1 and therefore is assigned a toxicity equivalence factor (TEF) of 0.6, as opposed to 1.0 (OA, DTX1). The theoretical maximum permitted level of DTX2 in a sample is therefore approximately 267 ppb. The perfect cross-reactivity to ensure the assay is fit for purpose for regulatory monitoring should detect samples containing 160 ppb OA/DTX1 and 267 ppb DTX2. In reality, however, there are no reported cases of DTX2 occurring in isolation to the authors' knowledge. DTX3 cannot be detected directly via analytical, enzyme-inhibition or immunoassays. The hydrolysis step was therefore required to ensure detection of DTX3 esters which may be present in samples. This alkaline hydrolysis converts DTX3 esters back into parent toxin forms (OA, DTX1 or DTX2), which can be detected.

6. Materials

Fresh shellfish (common mussels, king scallops, pacific oysters, surf clams) were obtained locally. Shellfish were shucked and tissue combined and homogenized in a blender. Shellfish were held refrigerated (2 - 8 ^{o}C) before use; or frozen (- 20 ^{o}C) if not used on the same day.

LC-MS/MS verifications were carried out on in-house preparations of shellfish homogenates to ensure samples were free of OA-group toxins, prior to spiking. Certified negative common mussel tissue, CRM-Zero-Mus (NRCC), was also used as the negative materials for spiked matrix studies.

Naturally contaminated / incurred shellfish homogenates were obtained from different countries (US, Canada, Europe).

Shellfish homogenates were aliquoted into 2.0 g amounts into filter extraction bags prior to spiking and/or extractions. Certified Reference Materials (CRMs) were obtained from the National Research Council, Canada (NRCC) for all spiked buffer and matrix studies. These included CRM-OA-c (17 µM), CRM-DTX1 (18.5 µM) and CRM-DTX2 (9.7 µM).

To ensure spiking and extraction efficiencies, the accepted analytical methodology (LC-MS/MS) has also been used to determine DSP toxin level for key studies (spiked or incurred). Unless otherwise stated, the analytical procedure employed followed the EU Harmonised Standard Operating Procedure for determination of OA-Group toxins by LC-MS/MS [1]

7. Methods Validation

7.1. Extraction efficiency of toxins

Accepted extraction methods require centrifugation and usually 0.2/0.45 uM filtration, which are not desired for a rapid LFD assay. Therefore studies were conducted to simplify accepted extraction methods without sacrificing the extraction recoveries of key toxins.

1. Study A (Oysters containing OA and DTX3 esters)

Four oyster samples known to contain high levels of DTX3 esters (> 90%) of OA were employed for the study. Sample extracts were quantified using analytical methods. Key changes of the LFD extraction is the absence of centrifugation and a lower sample:solvent ratio.

Results are shown in Table 1 and demonstrate good equivalence of results with a mean recovery of 102.2% (n = 4), indicating no clear impacts on recovery of OA/DTX3 from samples. The large standard deviations are most likely due to several reasons, including sample homogeneity and also that the LC-MS/MS and LFD extractions were carried out in two different laboratories in 2008 and 2012, respectively.

2. Study B (Mussels and Clams containing DTX1 and DTX3 esters)

Samples known to contain primarily DTX1 and DTX3 esters of DTX1 were employed for the study (mussels, clams). Extracts prepared using LC-MS/MS methodologies were compared to those prepared via the LFD extraction method (n=2 extractions per each condition). All extractions were carried out at the same site.

Results are shown in Table 1 and demonstrate a high level of equivalence with a mean LFD recovery of 114% (n = 5 samples). The mean LFD recovery for the three samples within the standard working range of LCMS was 105%.

Table 1. The extraction efficiencies of LFD extracts in comparison to the LCMS extracts.

Study	Sample	LCMS extract (ppb)	LFD extract (ppb)	Recovery (%)	Mean recovery (%)
Study A	**Sample 1**	190	130	68.4%	**102.2%**
	Sample 2	280	350	125.0%	
	Sample 3	410	230	56.1%	
	Sample 4	440	700	159.1%	
Study B	**Sample 5**	483	513	106.2%	**113.8%**
	Sample 6	822	866	105.4%	
	Sample 7	540	556	103.0%	
	Sample 8*	1382	1863	134.8%	
	Sample 9*	1983	2372	119.6%	
* outwith LCMS standard working range					

7.2. Sensitivity and cross-reactivity

7.2.1. Spiked Matrix

A spiked matrix study was conducted to confirm the sensitivity and cross-reactivity in a variety of key matrices (mussels, scallops, oysters and clams). Based on previous validations (Reveal DSP version 1) it was not expected to encounter any matrix effects due to the dilutions of shellfish employed. Shellfish were spiked at various levels of toxins (OA, DTX1, DTX2) ranging from 0 – 533 ppb.

Results

Results indicated a cross-reactivity profile deemed acceptable for regulatory screening and monitoring purposes with OA and DTX1 data indicating 100% accuracy at action limit levels, with DTX2 data indicating 100% accuracy containing approximately 1.5 times the action limits.

Okadaic acid (Table 2): 100% accuracy at correctly identifying negative samples spiked at 0 and 40 ppb was obtained (n=40). 95% accuracy at correctly identifying 60 ppb samples as negative was observed. At 60 ppb one out of five replicates for the scallop sample gave a positive response, all other conditions obtained a negative result. Samples containing 120 ppb OA generated a mix of positive and negative results (85% positive results). All matrices spiked at ≥ 140 ppb obtained a positive result.

DTX1 (Table 3): 100% accuracy at correctly identifying negative samples spiked at 0 and 40 ppb was obtained (n=40). At 100 ppb, the accuracy to correctly generate a negative result was 95% as one device obtained a positive result (scallops). 50% accuracy was observed at 120 ppb, with 95% accuracy to generate positive results at 140 ppb. All matrices containing ≥ 160 ppb DTX1 obtained a positive result.

DTX2 (Table 4): All blank samples provided a negative result (n=20). At 133 ppb, the accuracy to detect negatives was 95%, as one from five replicate Reveal 2.0 DSP devices generated a positive response (scallops). The overall accuracy to detect 267 ppb was 50%. All matrices evaluated at ≥ 400 ppb obtained a positive result.

Table 2: Dose response of Reveal 2.0 DSP with OA

	Species				
Level (ppb OA)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**% positive**
0, 40	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
60	-	-	-	-	5
	-	-	-	-	
	-	-	-	+	
	-	-	-	-	
	-	-	-	-	
120	+	+	+	+	85
	+	-	+	+	
	+	-	+	+	
	+	-	+	+	
	+	+	+	+	
140, 160, 320	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

Table 3: Dose response of Reveal 2.0 DSP with DTX1

	Species				
Level (ppb)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**Overall % positive**
0, 40	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
100	-	-	-	-	5
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	+	
120	-	-	-	-	50
	+	+	-	-	
	+	+	-	+	
	+	+	-	+	
	-	+	-	+	
140	+	+	+	+	95
	+	+	+	+	
	+	+	+	+	
	-	+	+	+	
	+	+	+	+	
160, 320	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

Table 4: Dose response of Reveal 2.0 DSP with DTX2

	Species				
Level (ppb)	**Mussels**	**Oysters**	**Clams**	**Scallops**	**Overall % positive**
0	-	-	-	-	0
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
133	-	-	-	-	5
	-	-	-	-	
	-	-	-	-	
	-	-	-	-	
	-	-	-	+	
267	-	+	-	-	50
	-	+	-	+	
	+	+	+	+	
	+	-	-	+	
	-	-	-	+	
400, 533	+	+	+	+	100
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	
	+	+	+	+	

*Note: no data on clams at 400 ppb

7.3. Robustness

Methods

A single-laboratory validation study was conducted to measure robustness of the Reveal 2.0 DSP method. Bulk mussel homogenate was separated into 180 portions of 2.0 g each into extraction bags and spiked to prepare samples at levels ranging from 0 to 534 ppb (table 5).

Table 5: Sample preparation to fulfill Methods Robustness Protocol

Toxin	Level (ppb)	Number of samples prepared
Blank	0	18
OA	40	18
	120	18
	160 (action limits)	18
DTX1	40	18
	120	18
	160 (action limits)	18
DTX2	133	18
	267 (action limits)	18
	534 (2 * action limits)	18

Spiked samples were also sent for LC-MS/MS extraction and analysis to ensure levels of toxins were acceptable using the reference method. The results are shown in Table 6. LC-MS analysis of samples indicated that DTX1 (160 ppb) and OA (160 ppb) may be present at lower levels than intended with recoveries of 87% (139 ppb ± 36 ppb) and 77% (123 ppb ± 32 ppb), respectively. Based on the spiked matrix studies in the section above, the possibility of a low level of negative results with the LFD method were expected on these '160 ppb' samples.

Samples were randomized, with each operator provided 20 samples each day consisting of:

- 2 x blank
- 2 x OA at 40 ppb, 2 x OA at 120 ppb and 2 x OA at 160 ppb
- 2 x DTX1 at 40 ppb, 2 x DTX1 at 120 ppb and 2 x DTX1 at 160 ppb
- 2 x DTX2 at 133 ppb, 2 x DTX2 at 267 ppb and 2 x DTX2 at 534 ppb

Each sample was extracted according to the LFD hydrolysis procedure. Each extract was tested with one device each from three lots of Reveal 2.0 DSP LFDs. 60 test results were to be collected from each operator. In total, there were 3 operators testing over three days, which equated to a total of 540 test strips. However, due to errors in extraction (mainly operator 3, day 1), data from 12 sample extracts were not obtained.

Table 6: Results of assay method robustness for the Reveal 2.0 DSP test

					Number of positives (n = 3 lateral flows, one from each of three batches)			Total Positives (%)
Toxin	**Level ppb**	**LC-MS/MS analysis ppb (uncertainty at 26%)**	**Extraction**	**Operator**	**Day1**	**Day2**	**Day3**	
Blank	0	< LOD (< 25 ppb)	1	1	0	0	0	0
			2		0	0	0	
			1	2	0	0	0	
			2		0	0	0	
			1	3	0	0	0	
			2		No data	0	0	
OA	40	35	1	1	0	1	0	13
			2		0	1	0	
			1	2	1	0	0	
			2		2	0	0	
			1	3	No data	0	0	
			2		1	0	1	
	120	95	1	1	3	1	1	67
			2		1	0	2	
			1	2	3	0	3	
			2		2	3	3	
			1	3	No data	2	3	
			2		No data	2	3	
	160	123	1	1	3	3	3	98
			2		2	3	3	
			1	2	3	3	3	
			2		2	1	3	
			1	3	No data	3	3	
			2		No data	3	3	

Table 6: Results of assay method robustness for the Reveal 2.0 DSP test

					Number of positives (n = 3 lateral flows, one from each of three batches)			Total Positives (%)
Toxin	**Level ppb**	**LC-MS/MS analysis ppb (uncertainty at 26%)**	**Extraction**	**Operator**	**Day1**	**Day2**	**Day3**	
DTX1	40	36	1	1	0	0	0	6
			2		0	0	0	
			1	2	0	0	0	
			2		2	0	0	
			1	3	2	0	0	
			2		1	0	0	
	120	117	1	1	0	1	0	51
			2		0	1	0	
			1	2	3	3	3	
			2		3	3	2	
			1	3	No data	1	No data	
			2		No data	0	3	
	160	139	1	1	3	No data	1	94
			2		3	2	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	3	3	3	
			2		3	3	3	
DTX2	133	95	1	1	2	0	0	31
			2		1	0	0	
			1	2	3	2	1	
			2		2	0	2	
			1	3	No data	0	0	
			2		1	1	1	
	267	296	1	1	2	2	2	90
			2		3	3	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	No data	3	2	
			2		3	2	3	
	534	571	1	1	3	3	3	100
			2		3	3	3	
			1	2	3	3	3	
			2		3	3	3	
			1	3	3	3	3	
			2		No data	3	3	

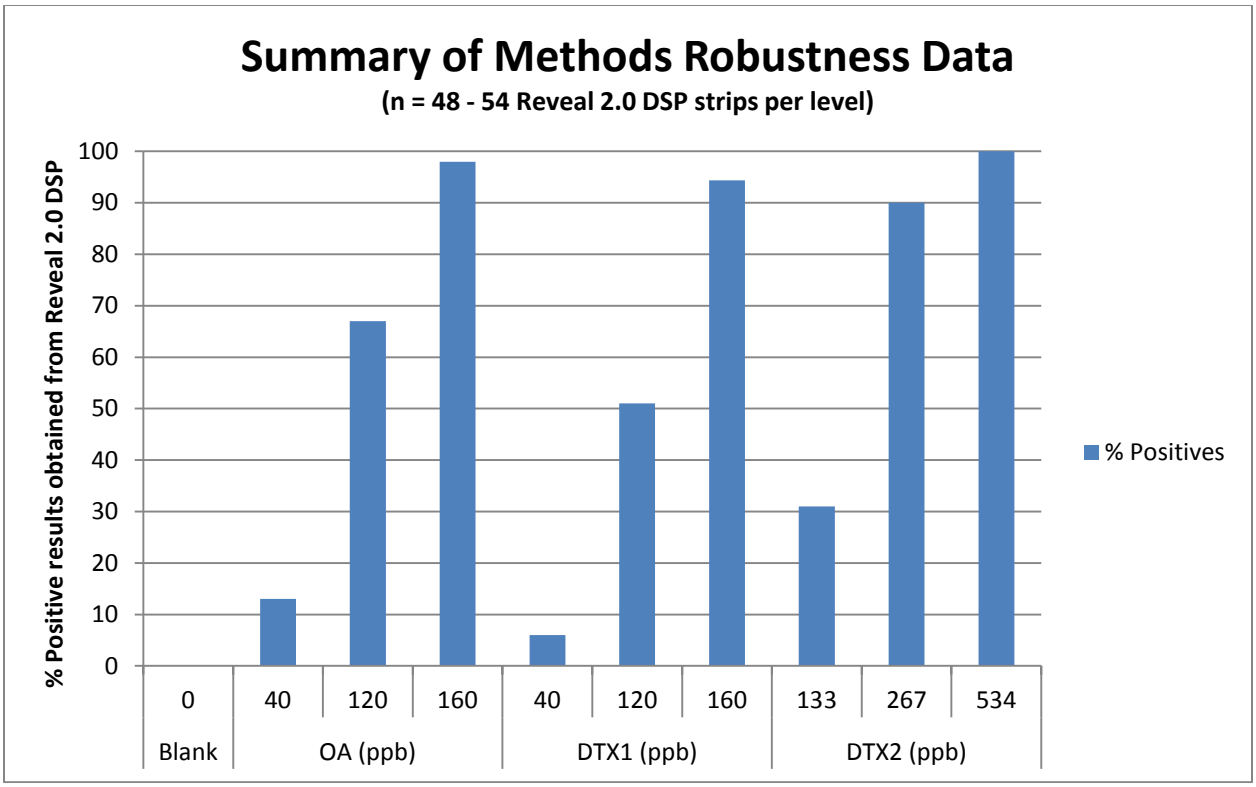


Figure 1. Summary of Reveal 2.0 DSP single laboratory robustness study

Results

Figure 1 shows a summary of the results. The study demonstrated that results were consistent across LFD lots, operators and days. The accuracy of the method to generate negative results from blank samples and positive results from samples containing 160 ppb (OA and DTX1) and 534 ppb (DTX2) was 98%. The accuracy of the assay to generate positive results with samples containing 267 ppb DTX2 was 90%. As expected, at 120 ppb OA eqs, both positive and negative results were obtained. The accuracy of samples containing 40 ppb of DTX1 and OA to generate negative results was 94% and 87%, respectively.

7.4. Post-robustness follow-up experiment

A follow-up experiment was carried out to gather more data at 40, 160 and 175 ppb OA or DTX1. The aim of this experiment was two-fold: (1) to determine whether the few false results obtained during methods robustness could be recreated when using certified negative material, and (2) to determine assay performance at toxin levels higher than the current regulatory limit. CRM-Zero-Mus samples were employed for the spiked matrix portion of this study. All samples were extracted and hydrolysed following the LFD protocol; and each extract was tested using three lateral flow devices. In addition, four naturally contaminated mussel samples were also evaluated with known levels of OA/DTX3 (around the regulatory limits), as characterized and quantified via a national reference laboratory (NRL) in the UK.

Table 7. Post-robustness study single operator study

Sample (Mussels)	Level (ppb OA eqs)	LFD results - positives (n = 3)
OA	40	0
	160	3
	175	3
	200	3
DTX1	40	0
	160	3
	175	3
	200	3
Incurred Sample A (OA/DTX3)*	80	2
Incurred Sample B (OA/DTX3)*	122	2
Incurred Sample C (OA/DTX3)	125	3
Incurred Sample D (OA/DTX3)	170	3

*Samples A and B negative when diluted 1:2 using negative mussel extracts

Results

Results are shown in Table 7. From the spiking data, at 40 ppb OA equivalents, all Reveal 2.0 DSP devices obtained a negative outcome; at 160 ppb OA equivalents and above, all devices were positive. Correlation between Reveal 2.0 DSP test results and levels of toxins measured in the naturally contaminated samples was good.

7.5. Naturally Contaminated / Incurred Samples (Neogen)

A total of 29 naturally contaminated samples were evaluated. These consisted of common mussel samples from the UK; native oysters (*Crassostrea virginica)*, clams and common mussels from the US; and common mussels and king scallop samples from Scotland (UK).

Samples were extracted and run according to hydrolysis LFD procedures. Analytical data was obtained via accepted analytical methods for most samples, as well as by PP2A*.

Results

Results are shown in Table 8. Samples containing OA equivalents at concentrations less than the limit of quantification by analytical means were all negative on the Reveal 2.0 DSP device. Samples quantified to contain ≤ 122 ppb OA equivalents obtained a negative result on the lateral flow device. All the remaining samples (≥ 125 ppb OA equivalents) were positive on Reveal 2.0 DSP.

* PP2A assay: OA is well known as a protein serine/threonine phosphatase inhibitor. Within a certain range, the inhibition of serine/threonine protein phosphatase type 2A (PP2A) is proportional to the concentration of OA and DTXs in solution [3]. Inhibition of this enzyme by OA standards of known concentration allows generation of a standard curve against which OA and DTXs in extracts from shellfish samples can be quantified in total OA equivalents. This method is based on the protein phosphatase inhibition assay using fluorescence substrates (FFPIA) first described by Vieytes *et. al.* (1997) [2]. The method incorporates a chemical clean-up procedure using solid phase extraction.

Table 8. Test of Naturally Incurred Samples

Sample Group	Sample Number	Quantitation Laboratory (LC-MS/MS unless otherwise stated)	Analytical Results (ppb OA equivalents)	Reveal 2.0 DSP Result + positive - negative
Mussels (UK) Various toxin profiles including OA, DTX1, DTX2 and DTX3 esters at different levels	Sample 1	EXTERNAL LAB 1	< LOD	-
	Sample 2	EXTERNAL LAB 1	81	-
	Sample 3	EXTERNAL LAB 1	122	-
	Sample 4	EXTERNAL LAB 1	125	+
	Sample 5	EXTERNAL LAB 1	170	+
	Sample 6	EXTERNAL LAB 1	269	+
	Sample 7	EXTERNAL LAB 1	336	+
	Sample 8	EXTERNAL LAB 1	386	+
	Sample 9	EXTERNAL LAB 1	415	+
	Sample 10	EXTERNAL LAB 1	569	+
	Sample 11	EXTERNAL LAB 1	569	+
	Sample 12	EXTERNAL LAB 1	618	+
	Sample 13	EXTERNAL LAB 1	< LOD	-
Oysters (US) Toxin profiles consisting of primarily OA, most of which as DTX3 esters (>90%).	Sample 14	EXTERNAL LAB 3	< LOD	-
	Sample 15	EXTERNAL LAB 3	190	+
	Sample 16	EXTERNAL LAB 3	376	+
	Sample 17	EXTERNAL LAB 3	410	+
	Sample 18	EXTERNAL LAB 3	< LOD	-
	Sample 19	EXTERNAL LAB 3	440	+
	Sample 20	EXTERNAL LAB 3	450	+
	Sample 21	EXTERNAL LAB 3	470	+
	Sample 22	EXTERNAL LAB 3	< LOD	-
Mussels and Clams (US) Toxin profiles consisting primarily of DTX1 and DTX3.	Sample 23 (clams)	Neogen	< LOD	-
	Sample 24 (clams)	EXTERNAL LAB 2	515	+
	Sample 25 (clams)	Neogen	< LOD	-
	Sample 26 (mussels)	EXTERNAL LAB 1	< LOD	-
	Sample 27 (mussels)	EXTERNAL LAB 2	524	+
	Sample 28 (mussels)	EXTERNAL LAB 2	944	+
	Sample 29 (mussels)	EXTERNAL LAB 2	~1700	+
	Sample 30 (mussels)	EXTERNAL LAB 2	~2400	+
Mussels and Scallops (Scotland)	Sample 31 (mussels)	Veromara (PP2A)	83-132	-
	Sample 32 (scallops)	Veromara (PP2A)	41-66	-
	Sample 33* (scallops)	Neogen	200	+
	Sample 34 (scallops)	Neogen	No detection	-

* Sample 33 contained OA & DTX2 (70% DTX3) as quantified by LCMS

7.6. Naturally Contaminated / Incurred Samples (External Lab)

A total of 40 naturally contaminated samples (mussels, scallops or oysters) were evaluated in a blind study. Samples were extracted and run according to both free toxin (unhydrolysed) and total toxin (hydrolysis) LFD procedures. Quantitative confirmatory data was obtained via accepted LCMS analytical methods for samples and/or by MBA.

Results

Results are shown in Table 9. Samples containing OA equivalents at concentrations less than the limit of quantification by analytical means were all negative. There were no false negative results with the hydrolysis procedure. The LFD generated two false positive results with respects to the action limits; samples 9 and 29, which were quantified to contain 130 and 83.7 ppb OA eqs, respectively. Sample 13 was negative by the free toxin LFD procedure and MBA, but positive with the hydrolysed LFD procedure and LCMS at 177 ppb OA eqs.
In addition, all positive results by the free toxin protocol were positive further to hydrolysis, as intended. This indicates that a screen can be completed effectively using the rapid extraction protocol with samples containing free toxins at levels significant to the action limits (without the requirement of testing hydrolysed extracts).

Table 9. LFD results in comparison to confirmatory reference methods (laboratory 2)

Sample	Sample Type	LFD Result (free toxins)	LFD Result (hydrolysis)	Confirmatory Result	Confirmatory Result (LCMS, where quantitation provided)
1	Mussel	Negative	Negative	Negative	151 ppb OA eqs
2	Mussel	Negative	Negative	Negative	149 ppb OA eqs
3	Mussel	Positive	Positive	Positive	180 ppb OA eqs
4	Mussel	Positive	Positive	Positive	189 ppb OA eqs
5	Mussel	Positive	Positive	Positive	176 ppb OA eqs
6	Mussel	Negative	Negative	Negative	Negative by LCMS
7	Mussel	Positive	Positive	Positive	181 ppb OA eqs
8	Mussel	Positive	Positive	Positive	176 ppb OA eqs
9	Mussel	Negative	Positive	Negative	130 ppb OA eqs
10	Mussel	Positive	Positive	Positive	176 ppb OA eqs
11	Mussel	Negative	Negative	Negative	61 ppb OA eqs
12	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
13	Scallops	Negative	Positive	Negative	DSP Negative by MBA ; ASP Positive LCMS: 177 ppb OA eqs OA eqs
14	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
15	Oyster	Negative	Negative	Negative	DSP Negative by MBA
16	Oyster	Negative	Negative	Negative	DSP Negative by MBA
17	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
18	Scallops	Negative	Negative	Negative	DSP Negative by MBA
19	Scallops	Negative	Negative	Negative	DSP Negative by MBA
20	Scallops	Negative	Negative	Negative	DSP Negative by MBA
21	Scallops	Negative	Negative	Negative	DSP Negative by MBA
22	Scallops	Negative	Negative	Negative	DSP Negative by MBA
23	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
24	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
25	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
26	Scallops	Negative	Negative	Negative	DSP Negative by MBA ; ASP Positive
27	Scallops	Negative	Negative	Negative	DSP Negative by MBA
28	Mussel	Positive	Positive	Negative	139.4 ppb OA eqs
29	Mussel	Negative	Negative	Negative	83.7 ppb OA eqs
30	Mussel	Positive	Positive	Positive	850 ppb OA eqs
31	Mussel	Positive	Positive	Positive	1141 ppb OA eqs
32	Mussel	Negative	Positive	Positive	215 ppb OA eqs
33	Mussel	Positive	Positive	Positive	2796 ppb OA eqs
34	Mussel	Positive	Positive	Positive	365 ppb OA eqs
35	Mussel	Positive	Positive	Positive	695 ppb OA eqs
36	Mussel	Negative	Positive	Positive	452 ppb OA eqs
37	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
38	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
39	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive
40	Scallops	Negative	Negative	Negative	Negative for DSP by MBA; ASP Positive

7.7. Inter-laboratory evaluations

Three sites were provided six mussel samples (*Mytilus edulis*) randomized blind and carried out extractions in duplicate. Results are shown in table 10. Accuracy to generate negative results with samples containing < 40 ppb OA eqs and positive results with samples containing 170 ppb was 100%.

Table 10. Summary of inter-laboratory data

Sample	Level (OA eqs)	Expected result	LFD	Actual LFD result		
				Site 1	Site 2	Site 3
A	120 ppb	Neg or Pos	Test 1	Positive	Positive	Positive
			Test 2	Positive	Positive	Positive
B			Test 3	Positive	Negative	Negative
			Test 4	Positive	Positive	Negative
C	< 40 ppb	Negative	Test 5	Negative	Negative	Negative
			Test 6	Negative	Negative	Negative
D			Test 7	Negative	Negative	Negative
			Test 8	Negative	Negative	Negative
E	170 ppb	Positive	Test 9	Positive	Positive	Positive
			Test 10	Positive	Positive	Positive
F			Test 11	Positive	Positive	Positive
			Test 12	Positive	Positive	Positive

7.8. LFD lot comparability

A spiked buffer study was conducted as a means to determine accuracy and lot comparability. The following levels were tested across multiple cards of three LFD lots; buffer only (representing 0 ppb), OA and DTX1 at 40, 80, 120 and 160 ppb and DTX2 at 134, 267, 400 and 534 ppb (n = 9 LFDs per sample).

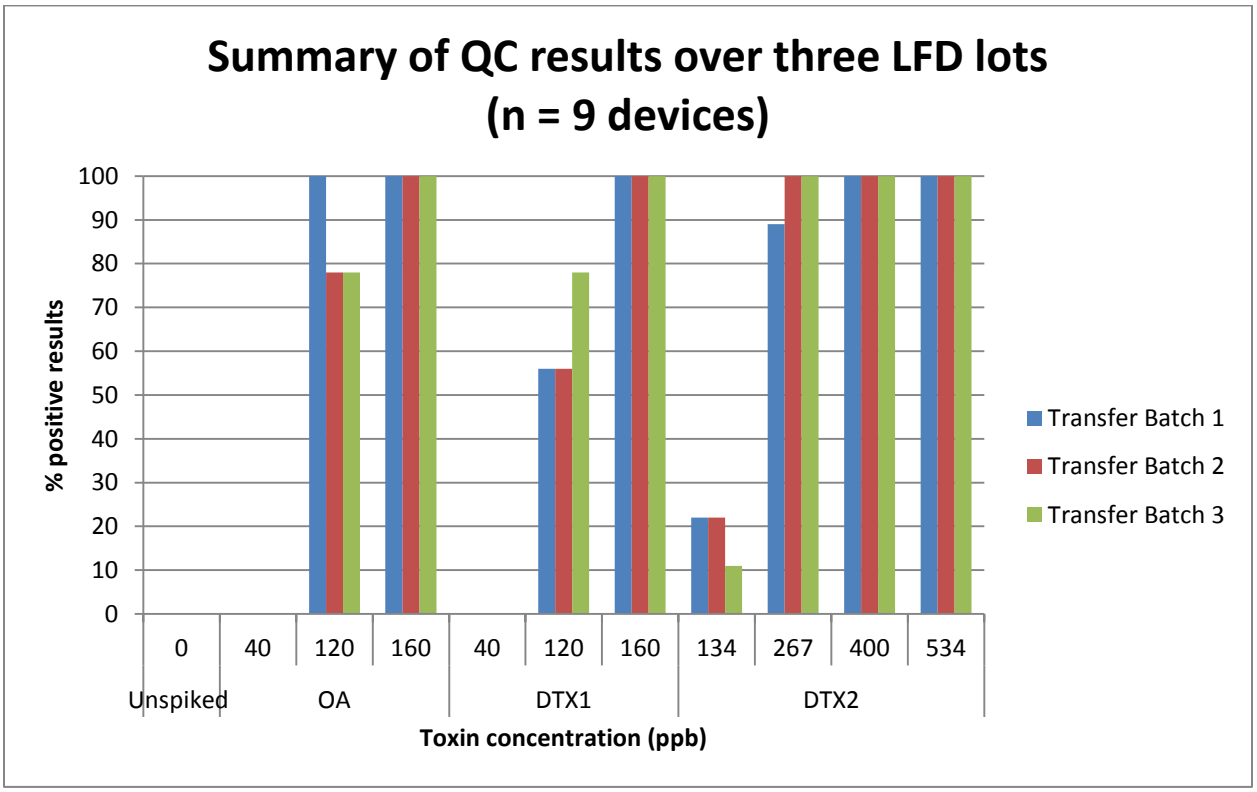


Figure 2. Summary of LFD lot comparability (n = 3 lots)

Results

Results are shown in Figure 2. The overall accuracy to generate negative results at 0 and 40 ppb OA eqs, and positive results at 160 ppb OA eqs (OA/DTX1) as well as 400/533 ppb DTX2; was 100% (n = 162) across all three lots. The overall accuracy to detect DTX2 at 267 ppb was 96% (n = 27), as one test produced a borderline negative result with lot 1 devices.

7.9. Reveal 2.0 DSP Stability

An 18 month real time stability trial of the Reveal 2.0 DSP assay is currently in process. Briefly, test strips from three lots were evaluated at different conditions including room temperature (22 – 25 °C) and incubated (37°C). Room temperature strips were tested at weeks 0, 1, 2, 3, 4, 8, 12,16, 20 and 26 (month 6). Further testing time points are planned for months 8, 10, 12, 14, 16 and 18. The accuracy of the device to date to generate negative results at 0 ppb and positive results at 160 ppb (OA/DTX1) and 534 ppb (DTX2) is 100% across all three lots.

Using the Arrhenius equation* to predict the shelf life of an assay using accelerated data, the predicted shelf life at room temperature of Reveal 2.0 DSP is currently at least 8 months. Stability trials of all lots will continue until a final shelf life can be assigned.

*Arrhenius equation:
For every 10^{o}C temperature rise above the normal storage temperature, the stability at the higher temperature is multiplied x 2^{x} to give the predicted stability where x is the number of 10^{o}C increments above the planned storage temperature. Therefore if the planned storage temperature is 25°C, accelerated testing at 45^{o}C allows a predicted shelf life of x 2^{2} = 4.

7.10. Ruggedness

The following three parameters were evaluated using spiked mussel samples (hydrolysed and rapid extracts) using LFDs from the robustness study lots of devices (n = 3):

- Extract stability (RT) post extraction (up to approximately 240 mins)
- Sample volume in microwell (100 uL ± 10 uL)
- Run time (15 mins ± 2 mins)

Results
No impacts on accuracy (to generate negative results at 0 and 40 ppb and positive results at 160 ppb OA) were observed with any of the parameters evaluated.

7.12. Quality Control Testing

Quality control (QC) testing of manufactured lots of the Reveal 2.0 DSP assay is performed at both in-process and finished product stages. In-process QC consists of testing every card with negative buffers A and B (n= 2) to check for line intensity, line position and intra-lot variability between cards on both the test and control lines. For finished product testing, strips from various cards representative of the batch are selected for positive testing. The following positive samples are tested: OA (40, 160 ppb), DTX1 (40, 160 ppb) and DTX2 (267 and 534 ppb). To ensure maximal performance of batches all tests containing 40 ppb must produce a negative result and all tests containing 160 ppb must produce a positive result. It is acceptable for DTX2 at 267 ppb to produce borderline negative results, although the majority of results are positive. This is due to the fact the DTX2 always co-occurs with OA. The final stage of testing involves testing using shellfish extracts containing approximately 40 ppb OA and 160 ppb OA (both rapid and hydrolysis extracts) to ensure negative and positive results are obtained, respectively.

8. Discussion and Summary

The use of LFDs has been previously exploited for detection of marine biotoxins. However, two major weaknesses for end-users have been the cross reactivity profile and the subjective nature of the interpretation of results. Reveal 2.0 DSP was designed as a reader-based assay to eliminate any subjective element, with a positive cut off for OA group toxins in shellfish relevant to regulatory limits of 160 ppb OA eqs. Sample homogenates could be screened in 20 min for free toxins (including extraction and assay time) while waiting for hydrolysis. If positive, then the hydrolysed extract would not need to be screened. If negative, the hydrolysed extract would be screened post-hydrolysis to determine presence of esters. Substantial validation data demonstrated the occurrence of no false negative results with naturally contaminated samples (various toxin profiles and matrices) containing 160 ppb OA eqs or greater and no false positives with incurred samples containing up to approximately 80 ppb OA eqs.

A robust and rapid LFD has been reported which demonstrates practical, simple and accurate screening for shellfish contaminated with OA group toxins. The assay can be utilised as a potential early warning detection system for use within the shellfish industry including shipboard or remote locations, providing added protection of shellfish consumers. Results demonstrate a rapid single-step assay, which requires minimal materials, that provides the simplest OA extraction and detection system reported to date. It allows either/both free toxins and total toxins to be rapidly screened from the same original sample extracts. More importantly, the assay has demonstrated high performance characteristics with respect to accuracy, recovery, cross-reactivity, specificity, matrix effects, robustness, ruggedness, reproducibility and stability. There is also a significant potential for this method to be used in regulatory laboratories to replace the costly LC-MS/MS or MBA based tests that are routinely employed as part of national monitoring programmes.

References

1. Community Reference Laboratory for Marine biotoxins (CRLMB)., Agencia Española de Seguridad Alimentaria y Nutrición (AESAN). (2009). EU Harmonised Standard Operating Procedure for determination of OA-Group Toxins by LC-MS/MS. Version 1. http://www.aesan.msps.es/en/CRLMB/web/procedimientos_crlmb/crlmb_standard_operating_procedures.shtml
2. Vieytes, M. R., Fontal, O. I., Leira, F., Baptista de Sousa, J. M. V., and Botana, L. M. (1997) A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins. *Anal. Biochem.* **248,** 258-264.

Acknowledgements

We thank Nate Banner, Sharon Graham, James Clarke, Steve Schadler and Frank Klein of Neogen for all their help throughout this study.

SAMPLE PREPARATION AND PRELIMINARY EXTRACTION

The sample to be tested should be collected according to accepted sampling techniques.

1. Obtain a representative sample. Shell the samples.
2. Thoroughly rinse the samples with distilled or deionized water, and allow any excess water to drain.
3. Homogenize (e.g., blend, puree) the shellfish in a high-speed blender.
   NOTE: A good homogenate is essential in order to obtain an accurate result.
4. Number both sides of an extraction bag using a marker, so that one side is labeled "1" and the opposite side labeled "2."
   NOTE: The extraction bag contains a mesh filter which allows for partial filtration of the sample. All samples/solution should only ever be added to side "1".
5. Weigh out 2 g (± 0.1 g) of homogenized sample in the bottom of the extraction bag on side "1."
   IMPORTANT: Ensure the entire sample is at the bottom of the bag prior to next step.
6. Add 8 mL of analytical grade methanol to side "1" of the extraction bag containing the sample.
7. Ensuring that the sample and methanol remain in the lower half of the extraction bag, position and hold the green straw approximately half-way down from the top of the bag. Fold the upper edge of the bag over the green straw. Firmly clip on the white clip to prevent leakage of the sample.
8. Place the extraction bag on a firm surface and press the roller firmly on the sample extraction bag, pushing the roller back and forth for **30 seconds** to aid in obtaining a homogenous sample extract.
9. Slide out the green straw and remove the white bag clip.
10. Remove the bag contents from side "2" into a suitable container (there may be small pieces of shellfish remaining on side "1"). Discard the used extraction bag.
11. Ensuring the sample extract is well mixed, pour into the barrel of a Neogen filter syringe until it is almost half full. Place the plunger on top, and filter sample extract into a collection tube.
    IMPORTANT: The filtered solution should be transparent/clear and not cloudy. Should the filter syringe block or should the filtered extract not be clear, pour the unfiltered contents into a fresh syringe filter to ensure a clear solution.

NOTE: Use **Procedure 1 – Rapid screen (to detect OA, DTX-1 and DTX-2**) or **Procedure 2 – Hydrolysis (to detect OA, DTX-1, DTX-2 and DTX-3)**, prior to proceeding to the test procedure.

EXTRACTION PROCEDURE 1 – RAPID SCREEN (TO DETECT OA, DTX-1, DTX-2)

1. Remove 100 µL of the sample extract using a disposable pipettor* provided (or alternatively, by use of a standard pipettor), and add into a vial of DSP buffer B (white cap).
   *To use the disposable pipettors, firmly press the top bulb of the pipettor, insert the tip into the solution, slowly release the top bulb to draw up the sample extract. Excess volume (e.g., above 100 µL) will overflow into the lower bulb, ensuring 100 µL is ready to dispense. Press the top bulb firmly and release slowly to dispense. Discard the used pipettor.
2. Proceed to **Test procedure**.

EXTRACTION PROCEDURE 2 – HYDROLYSIS (TO DETECT OA, DTX-1, DTX-2, DTX-3)

1. Switch on heater block to 76°C.
2. Ensuring that the filtered extract is well-mixed, transfer 800 µL into a glass vial.
3. Add 100 µL of NaOH (2.5M) and cap tightly. Mix using a vortex on full speed for **30 seconds**.
4. Heat the vial in the heater block at 76°C for **40 minutes**.
5. After 40 minutes, remove the vial from the heater block and allow it to cool to room temperature. Alternatively, the sample vial can be placed in ice to cool faster.
6. Add 100 µL of HCl (2.5M) and mix using a vortex on full speed for **30 seconds**.
7. Remove 100 µL of the sample extract and add into a vial of DSP buffer A (gray cap).
8. Proceed to **Test procedure**.

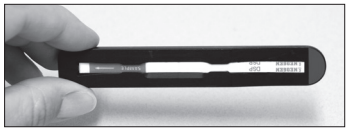
TEST PROCEDURE

1. Remove the appropriate number of microwells and place into the microwell holder.
2. Shake the vial containing sample extract and buffer vigorously by hand or with a vortex on full speed for **30 seconds**.
3. Remove 100 µL of the diluted sample extract and add to a microwell.
4. Remove the required number of DSP strips from the lateral flow stay dry tube and immediately close the tube.
5. Place the DSP strip with the sample end down into the well containing the filtered extract solution. Set a timer for **15 minutes** to allow the lines on the strip to develop.
6. After 15 minutes, remove the test strip and immediately interpret the results using AccuScan Pro reader.

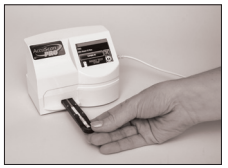
READING TEST RESULTS

Test strips should be read within **1 minute** of completion of the 15 minute incubation. Refer to **AccuScan Pro Reader Set Up** for test selection and set up information.

1. Fully insert the Reveal 2.0 for DSP test strip into the black R cartridge adapter with the sample end first and results facing out.



2. Insert the cartridge with test strip side up in the AccuScan Pro. The reader will automatically begin analyzing the cartridge. **CAUTION:** Removing cartridge prior to completion can result in invalid readings.
3. The AccuScan Pro reader will analyze the test strip and results will be displayed and stored in the reader.



NOTES

1. Ensure device is fully inserted into cartridge.
2. Readings should be made between **15–16 minutes**. Readings after 16 minutes may be inaccurate due to overdevelopment of the device.
3. The strips must be read using Neogen's AccuScan Pro reader.

PERFORMANCE CHARACTERISTICS

1. Reveal DSP is designed to screen for OA group toxins (OA and DTXs) in shellfish.

VALIDATED MATRICES

Mussels, scallops, oysters, clams and cockles.

NOTE: Neogen continues to validate new commodities. Please contact a representative for the latest validated commodity list.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

NEOGEN® CORPORATION

North America
Neogen Headquarters
620 Lesher Place, Lansing, MI 48912 USA
800/234-5333 (USA/Canada) or 517/372-9200
Fax: 517/372-2006 • foodsafety@neogen.com
www.neogen.com

Mexico
Neogen Latinoamérica
Darwin No. 83, Col. Anzures, México, 11590 D.F.
(55) 5254-8235, (55) 5203-0111, (55) 5531-2837
Fax: (55) 5531-1647
informacion@neogenlac.com • www.neogen.com

Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Brazil
Neogen do Brasil
Rua: Alberto Guizo 760, Distrito Industrial João Narezzi, Indaiatuba – SP Brasil, Cep: 13.347-402
Tel: +55 19 3935.3727
info@neogendobrasil.com.br • www.neogen.com

NE1590 R2.0_DSP_0113

Read instructions carefully before starting test

Reveal® 2.0 for DSP

For use with the Reveal AccuScan® Pro reader

Store at 18–30°C (64–86°F) • Do not freeze.

THE TOXINS
Toxins that cause diarrhetic shellfish poisoning (DSP) include the okadaic acid (OA) group of toxins. OA is produced by marine dinoflagellates such as *Dinophysis*, and has structural analogs referred to as the dinophysistoxins (DTXs). Clinical toxicological effects attributed to DSP following consumption of contaminated seafood includes diarrhea, nausea and vomiting. Human cases have been reported since the early 1960s in Norway and elsewhere on a global scale. The established European Union maximum permitted levels are 160 µg OA equivalents (OA, DTXs, pectenotoxins) per kg shellfish meat (160 ppb). The U.S. Food and Drug Administration action limits are 160 µg (160 ppb) OA equivalents (OA, DTXs) in shellfish.

INTENDED USE
Reveal 2.0 for DSP is an immunochromatographic lateral flow assay used for the rapid and practical qualitative analysis of shellfish possibly contaminated by OA group toxins (OA, DTX-1, DTX-2, and DTX-3). The test can detect as little as 160 ppb of OA equivalents in shellfish samples.

INTENDED USER
The test kit is designed for use by quality control personnel and other personnel familiar with handling shellfish possibly contaminated by OA toxins.

ASSAY PRINCIPLES
Reveal 2.0 for DSP is a single-step lateral flow device based on a competitive immunoassay format. In summary, the shellfish extract is wicked through a reagent zone, containing antibodies specific for OA-group toxins that have been conjugated to colored particles. If the toxins are present in the sample, the toxin will be captured by the particle-antibody complex. The complex then is wicked onto a membrane, which contains a stationary capture zone of a toxin-protein conjugate. This zone captures any uncomplexed toxin particle-antibody. Therefore, as the concentration of toxins in the sample increases, the test line intensity decreases. The membrane also contains a stationary control zone which always will form regardless of the level of toxins.

STORAGE REQUIREMENTS
Store kit components at room temperature (18–30°C, 64–86°F) to ensure full shelf life. Test strips should remain capped in their original sample tubes until used to ensure optimal performance.

MATERIALS PROVIDED
Reveal 2.0 for DSP (Neogen item 9561)
1. 24 Reveal 2.0 for DSP lateral flow test strips
2. 24 wells
3. 24 vials of DSP buffer A (gray cap)
4. 24 vials of DSP buffer B (white cap)
5. 25 extraction bags
6. 48 disposable 100 µL pipettors

MATERIALS RECOMMENDED BUT NOT PROVIDED
1. Marine Biotoxins Starter Kit (Neogen item 9563)
   - Microwell holder
   - 1 roller
   - 1 bag clip (white clip and green straw)
2. Distilled water
3. Methanol (analytical grade recommended, VWR 20864.320)
4. Filter syringes (Neogen item 9420)
5. Sample collection cups with lids (Neogen items 9428, 9428B)
6. Blender (Neogen items 9493, 9477 or 9495)
7. Scale capable of weighing 0.5–400 g ± 0.1 g (Neogen item 9427)
8. Timer (Neogen item 9452)
9. Graduated cylinder, 50 mL (Neogen item 9447)
10. Sample collection tubes with caps, 5 mL (Neogen item 9421, 9421B)
11. AccuScan Pro reader (Neogen item 9565)

MATERIALS REQUIRED IF PERFORMING A HYDROLYSIS EXTRACTION
1. DSP Hydrolysis Pack (Neogen item 9554)
   - 2.5 M sodium hydroxide (NaOH) solution (5 mL)
   - 2.5 M hydrochloric acid (HCl) solution (5 mL)
2. Pipettor, 100–1000 mL, and pipette tips (Neogen item 9463, 9464)
3. Heater block (capable of holding 76 ± 2°C (recommended Stuart, SBH130D)
4. Glass sample vials (Recommended Water's P/N 600000751CV)
5. 2.5 M NaOH and 2.5M HCL solutions (Neogen item 9561)
6. Vortex (~ 3000 rpm, recommended Fisher Scientific 11726477)

PRECAUTIONS
1. The test strips must remain inside the stay dry tube before use.
2. Store test kit at room temperature (18–30°C, 64–86°F) when not in use. Do not freeze.
3. Do not use kit contents beyond expiration date.
4. Treat all liquids, including sample extract, and used components as if contaminated with toxin. Gloves and other protective apparel should be worn at all times.
5. To avoid cross-contamination, use clean pipettors, extraction bags and fresh extraction solutions for each sample.

ACCUSCAN PRO READER SET UP
1. Enter the lot-specific QR code by selecting the QR code icon on the reader. Place the QR code into the cartridge and insert the cartridge into the reader. **NOTE**: For instructions on manually entering sample IDs, see the AccuScan Pro user manual.
2. Return to the home screen and select the test strip icon. Touch the **Marine Biotoxins** category, then select the **DSP** test type.

MATERIAL SAFETY DATA SHEET

Section 1. Company Identification and Product Information			
Product Name or Identity:	**Reveal® 2.0 for DSP**		
Manufacturer's Name:	**Neogen Europe, Ltd.**	**Fax No.:**	UK, 01292 525 601 International: ++44 (0) 1292 525 601
	The Dairy School	**Phone No.:**	UK, 01292 525 600 International: ++44 (0) 1292 525 600
	Auchincruive, Ayr, KA6 5HW, Scotland, UK	**e-mail:**	info@neogeneurope.com
Date Prepared or Revised: February 2013		**Chemtrec: (800) 424-9300** **Outside US and Canada: (703) 527-3887**	

Section 2. Composition / Information on Hazardous Ingredients			
This product is a mixture of the substances listed below with the addition of nonhazardous materials.			
Hazardous Components Specific Chemical Identity:	**CAS-No.**	**%**	**Hazard Symbol**
This product contains no hazardous constituents, or the concentration of all chemical constituents are below the regulatory threshold limits described by Occupational Safety Health Administration Hazard Communication Standard 29 CFR 1910.1200 and the European Directive 91/155/EEC, and 93/112/EC.	NA	NA	NA

Section 3. Health Hazard Identification	
Health Hazards: *(Acute and Chronic)*	**Information pertaining to particular dangers for man and environment.** When used and handled according to specifications, the product does not have harmful effects according to the information provided to us. May cause minor irritation of the eyes and skin.

Section 4. First Aid Measures	
Emergency / First Aid Procedures:	**Ingestion:** If swallowed, seek medical attention immediately. Wash out mouth with water, provided person is conscious. Show physician product label.
	Inhalation: If inhaled, supply fresh air or oxygen. Seek medical attention if breathing is labored or becomes difficult. If not breathing, apply artificial respiration.
	Eye Contact: Rinse opened eye for at least 15 minutes under running water, lifting lower and upper eyelids occasionally. Seek medical attention.
	Skin Contact: Remove contaminated clothing. Immediately wash with plenty of soap and water for at least 15 minutes. Seek medical attention if irritation develops. Wash clothing before reuse.

Section 5. Fire and Explosion Hazard Data	
Flash Point (Method Used): N/A	**Flammable Limits**: LEL – N/A UEL – N/A
Extinguishing Media: Use alcohol foam, dry chemical, or carbon dioxide. Water may be ineffective.	
Protective Equipment: Firefighters should wear protective equipment and self-contained breathing apparatus.	
Unusual Fire and Explosion Hazards: During heating or in case of fire, poisonous gases are produced. Fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source, is a potential dust explosion hazard.	

Section 6. Accidental Release Measures

Personal Precautions: Wear self-containing breathing apparatus, rubber boots, and heavy rubber gloves. Place contaminated material in a chemical waste container.

Environmental Precautions: Prevent dispersion of material. Wipe up with damp sponge or mop.

Clean-up Methods: Contact safety officer if questions arise and ventilate area.

Refer to Section 7 for Handling Information.

Refer to Section 8 for Person Protection Equipment.

Refer to Section 13 for Disposal Information.

Section 7. Handling and Storage

Handling: Protect against physical damage. Ensure good ventilation / exhaustion and do not breathe vapor. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

Storage: Keep container tightly closed. Keep away from heat, sparks, flame and incompatible material. Storage area should be cool, dry, and away from incompatible materials. Containers of this material may be hazardous when empty since they retain product residues. Store at 18 - 30˚C.

Other Precautions: N/A

Section 8. Exposure Controls / Personal Protection

Components with limit values that require monitoring: Not Applicable

OSHA–PEL: N/A	**TLV:** N/A

Additional Information: Personal Protection listed below are general requirements for laboratory personnel. Follow the usual precautionary measures for handling chemicals / powder. Avoid contact with eyes, skin, and clothing.

Personal Protective Equipment:
Keep away from food, beverages, and feed.
Wash hands before and after entering laboratory.

Breathing Equipment: In case of brief exposure, use a chemical fume hood or a NIOSH/MSHA-approved respiratory.

Hand Protection: Use chemical resistant gloves.

Eye Protection: Wear safety glasses.

Body Protection: Wear lab coat or other protective work clothing.

Section 9. Physical and Chemical Properties

Appearance and Odor: N/A

Boiling Point: Not determined

Melting Point: Not determined

Density: Not determined

Section 10. Stability and Reactivity

Stability:	Unstable		
	Stable	X	Conditions to Avoid: Stable under normal storage conditions.

Incompatibility *(Materials to Avoid)*: None known.

Hazardous Decomposition or Byproducts: Carbon dioxide (CO_2), Carbon monoxide (CO), or Nitrogen oxides (NOx).

Hazardous Polymerization:	May Occur		Conditions to Avoid: No dangerous reactions known.
	Will Not Occur	X	

NEOGEN CORPORATION

Section 11. Toxicological Information

LD/LC50 values that are relevant:
Acute Toxicity: When used and handled according to specifications and according to information provided for us, this product is not known to be toxic or hazardous at use concentrations.
Carcinogenicity Classification: Not Applicable
IARC (International Agency for Research on Cancer) – Not Listed
NTP (National Toxicology Program) Not Listed

Chronic: Prolonged or repeated skin contact may cause dermatitis.
Additional toxicological information: Any toxin(s) present in this kit are at concentration levels below the regulatory threshold limits which require registration under the Select Agent Program in as detailed in 42 CFR Part 73, 9 CFR Part 121, and 7 CFR Part 331.

Section 12. Ecological Information

Ecotoxicity Tests: The ecological effects have not been thoroughly investigated, but currently none have been identified.

Section 13. Disposal Considerations

Waste Disposal Method: Dispose in accordance with all applicable federal, state, and local environmental regulations.
RCRA P-Series: None listed.
RCRA U-Series: None listed.
Contact a licensed professional waste disposal service to dispose of this material if questions arise.

Container Information: Do not remove labels from containers until they have been cleaned.

Section 14. Transport Information

DOT Regulations: Not Regulated

Land Transport ADR/RID (cross-border): Not Regulated

Maritime Transport IMDG: Not Regulated

Air Transport ICAO-TI and IATA-DGR: Not Regulated

Section 15. Regulatory Information

EU Regulations, Hazard Symbol(s): N/A

Section 16. Other Information

This document is believed to be correct, but does not purport to be all inclusive and shall be used only as a guide. Neogen Corporation shall not be held liable for any damage resulting from handling or from contact with the above product. These suggestions should not be confused with state, municipal or insurance requirements, and constitute NO WARRANTY.

Read full Reveal 2.0 for DSP (Neogen item 9561) kit instructions carefully before starting test

Reveal® 2.0

DSP Hydrolysis Pack

INTENDED USE
DSP Hydrolysis Pack is intended to be used as an accessory to the Reveal 2.0 for DSP test kit for the extraction of OA and DTXs in shellfish samples.

MATERIALS PROVIDED

DSP Hydrolysis Pack (Neogen item 9554)

1. 2.5 M sodium hydroxide (NaOH) solution (5 mL)
2. 2.5 M hydrochloric acid (HCl) solution (5 mL)

STORAGE
DSP Hydrolysis Pack may be stored at room temperature but should not be frozen or exposed to high temperatures (> 90°F) for more than two weeks. For best results, do not store in direct sunlight.

DISPOSAL
Dispose in accordance with all applicable federal, state and local environmental regulations.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

Proposal No. 13-113
Product 9554

Read full Reveal 2.0 for DSP (Neogen item 9561) kit instructions carefully before starting test

Reveal® 2.0

DSP Hydrolysis Pack

INTENDED USE
DSP Hydrolysis Pack is intended to be used as an accessory to the Reveal 2.0 for DSP test kit for the extraction of OA and DTXs in shellfish samples.

MATERIALS PROVIDED

DSP Hydrolysis Pack (Neogen item 9554)

1. 2.5 M sodium hydroxide (NaOH) solution (5 mL)
2. 2.5 M hydrochloric acid (HCl) solution (5 mL)

STORAGE
DSP Hydrolysis Pack may be stored at room temperature but should not be frozen or exposed to high temperatures (> 90°F) for more than two weeks. For best results, do not store in direct sunlight.

DISPOSAL
Dispose in accordance with all applicable federal, state and local environmental regulations.

CUSTOMER SERVICE
Neogen Customer Assistance and Technical Services can be reached by using the contact information on the back of this booklet. Training on this product, and all Neogen test kits, is available.

MSDS INFORMATION AVAILABLE

Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY

Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

NEOGEN® CORPORATION

North America
Neogen Headquarters
620 Lesher Place, Lansing, MI 48912 USA
800/234-5333 (USA/Canada) or 517/372-9200
Fax: 517/372-2006 • foodsafety@neogen.com
www.neogen.com

Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Mexico
Neogen Latinoamérica
Darwin No. 83, Col. Anzures, México, 11590 D.F.
+52 (55) 5254-8235, +52 (55) 5203-0111,
+52 (55) 5531-2837
Fax: +52 (55) 5531-1647
informacion@neogenlac.com • www.neogen.com

Brazil
Neogen do Brasil
Rua: Alberto Guizo 760, Distrito Industrial João
Narezzi, Indaiatuba – SP Brasil, Cep: 13.347-402
Tel: +55 19 3935.3727
info@neogendobrasil.com.br • www.neogen.com

MSDS INFORMATION AVAILABLE
Material safety data sheets (MSDS) are available for this test kit, and all of Neogen's test kits, on Neogen's website at www.neogen.com, or by calling Neogen at 800/234-5333 or 517/372-9200.

WARRANTY
Neogen Corporation makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made are of standard quality. If any materials are defective, Neogen will provide a replacement of the product. Buyer assumes all risk and liability resulting from the use of this product. There is no warranty of merchantability of this product or of the fitness of the product for any purpose. Neogen shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product.

NEOGEN® CORPORATION

North America
Neogen Headquarters
620 Lesher Place, Lansing, MI 48912 USA
800/234-5333 (USA/Canada) or 517/372-9200
Fax: 517/372-2006 • foodsafety@neogen.com
www.neogen.com

Europe, Middle East and Africa
Neogen Europe
The Dairy School, Auchincruive, Ayr
KA6 5HW Scotland, UK
+ 44 (0) 1292 525 600
Fax: + 44 (0) 1292 525 601
info_uk@neogeneurope.com
www.neogeneurope.com

Mexico
Neogen Latinoamérica
Darwin No. 83, Col. Anzures, México, 11590 D.F.
+52 (55) 5254-8235, +52 (55) 5203-0111,
+52 (55) 5531-2837
Fax: +52 (55) 5531-1647
informacion@neogenlac.com • www.neogen.com

Brazil
Neogen do Brasil
Rua: Alberto Guizo 760, Distrito Industrial João
Narezzi, Indaiatuba – SP Brasil, Cep: 13.347-402
Tel: +55 19 3935.3727
info@neogendobrasil.com.br • www.neogen.com

©Neogen Corporation, 2013. Neogen and Reveal are registered trademarks of Neogen Corporation. All other brand and product names are trademarks or registered trademarks of their respective companies.

NE1809 DSP Hydrolysis Pack_0113

SAFETY DATA SHEET

Section 1. Identification of the Substance/Mixture and of the Company/Undertaking			
1.1 Product Identifiers:	**DSP Hydrolysis Pack** Product #9554		
1.2 Relevant Identified Uses of the Substance or Mixture and Uses Advised Against		Identified Uses: Laboratory chemicals	
1.3 Details of the Supplier of the Safety Data Sheet	**Neogen Europe, Ltd.**	**Fax No.:**	UK, 01292 525 602 International: ++44 (0) 1292 525 601
	The Dairy School	**Phone No.:**	UK, 01292 525 610 International: ++44 (0) 1292 525 600
	Auchincruive, Ayr, KA6 5HW, Scotland, UK	**e-mail:**	info@neogeneurope.com
1.4 Emergency Telephone Number			
Date Prepared or Revised: February 2013		**Chemtrec (US): (800) 424-9300** **Outside US and Canada: (703) 527-3887** **Poison Control (UK): 08454 24 24 24**	

Section 2. Hazards Identification		
2.1 Classification of the Substance or Mixture		
Specific Chemical Identity:	**Regulation (EC) No 1272/2008**	**EU Directives 67/548/EEC or 1999/45/EC**
Sodium Hydroxide Solution	-Skin corrosion (Category 1A)	R35: Causes severe burns
Hydrochloric Acid Solution	-Skin corrosion/Irritation (Category 2) -Serious eye damage/Eye irritation (Category 2) -Specific target organ systemic toxicity (single exposure) (Category 3)	R36/37/38: Irritating to the eyes, respiratory system and skin.
2.2 Label Elements	**Labelling according to Regulation (EC) No 1272/2008 [CLP]**	
Pictogram		
Signal Word	Danger	
Hazard Statements	H314: Causes severe skin burns and eye damage H335: May cause respiratory irritation	
Precautionary Statements	P280: Wear protective gloves/protective clothing/eye protection/face protection. P305/351/338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P310: Immediately call a POISON CENTER or doctor/physician.	
Other Hazards	None known	

Section 3. Composition/Information on Ingredients		
3.2 Mixtures:	*(For the full text of the H-Statements and R-phrases mentioned in this Section, see Section 16.)*	
Components		**Classification**
Sodium hydroxide solution Concentration: 62%	CAS No. 1310-73-2 EC No. 215-185-5 Index No. 011-002-00-6	Skin Corr. 1A; H314 C; R35
Hydrochloric acid solution Concentration: 50%	CAS No. 7647-01-0 EC No. 231-595-7 Index No. 017-002-01-X	Skin Corr. 1B; H314; STOT SE 3; H335 C; R34; X1; R37

Section 4. First Aid Measures	
4.1 Description of First Aid:	**Ingestion:** DO NOT INDUCE VOMITING. If swallowed, seek medical attention immediately. Wash out mouth with water, provided person is conscious. Show physician product label.
	Inhalation: If inhaled, supply fresh air or oxygen. Seek medical attention if breathing is laboured or becomes difficult. If not breathing, apply artificial respiration.
	Eye Contact: Rinse opened eye for at least 15 minutes under running water, lifting lower and upper eyelids occasionally. Immediate medical attention is required.
	Skin Contact: Remove contaminated clothing. Immediately wash with plenty of soap and water for at least 15 minutes. Seek medical attention immediately. Wash clothing before reuse.
4.2 Most Important Symptoms and Effects, Both Acute and Delayed	Burning sensation, cough, wheezing, laryngitis, shortness of breath, spasm, inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema. Material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes and skin.
4.3 Indication of Any Immediate Medical Attention and Special Treatment Needed	No data available

Section 5. Firefighting Measures
5.1 Extinguishing Media Suitable extinguishing media: Use alcohol-resistant foam, dry chemical, or carbon dioxide. Unsuitable extinguishing media: None known
5.2 Special Hazards Arising from the Substance or Mixture: Contact with metals may evolve flammable hydrogen gas. Thermal decomposition can lead to release of irritating gases and vapours.
5.3 Advice for Firefighters: Firefighters should wear protective equipment and self-contained breathing apparatus.

Section 6. Accidental Release Measures
6.1 Personal Precautions, Protective Equipment and Emergency Procedures: Use personal protective equipment. Place contaminated material in a chemical waste container. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas.
6.2 Environmental Precautions: Prevent dispersion of material. Do not let produce enter drains.
6.3 Methods and Materials for Containment and Cleaning Up: Soak up with inert absorbent material and dispose of as hazardous waste. Keep in suitable, closed containers for disposal. If questions arise contact safety officer.
6.4 Reference to Other Sections: For disposal see section 13.

Section 7. Handling and Storage
7.1 Precautions for Safe Handling: Wear appropriate personal protective equipment. Ensure good ventilation / exhaustion and do not breathe vapour or mist. Avoid contact with eyes, skin, and clothing.
7.2 Conditions for Safe Storage, Including Any Incompatibilities: Keep container tightly closed in a cool, well-ventilated area. Do not store in metal containers.
7.3 Specific End Uses: No data available

Section 8. Exposure Controls / Personal Protection
Components with limit values that require monitoring: Not Applicable
8.1 Control Parameters (Exposure Limits)
Sodium hydroxide solution: STEL: 2 mg/m^3 (UK EH40 WEL – Workplace Exposure Limits)
Hydrochloric acid solution: STEL: 8 mg/m^3 (UK EH40 WEL – Workplace Exposure Limits)
8.2 Exposure Controls **Appropriate Engineering Controls:** Personal Protection listed below are general requirements for laboratory personnel. Follow the usual precautionary measures for handling chemicals / liquid. Avoid contact with eyes, skin, and clothing. Wash hands before breaks and at the end of the workday. Ensure eyewash stations and safety showers are close to the workstation location.
Personal Protective Equipment: **Eye/Face Protection:** Tightly fitting safety goggles or face shield. Use equipment for eye protection tested and approved under appropriate government standards such as EN 166. **Skin and Body Protection:** Protective gloves that satisfy the specifications of EU Directive 89/686/EEC and the standard EN 374 derived from it. Use proper glove removal techniques to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands. Wear long-sleeved clothing. Wash contaminated clothing before reuse. **Respiratory Protection:** When workers are facing concentration above the exposure limit, they must use appropriate certified respirators.

Section 9. Physical and Chemical Properties		
9.1 Information on Basic Physical and Chemical Properties		
a) Appearance	Colourless liquid	
b) Odour	Hydrochloric Acid: Sharp, disagreeable odour	
c) Odour threshold		
	Sodium hydroxide solution	**Hydrochloric acid solution**
d) pH:	14.0	0.1
e) Freezing point:	-12 - 10°C	-17°C
f) Boiling point:	105 - 140°C	81. - 110°C
g) Flash point:	Not applicable, not flammable	Not applicable, not flammable
h) Evaporation rate	No information available	No information available
i) Flammability (solid, gas)	Not applicable, liquid	Not applicable, liquid
j) LEL/UEL	Not flammable	Not flammable
k) Vapour pressure	< 24 hPa at 20°C	160 mm Hg at 20°C
l) Vapour density	1.38 (Air = 1.0)	1.26 (Air = 1.0)
m) Relative density	1.327	1.05-1.12
n) Water solubility	Completely miscible, soluble	Soluble

Section 9. Physical and Chemical Properties *(cont'd)*		
9.1 Information on Basic Physical and Chemical Properties *(cont'd)*		
	Sodium hydroxide solution *(cont'd)*	**Hydrochloric acid solution** *(cont'd)*
o) Partition coefficient	No information available	No information available
p) Autoignition temperature	No information available	No information available
q) Decomposition temperature	No information available	No information available
r) Viscosity	No information available	No information available
s) Explosive properties	No information available	No information available
t) Oxidizing properties	No information available	No information available
9.2 Other information: No information available		

Section 10. Stability and Reactivity
10.1 Reactivity: No data available
10.2 Chemical Stability: Stable under normal conditions
10.3 Possibility of Hazardous Reactions: May react with metals and lead to form flammable hydrogen gas
10.4 Conditions to Avoid: Excess heat; incompatible substances
10.5 Incompatible Materials: Acids, aldehydes, aluminium, bases, chlorinated solvents, metals, organic materials, oxidizing agents, phosphorus, reducing agents, tin/tin oxide, zinc
Hazardous Decomposition Products: Hydrogen chloride gas

Section 11. Toxicological Information		
11.1 Information on Toxicological Effects		
	Sodium hydroxide solution	**Hydrochloric acid solution**
a) Acute toxicity	Oral, rat LD_{50}: >90 mL/kg	Oral, rat LD_{50}: 700 mg/kg
b) Skin corrosion/irritation	Skin, rabbit: 500 mg/24hr severe	No data available
c) Serious eye damage/irritation	Eye, rabbit: 1 mg/24hr severe	No data available
d) Respiratory or skin sensitisation	No data available	No data available
e) Germ cell mutagenicity	No data available	Mutagenic effects have occurred in experimental animals
f) Carcinogenicity	Not listed (IARC, ACGIH, NTP)	IARC Group 3
g) Reproductive toxicity	No data available	Experiments have shown reproductive toxicity effects on laboratory animals
h) STOT-single exposure	No data available	Skin, eyes
i) STOT-repeated exposure	No data available	Respiratory system, skin, eyes, gastrointestinal tract (GI), liver, kidney, blood, teeth
j) Aspiration hazard	No data available	No data available

Potential health effects

Inhalation: May be harmful if inhaled. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.

Ingestion: May be harmful if swallowed. Causes burns.

Skin: May be harmful if absorbed through the skin. Causes skin burns.

Eyes: Causes eye burns.

NEOGEN CORPORATION

Section 11. Toxicological Information *(cont'd)*
11.1 Information on Toxicological Effects *(cont'd)*
Signs and symptoms of exposure Burning sensation, cough, wheezing, laryngitis, shortness of breath, spasm, inflammation and oedema of the larynx, spasm, inflammation and oedema of the bronchi, pneumonitis, pulmonary oedema. Material is extremely destructive to the tissue of the mucous membranes and upper respiratory tract.

Section 12. Ecological Information
12.1 Toxicity: Hydrochloric Acid: Fresh water fish 282 mg/L LC_{50} 96h Sodium Hydroxide: No data available
12.2 Persistence and Degradability: No data available
12.3 Bioaccumulative Potential: No data available
12.4 Mobility in Soil: No data available
12.5 Results of PBT and vPvB Assessment: No data available
12.6 Other Adverse Effects: None known

Section 13. Disposal Considerations
13.1 Waste Treatment Methods: Product should be disposed of with a licensed waste disposal company, while following all regional, local and national regulations.

Section 14. Transport Information			
	ADR/RID	**IMDG**	**IATA**
14.1 UN Number	1760	1760	1760
14.2 UN Proper Shipping Name	Corrosive, liquid, n.o.s.	Corrosive, liquid, n.o.s.	Corrosive, liquid, n.o.s.
14.3 Transport Hazard Class	8	8	8
14.4 Packing Group	II	II	II
14.5 Environmental Hazards	No	Marine pollutant: No	No
14.6 Special Precautions for User	No information available		

NEOGEN CORPORATION

Section 15. Regulatory Information

15.1 Safety, Health and Environmental Regulations/Legislation Specific for the Substance or Mixture

International Inventories:

Hydrochloric Acid:

EINECS	ELINCS	TSCA	DSL	NDSL	PICCS	ENCS	CHINA	AICS	KECL
231-595-7	-	T	X	-	X	X	X	X	KE-20189

Legend:

EINECS/ELINCS – European Inventory Lists

TSCA – United States Toxic Substances Control Act Inventory, Section 8(b)

DSL/NDSL – Canadian Domestic Substances List/Non-Domestic Substances List

PICCS – Philippines Inventory of Chemicals and Chemical Substances

ENCS – Japan Existing and New Chemical Substances

CHINA – China Inventory of Existing Chemical Substances

AICS – Inventory of Chemical Substances

KECL – Existing and Evaluated Chemical Substances

15.2 Chemical Safety Assessment

No data available

Section 16. Other Information

H314: Causes severe skin burns and eye damage
H335: May cause respiratory irritation

R35: Causes severe burns
R37: Irritating to respiratory system
R36/37/38: Irritating to eyes, respiratory system and skin.

This safety data sheet complies with the requirements of Regulation (EC) No. 1907/2006.

This document is believed to be correct, but does not purport to be all inclusive and shall be used only as a guide. Neogen Corporation shall not be held liable for any damage resulting from handling or from contact with the above product. These suggestions should not be confused with state, municipal or insurance requirements, and constitute NO WARRANTY.

Proposal No. **13-114**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Darcie Couture
Affiliation	Resource Access International
Address Line 1	710 River Road
Address Line 2	
City, State, Zip	Brunswick, ME 04011
Phone	207-266-8984
Fax	None
Email	darcie.couture@att.net
Proposal Subject	Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas. 11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	4. Approved Limited Use Methods for Marine Biotoxin Testing This submission presents the 'Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination' for consideration as an NSSP Approved Limited Use Method. The RBA is a competition-based assay that employs radiolabeled saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity. The RBA offers a high-throughput, sensitive, and quantitative alternative to the mouse bioassay (MBA), which has been the long-standing reference method for PSP toxicity. Further, the RBA eliminates the use of live animals for detection of these toxins. While the RBA still uses receptors prepared from animals, the number of animals required for analysis is significantly reduced. Using native receptors as the analytical recognition elements for the assay allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods that require conversion factors of equivalent toxicity to calculate the overall toxicity. The RBA has undergone AOAC single- and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27). Results from those studies, and additional data, are included in this proposal submission for the RBA to be considered for approval as an NSSP Approved Limited Use Method for Marine Biotoxin Testing.
Public Health Significance	Paralytic shellfish poisoning intoxications result from the consumption of seafood (primarily bivalve molluscs) contaminated with neurotoxins known as paralytic shellfish toxins (PSTs). This suite of toxins binds to voltage-gated sodium channels and may result in paralysis if enough toxin is consumed. In extreme cases when respiratory support is not available to the patient, the intoxication may prove fatal. Since the toxins cannot be destroyed during cooking and there is no way to remove the toxins from seafood, the best control strategy is to ensure that contaminated product never reaches the market. To protect public health, harvesting closures are implemented when toxicity exceeds the guidance level of 80 micrograms saxitoxin equivalents per 100 grams of shellfish tissue. As such,

	accurate analytical methods are needed to monitor shellfish toxicity for making decisions regarding opening and closing shellfish growing areas accordingly. Acceptance of the RBA as an NSSP Approved Limited Use Method for PSP toxicity determination would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA, thereby providing an early warning system for monitoring programs as toxin levels begin to rise.
Cost Information	The estimated cost for a full 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitative results would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. Further, the filter plates used in the RBA differ from ELISA plates in that all reagents are added to each well as needed rather than already being a component of the plate, making it more practical and cost-effective to analyze samples when there is less than a full plate.
Action by 2013 Laboratory Methods and Quality Assurance Review Committee	1. Recommended approval of this method as an alternative to the mouse bioassay for PSP in mussels. 2. Recommended approval of this method for Limited Use for clams and scallops for the purpose of screening and precautionary closure for PSP. 3. Recommended referral of this proposal to an appropriate committee as determined by the Conference Chairman to address this method in oysters. 4. Recommended Executive Office sends a letter to submitter to request a checklist for evaluation of labs using this method with said checklist to be submitted within three (3) months.
Action by 2013 Task Force I	Recommended adoption of Laboratory Method Review and Quality Assurance Committee recommendation on Proposal 13-114.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-114.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-114.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-114 to an appropriate committee as determined by the Conference Chair until additional data for oyster matrix are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-114.
Action by 2015 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-114.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-114.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination**
Name of the Method Developer	**Dr. Fran Van Dolah**
Developer Contact Information	**Tel: (843) 725-4864** **Email: Fran.vandolah@noaa.gov**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Paralytic shellfish poisoning (PSP) is the human intoxication that results from the consumption of seafood, primarily bivalve molluscs, contaminated with natural, algal-derived toxins known as paralytic shellfish toxins (PSTs) or the saxitoxins (STXs). This family of neurotoxins binds to voltage-gated sodium channels, thereby attenuating action potentials by preventing the passage of sodium ions across the membrane. Symptoms include tingling, numbness, headaches, weakness, and difficulty breathing. Medical treatment is to provide respiratory support, without which the prognosis can be fatal. To protect human health, seafood harvesting bans are implemented when toxins exceed a safe guidance level (80 μg STX equivalents per 100 g tissue or 800 μg STX equivalents per kg). Successful monitoring and management programs are attributed with minimizing the number of PSP cases and associated deaths. The mouse bioassay (MBA) has long-served as the gold standard method for detecting PSP in regulatory environments. Even though the MBA is an NSSP Approved Method for Marine Biotoxin Testing, there are numerous reasons for considering alternative methods for PSP detection. Disadvantages of the MBA include high variability and the use of live animals. Given these limitations of the MBA, particularly the ethical concerns of using live animals, there have been great strides in method development and validation for alternative approaches. Recently, the post-column oxidation liquid chromatographic method (PCOX) for PSP detection was accepted as an NSSP Approved Limited Use Method, providing an alternative to the MBA. While some laboratories are in the process of transitioning to this

		method, implementation requires costly instrumentation and skilled personnel. Furthermore, the PCOX method identifies and quantifies individual PSP toxins. Toxicity equivalency factors must then be taken into consideration to calculate the expected overall toxicity in μg STX equivalents per 100 g tissue. The proposed receptor binding assay (RBA) addresses the major shortcomings of the PCOX and MBA by quantitatively measuring the overall PSP toxicity and doing so without the need of live animals, respectively. The RBA relies on the interaction of the toxins with the native receptor site (i.e., voltage-gated sodium channels). In this functional assay toxins bind to their receptors according to their affinity, yielding an integrated toxic potency. The RBA is more sensitive than the MBA, allowing monitoring laboratories earlier warning capabilities as toxins become elevated. The RBA has successfully undergone AOAC single laboratory validation (Van Dolah et al. 2009 – Appendix II) and a full collaborative study (Van Dolah et al. 2012 – Appendix III). The RBA is now considered an AOAC Official Method of Analysis (OMA 2011.27 – Appendix IV). This proposal provides data from the AOAC studies as well as additional data to seek consideration for the RBA to be an NSSP Approved Limited Use Method.
2. What is the intended purpose of the method?	Y	This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish. Applications include: (1) Growing Area Survey & Classification and (2) Controlled Relaying. The RBA serves as an alternative to the MBA in these applications, offering a measure of integrated toxicity with high throughput and the elimination of live animal testing.
3. Is there an acknowledged need for this method in the NSSP?	Y	Yes, there is an acknowledged need for this method in the NSSP. Even though the MBA and PCOX methods have been respectively NSSP Approved and Approved for Limited Use, there remains a need for the proposed method. The RBA would provide an alternative to (1) the MBA, which uses live animals, and (2) the PCOX method, which requires costly equipment and skilled personnel and offers low throughput.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Molecular. The RBA is a functional assay, whereby toxins present in the standard/sample bind to sodium channel preparations in the assay. Radiolabeled toxins are added to solution to compete with toxins present in the standard/sample for binding sites, and thus a decrease in signal from radiolabeled toxins represents an increase in standard/sample toxicity. This competitive RBA allows for quantitation that directly relates to the composite toxicity of the sample.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
Method Scope	Y	The RBA provides a high throughput, sensitive, accurate, quantitative assay for PSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Limited Use Method for the purposes of screening for PSP toxicity.

References	Y	Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. Journal of AOAC International 92(6): 1705-1713. See Appendix II. Van Dolah et al. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor binding assay: Collaborative study. Journal of AOAC International 95(3): 795-812. See Appendix III. OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org. See Appendix IV.
Principle	Y	This assay is based on the interaction between the toxins and their native receptor, the voltage-gated sodium channels. All PSTs bind to site 1 of the voltage-gated sodium channels according to their potency, resulting in a measure of integrated potency (independent of knowing which toxin congeners are present) similar to mouse intraperitoneal potency. In the RBA, tritiated saxitoxin (^{3}H-STX) competes with unlabeled PSTs in the homogenized and extracted shellfish sample for a finite number of available receptor sites in a rat brain membrane preparation. After a binding equilibrium is reached, unbound ^{3}H-STX is removed by filtration and the remaining ^{3}H-STX is measured with a scintillation counter (as counts per minute or CPM). The amount of ^{3}H-STX present is indirectly related to the amount of unlabeled PSTs in the sample. Scintillation counting can be conducted using traditional scintillation counters or microplate counting. However, the microplate format is preferred as it minimizes sample handling and the amount of radioactivity used.
Any Proprietary Aspects	N	None. All reagents can be prepared or purchased.
Equipment Required	Y	The following list identifies the equipment and supplies needed for conducting the RBA. For the assay: (a) Scintillation counter (traditional or microplate) (b) An 8-channel pipettor (5-200 µl variable volume and disposable tips) (c) Micropipettors (1-1000 µl variable volumes and disposable tips) (d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50) (e) MultiScreen vacuum manifold (Millipore; Cat. No. NSVMHTS00) (f) Vacuum pump (g) Centrifuge tubes (15 and 50 ml, conical, plastic) (h) Mini dilution tubes in 96-tube array (i) Reagent reservoirs (j) Ice bucket and ice (k) Vortex mixer (l) Sealing tape (Millipore; Cat. No. MATA HCL00) (m) Volumetric flask or graduated beaker (1 L) (n) -80 °C freezer (o) Refrigerator

		Additional supplies when using a traditional scintillation counter (as opposed to a microplate counter): (p) MultiScreen punch device (Millipore; Cat. No. MAMP 096 08) (q) MultiScreen disposable punch tips (Millipore; Cat. No. MADP 196 10) (r) MultiScreen punch kit B for 4 ml vials (Millipore; Cat. No. MAPK 896 0B) (s) Scintillation vials (4 ml) For sample extraction: (t) Blender or homogenizer for sample homogenization (u) Pipets (v) Centrifuge tubes (15 ml, conical, plastic) (w) pH meter or pH paper (x) Hot plate or water bath (y) Graduated centrifuge tubes (15 ml) (z) Centrifuge and rotor for 15 ml tubes For rat brain isolation: (aa) Teflon/glass homogenizer (Motorized tapered Teflon pestle and glass tune (15 ml) (bb) Motorized tissue homogenizer (Polytron or small handheld blender) (cc) High-speed centrifuge and fixed angle rotor (20 000 x g rcf) (dd) Centrifuge tubes (12-15 ml, rated for 20 000 x g) (ee) plastic cryovials (2 ml) (ff) Graduated beaker (300 or 500 ml) (hh) Pipets (5-10 ml, disposable) (ii) Forceps (jj) Ice bucket and ice (kk) top loading balance
Reagents Required	Y	For the assay: (a) STX diHCl standards (NIST RM 8642; available through the National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA] (b) ^{3}H-STX (0.1 mCi per ml, ≥10 Ci per mmol, ≥90% radiochemical purity; available through American Radiolabeled Chemicals, St. Louis, MO) (c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent]) (d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent]) For microplate counter only: (e) Ultima Gold liquid scintillation cocktail (PerkinElmer Inc.; Waltham, MA; Cat. No. 6013321 [or equivalent]) For traditional counter only: (f) Scintiverse BD liquid scintillation cocktail (Fisher Scientific; Waltham, MA; Cat. No. SX-18 [or equivalent]) For sample extraction: (g) Hydrochloric acid (HCl; 1.0 and 0.1 M) (h) Sodium hydroxide (0.1 M) (i) Water (distilled or deionized [18 μΩ]) For rat brain isolation: (j) 20 rat brains (male, 6-week old Sprague-Dawley;

		available through Hilltop Lab Animals, Inc., Scottdale, PA; www.hilltoplabs.com [or equivalent]) (k) MOPS, pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G [or equivalent]) (l) Choline chloride (100 mM; Sigma; Cat. No. C7527-500G [or equivalent]) (m) Phenyl methylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO: Cat. No. P7626) (n) Isopropanol (o) Micro bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL)
Sample Collection, Preservation and Storage Requirements	Y	A representative shellfish sample should include 12 market size organisms pooled together (should be at least 100 g). Clean the outside of shellfish with running tap water. Open the shell by cutting into the adductor muscle, being careful to not cut or damage the viscera. Rinse the inside to remove sand and dirt and remove tissue from ~12 organisms. Collect the tissue on a number 10 sieve and allow to drain for ~5 minutes. Remove any obvious pieces of shell or debris. Transfer meat to blender or homogenizer and blend until homogeneous. This homogenate is then extracted for toxins. For the detailed sample extraction procedure see Sample Extraction in Appendix A. Shellfish homogenates must be tested immediately or stored frozen prior to analysis. Saxitoxin standards must be stored refrigerated and ^{3}H-STX must be stored at -80 °C. The rat brain preparation can be produced in bulk, partitioned into aliquots, and stored long-term at -80 °C until use.
Safety Requirements	Y	General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Even though low levels of radiation are used for this assay, users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of isotopes. Please see Appendix C for further safety requirements.
Clear and Easy to Follow Step-by-Step Procedure	Y	The protocol is very clear and easy to follow. Please see the detailed protocol below in Appendix A.
Quality Control Steps Specific for this Method	Y	Quality control steps are in place to determine if assay results are acceptable: (a) The slope of the standard curve must be between -0.8 and -1.2 (theoretical slope is -1). If the slope of a standard curve from a given assay falls outside of this range, the data should be considered unacceptable and the assay must be rerun. (b) The RSDs of triplicate counts per minute (CPMs) for the standards must be below 30%. (c) If the IC_{50} (inhibitory concentration at which CPM is 50% max) is out of the acceptable range (2.0 nM ± 30%), the data should be considered unacceptable and the assay should be rerun. (d) A QC sample should always be included and found to be in range. Typically a 1.8 x 10^{-8} M STX concentration

		(3 nM STX in-well concentration) is run as a QC and should be within 30%. Results outside of this range should trigger consideration of assay acceptance. The following criteria must be met to accept sample measurement: (e) For sample measurement, quantitation should only be done on sample dilutions that fall within the linear range. As such, binding (B, measured as counts per minute) scaled by the maximum binding (B_0) should be between 0.2-0.7 for sample quantitation to be performed (any sample falling outside of this range is considered out of the dynamic range). If $B/B_0 > 0.7$, the concentration is too low to be quantified and should be reported as below the limit of detection (LOD). If $B/B_0 < 0.2$, the sample should be diluted and rerun if quantitation is needed. (f) The RSDs for the sample CPMs should be $\leq 30\%$. These quality control criteria are also stated in section H in Appendix IV.
C. Validation Criteria		
1. Accuracy / Trueness	Y	Validation data presented in Section C are from both the SLV (Van Dolah et al. 2009) and the collaborative study (Van Dolah et al. 2012). Nine laboratories from six countries completed the collaborative study. There were a total of 21 shellfish homogenates tested in three different assays on independent days. Different shellfish species from a range of geographical locations were used in the study: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surfclam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast. Samples included those that were naturally contaminated, those that were spiked, and another that served as a negative control. Accuracy was evaluated based on recovery. As also stated under Section C. 4., Recovery of the QC check sample (3 nM in-well solution) was 99.3% (Appendix II). During the SLV recovery was evaluated for STX standard spiked into mussel tissue at concentrations below, at and above the regulatory guidance level. Recovery for the nominal spike at 40 μg STX eq 100 g^{-1} was 115%. At 80 μg STX eq 100 g^{-1}, recovery was found to be 129%. At a nominal spike of 120 μg STX eq 100 g^{-1}, recovery was 121% (Appendix II). During the collaborative study, recovery of PSTs from shellfish was found to be 84.4% (when spiked with 20 μg STX eq 100 g^{-1}), 93.3% (when spiked with 50 μg STX eq 100 g^{-1}), and 88.1% (when spiked with 120 μg STX eq 100 g^{-1}). See Appendix III.
2. Measurement Uncertainty	Y	ND

3. Precision Characteristics (repeatability and reproducibility)	Y	Repeatability (RSD_r) was determined during the SLV on six naturally contaminated shellfish samples on five independent days and was found to be 17.7%. See Appendix II. The reproducibility (RSD_R) during the collaborative study was found to be 33.2% for all laboratories. However, upon removing the results from the one laboratory that had no previous RBA experience, the RSD_R was 28.7%. If data from routine users of the RBA were evaluated, the RSD_R was 23.1%. See Appendix III. Repeatability (RSD_r) during the collaborative study ranged from 11.8-34.4%. For routine users of the RBA, the average RSD_r = 17.1%, consistent with the RSD_r obtained during the SLV. See Appendix III.
4. Recovery	Y	Recovery of the QC check sample (3 nM in-well solution) was 99.3% (Appendix II). During the SLV recovery was evaluated for STX standard spiked into mussel tissue at concentrations below, at and above the regulatory guidance level. Recovery for the nominal spike at 40 μg STX eq 100 g^{-1} was 115%. At 80 μg STX eq 100 g^{-1}, recovery was found to be 129%. At a nominal spike of 120 μg STX eq 100 g^{-1}, recovery was 121% (Appendix II). During the collaborative study, recovery of PSTs from shellfish was found to be 84.4% (when spiked with 20 μg STX eq 100 g^{-1}), 93.3% (when spiked with 50 μg STX eq 100 g^{-1}), and 88.1% (when spiked with 120 μg STX eq 100 g^{-1}). See Appendix III.
5. Specificity	Y	The RBA is specific to toxins that bind to site 1 of voltage-gated sodium channels. This includes all PSP congeners, whereby binding affinity is proportional to potency. Tetrodotoxin also binds to site 1 of the sodium channels, yet the typical combinations of sources, vectors, and geographical regions of tetrodotoxin and the saxitoxins differ.
6. Working and Linear Ranges	Y	The dynamic range of the assay was determined to be 1.2-10.0 nM in-well concentration (Appendix II). Linearity assessment was conducted with three calibration standards (1.5, 3.0, and 6.0 nM STX in -well concentration) on five independent days. The linear regression yielded a slope of 0.98 and an r^2 = 0.97 (Appendix II). During the collaborative study, the assay was set for the critical range of shellfish toxicities below, near and just above the regulatory guidance level (~15-240 μg STX eq 100 g^{-1} or ~150-2400 μg STX eq kg^{-1}). Appendix III.
7. Limit of Detection	Y	The LOD, as determined in the collaborative study, is 4.5 μg STX eq 100 g^{-1} or 45 μg STX eq kg^{-1} See Appendix III.
8. Limit of Quantitation / Sensitivity	Y	The limit of quantitation (LOQ) was empirically determined as the concentration in a 10-fold diluted sample that resulted in a in a B/B0 of 0.7 (more conservative than the 0.8 typically used as the cut off for such assays). The LOQ was determined to be 5.3 μg STX eq 100 g^{-1} during the SLV (Appendix II).

		The LOQ of the RBA is 12.6 μg STX eq 100 g^{-1} or 126 μg STX eq kg^{-1}, as compared to the MBA LOQ of ~40 μg STX eq 100 g^{-1} (or ~400 μg STX eq kg^{-1}). See Appendix III.
9. Ruggedness	Y	Ruggedness was addressed and critical steps were noted that could affect precision and accuracy. It was deemed important to clarify the shellfish extracts by centrifugation prior to performing the assay, particularly if the sample was refrigerated or frozen. The rat brain preparations should be vortexed frequently to ensure the synaptosomes are in suspension, and the buffer should be ice cold to ensure that toxins are not released from the receptor. Assay plate filtration should be at a rate of 2-5 seconds. Lastly, a minimum of 30 minutes should be allowed before reading the plates after scintillation liquid is added such that scintillant can penetrate the filters. For more detail please refer to Appendix II and Appendix III.
10. Matrix Effects	Y	No matrix effects were reported. Minimum dilutions of shellfish extracts were 10-fold and were found to be sufficient to eliminate matrix effects. See Appendix III.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	The RBA was compared to the MBA and the pre-column oxidation (Pre-COX) liquid chromatography with fluorescence detection (LC-FD) approach during the SLV. RBA results compared well to those obtained by the MBA in two separate studies. In one component of the SLV, six naturally contaminated samples (clams, mussels, and sea scallops) were tested by RBA and MBA. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 = 0.98 was obtained, with a slope of 1.29. In the second component of the SLV, which included 110 naturally contaminated shellfish, an r^2 = 0.88 and a slope of 1.32 was obtained (Appendix II). Nine naturally contaminated samples (six blue mussels and three scallops) were extracted and analyzed by RBA and Pre-COX. Samples were analyzed using the RBA following the typical extraction (0.1 M HCl), but also following the extraction procedure used for the Pre-COX method (1% acetic acid). A good correlation was found between the two methods for both extraction methods. When the RBA samples were extracted with HCl, the RBA compared to the Pre-COX yielded an r^2 = 0.98 and a slope of 1.39. When samples were extracted the same for both methods (acetic acid), the correlation was slightly improved with an r^2 = 0.99 and a slope of 1.32 (Appendix II). During the collaborative study, ten laboratories from seven countries performed the RBA. Additionally three of the laboratories conducted the MBA, and one laboratory tested the samples using the Pre-COX LC-FD. The MBA and RBA data comparison yielded an r^2 = 0.84 and a slope of 1.63. The LC-FD and RBA data comparison yielded an r^2 = 0.92 and a slope of 1.20. Both RBA and LC-FD methods generally report higher toxicity in shellfish, especially at or near the guidance level, relative to the MBA. This provides a conservative measure and allows for an earlier warning of developing

		toxicity. See Appendix III.
D. Other Information		
1. Cost of the Method	Y	The estimated cost per 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample [ranging from 3.5-600 μg STX eq 100 g^{-1}] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced.
2. Special Technical Skills Required to Perform the Method	Y	General laboratory training is necessary (this would include being able to prepare reagent solutions, pipetting, centrifugation, and simple calculations). Additional training for working with low levels of radioactive material is required.
3. Special Equipment Required and Associated Cost	Y	A microplate scintillation counter is needed and the cost is ~$60-100K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K.
4. Abbreviations and Acronyms Defined	Y	A list of abbreviations and acronyms is provided below in Appendix I.
5. Details of Turn Around Times (time involved to complete the method)	Y	Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turn around time for data in approximately 3 hours. Up to six plates per analyst are possible in one day, yielding a throughput of 42 samples per day.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	The Center for Food Safety and Applied Nutrition (CFSAN) Quality System (QS) provides guidance to (1) design and develop processes, products, and services related to CFSAN's mission, the FDA's regulatory mission, and critical management and administrative support services, and (2) continually improve and strengthen product and service quality. The Laboratory Quality Assurance program serves as CFSAN's logical application of QS to Center laboratories and lab-based activities. The third edition (October 2009) of the Laboratory Quality Manual was followed. Standard reference materials for saxitoxin are obtained through the National Institute of Standards and Technology (NIST) and are accompanied by a Report of Investigation (See Appendix V). The standard reference saxitoxin used in the RBA is the same as that employed with the MBA. The 3H-STX is obtained through American Radiolabeled Chemicals, Inc., and is accompanied by a Technical Data Sheet with lot specifications (Appendix VI).
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** – The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** – the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Appendix A: RBA Step-by-Step Procedure

A. Sample Extraction
   a. The extraction detailed below represents a small scale MBA extraction procedure. The actual MBA extraction could be used instead of the small scale version described here.
   b. Accurately weigh 5.0 g of tissue homogenate into a tared, labeled 15 ml conical tube.
   c. Add 5.0 ml of 0.1 M HCl, vortex, and check pH.
      i. If necessary, adjust pH to 3.0-4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing.
   d. Place the tube in a beaker of boiling water on hot plate (or in a water bath) for 5 min with the caps loosened.
   e. Remove and cool to room temperature.
   f. Check pH and, if necessary, adjust cooled mixture to 3.0-4.0 as described above.
   g. Transfer entire contents to a labeled, graduated centrifuge tube and dilute volumetrically to 10 ml.
   h. Gently stir contents to homogeneity and then allow to settle until a portion of supernatant is translucent and can be decanted free of solids.
   i. Pour 5-7 ml of the translucent supernatant into a labeled centrifuge tube.
   j. Centrifuge at 3000 x *g* for 10 min.
   k. Retain clarified supernatant and transfer to a clean, labeled centrifuge tube.
   l. Store extracts at -20 °C until tested in RBA.

B. Preparation of Stock Solutions and Standards
   a. Assay buffer: 100 mM MOPS/100 mM choline chloride, pH 7.4
      i. Weigh 20.9 g MOPS and 13.96 g choline chloride and add to 900 ml distilled or milli-Q water.
      ii. Adjust pH to 7.4 with NaOH while stirring.
      iii. Bring to a final volume of 1 L with distilled or milli-Q water.
      iv. Store at 4 °C.
   b. Radioligand solution: ^{3}H-STX
      i. Calculate the concentration of ^{3}H-STX stock provided by the supplier. Suppliers generally provide specific activity in Ci/mmol (~10-30 Ci/mmol) and activity in mCi/ml (~0.05-0.1 mCi/ml), from which the molar concentration can be calculated.
      ii. Prepare 4 ml of a 15 nM working stock of ^{3}H-STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate.
      iii. Measure total counts of each working stock prior to running an assay. Add 36 µl of working stock ^{3}H-STX in buffer to a liquid scintillation counter vial with 4 ml scintillant and count on a traditional liquid scintillation counter to confirm correct dilution. The CPM should be consistent and within 15% of expected value.

c. Unlabeled STX standard working solution: The STX diHCl standard (NIST RM 8642 STX diHCl) is provided at a concentration of 268.8 μM (100 μg/ml).
    i. A bulk standard curve can be made up in advance and stored at 4 °C for up to one month. The use of a bulk standard curve minimizes time needed for routine analyses and improves repeatability.
    ii. Make up 3 mM HCl (e.g., from a 3 M stock, 50 μl in 50 ml) and use for the serial dilutions.
    iii. Serial dilutions should result in the following stock concentrations (M):
        1. 6×10^{-6} [100 μl 268.8 μM STX + 4.38 ml 0.003 M HCl]
        2. 6×10^{-7} [500 μl 6×10^{-6} M STX + 4.5 ml 0.003 M HCl]
        3. 1.8×10^{-7} [1.5 ml 6×10^{-7} M STX + 3.5 ml 0.003 M HCl]
        4. 6×10^{-8} [500 μl 6×10^{-7} M STX + 4.5 ml 0.003 M HCl]
        5. 1.8×10^{-8} [500 μl 1.8×10^{-7} M STX + 4.5 ml 0.003 M HCl]
        6. 6×10^{-9} [500 μl 6×10^{-8} M STX + 4.5 ml 0.003 M HCl]
        7. 6×10^{-10} [500 μl 6×10^{-9} M STX + 4.5 ml 0.003 M HCl]
        8. 5 ml 0.003 M HCl.

d. Interassay calibration standard (QC check): Reference standard STX (1.8×10^{-8} M STX) in 3 mM HCl. For long-term storage keep at -80 °C; for routine use (up to one month), store at 4 °C.

e. Rat brain membrane preparation: Prepare bulk rat brain membrane preparations (Appendix B) and store at -80 °C.
    i. Thaw an aliquot of rat brain preparation on ice.
    ii. Dilute membrane preparation with cold (4 °C) 100 mM MOPS/100 mM choline chloride, pH 7.4 to yield a working stock with a protein concentration of 1.0 mg/ml.
    iii. Vortex vigorously to achieve a visibly homogeneous suspension.
    iv. Keep the diluted membrane preparation on ice.

C. Performing the Assay

a. Plate setup: When possible use a multichannel pipet to minimize effort and increase consistency.
    i. Run standards, samples, and QC check in triplicate.
    ii. For quantitation, multiple dilutions per extract should be analyzed in order to obtain a value that falls within the dynamic range of the assay. A minimum sample extract dilution of 1:10 is recommended to minimize potential matrix effects.
    iii. Use of a standard plate layout (Figure 1) is recommended. This will improve ease of analysis and can help maximize the number of samples/standards that can be analyzed per plate.

b. Addition of samples/standards: Add in the following order to each well-
    i. 35 μl assay buffer
    ii. 35 μl STX standard/QC check/sample extract
    iii. 35 μl ^{3}H-STX
    iv. 105 μl membrane preparation (ensure solution is homogeneous)
    v. Cover the plate and incubate at 4 °C for 1 h.

c. Assay filtration: Use the vacuum manifold attached to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process.
    i. Set the vacuum pressure gauge on the pump or manifold to ~4-8" Hg (~135-270 millibar).
    ii. Place the 96-well plate on the vacuum manifold.
    iii. Fill any empty wells with 200 µl MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate.
    iv. Turn on vacuum. Optimum vacuum will pull the wells dry in 2-5 s.
    v. With vacuum pump running, quickly rinse each well twice with 200 µl ice cold MOPS/choline chloride buffer using a multichannel pipet. Maintain vacuum until liquid is removed.
d. Preparation of the assay for counting: Remove the plastic bottom from the plate and blot the plate bottom once on absorbent towel.
    i. For counting in microplate scintillation counter:
        1. Seal the bottom of a counting cassette with sealing tape.
        2. Place the microplate in the counting cassette.
        3. Add 50 µl scintillation cocktail per well using multichannel pipet.
        4. Seal the top of the plate with sealing tape.
        5. Incubate for 30 min at room temperature.
        6. Place the plate in the scintillation counter and count for 1 min per well.
    ii. For counting in traditional scintillation counter:
        1. Place the microplate in the MultiScreen punch system apparatus and place the disposable punch tips on top of the microplate.
        2. Punch the filters from the wells into scintillation vials and fill with 4 ml scintillation cocktail.
        3. Place caps on the vials and vortex.
        4. Allow vials to sit overnight in the dark.
        5. Count using a tritium window in a traditional scintillation counter.

D. Analysis of Data
    a. Curve fitting: Perform curve fitting using a four-parameter logistic fit (sigmoidal dose response curve with variable slope).
        i. $y = \text{min} + (\text{max-min})/1+10^{(\text{x-log IC50})\text{Hill slope}}$
        ii. where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX (also known as B_0); min is the bottom plateau, equal to nonspecific binding in CPM in the presence of saturating nonradiolabeled STX; IC50 is the inhibitory concentration at which CPM are 50% of max-min); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is the total ligand binding in CPM (B/B_0).
    b. Sample quantification: Sample quantification is only carried out on dilutions having a B/B_0 in the range of 0.2-0.7.
        i. Where B represents the bound ^{3}H-STX in CPM in the sample and B_0 represents the max bound ^{3}H-STX in the sample.

ii. Sample concentration is calculated in μg STX diHCl equivalents (eq)/kg shellfish as described below:

(nM STX eq) x (sample dilution) x [(210 μl total volume)/35 μl sample]
= nM STX eq in extract

(nM STX diHCl eq in extract) x (1 L/1000 ml) x (372 ng/nmol) X (1 μg/1000 ng)
= g STX diHCl eq/ml

μg STX diCHl eq/ml x (ml extract/g shellfish) x (1000g/kg)
= μg STX diHCl eq/kg

Figure 1. Example plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

Concentrations indicate those of the STX standard curve; REF = reference; QC = quality control; U = unknown sample (with dilutions indicated). The same standard curve made be used for additional plates run on the same day using the same reagents (i.e., 11 samples can be run on subsequent plates).

Appendix B: Rat Brain Membrane Preparations

A. Equipment/Supplies
    a. Teflon/glass homogenizer: Tapered Teflon pestle and glass tube, 15 ml
    b. Motorized tissue homogenizer: Polytron or small hand-held blender
    c. High-speed centrifuge and fixed angle rotor: capable of 20,000 x *g*
    d. Centrifuge tubes: 12-15 ml, rated for >20,000 x *g*
    e. Plastic cryovials: 2 ml
    f. Glass beaker: 300-500 ml
    g. Pipets: disposable 5 and 10 ml
    h. Forceps.
B. Reagents
    a. 20 rat brains: male, 6-week old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA) or equivalent
    b. MOPS: pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G)
    c. Choline chloride: 100 mM (Sigma; Cat. No. C7527-500G)
    d. Phenyl methylsulfonyl fluoride (PMSF): (Sigma; Cat. No. P7626)
    e. Isopropanol.
C. Procedure
    a. Prepare 1 L of 100 mM MOPS, pH 7.4, containing 100 mM choline chloride (as described in Appendix A) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol: dissolve 0.174 g PMSF in 10 ml isopropanol to make 100 mM stock. Aliquot stock and store at -20 °C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh in the day of use.
    b. Remove the medulla and cerebellum from each brain using forceps and discard. Place cerebral cortex in a small amount of ice-cold buffer and place on ice.
    c. Place one cerebral cortex in 12.5 ml MOPS/choline Cl/PMSF, pH 7.4, in glass/Teflon homogenizer. Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes and ensure there are no visible chinks remaining in the homogenate. Keep tube in ice at all times. Pour homogenized tissue into 250 ml beaker on ice and repeat procedure with remaining cortices.
    d. Transfer pooled homogenate tissue to centrifuge tubes, balance the tubes (pairwise: using ice-cold buffer to balance), and centrifuge at 20,000 x *g* for 15 min at 4 °C.
    e. Aspirate the supernatant and resuspend pellets in ice-cold MOPS/choline Cl/PMSF, using an adequate amount to fully resuspend the pellet (5-10 ml per brain).
    f. Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume up to 200 ml (keep on ice).
    g. Keeping the beaker on ice, polytron (or homogenize with small handheld blender) at 70% full speed for 20 s to obtain a homogeneous solution.
    h. Aliquot 2 ml per tube into cryovials. It is critical to keep the preparation well mixed while dispensing. Keep cryotubes on ice.
    i. Freeze and store at -80 °C. This preparation is stable for at least 6 months.

D. Protein Assay
    a. Determine the protein concentration of the membrane preparation using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) or equivalent. The above protocol should yield ~6-8 mg protein/ml of rat membrane preparation.
    b. Determine the membrane dilution needed for the assay. The protein concentration in the daily working stock should be 1 mg/ml (which yields a diluted concentration of 0.5 mg/ml in-assay concentration). Based on the protein concentration determined using the protein assay, dilute rat membrane preparation with buffer to 1 mg/ml. It is this diluted membrane preparation that is used in the assay.
    c. Protein concentrations must be determined and new dilutions calculated accordingly for each new batch of membranes prepared.

Appendix C: Radiation Safety Requirements

A. All users must follow all local, state, and federal laws and procedures regarding receipt, use and disposal of isotopes.
B. All users must be trained in and follow all in-house safety procedures for working with radiolabeled materials.
C. All isotopes and work stations where isotopes are used should be controlled access areas. Any one with access to the area must also receive radiation safety training.
D. Freezers where the isotopes are stored must be locked.
E. Personal protective equipment must include lab coats (designated specifically for use with radioactive materials), safety glasses, and gloves.
F. Radioactive materials will only be handled and manipulated in designated areas, which have been clearly identified and labeled accordingly.
G. Work with source radiation material must be conducted in a fume hood.
H. Radioactive materials will be stored and/or carried in secondary containment.
I. When possible, disposable supplies such as pipet tips, absorbent paper, and kim wipes will be used so that contaminated supplies can be readily disposed of as radioactive waste.
J. Wipe surveys will be conducted at the end of each experiment as well as monthly to ensure that there is no contamination in the laboratory.
K. The filter plates used in the assay will be designated as solid radioactive waste, while the washes from the filter plates (containing buffer and unbound ^{3}H-STX) will be handled as liquid radioactive waste. There will be a dry active waste container to hold contaminated items such as the plates, gloves, absorbent paper and kim wipes. There will be a liquid waste jug to hold the contaminated liquid radioactive waste.
L. All wastes must be disposed of according to state and local laws.

Appendix I. Abbreviations and Acronyms

^{3}H-STX	Tritiated saxitoxin
AOAC	Association of Analytical Communities
ARC	American Radiolabeled Chemicals
B	Bound CPM
B_o	Maximum bound CPM
CFSAN	Center for Food Safety & Applied Nutrition
CPM	Counts per minute
diHCl	Dihydrochloride
Eq	Equivalents
HCl	Hydrochloric acid
IC_{50}	Inhibitory concentration at which CPMs are at 50% max
LC-FD	Liquid chromatography with fluorescence detection
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Mouse bioassay
MOPS	3-Morpholinopropanesulfonic acid
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NSSP	National Shellfish Sanitation Program
OMA	Official method of analysis
PMSF	Phenyl methylsulfonyl fluoride
PCOX	Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX	Pre-column oxidation liquid chromatography with fluorescence detection
PSP	Paralytic shellfish poisoning
PSTs	Paralytic shellfish toxins
QC	Quality control
QS	Quality System
RBA	Receptor binding assay
RSD	Relative standard deviation
SLV	Single laboratory validation
STX	Saxitoxin

Appendix II

FOOD CHEMICAL CONTAMINANTS

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

FRANCES M. VAN DOLAH, TOD A. LEIGHFIELD, and GREGORY J. DOUCETTE
NOAA National Ocean Service, Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412
LAURIE BEAN
Maine Department of Natural Resources, McKown Point, W. Boothbay Harbor, ME 04575
BARBARA NIEDZWIADEK and DOROTHEA F.K. RAWN
Health Canada, Food Research Division, Sir Frederick Banting Research Centre, Ottawa, ON, Canada K1A 0L2

A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX $\pm$ 0.3 (RSD = 10.8%) with a slope of 0.96 $\pm$ 0.06 (RSD = 6.3%) and a dynamic range of 1.2–10.0 nM. The LOQ was 5.3 μg STX equivalents/100 g shellfish. Linearity, established by quantification of three levels of purified STX (1.5, 3, and 6 nM), yielded an r^2 of 0.97. Recovery from mussels spiked with three levels (40, 80, and 120 μg STX/100 g) averaged 121%. Repeatability (RSD_r), determined on six naturally contaminated shellfish samples on 5 independent days, was 17.7%. A method comparison with the AOAC mouse bioassay yielded r^2 = 0.98 (slope = 1.29) in the SLV study. The effects of the extraction method on RBA-based toxicity values were assessed on shellfish extracted for PSP toxins using the AOAC mouse bioassay method (0.1 M HCl) compared to that for the precolumn oxidation HPLC method (0.1% acetic acid). The two extraction methods showed linear correlation (r^2 = 0.99), with the HCl extraction method yielding slightly higher toxicity values (slope = 1.23). A similar relationship was observed between HPLC quantification of the HCl- and acetic acid-extracted samples (r^2 = 0.98, slope 1.19). The RBA also had excellent linear correlation with HPLC analyses (r^2 = 0.98 for HCl, r^2 = 0.99 for acetic acid), but gave somewhat higher values than HPLC using either extraction method (slope = 1.39 for HCl extracts, slope = 1.32 for acetic acid). Overall, the excellent linear correlations with the both mouse bioassay and HPLC method and sufficient interassay repeatability suggest that the RBA can be effective as a high throughput screen for estimating PSP toxicity in shellfish.

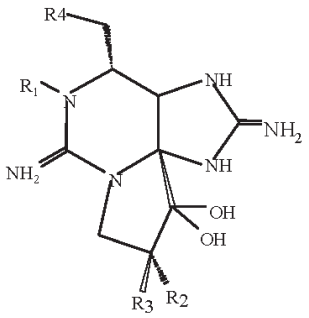
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fran.vandolah@noaa.gov

Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 μg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/μmol
Carbamate	STX	H	H	H	$OCONH_2$	2483
	Neo STX	OH	H	H	$OCONH_2$	2295
	GTX1	OH	OSO3-	H	$OCONH_2$	2468
	GTX2	H	OSO3-	H	$OCONH_2$	892
	GTX3	H	H	OSO3-	$OCONH_2$	1584
	GTX4	OH	H	OSO3-	$OCONH_2$	1803
Sulfocarbamoyl	GTX5 (B1)	H	H	H	OCONHSO3-	160
	GTX6 (B2)	OH	H	H	OCONHSO3-	-
	C1	H	OSO3-	H	OCONHSO3-	15
	C2	H	H	OSO3-	OCONHSO3-	239
	C3	OH	OSO3-	H	OCONHSO3-	33
	C4	OH	H	OSO3-	OCONHSO3-	143
Decarbamoyl	dcSTX	H	H	H	OH	1274
	dcNeoSTX	OH	H	H	OH	-
	dcGTX1	OH	OSO3-	H	OH	-
	dcGTX2	H	OSO3-	H	OH	1617
	dcGTX3	H	H	OSO3-	OH	1872
	dcGTX4	OH	H	OSO3-	OH	-
Deoxydecarbamoyl	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO3-	H	-
	doGTX3	H	OSO3-	H	H	-

Figure 1. Structures and toxic potency of 21 saxitoxin congeners. Toxic potency is listed as mouse units (MU)/μmole, where a mouse unit is defined as the minimum amount required to kill a 20 g mouse in 15 min when administered by IP injection. The table is modified from ref. 15.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200
B	10^{-7}	10^{-7}	10^{-7}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
C	3 x 10^{-8}	3 x 10^{-8}	3 x 10^{-8}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
D	10^{-8}	10^{-8}	10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
E	3 x 10^{-9}	3 x 10^{-9}	3 x 10^{-9}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
F	10^{-9}	10^{-9}	10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
G	10^{-10}	10^{-10}	10^{-10}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
H	10^{-11}	10^{-11}	10^{-11}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			

U = unknown sample

Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(**a**) *Microplate scintillation counter*.—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(**b**) *Microplate filtration manifold*.—Millipore (Bedford, MA).

(**c**) *Hot plate*.—Fisher Scientific (Suwannee, GA).

(**d**) *Countertop centrifuge*.—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(**e**) *Microtiter filter plates (96 well) with 1.0* μ*m pore size type FB glass fiber filter/0.65* μ*m pore size Duropore support membrane*.—Cat. No. MSFB N6B 50 (Millipore Corp., Billerica, MA).

(**f**) *Microplate sealing tape*.—Cat. No. MATA HCL00 (Millipore Corp.).

(**g**) *Vortex mixer*.—Daigger Vortex Genie II (Daigger Scientific, Vernon Hills, IL).

(**h**) *Teflon/glass tissue homogenizer*.—Wheaton (Millville, NJ).

(**i**) *Polytron homogenizer*.—Brinkmann Instruments (Westbury, NY).

Reagents

(**a**) *Hydrochloric acid (HCl)*.—0.1 M.

(**b**) *[^{3}H]STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (International Isotopes Clearinghouse, Leawood, KS).

(**c**) *STX diHCl*.—FDA reference standard (Office of Seafood, Laurel, MD) or National Research Council (NRC) of Canada Institute of Marine Biosciences (Halifax, NS, Canada).

(**d**) *Assay buffer*.—75 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat. No. H9136]/140 mM NaCl, pH 7.5 (Sigma, St. Louis, MO).

(**e**) *Liquid scintillation cocktail*.—Optiphase (PerkinElmer Life Sciences, Downers Grove, IL).

Preparation of Samples (0.1 M HCl Extraction)

Shellfish samples were shucked and homogenized according to the AOAC mouse bioassay protocol (1). For the HCl extraction method, 5.0 (±0.1) g of tissue homogenate was transferred to a tared 15 mL conical polypropylene centrifuge tube. A 5.0 mL volume of 0.1 M HCl was added, and the sample was mixed on a Vortex mixer. The pH was checked to

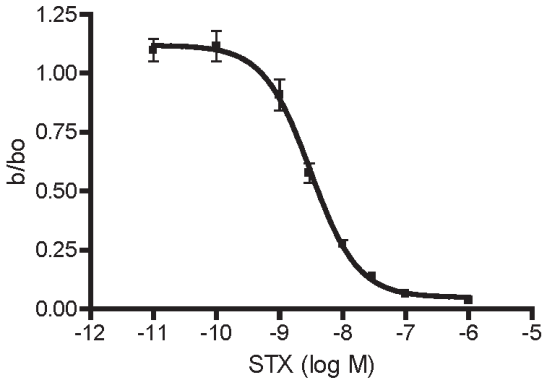


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

confirm it was between 3.0 and 4.0 in order to avoid alkalinization and destruction of the toxin, and adjusted with 1 M HCl or 0.1 M NaOH as needed. Tubes were placed in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Following removal from the boiling water bath, samples were allowed to cool to room temperature, and the pH was again confirmed to be between 3.0 and 4.0. The entire contents were then transferred to a graduated cylinder, diluted volumetrically to 10 mL, and centrifuged for 5 min at 1000 × *g*. The supernatant was transferred to a clean tube.

Preparation of Samples (Acetic Acid Extraction Method)

In a 50 mL plastic centrifuge tube, 5.0 ± 0.1 g homogenate was mixed with 3.0 mL 1% acetic acid on a vortex mixer. Tubes were capped loosely to avoid pressure buildup and placed in a boiling water bath for 5 min. Following removal from the water bath, samples were cooled in a beaker of cold water for 5 min, and then centrifuged for 10 min at 3000 × *g*. The supernatant was transferred to a 15 mL graduated conical test tube. A 3 mL amount of 1% acetic acid was added to the original tube with solid residue, mixed well on a vortex mixer, and centrifuged again for 10 min at 3000 × *g*. The second supernatant was combined with the first and diluted to 10 mL with water.

Preparation of Stock Solutions, Standards, and Reagents for Assay

(**a**) *Radioligand solution.*—[^{3}H]STX stock is provided in 50 μCi ampules, 24 Ci/mmol, 0.1 mCi/mL (4.17 μM). A 15 nM working stock of [^{3}H] STX was prepared fresh daily in 75 mM HEPES/140 mM NaCl (for 2.5 nM final in-well concentration).

(**b**) *STX standard curve.*—FDA STX dihydrochloride reference standard (100 μg/mL or 268.8 μM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6×10^{-6}, 6×10^{-7}, 1.8×10^{-7}, 6×10^{-8}, 1.8×10^{-8}, 6×10^{-9}, 6×10^{-10}, 6×10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total [^{3}H]STX binding in the absence of unlabeled STX.

Table 1. RBA measurements of calibration standards for assay linearity assessment (nM STX; *n* = 5)

Nominal	Mean	SD	RSD
1.5	1.7	0.16	10
3.0	3.0	0.52	17
6.0	6.0	0.34	6

(**c**) *Calibration standard (QC check).*—A reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquotted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 μL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor.

(**d**) *Rat brain membrane homogenate.*—Cerebral cortices from 6-week-old male Holzman rats (Harlan Bioproducts, Indianapolis, IN) were homogenized on ice in a glass/Teflon tissue homogenizer in 75 mM HEPES/140 mM NaCl, pH 7.5, containing 0.1 mM PMSF (phenylmethanesulfonylfluoride;12.5 mL/brain) at 385 rpm for 10 strokes. Pooled homogenates were centrifuged at 20 000 × *g* for 15 min at 4°C and the pellet was resuspended in HEPES buffer (12.5 mL/brain) and rehomogenized on ice using a Polytron homogenizer set at 70% power for 20 s to ensure a fine suspension. The brain homogenate was aliquotted 2 mL/tube in cryovials and stored at –80°C. The protein concentration of the brain homogenate was determined using the Micro bicinchoninic acid (BCA) Assay (Pierce, Rockford, IL). For each assay, an aliquot of brain homogenate was thawed on ice and diluted with ice cold 75 nM HEPES/150 mM NaCl, pH 7.5, to yield a final protein concentration of 0.5 mg/mL in the assay.

Table 2. Recovery of analyte from spiked samples (μg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD_r	Recovery, %
0	<dl[a]			
40	47	8.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

[a] <dl = Less than LOQ (5 μg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; *n* = 5) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	65	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

Assay Procedure

(**a**) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(**b**) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equialents/100 g shellfish using the following formulas:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM equiv. STX in extract}$$

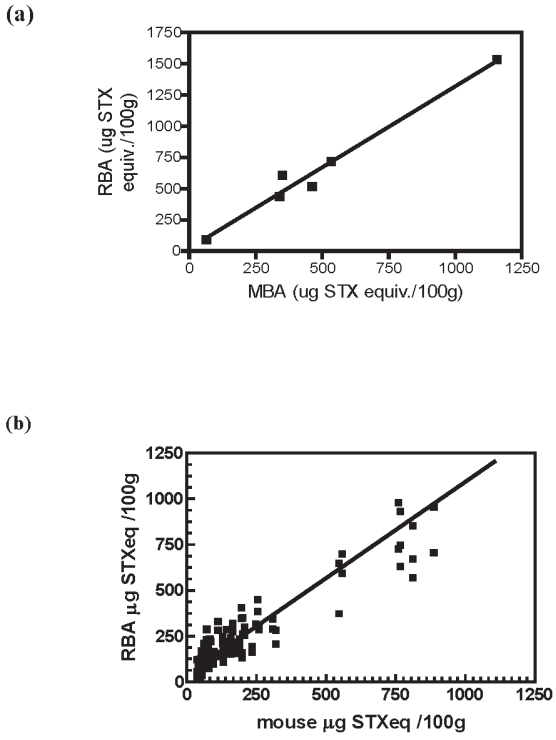


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

$$(\text{nm equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, $b/b_o < 0.2$), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., $b/b_o > 0.7$) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD_r = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

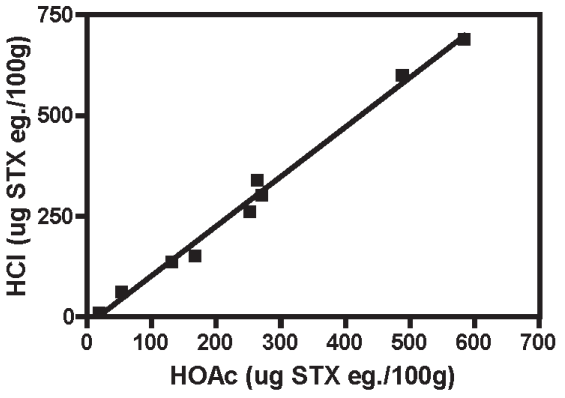


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.6	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Plactopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

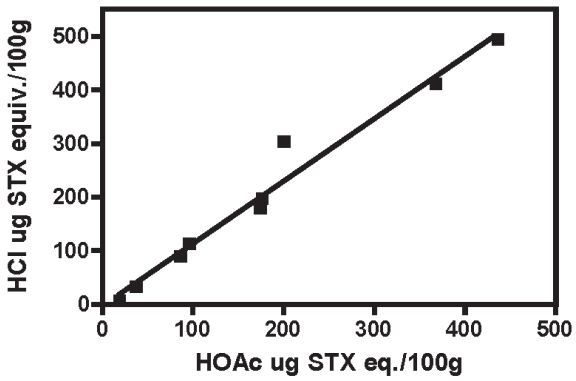


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

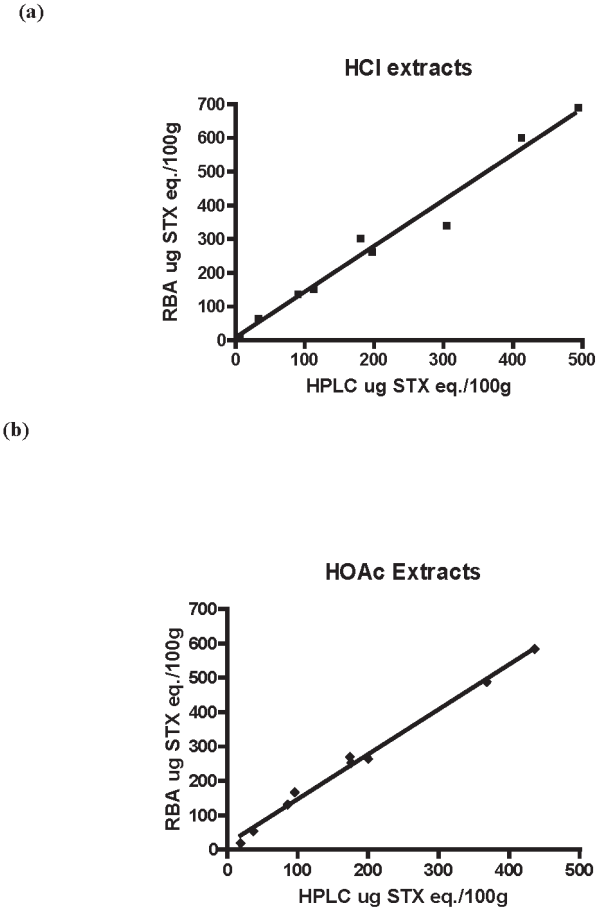


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

Acknowledgments

We thank Karen Mao and Steven Eaker for excellent technical support in the conduct of this study.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2006) *J. AOAC Int.* **88**, 1714–1732

(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(4) Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009) *J. AOAC Int.* **92**, 190–207

(5) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(6) Hall, S., Strichartz, G., Moczydlowshi, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(7) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636

(8) Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(9) Powell, C.L., & Doucette, G.J. (1999) *Nat. Toxins* **7**, 393–400

(10) Suarez-Isla, B.A., & Valez, P. (2000) in *Seafood and Freshwater Toxins,* L.M. Botana (Ed.), Marcel Dekker Inc., New York, NY, pp 187–202

(11) Llewellyn, L.E., Doyle, J., Jellett, J., Barrett, R., Alison, C., Bentz, C., & Quilliam, M. (2001) *Food Addit. Contam.* **18**, 970–980

(12) Ruberu, S.R., Liu, Y.-G., Wong, C.T., & Perera, S.K. (2003) *J. AOAC Int.* **86**, 1–9

(13) Usup, G., Leaw, C.-P., Cheah, M.-Y., Ahmad, A., & Ng, B.-K. (2004) *Toxicon* **44**, 37–43

(14) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667

(15) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

FOOD CHEMICAL CONTAMINANTS

Appendix III

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

FRANCES M. VAN DOLAH, SPENCER E. FIRE, TOD A. LEIGHFIELD, CHRISTINA M. MIKULSKI, and GREGORY J. DOUCETTE
Center for Coastal Environmental Health and Biomolecular Research, NOAA Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412

Collaborators: Å. Andersson; L. Bean; D. Couture; S. DeGrasse; A. DeLeon; V. Dell'Ovo; L. Flewelling; P. Holland; G. Langlois; R. Lewis; M. Masuda; P. McNabb; C. Mikulski; B. Niedzwiadek; B. Porntepkasemsan; D. Rawn; E. Sombrito; K. Srisuksawad; B. Suarez; S. Subsinserm; A. Tubaro

A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

Submitted for publication December 3, 2011.
The recommendation was approved by the Method Committee on Paralytic Shellfish Toxins as First Action. *See* "Methods News," (2011) *Inside Laboratory Management*, January/February issue.

Corresponding author's e-mail: Fran.vandolah@noaa.gov
DOI: 10.5740/jaoacint.CS2011_27

Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
2	MLV06	California mussel (*Mytilus californianus*) from the U.S. west coast	x
3	MLV08	Green mussel (*Perna canaliculus*) from New Zealand	
4	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
5	MLV12	Blue mussel (*M. edulis*) east coast U.S., spiked with 200 µg/kg STX diHCl	
6	MLV14	Blue mussel (*M. edulis*) east coast U.S., spiked with 1200 µg/kg STX diHCl	
7	MLV16	Almeja clam (*Venus antique*) from Chile	
8	MLV01	Surf clam (*Spisula solidissima*) from the U.S. east coast	
9	MLV02	Chorito mussel (*M. chiliensis*) from Chile	
10	MLV04	Scallop (*Placopecten magellanicus*) from the U.S. east coast	
11	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
12	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
13	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
14	MLV13	Blue mussel (*M. edulis*) east coast U.S., spiked with 500 µg/kg STX diHCl	
15	MLV03	Chorito mussel (*M. chiliensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
17	MLV06	California mussel (*M. californianus*) from the U.S. west coast	x
18	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
19	MLV10	Butterclam (*Saxidomus gigantea*) from the U.S. west coast	
20	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
21	MLV15	Blue mussel (*M. edulis*) negative control, east coast U.S.	

[a] Sample number identifies the individual samples analyzed in the assays, with 1–7 analyzed in assay 1, 8–14 in assay 2, and 15–21 in assay 3. Sample identification (MLV for multilaboratory validation) describes the 16 unique samples, among which five were assayed as blind duplicates, to make a total of 21 samples. Blind duplicates, run in different assays, are identified by an "x."

metabolized in shellfish that accumulate them, making analytical determination of paralytic shellfish toxins (PST) in shellfish complex. The long-standing regulatory method for PST is the AOAC mouse bioassay (1; AOAC Method **959.08**), with a regulatory limit of 800 µg STX di HCl equiv./kg shellfish generally applied, but established at 400 µg STX diHCl equiv./kg in certain countries (e.g., the Philippines). However, at concentrations near the regulatory limit, the mouse bioassay can significantly underestimate PST in shellfish (2). This, in addition to increasing resistance to live animal testing in both the United States and the European Union (EU), has increased the need to develop alternative methods suitable for use in a high-throughput monitoring or regulatory setting.

In the past decade, several alternatives to the mouse bioassay have been developed. In the EU, the mouse bioassay remains the reference method for PST in shellfish, but European Commission (EC) Regulation 1664/2006 specifies that other internationally recognized methods may be used. Two HPLC methods, a precolumn oxidation method (3, 4; AOAC Method **2005.06**) and a postcolumn oxidation method (5; AOAC Method **2011.02**), have been approved by AOAC as *Official Methods*SM for PSP toxin analysis. The EC directive recognizes the precolumn oxidation HPLC method (AOAC Method **2005.06**) as an alternative to the mouse bioassay, but retains the mouse bioassay as the reference method in instances where results are challenged. HPLC methods separate and quantify individual STX congeners, which are then recombined according to their toxic equivalencies to yield a composite PST toxicity value. Although the HPLC methods perform well quantitatively, a high-throughput screening method capable of reporting toxic potency directly is still desirable for monitoring programs that often screen large numbers of negative samples. A qualitative lateral flow antibody test for PST with a reported detection limit of 400 µg STX equiv./kg was developed by Jellett Rapid Testing Ltd (Chester Basin, NS, Canada) and approved by the U.S. Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration as a screening method in specific circumstances. This method performed well in a comparison study with the mouse bioassay (6), but is not fully quantitative and has not been subjected to a full AOAC collaborative trial. To date, a suitable quantitative, high-throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The receptor binding assay (RBA) for PST is an excellent candidate for fulfilling the requirements of a high-throughput, quantitative assay that directly reports a composite toxic potency.

The basis of the RBA is the interaction between the toxins and their pharmacological target. All STX congeners bind to site 1 on the alpha subunit of the voltage-gated sodium channel with binding affinities proportional to their toxic potency (7). Therefore, an RBA can quantitatively measure the combined toxic potency of mixtures of STX congeners in a sample,

independent of the toxin congeners present (8). In the RBA for PST, tritiated STX ([^{3}H] STX) competes with unlabeled STX and/or its congeners for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H] STX is removed by filtration and receptor bound [^{3}H] STX quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unlabeled toxin present. A standard curve is generated using increasing concentrations of nonradiolabeled STX standard from 10^{-10} to 10^{-6} M STX. The concentration of toxin in samples is determined in reference to the standard curve.

The assay being tested in this collaborative trial is a modification of the method of Doucette et al. (9) to incorporate a 96-well microtiter plate format, which increases sample throughput and minimizes error by reducing sample handling and pipetting steps. This microplate PST RBA was evaluated in a single-laboratory validation (SLV) study (10), which established an interassay repeatability (RSD_r) of 17.7% and good correlation with the mouse bioassay and precolumn oxidation HPLC methods. The toxin concentrations in shellfish tested in the SLV study ranged from near to well above the regulatory limit (approximately 900–15 000 μg STX diHCl equiv./kg). The current study focuses more specifically on the performance of the RBA in the critical range of shellfish toxicities below, near, and slightly above the regulatory limit (approximately 150–2400 μg STX diHCl equiv./kg).

The results of the collaborative study suggest that the RBA for PST is a suitable high-throughput screen for PST in shellfish. Although HPLC methods offer quantitative information on congener composition of samples, often the desired information is composite toxic potency, which requires the summation of individual congeners, corrected for their individual toxic equivalencies. The RBA provides a single integrated toxic potency value that reflects activity of all known and potential unknown congeners present in the sample. Use of the microtiter plate format, in conjunction with microplate scintillation counting, provides the ability to screen multiple samples simultaneously in a total assay time of less than 3 h. The assay format described in the current study provides for the quantitative determination of composite PST toxicity in seven shellfish extracts per 96-well microplate, each run in triplicate at three dilutions, covering toxicity ranges of approximately 35–6000 μg STX diHCl equiv./kg. In a high-throughput assay setting, multiple plates can be set up simultaneously, so that six assay plates can easily be accommodated each day by a single analyst, for a throughput of 42 samples/day. This compares favorably to an estimated throughput of 20–25 samples a day by the precolumn HPLC method (B. Niedzwiadek, Health Canada, personal communication) or 30–35 by mouse bioassay (B. Suarez, University of Chile, personal communication).

Collaborative Study

The focus of this study was to assess the performance of the RBA to determine PST toxicity in samples of commercially important shellfish at a range of concentrations below and above the regulatory limit. Twenty-one shellfish homogenates were included in the study, which represented 16 unique samples (Table 1). The homogenates included 12 naturally contaminated shellfish samples of different species collected from several geographic regions: blue mussel (*M. edulis*) from the U.S. east and west coasts, California mussel (*M. californianus*) from the U.S. west coast, chorito mussel (*M. chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method **959.08**), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**); however, final concentrations in µg/kg and µg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well CVs for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_o), and calculated values for these samples in the well (nM), in the extract (µg STX equiv./mL), and in the shellfish tissue (µg STX equiv./kg). Participants were also asked to report all raw count data so that all results could be analyzed by the coordinating laboratory using identical software (GraphPad Prism 4.0) to assess whether systematic differences in quantification arose from using different curve-fitting software. All data were reported via e-mail to the coordinating laboratory.

The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in dilutions and calculations and for use of the prescribed curve-fitting model. Obvious errors were corrected and the participant laboratory was consulted for concurrence. The reviewed results were then used for evaluation in the collaborative study.

Statistical Evaluation of the Collaborative Study

For each sample analyzed, outliers were first determined using the Grubbs test at a probability value of 1% (www.graphpad.com), with no more than one outlier removed, so that valid data remained from a minimum of eight laboratories. The mean, S_R, and RSD_R, and HorRat values were then calculated for each sample. For blind duplicates, the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0, was used to further evaluate for outliers and determine S_r and RSD_r. GraphPad Prism was used to determine correlation among the RBA, mouse bioassay, and HPLC results.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration.

All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, or International Isotopes Clearinghouse, Leawood, KS).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—See *Appendix*.

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer*.—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution*.—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation*.—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \cdot EC50\ Hill\ slope)}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (GraphPad Software, Inc.) is recommended. For the microplate counter users, receptor

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	–0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

$$(\text{nM STX equiv}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCL equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration). Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

	Microplate column											
Microplate row	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

Results and Discussion

Sample Characterization

All shellfish homogenates (MLV1–16) were analyzed by HPLC using the precolumn oxidation method (AOAC Method **2005.06**) to determine toxin congener profiles and quantify total PST as μg STX diHCl equiv./kg prior to initiation of the study (Table 2). It is noteworthy that the clear majority of samples, irrespective of shellfish species and location, were dominated largely by STX and GTX2,3 whereas the N1-hydroxylated congeners NEO and GTX1,4 were virtually absent, except in blue mussel from the U.S. west coast. The most unusual profile was observed in green mussel, which was dominated by the weakly toxic N-sulfo-carbamoyl congeners C1,2. The samples were analyzed by the AOAC mouse bioassay (AOAC Method **959.08**) by three laboratories that routinely perform the mouse bioassay for regulatory purposes (Table 3). The mouse bioassay detection limit is approximately 400 μg STX diHCl equiv./kg (one laboratory reported values as low as 290 μg STX equiv./kg). Because the study design included samples that bracketed the lower regulatory limit of 400 μg STX diHCl equiv./kg, several samples were reported as being below the mouse bioassay detection limit. For samples in which all values were above the detection threshold, the between-laboratory RSD_R of the mouse bioassay was 18.9%.

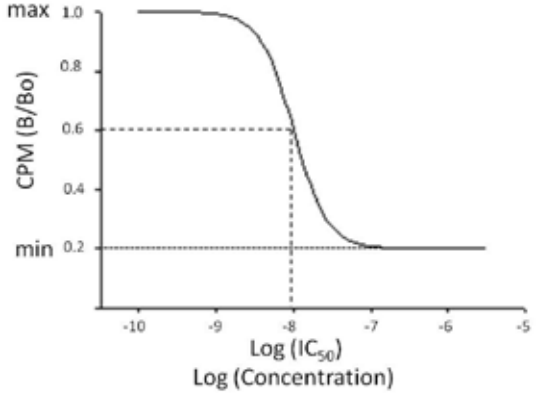


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

Data Reporting and Initial RBA Data Review

Nine of the 10 laboratories that received the study materials completed the study and reported results. All nine carried out the practice assay and reported results to the coordinating laboratory, which evaluated the results and provided feedback to the participating laboratories before initiating the full study. Following completion of the full study, the participating laboratories provided all raw and calculated data for each of

the three assays performed via e-mail to the coordinating laboratory. The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in sample dilutions and calculations, and for the use of the prescribed curve-fitting model. One laboratory used a sigmoidal curve-fitting model with the slope set to 1 (one-site binding curve in Prism), rather than the prescribed four-parameter logistic fit. In this case, the raw data were reanalyzed by the coordinating laboratory using the prescribed method. Obvious errors in calculation were corrected, such as accounting for the two-fold sample dilution resulting from the extraction process. In some cases, the participating laboratory carried out a fourth assay due to variability or inconsistency among dilutions for selected samples. In these cases, the value reported from the repeat (fourth) assay was used. One laboratory had consistent disagreement between the 1/50 and 1/200 dilutions when both fell within B/B_0 0.2–0.7. In all cases the 1/200 dilution overestimated almost two-fold relative to the 1/50 dilution, suggesting a systematic dilution error. In standard practice, these samples should be rerun. However, the instructions did not direct the participants to do so. Therefore, where there was corroborative evidence for the value reported by the 1/50 dilution, based on the 1/10 dilution, the 1/200 dilution was omitted. Where there was no basis on which to exclude the 1/200 value, an average value was calculated. This tended to result in an overestimate, and in two cases resulted in statistical outliers.

Overall Performance of the Method: Reproducibility

Table **2011.27A** summarizes the results obtained for 21 individual shellfish samples analyzed in three RBAs, determined by nine participating laboratories. Samples 1–7 were analyzed in the first assay, samples 8–14 in the second assay, and samples 15–21 in the third assay. Among these samples were five blind duplicates, treated here as individual unknown samples. One sample (marked by an footnote a in Table **2011.27A**) had a high variability in CPM between wells that was not attributable to any known cause, and was, therefore, omitted from analysis. Outliers identified by Grubbs test ($P < 0.01$) were excluded from the analysis (marked by footnote b in Table **2011.27A**). The overall RSD_R among all 21 independent samples was 33.2%, resulting in an average HorRat value of 2.0 (Table **2011.27A**). The HorRat values on individual samples ranged from 1.4 to 3.3, with a median value of 1.8. There was no apparent trend in reproducibility according to sample concentration or among shellfish species. If only the laboratories that are routine users of the RBA for PST (Laboratories 1–5) are included in the analysis, the average RSD_R is 23.1%, resulting in an average HorRat value of 1.4. Laboratory 9 tended to report the lowest values among the participating laboratories (14 of 21 samples), and although its individual sample values were not found to be statistical outliers, removing the results of this laboratory reduces all but one HorRat value (which remains unchanged), yielding an average HorRat value of 1.8 (range 1.0–2.8; Table **2011.27A**). Removal of any other single laboratory's results does not appreciably change the overall study performance. The reason for the systematically low values reported by Laboratory 9 is not clear, since the assay parameters fall well within those reported by the other laboratories. Given that assay parameters are within normal range, one possible source of systematic error could be incomplete extraction or pH adjustment of extracts, either of which would result in lower toxicity values.

A comparison of the RBA reproducibility with that of existing AOAC *Official Methods* is instructive. The AOAC collaborative study of the mouse bioassay (11), which entailed the analysis of seven samples representing three levels of STX-spiked shellfish by 11 participating laboratories, yielded a similar average RSD_R of 22%. More recent proficiency tests of the mouse bioassay performed in European regulatory laboratories report RSD_R of 2.3–38.3% on three samples run by eight laboratories (2) and RSD_R of 18.1–44.8% on two samples run by 20 laboratories (12). The mouse bioassay RSD_R values obtained in the current study ranged from 1.1 to 46.3% (average 19%) for three laboratories. The collaborative studies of the HPLC methods report reproducibility values for individual PST congeners, but do not report reproducibility of the composite toxic potency values. Collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an average RSD_R of 27.0% and HorRat value of 1.3 (range 0.8–2.1) for STX following C18 cleanup, but the reproducibility of other congeners varied considerably, with the maximum HorRat value (4.7), exceeding the highest HorRat value obtained by RBA (3.3).

Because composite toxic potency values were not reported in the studies of the HPLC methods, it is uncertain how this variability influences the composite toxic potency calculated from these methods. The average and ranges of HorRat values obtained for different congeners were: neoSTX–1.7 (range 1.2–2.5); dcSTX–1.1 (range 0.6–2.1); GTX1,4–1.9 (range 1.1–4.2), GTX2,3–1.4 (range 0.8–1.9); B1–1.1 (range 0.7–1.9); and C1,2–1.6 (range 0.9–4.5). Because of the variability obtained in neoSTX, GTX1,4, C3,4, and B2, AOAC Method **2005.06** calls for a second SPE-COOH cleanup of samples suspected of containing these congeners, after which reproducibility improved somewhat: neoSTX–1.8 (range 1.3–2.1); GTX1,4–1.3 (range 1.0–2.1); and C3,4–1.2 (range 0.8–1.8). The postcolumn oxidation HPLC method (AOAC Method **2011.02**) reported an average HorRat value of 0.6 for STX. In this method, neoSTX with an average HorRat of 1.9 (range 0.6–4.0) and GTX4 with an average HorRat of 1.6 (range 1.0–2.9) had reproducibility values that may affect the overall composite potency values. The maximum HorRat value (4.0) reported in this study also exceeded the maximum value reported in the RBA.

In summary, with the removal of Laboratory 9, the overall reproducibility of the RBA falls within the performance measures achieved by the established AOAC *Official Methods* for PST. The difference in reproducibility achieved by the laboratories that are routine users of the assay and participants who are not routine users of the method highlights the importance of training if this method were to be implemented in a regulatory setting.

Within-Laboratory Repeatability

Within-laboratory variability (RSD_r) was determined on five samples that were provided as blind duplicates. Participants were unaware that blind duplicates were included among the coded samples received. The duplicate samples were coded so that they were analyzed in separate assays, with different duplicate pairs falling into different assays (Table 1). One outlier was found among the results of the blind duplicates by Cochran's

test, $P < 0.025$ (Laboratory 7, sample MLV11) using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0. An overall RSD_r of 25.1% was observed, with an RSD_R of 32.9%, yielding a HorRat value of 2.0, similar to that of the overall study (Table **2011.27B**). When the performance of individual laboratories was evaluated separately, the average RSD_r was 22.2%, with individual laboratories varying from 11.8 to 34.4% (Table **2011.27C**). Routine users of the microplate format of the PST RBA (Laboratories 1–5) obtained an average RSD_r of 17.1%, which is similar to that obtained in the SLV study (10), and lower than that obtained by nonroutine users (Laboratories 6–9), which averaged 26.1% and ranged as high as 34.4%. The AOAC collaborative study of the mouse bioassay (11) did not report RSD_r; however, analysis of the data from that study using AOAC INTERNATIONAL's Interlaboratory Study Workbook for Blind Duplicates results in an average RSD_r of 16.5% for three STX-spiked samples. Proficiency testing of the mouse bioassay performed in eight French laboratories reported an average RSD_r of 8.3% on three samples (2). The analysis of blind duplicates in the collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an RSD_r of 15.2% for STX following SPE C18 cleanup and an average RSD_r of 16.4% across all congeners, which ranged from 6.0 to 31.7%. Following SPE–COOH cleanup, repeatability was similar, with RSD_r of 17.2% across all congeners. The intralaboratory repeatability values obtained in the postcolumn oxidation HPLC method (AOAC Method **2011.02**) averaged 6.4% for STX; most other congeners were similar, with neoSTX being the only congener that showed a somewhat higher RSD_r of 23.3%.

In summary, the within-laboratory repeatability of the RBA was found to be acceptable, with all but two laboratories achieving an RSD_r of 23.3% or less, and the routine users of the assay achieving an average RSD_r of 17.1%.

Spike Recovery

Three samples included in the study were homogenates of blue mussel spiked with STX diHCl at concentrations intended to bracket the regulatory limits of 800 μg STX equiv./kg used by most countries and 400 μg STX equiv./kg imposed in the Philippines. Nominal concentrations in the spiked samples were 200, 500, and 1200 μg STX equiv./kg. Also included in the study was the blue mussel homogenate to which the STX spikes had been added, which was determined to be negative for STX by the precolumn oxidation HPLC method. The negative control homogenate was reported as nondetectable by eight of nine laboratories. Recovery of spiked STX by the RBA was 84.4, 93.3, and 88.1%, respectively, for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, and yielded a slope of 0.87 and r^2 of 0.86 (Figure 2). In the current study, the mouse bioassay reported < detection limit, and 68.6 and 40.5% recovery for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels. The AOAC collaborative study of the mouse bioassay (11) reported recoveries of 62.3% at spike levels similar to those in the current study (equivalent to 1000 μg STX diHCl equiv./kg) but higher recoveries of 81.5 and 96.0% were achieved at higher spike levels equivalent to 4000 and 8000 μg STX diHCl equiv./kg.

The observed poor recovery in the mouse bioassay at concentrations near and below the regulatory limit has been observed in other studies (2), and has been attributed to a salt or protective effect of the shellfish matrix, which, for concentrations at or below the regulatory limit of 800 μg/kg, is injected undiluted into the mouse. The spike recovery observed in the precolumn HPLC method in this study is also somewhat low, with 54.0, 62, and 51.5% recovery at the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, respectively. The AOAC collaborative study of the precolumn HPLC method reported 74.4–76.8% at similar spike levels following SPE C18 cleanup and 63.7–68.2% following SPE-COOH cleanup (3, 4). In comparison, the postcolumn HPLC method reported 88–104% recovery of STX spiked at levels somewhat lower than the current study. The higher recovery of the RBA than the HPLC method in the current study may reflect the use of the 0.1 M HCl extraction method in the RBA as compared to the acetic acid extraction used in the HPLC methods.

We previously established in the SLV study that the RBA performs well with shellfish extracted using either method (10). In that study, the RBA reported slightly higher toxicity values for shellfish extracts made using the 0.1 M HCl method than the acetic acid extraction, yielding a correlation of 0.99 with a slope of 1.23 (10). The higher toxicity reported by the RBA in 0.1 M HCl extracts may reflect the hydrolysis of less toxic congeners to more toxic congeners.

Assay Parameters and Quality Metrics

Table **2011.27D** summarizes the assay parameters and quality metrics for all laboratories. Eight of nine laboratories used microplate scintillation counters. Laboratory 4 used the manual counting method in which the microplate well filters are punched out, using an eight-place punch system, into traditional 4 mL scintillation vials and counted. Its performance using the manual counting method (RSD_r 17.4%) was similar to or better than that of the laboratories using the microplate method, indicating that using the manual counting method does not affect the performance of the assay. Similarly, there was no apparent difference in assay parameters when the Packard Top Count (single detector) was used, compared to the Wallac Microbeta (coincidence detector), although the reference CPM values obtained on the Top Count generally were somewhat lower due to differences in counting efficiency inherent in the differences in detector geometry. Eight of nine laboratories used GraphPad Prism for curve-fitting, while only Laboratory 5 used Wallac MultiCalc software. Values reported by Laboratory 5 fell well within the range of values reported by laboratories using Prism.

All assays resulted in slopes between –0.8 and –1.2, as specified in the protocol. This specification reflects the fact that in a competitive binding assay for a ligand that interacts specifically at a single receptor site, the slope of the resulting standard curve should theoretically be 1.0. Although curve-fitting software packages often include a one-site binding curve that fixes the slope at 1.0, we specified in the protocol the use of the four-parameter logistic fit (also known as sigmoidal dose-response with variable slope), because it more readily identifies problems with the standard curve that may skew results. Laboratory 9 reported results using a one-site binding curve fit; in this case, the coordinating laboratory recalculated their raw data using the four-parameter logistic fit. The protocol also calls for RSD% < 30 on all standards. Most analysts did not experience variability problems in the standard wells. Infrequent high RSDs were most often associated with the well

Table 2. Congener profiles in shellfish homogenates included in the collaborative study[a]

Sample name	Species	STX	NEO	dcSTX	GTX1,4	GTX2,3	dcGTX2,3	B1	C1,2	C3,4	Total PSP	µg STX diHCl equiv./kg
MLV01	Surf clam	639.8		74.0		226.2	207.0				1146.9	894.3
MLV02	Almeja clam	298.3				1290.1		266.6			1855.0	802.1
MLV03	Chorito mussel	77.6				310.4					388.0	195.5
MLV04	Atlantic sea scallop	831.6				2785.6					3617.3	1890.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.9	1070.9
MLV10	Butter clam	1763.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	684.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHCl equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	890	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method **2006.02**, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10×SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0 <0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table **2011.27D**. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3×SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10×SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_R	RSD_R,%	Recovery, %
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

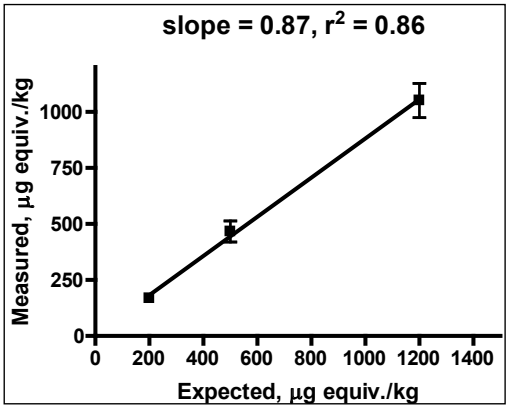


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table **2011.27E**). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8″ Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_o 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_o 0.2–0.7. When corrected for dilution, serial sample dilutions should yield similar quantification. The absence of linearity between sample dilutions indicates either error in dilution or sample matrix interference; however, at the minimum sample dilutions recommended in the proposed *Official Method*, matrix effects from shellfish homogenates have not been encountered (10). In the current study, the nonlinearity of dilutions experienced in several samples by Laboratory 8 was not observed by the other laboratories, suggesting a systematic sample dilution issue rather than a sample matrix problem. Although experienced in RBAs in general, Laboratory 8 had not previously run the microplate filtration format of the assay for PST.

Laboratory 9, which reported generally lower values than the other laboratories, although familiar with the assay, had not performed it in more than a year. The lower values reported do not appear to be associated with conduct of the assay, or scintillation conduct of the assay, or scintillation counting, since the assay metrics are well within the averages reported by the other laboratories. Insufficient boiling or pH adjustment of sample extracts are a possible explanation. These points identified by the study participants should be added to the critical steps identified in the SLV study (10) that can affect precision and accuracy of the assay results, including: (*1*) ensure that the water is strongly boiling during extraction; (*2*) carefully adjust pH of extracts; (*3*) ensure even distribution of the membrane preparation across the microplate by frequent vortex-mixing or pipetting before and during its addition to the plate; (*4*) the wells must clear within 2–5 s during filtration; (*5*) the wash buffer should be ice-cold to minimize the rate of toxin release from the receptor; and (*6*) following addition of scintillant to the wells, incubate a minimum of 30 min to ensure that the scintillant fully penetrates the filters before counting.

Recommendations

The collaborative study of the RBA for PST was completed by nine laboratories representing six countries. Collaborators quantified PST as a composite toxicity value reported in µg STX di HCl equiv./kg in a variety of shellfish species from different regions of the world, containing varied toxin congener profiles. The study included laboratories with extensive experience as well as others with little or no previous experience. The study also included both microplate and scintillation counters as end points, because either instrument type could potentially be used by test laboratories. The study demonstrates that the RBA yields adequate repeatability, reproducibility, and recovery for routine determination and monitoring of PST in shellfish. The greater precision attained by laboratories that received prior training on the RBA and routinely implement this assay suggests that

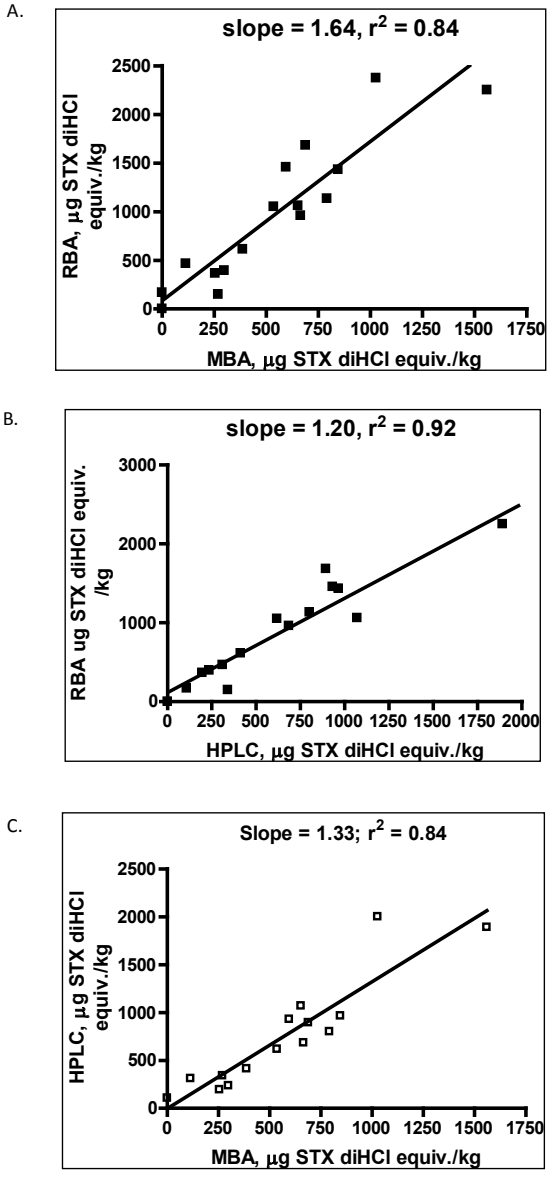


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

the overall interlaboratory reproducibility can be further improved. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the determination of PST in shellfish.

Acknowledgments

We would like to thank Laurie Bean (Maine Department of Marine Resources), Stacey DeGrasse (U.S. Food and Drug Administration), Patrick Holland (Cawthron Institute), Gregg Langlois (California Department of Health Services), Bob Lona (Washington State Department of Health), and Benjamin Suarez (University of Chile) for providing the shellfish material used in the study. We are grateful to Barbara Niedzwiadek and Dorothea Rawn (Health Canada) for HPLC analyses and to Laurie Bean and Darcie Couture (Maine Department of Marine Resources), Benjamin Saurez, and Supanoi Subsinserm (Thailand Department of Fisheries) for performing mouse bioassays. We are indebted to the following collaborators for their dedication and perseverance in carrying out the RBAs for this study:

Leanne Flewelling, Florida Wildlife and Marine Research Institute, St. Petersburg, FL

Stacey DeGrasse, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD

Patrick Holland and Paul McNabb, Cawthron Institute, Nelson, New Zealand

Gregg Langlois and Melisa Masuda, California Department of Public Health, Richmond, CA

Richard Lewis and Åsa Andersson, University of Queensland, Queensland, Australia

Christina Mikulski, NOAA Marine Biotoxins Program, Charleston, SC

Elvira Sombrito and Aileen DeLeon (Philippine Nuclear Research Institute, Manila, the Philippines

Kanitha Srisuksawad and Boonsom Porntepkasemsan, Thailand Nuclear Research Institute, Bangkok, Thailand

Aurelia Tubaro and Valeria Dell'Ovo, Dipartimento dei Materiali e Risorse Naturali, Trieste, Italy

This project was funded by the International Atomic Energy Agency Interregional Project INT7017 9001 and NOAA Marine Biotoxins Program Project 02E0017. FDA saxitoxin diHCl used for tritiation by IICH and STX diHCl reference standard (NIST RM 8642) were provided by Sherwood Hall, FDA Office of Seafoods.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) LeDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(3) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(5) *Official Methods of Analysis* (2011) AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.02**

(6) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425. http://dx.doi.org/10.1016/S0041-0101(02)00153-8

(7) Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(8) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667. http://dx.doi.org/10.1021/tx050277i

(9) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636. http://dx.doi.org/10.1016/S0041-0101(96)00189-4

(10) Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., & Rawn, D.F.K. (2009) *J. AOAC Int.* **92,** 1705–1713

(11) McFarran, E.F. (1959) *J. AOAC Int.* **42**, 263–271

(12) EU Reference Laboratory for Marine Biotoxins (2010) *Report*

on the EURLMB 2010 proficiency testing on saxitoxin group (PSP) toxins determination, Vigo, Spain

(13) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics,* http://www.eurachem.org/index.php/publications/guides/mv

(14) Kleivdahl, H., Kristiansen, S.-I., Nilson, M.A., Goksoyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(**a**) *Teflon/glass homogenizer*.—Motorized tapered Teflon pestle and glass tube, 15 mL.

(**b**) *Motorized tissue homogenizer*.—Polytron or small hand-held blender.

(**c**) *High-speed centrifuge and fixed angle rotor.*—Capable of 20 000 × *g* (rcf).

(**d**) *Centrifuge tubes*.—12–15 mL rated for >20 000 × *g* (rcf).

(**e**) *Plastic cryovials*.—2 mL.

(**f**) *Graduated beaker*.—300 or 500 mL.

(**g**) *Pipets*.—Disposable 5 and 10 mL.

(**h**) *Forceps*.

B. Reagents

(**a**) *20 Rat brains*.—Male, 6-week-old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA; http://hilltoplabs.com) or equivalent.

(**b**) *MOPS*.—pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G).

(**c**) *Choline chloride*.—100 mM (Sigma; Cat. No. C7527-500G).

(**d**) *Phenyl methylsulfonyl fluoride (PMSF)*.—Sigma; Cat. No. P7626.

(**e**) *Isopropanol*.

C. Procedure

(*1*) Prepare 1 L 100 mM MOPS buffer, pH 7.4, containing 100 mM choline chloride (detailed protocol in **E**, above) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol; dissolve 0.174 g PMSF in 10 mL isopropanol to make 100 mM stock. Aliquot and store at –20°C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh on the day of use.

(*2*) Remove medulla and cerebellum from each brain using forceps and discard. Place the cerebral cortex (*see* Figure 1) in a small amount of ice-cold buffer and place on ice.

(*3*) Place one cerebral cortex in 12.5 mL MOPS/choline Cl/PMSF, pH 7.4, in glass/teflon homogenizer (two brains in 25 mL buffer will fit into 30 mL homogenizer tube). Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes (more if necessary to homogenize brain; there should be no visible chunks remaining in the homogenate). Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

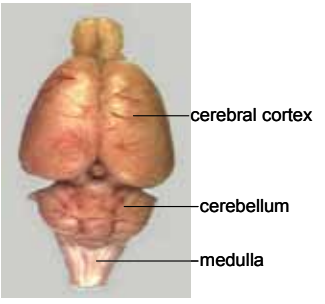


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20 000 × g for 15 min at 4°C.

(*5*) Aspirate the supernatant and resuspend the pellets in ice-cold MOPS/choline Cl/PMSF buffer, using an adequate amount (~5 mL) to fully resuspend the pellet (can use clean glass stir rod to break up pellet), not exceeding 10 mL per brain.

(*6*) Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume to 200 mL total (keep on ice).

(*7*) Keeping the beaker on ice, Polytron (or use a small hand-held blender at low speed) at 70% full speed for 20 s to obtain a consistent homogenate.

(*8*) Aliquot 2 mL/tube into cryovials. It is critical to keep the preparation well mixed while dispensing, e.g., prior to each aliquot to ensure equal allocation of protein/receptors to each vial. Keep cryotubes on ice.

(*9*) Freeze and store at –80°C. This preparation is stable for at least 6 months. Use a permanent marker to label the preparation date on the storage container.

D. Protein Assay

(**a**) Determine protein concentration of membrane preparation using Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) protein assay kit or equivalent protein assay (Thermo Fisher, Rockford, IL). The above protocol should yield 6–8 mg protein/mL of rat membrane preparation.

(**b**) Determine membrane dilution needed for the assay. The protein concentration in the daily working stock for the assay should be 1 mg/mL (this is diluted in the assay to yield 0.5 mg/mL in-assay concentration). Based on the protein concentration determined in the protein assay, determine the dilution needed to achieve 1 mg/mL. This is the dilution used in section **E**(**e**) above for all assays using this lot of membrane preparation. The protocol above typically yields a protein concentration that requires a dilution of 1/6–1/8. (Do not use less than 1/4 dilution or filtration wells may become clogged.) Protein concentration will need to be determined for each new batch of membrane preparation.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration. All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA, USA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, USA, or International Isotopes Clearinghouse, Leawood, KS, USA).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO, USA; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—*Appendix 1* [*J. AOAC Int*. (future issue)].

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA, USA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL, USA; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation*.—Prepare rat brain membrane preparation in bulk [*Appendix 1*; *J. AOAC Int*. (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Laboratory	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter*.—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)\,\text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	-0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

$$(\text{nM STX equiv.}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv. in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

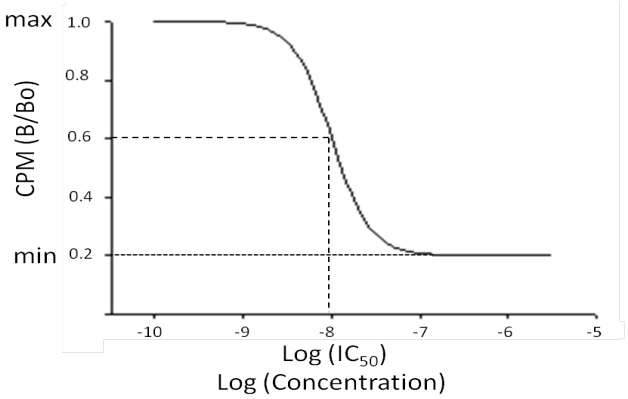


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int*. (future issue)

National Institute of Standards & Technology

Report of Investigation

Reference Material 8642

FDA Saxitoxin Dihydrochloride Solution

This Reference Material (RM) is intended for use in calibrating the mouse bioassay used in AOAC International Official Method 959.08 Paralytical Shellfish Poison [1] and for other similar uses. RM 8642 FDA Saxitoxin Dihydrochloride Solution was prepared by the U.S. Food and Drug Administration's (FDA's) Center for Food Safety and Applied Nutrition (CFSAN), where it was identified as Lot 089. The RM is saxitoxin dihydrochloride (CAS No. 35554-08-6) in a solution containing a hydrochloric acid concentration of 5 mmol/L in 20 % ethanol in water (volume fraction). A unit of RM 8642 consists of ten amber, borosilicate glass ampoules, each containing approximately 1.2 mL of solution.

Reference Mass Fraction Value: The reference value for the mass fraction of saxitoxin hydrochloride in solution in RM 8642, identified by FDA as lot 089, is 103 µg/g with an expanded uncertainty of 4 µg/g. Reference values are noncertified values that are estimates of the true value; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [2]. The reference mass fraction value is based on the gravimetric preparation of a stock solution and gravimetric dilution to produce the final material, and uncertainties associated with the associated weighings. The uncertainty is expressed as an expanded uncertainty, $U = ku_c$, at the 95 % level of confidence, $k = 2$, and includes a 2 % Type B purity uncertainty component as well as the gravimetric uncertainty [3]. Values are reported on an "as-received" basis in mass fraction units [4].

Expiration of Value Assignment: The reference value for RM 8642 is valid, within the measurement uncertainty specified, until **01 July 2013**, provided the RM is handled and stored in accordance with instructions given in this report (see "Instructions for Use"). This report is nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the value assignment before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The technical and support aspects involved in the preparation and issuance of this Reference Material were coordinated through K.E. Sharpless of the NIST Analytical Chemistry Division and M.P. Cronise of the NIST Measurement Services Division.

The solution was prepared and characterized by S. Hall of the Division of Bioanalytical Chemistry, Office of Regulatory Science, CFSAN, FDA.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Robert L. Watters, Jr., Chief
Measurement Services Division

Gaithersburg, MD 20899
Report Issue Date: 09 December 2010
Report Revision History on last page.

NOTICE AND WARNING TO USERS

Warning: For laboratory use only.

Storage: Unopened ampoules should be stored upright under normal laboratory conditions inside the original container supplied by NIST.

INSTRUCTIONS FOR USE

Gently tap the ampoule prior to opening to allow any solution in the tip to drain into the body of the ampoule.

Prepare a working solution as follows: On a top-loading balance, record the tare weight of an appropriate plastic bottle to 0.1 g or better. To the bottle, add approximately 100 mL water that has been acidified to pH 3 with hydrochloric acid. To minimize error due to evaporation, be prepared to immediately transfer the RM solution to this bottle after opening the ampoule. To open, hold the ampoule steady and grasp the stem at the metallic band with thumb and forefinger; **minimal** thumb pressure should be applied to the stem to snap it. Correctly done, the stem should break easily where pre-scored. Aspirate the RM solution into a dry, clean, disposable plastic syringe, 2 mL to 5 mL capacity, fitted with a suitable needle (such as 18 G × 1 ½″), weigh the syringe and its contents to 1 mg or better, and dispense the solution into the bottle of acidified water. Do not rinse the syringe. Reweigh the emptied syringe to determine the mass of RM solution transferred to the bottle. Add sufficient acidifed water (pH 3, HCl) to adjust the concentration to 1 µg/g. Weigh the bottle and its contents to determine the mass of solution prepared and the exact concentration of the working solution.

Because of the volatility of ethanol, the reference value is not applicable to material in ampoules that have been previously opened. The concentration of the working solution should be stable for more than one month if the solution is protected from evaporation. Dilution by mass is preferred but, if dilution by volume must be performed, the density of the solution is 0.971 g/mL and the concentration of this standard is 100 µg/mL with an expanded uncertainty of 4 µg/mL. This uncertainty is calculated as described above.

Source and Preparation of Material: Saxitoxin was extensively purified on three low-pressure preparative columns, each containing a different stationary phase. The saxitoxin was converted to the dihydrochloride form by passage through an ion exchange resin in the chloride form. Purity was assessed at FDA by proton nuclear magnetic resonance spectroscopy, combustion analysis, and optical rotation. RM 8642, identified by FDA as lot 089, was prepared by dissolving the saxitoxin dihydrochloride in a solution of hydrochloric acid (5 mmol/L) in 20 % ethanol in water (volume fraction).

REFERENCES

[1] AOAC International; *Official Methods of Analysis of AOAC International*, 18th Edition, Gaithersburg, MD (2005).

[2] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at http://ts.nist.gov/MeasurementServices/ReferenceMaterials/PUBLICATIONS.cfm (accessed Nov 2010).

[3] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Nov 2010); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://www.nist.gov/physlab/pubs/index.cfm (accessed Nov 2010).

[4] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at: http://ts.nist.gov/WeightsAndMeasures/Metric/mpo_pubs.cfm (accessed Nov 2010).

Report Revision History: 09 December 2010 (Extension of the period of validity; editorial changes.); 09 June 2010 (Original report date).

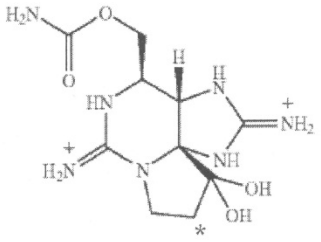
Users of this RM should ensure that the Report of Investigation in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

American
Radiolabeled
Chemicals, Inc.

101 ARC Dr.
St. Louis, MO 63146 U.S.A.
Ph. (314) 991-4545 or (800) 331-6661
Fax (314) 991-4692 or (800) 999-9925
Web: http://www.arc-inc.com
E-mail: arcinc@arc-inc.com

TECHNICAL DATA SHEET
ART 1301 Saxitoxin [11-^{3}H]

LOT SPECIFIC TECHNICAL DATA:



Lot number: 120814
Specific activity: estimated 20-30 Ci/mmol
Solvent: Methanol
Radioactive concentration: 0.05 mCi/ml
Molecular weight: 299.2

PACKAGING INFORMATION:

ART 1301 is packaged as a solution in methanol in a sealed ampoule. It is shipped in dry ice.

STABILITY AND STORAGE RECOMMENDATIONS:

A working stock of 1/50 dilution in methanol can be stored at 4°C. Long-term storage should be carried out at -80°C, based on the previous commercially available Saxitoxin [^{3}H], which was not stable at -20°C. The rate of degradation at -80°C is approximately 0.3-1% for the first month.

RADIOCHEMICAL AND CHEMICAL PURITY:

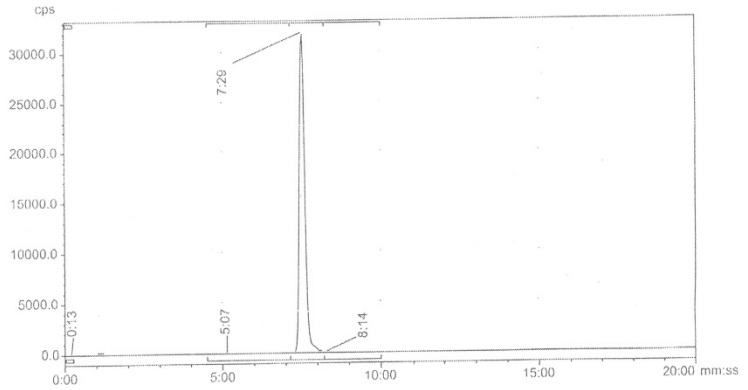
Radiochemical Purity: 99.56%

Column: Zorbax SB-AQ (250 x 3.0mm)

Mobile Phase: water:isopropanol: heptafluorobutyric acid (99.5 : 0.5 : 0.005)

Flow Rate: 0.5 ml/min

Detector: β-RAM [^{3}H]



Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (cps)	Area (Counts)	%ROI (%)
Bkg 1	0:02	0:16	0:13	183.0		
Region 1	4:31	7:08	5:07	158.0	1375.5	0.36
Region 2	7:08	8:11	7:29	32767.0	384313.4	99.56
Region 3	8:11	9:58	8:14	136.0	320.3	0.08
3 Peaks					386009.1	100.00

Proposal No. 13-116

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Florida Department of Agriculture and Consumer Services
Affiliation	Florida Department of Agriculture and Consumer Services
Address Line 1	1203 Governor's Square Blvd.
Address Line 2	Suite 501
City, State, Zip	Tallahassee, FL 32301
Phone	850-617-7600
Fax	850-617-7601
Email	Kimberly.Norgren@freshfromflorida.com
Proposal Subject	Shellfish Quarantine Guidance Document
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control Section IV. Guidance Documents Chapter II. Growing Areas .02 Guidance for Developing Marine Biotoxin Contingency Plans
Text of Proposal/ Requested Action	Model Ordinance Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control Section A. (4) describes agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting during marine Biotoxin closures under specific, controlled conditions. The State of Florida has successfully implemented such an agreement to address Neurotoxic Shellfish Poisoning (NSP) for over a decade. This pilot project, developed in consultation with FDA, has resulted in zero cases of NSP in commercially harvested shellfish from Florida waters. NSP may affect any Gulf or South Atlantic state and therefore Florida wishes to provide ISSC member states with a proven quarantine protocol template for incorporation into the Model Ordinance Section IV. Guidance Documents. Guidance Documents Chapter II. Growing Areas .02 Guidance for Developing Marine Biotoxin Contingency Plans. Text of the proposed guidance is as follows: Example Protocol for Quarantine Harvest of Shellfish from Aquaculture Leases During *Karenia brevis* Closures: A. Closure of an entire shellfish growing area due to *Karenia brevis* shall be in accordance with Model Ordinance Chapter IV. @.04 C. (1). B. When a shellfish growing area is closed due to *Karenia brevis*, the Authority may allow harvest of shellfish from selected aquaculture leases within a specific zone by authorized harvesters and subsequent controlled quarantine at a certified shucker packer or shellstock shipper. This option would not be available if any Authority collected water samples in the specific zone exceeded 200,000 cells per liter of *Karenia brevis*. Zone is defined as an

Authority delineated geographic area within a Conditionally Approved or Approved classified shellfish growing area.

Controlled quarantine conditions:

The Authority will determine and plot the specific zones. Certified processors possessing a valid shellfish processing plant certification license must have written permission from the Authority to engage in this activity. To be eligible for participation in the quarantine program, the certified processor must:

(1) Provide the Authority with written and signed agreements the processor has with shellfish aquaculture leaseholders who would be supplying the shellfish and;
(2) Notate on their application letter which FDA-approved marine Biotoxin laboratory will be used to conduct the approved mouse bioassay and;
(3) Provide the Authority with the cooler capacity, physical address and current certification number of the facility to be used for controlled quarantine of shellfish. All quarantine coolers must be non-mobile, secure from unauthorized access and equipped with warning signs in a language readily understood by all employees.

Participation in each week's quarantine program is only possible for certified processors who:

(1) Have written permission on file with the Authority and are on an Authority-controlled document listing current approved quarantine program processors and;

(2) Possess emailed permission granted by the Authority the day before harvest for that one specific quarantine and;

(3) Propose harvesting a quantity of shellfish that meets the Authority established minimum number but does not exceed the maximum allowed number of shellfish of one specific species for that day.

Under no circumstances may any approved processor participate in any quarantine until they possess written (emailed) documentation sent by the Authority before each specific quarantine event.

- The authorization email sent by the Authority shall explicitly state the permissible species that may be harvested by that approved processor.
- The Authority will notify the appropriate law enforcement entity in charge of patrol of shellfish growing areas with a list of participants in that specific day's harvest.
- Persons harvesting a species not authorized for that day's harvest will be subject to seizure of that harvest by the Authority. In addition, the Authority will immediately seize and destroy product which is improperly tagged, violates any National Shellfish Sanitation Program (NSSP) Model Ordinance regulations, state laws or is from non-authorized participants.
- Co-mingling of species is not allowed to make up an individual

lot.

Violation of the terms of this protocol may result in the termination of the participant's future eligibility in the quarantine program, as determined by the Authority.

Prior to being considered for participation in any specific quarantine event, approved processors shall be contacted by the Authority and asked to provide the name of the species they plan to harvest and the quantity they plan on harvesting. Quantities shall be described as approximate total number by species in addition to total number of baskets, containers, bags, etc. with specific weights (if applicable) for those baskets, containers, bags, etc.

Eligible processors should be aware that daily implementation of this program is contingent on marine Biotoxin laboratory availability as well as Authority staffing considerations given staff time necessary to fulfill the requirements of the program.

Regulatory considerations on behalf of the Authority and staffing considerations on behalf of the marine Biotoxin lab necessitate an Authority developed maximum number of samples that could be potentially tested on any given week.

The Authority may implement a lottery, random rotation or similar procedure to ensure a fair distribution of testing opportunities among the eligible processors. It is suggested that the Authority develop this procedure with industry involvement.

Once specific permission is received from the Authority, the processor:

(2) May receive properly tagged shellfish from eligible aquaculturists only as indicated in the Authority's authorization email;
(3) Must upon receipt of shellfish, separate and maintain the shellfish into specific lots [A Lot is defined as shellfish of one species from no more than one day's harvest from a specific zone within a shellfish growing area];
(4) Must place shellfish under proper controls and quarantine; Proper controls and quarantine are defined by bold, clear, warning signage signaling the properly tagged and segregated shellfish within the processor's cooler are under quarantine and must not be moved until Authority permission is obtained pending outcome of laboratory testing. The signage should be such that it is clear to anyone entering the cooler (including facility employees and/or regulatory inspectors) that the affected shellfish are under quarantine. Wrapping of the entire lot with a single bright red or yellow ribbon or equivalent attached to the bold warning sign will further reinforce the warning message.
(5) Must allow the Authority to take two (2) random samples [minimum of twenty (20) shellfish per each sample] from each lot and deliver to the approved laboratory for approved mouse bioassay;
(6) Must hold all shellfish in quarantine at the approved processor's

	certified facility until receiving official written test result notice from the Authority via email or fax that the shellfish are cleared for sale; (7) Must either return shellfish to aquaculture lease(s) in the zone(s) from where harvested if any sample in a lot is 20 Mouse Units / 100 grams or greater or destroy the shellfish, both activities of which must be witnessed and documented by the Authority; (8) Must cease this activity if any Authority collected red tide cell counts in the specific zone exceeds 200,000 cells per liter of *Karenia brevis*; and (9) Must document all of the requirements listed above in the approved facility HACCP plan. C. If cell counts in all water samples fall to 5,000 cells/L or less Karenia brevis in the entire area, the Authority will collect shellfish meat samples for toxicity testing and the entire Shellfish Harvesting Area will be reopened if results of all samples are <20 MU/100g. I _____(print name) have received a copy of this quarantine protocol and I agree to abide by all terms and conditions. I understand I am bound by the terms of this agreement during the period of time that I am processing shellfish from a shellfish growing area that is currently in the closed status due to Karenia brevis. _____ Signed Date
13. Public Health Significance	Closures of shellfish growing areas due to Neurotoxic Shellfish Poisoning (NSP) may occur at any time in the Gulf of Mexico and to a lesser degree, the Atlantic coast. Well established procedures for detecting and responding to *Karenia brevis* blooms have safeguarded public health. Clear early warning signs, a cell count action level with a high factor of safety and established sampling networks provide excellent public health protection. A very real impact of *Karenia brevis* blooms is the resulting long-term closures of shellfish growing areas and severe economic impact to commercial shellfish operations. Florida addressed this issue after studying years of water quality samples and mouse bioassay results from shellfish growing areas. Hydrodynamic studies linked to water samples obtained from fixed stations over an extended period of time established clear patterns in distribution of *Karenia brevis*. Working in conjunction with harmful algal bloom researchers, shellfish growing area managers, FDA and industry, Florida developed a NSP quarantine protocol that has resulted in the retention of a shellfish industry in one of the most severely impacted HAB regions of the Gulf while protecting public health as required by the Model Ordinance. An enormous amount of data has been generated and reviewed during the years this protocol has been used. Repeated mouse bioassay testing on shellfish exposed to different levels of *Karenia brevis* has provided Florida with sufficient data to refine the protocol into a powerful management tool. Florida's experience pre-quarantine protocol was unfortunate, as several fledgling businesses failed due to repeated NSP closures. It was this economic damage that spurred the aforementioned collaborative effort between leading edge HAB researchers, shellfish growing area managers, FDA and industry. If adopted, shellfish producing states impacted by *Karenia brevis* could reference this protocol in the Guidance Document and use it to effectively manage NSP closures.

Cost Information	The estimated cost for a full 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitative results would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. Further, the filter plates used in the RBA differ from ELISA plates in that all reagents are added to each well as needed rather than already being a component of the plate, making it more practical and cost-effective to analyze samples when there is less than a full plate.
Action by 2013 Task Force I	Recommended referral of Proposal 13-116 to an appropriate committee as determined by the Conference Chairman
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-116.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-116.
Action by 2015 Biotoxin Committee	Recommended adoption of Proposal 13-116 with substitute language as follows: (4) The plan may include agreements or memoranda of understanding, between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting in designated parts of a state growing area while other parts of the same ~~the~~ growing area are placed in the closed status. Such controlled harvesting shall be conducted with strict assurances of safety. In state growing areas or designated portions of state growing waters that are closed, the authority may allow for harvesting if an end product testing program is developed and, ~~such as by batch release of shellfish lots only after~~ samples of each lot are tested and found to be below the action levels specified in Section C. The program must include at a minimum: i. Establishment of appropriate pre-harvest screening levels; ii. Establishment of appropriate screening and end product testing methods; iii. Establishment of appropriate laboratories/analysts to conduct screening and end product testing methods; iv. Establishment of representative sampling plan for both i. and ii. above; and v. Other controls as necessary to ensure that shellstock are not released prior to meeting all requirements of the program. Should the above amended proposal be adopted by the conference, then the Biotoxin Committee should develop a Guidance Document that includes guidance for development of end-product testing programs to address biotoxins in closed state waters.
Action by 2015 Task Force I	Recommends adoption of Biotoxin Committee recommendation on Proposal 13-116.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-116.

Proposal No. **15-102**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Growing Area Classification Committee
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite #1
City, State, Zip	Columbia, SC 29223
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Using Male-Specific Coliphage as a Tool to Refine Determinations of the Size of the Areas to be Classified as Prohibited Adjacent to Each Outfall
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas
Text of Proposal/ Requested Action	@.01 Sanitary Survey. A. General. (1) The sanitary survey is the written evaluation report of all environmental factors, including actual and potential pollution sources, which have a bearing on water quality in a shellfish growing area. The sanitary survey shall include the data and results of: (a) A shoreline survey; (b) A survey of the ~~bacteriological~~ microbiological quality of the water and in growing areas adjacent to wastewater system discharges the State Shellfish Control Authority may utilize MSC results from analysis of shellfish meat samples and the analysis of the data will be included in the sanitary survey report; (c) An evaluation of the effect of any meteorological, hydrodynamic, and geographic characteristics on the growing area; (d) An analysis of the data from the shoreline survey, the bacteriological and the hydrodynamic, meteorological and geographic evaluations; (e) A determination of the appropriate growing area classification. B. Sanitary Survey Required… C. Sanitary Survey Performance. (5) On an annual basis, the sanitary survey shall be updated to reflect changes in the conditions in the growing area. The annual reevaluation shall include: (a) A field observation of the pollution sources which may include: (i) A drive-through survey; (ii) Observations made during sample collection; and (iii) Information from other sources.

(b) Review, at a minimum, of the past year's water quality sample results by adding the year's sample results to the data base collected in accordance with the requirements for the bacteriological standards and sample collection required in Section .02;

(c) Review of available inspection reports and effluent samples collected from pollution sources;

(d) Review of available performance standards for various types of discharges that impact the growing area; ~~and~~

(e) A brief report which documents the findings of the annual reevaluation~~.~~; and

(f) The SSCA may use MSC meat sampling data and/or MSC waste water sampling data in the annual reevaluation of (5) (b), (c), and (d) above to evaluate the viral contributions of the performance standards of waste water system discharge (WWSD) impacts on shellfish growing areas.

(g) If MSC meat and/or water data is being used, the SSCA shall conduct annual sample collection and analysis in determining performance standards.

D. Shoreline Survey Requirements…

@.02 ~~Bacteriological~~ Microbiological Standards.

Note: The NSSP allows for a growing area to be classified using either a total or fecal coliform standard. The NSSP further allows the application of either standard to different water bodies within the state. The NSSP also allows for two (2) sample collection strategies for the application of the total or fecal coliform standard: adverse pollution condition and systematic random sampling. The 1992 Task Force II recommended that this portion of the Ordinance be codified in two (2) ways: a total coliform strategy and a fecal coliform strategy so that the state may choose sampling plans on a growing area basis. Within each strategy, provisions would appear for use of both systematic and adverse pollution condition sample collection. The Ordinance has been recodified in this manner. For maximum flexibility, a state may wish to adopt the use of both standards and both sampling strategies for each standard. This codification represents the fecal coliform standards. Additionally, states may choose to use MSC sample data in conjunction with total or fecal coliform data to evaluate areas impacted by waste water system discharges.

A.. General. Either the total coliform or fecal coliform standard shall be applied to a growing area. The SSCA may utilize MSC data in conjunction with bacteriological data to evaluate waste water system discharge (WWSD) impacts on shellfish growing areas.

B. Water Sample Stations…

C. Exceptions…

D. Standards for the Approved Classification of Growing Areas in the Remote Status…

E. Standard for the Approved Classification of Growing Areas Affected by Point Sources…

F. Standard for the Approved Classification of Growing Areas Affected by

Nonpoint Sources…

G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration…

H. Standard for the Restricted Classification of Growing Areas Affected by Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration…

@.03 Growing Area Classification.

A. General…
   (1) Emergency Conditions…
   (2) Classification of All Growing Areas…
   (3) Boundaries…
   (4) Revision of Classifications…
   (5) Status of Growing Areas…
      (a) Open Status…
      (b) Closed Status...
      (c) Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:
         (i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water; or
         (ii) For emergency closures ~~(not applicable for conditional closures)~~ of harvest areas caused by the occurrence of raw untreated sewage discharged from a large community sewage collection system or wastewater treatment plant, the analytical sample results shall not exceed background levels or a level of fifty (50) male-specific coliphage per 100 grams from shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted; or
         (iii) The requirements for Biotoxins or conditional area management plans as established in Section .04 and Section .03, respectively, are met; and
         (iv) Supporting information is documented by a written record in the central file.
      (d) Inactive Status…

(e) Remote Status…
(f) Seasonally Remote/Approved Status…

B. Approved Classification…

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(1) Survey Required. The sanitary survey meets the following criteria:

(a) The area will be in the open status of the conditional classification for a reasonable period of time. The factors determining this period are known, are predictable, and are not so complex as to preclude a reasonable management approach;

(b) Each potential source of pollution that may adversely affect the growing area is evaluated;

(c) ~~Bacteriological~~ Microbiological water quality correlates with environmental conditions or other factors affecting the distribution of pollutants into the growing area~~.~~; and

(d) For SSCAs utilizing MSC meat sample data, this data correlates with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.

(2) Management Plan Required. For each growing area, a written management plan shall be developed and shall include:

(a) For management plans based on wastewater treatment plant function, performance standards that include:

(i) Peak effluent flow, average flow, and infiltration flow;
(ii) Microbiological quality of the effluent;
(iii) Physical and chemical quality of the effluent;
(iv) Conditions which cause plant failure;
(v) Plant or collection system bypasses;
(vi) Design, construction, and maintenance to minimize mechanical failure, or overloading;
(vii) Provisions for monitoring and inspecting the waste water treatment plant; and
(viii) Establishment of an area in the prohibited classification adjacent to a wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;

(b) For management plans based on pollution sources other than waste water treatment plants:

(i) Performance standards that reliably predict when criteria for conditional classification are met; and
(ii) Discussion and data supporting the performance standards.

(c) For management plans based on waste water system discharge ~~treatment plant~~ function or pollution sources other than waste water system discharge ~~treatment plants~~, criteria that reliably predict when an area that was placed in the closed status because of failure to comply

with its conditional management plan can be returned to the open status. The minimum criteria are:

(i) Performance standards of the plan are fully met;

(ii) Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;

(iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. The study may establish criteria for reopening based on coliform levels in the water; ~~and~~

(iv) For Conditional Management Plans based on waste water system discharge performance and for SSCAs utilizing MSC, sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. Analytical sample results shall not exceed background levels or a level of 50 MSC per 100 grams. The study may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed; and

(v) Shellstock feeding activity is sufficient to achieve ~~coliform~~ microbial reduction.

(d) For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;

(e) For management systems based on marine Biotoxins, the procedures and criteria that reliably determine when the growing area may be placed in the open status;

(f) Procedures for immediate notification to the Authority when performance standards or criteria are not met;

(g) Provisions for patrol to prevent illegal harvest; and

(h) Procedures to immediately place the growing area in the closed status in 24 hours or less when the criteria established in the management plan are not met.

(3) Reevaluation of Conditional Classification…

(4) Understanding of and Agreement With the Purpose of the Conditional Classification and Conditions of Its Management Plan by All Parties Involved…

(5) Conditional Area Types…

(6) Conditionally Approved Classification…

	(7) Conditionally Restricted Classification... D. Restricted Classification… E. Prohibited Classification. (1) Exception… (2) General… (3) Sanitary Survey… (4) Risk Assessment… (5) Wastewater Discharges. (a) An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance. (b) The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria: (i) The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent; The SSCA may utilize MSC wastewater sample data in the determination of the performance of the sewage treatment plant; (ii) The decay rate of the contaminants of public health significance in the wastewater discharged; (iii) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and (iv) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries. NOTE: All references in Section II. Model Ordinance Chapter IV. Shellstock Growing Areas will be changed to Waste Water System Discharge (WWSD).
13. Public Health Significance	Male-specific Coliphage (MSC) is a RNA virus of E. coli present in high numbers in raw sewage (on the order of 105 PFU/100gm). MSC is similarly resistant to chlorine disinfection as are norovirus and hepatitis A viruses, which are the viral pathogens of concern in sewage. MSC is a good surrogate or marker for these enteric viruses and is a powerful tool to assess the impact on a growing area of raw, partially treated and treated sewage on adjacent growing areas. A better assessment of the risk of viral contamination at a particular location in an adjacent growing area can be ascertained directly using MSC assays of the shellstock. Performing and evaluating dye studies on waste water treatment plant outfall discharges, although effective, is expensive and complicated. Difficulties assessing ex-filtration and leakage from the sewage collection system are well known. Few tools and less guidance are available to adequately assess the performance of a particular waste water treatment plant design and its operation with respect to virus removal. There are advantages of using this specialty viral indicator to assess the overall impact of a municipal wastewater treatment system on a particular growing area. The ISSC held an MSC meeting in Charlotte on August 18-19, 2014 to discuss the

	available MSC science and knowledge. A panel of MSC experts provided MSC information and consensus regarding usage of MSC in the NSSP. (Click here to view, download, or print the MSC meeting report).
14. Cost Information	The use of MSC is not a requirement; rather, it is an option for States to use, so there would be no cost to States who do not choose to use it. For States that do choose to use MSC, the cost is discussed in the ISSC MSC Meeting Report, August 18-19, 2014, where it states: The MSC assay for shellfish is relatively easy to perform and the cost is roughly equivalent to that of performing fecal coliform testing. The initial cost to prepare laboratory to perform analysis, depends on the lab, and may be approximately $8000 to $10,000, if additional equipment is needed. There may also be cost associated with sample collection.
Action by 2015 Task Force I	Recommended adoption of Proposal 15-102 as amended. @.01 Sanitary Survey. A. General. (1) The sanitary survey is the written evaluation report of all environmental factors, including actual and potential pollution sources, which have a bearing on water quality in a shellfish growing area. The sanitary survey shall include the data and results of: (a) A shoreline survey; (b) A survey of the microbiological quality of the water and in growing areas adjacent to wastewater system discharges the State Shellfish Control Authority may utilize MSC results from analysis of shellfish meat samples and the analysis of the data will be included in the sanitary survey report; (c) An evaluation of the effect of any meteorological, hydrodynamic, and geographic characteristics on the growing area; (d) An analysis of the data from the shoreline survey, the bacteriological and the hydrodynamic, meteorological and geographic evaluations; (e) A determination of the appropriate growing area classification. B. Sanitary Survey Required… C. Sanitary Survey Performance. (5) On an annual basis, the sanitary survey shall be updated to reflect changes in the conditions in the growing area. The annual reevaluation shall include: (a) A field observation of the pollution sources which may include: (i) A drive-through survey; (ii) Observations made during sample collection; and (iii) Information from other sources. (b) Review, at a minimum, of the past year's water quality sample results by adding the year's sample results to the data base collected in accordance with the requirements for the bacteriological standards and sample collection

required in Section .02;

(c) Review of available inspection reports and effluent samples collected from pollution sources;

(d) Review of available performance standards for various types of discharges that impact the growing area;

(e) A brief report which documents the findings of the annual reevaluation; and

(f) The SSCA may use MSC meat sampling data and/or MSC waste water sampling data in the annual reevaluation of (5) (b), (c), and (d) above to evaluate the viral contributions of the performance standards of waste water system discharge (WWSD) impacts on shellfish growing areas.

(g) If MSC meat and/or water data is being used, the SSCA shall conduct annual sample collection and analysis in determining performance standards.

D. Shoreline Survey Requirements…

@.02 Microbiological Standards.

Note: The NSSP allows for a growing area to be classified using either a total or fecal coliform standard. The NSSP further allows the application of either standard to different water bodies within the state. The NSSP also allows for two (2) sample collection strategies for the application of the total or fecal coliform standard: adverse pollution condition and systematic random sampling. The 1992 Task Force II recommended that this portion of the Ordinance be codified in two (2) ways: a total coliform strategy and a fecal coliform strategy so that the state may choose sampling plans on a growing area basis. Within each strategy, provisions would appear for use of both systematic and adverse pollution condition sample collection. The Ordinance has been recodified in this manner. For maximum flexibility, a state may wish to adopt the use of both standards and both sampling strategies for each standard. This codification represents the fecal coliform standards. Additionally, states may choose to use MSC sample data in conjunction with total or fecal coliform data to evaluate areas impacted by waste water system discharges.

A. General. Either the total coliform or fecal coliform standard shall be applied to a growing area. The SSCA may utilize MSC data in conjunction with bacteriological data to evaluate waste water system discharge (WWSD) impacts on shellfish growing areas.

B. Water Sample Stations…

C. Exceptions…

D. Standards for the Approved Classification of Growing Areas in the Remote Status…

E. Standard for the Approved Classification of Growing Areas Affected by Point Sources…

F. Standard for the Approved Classification of Growing Areas Affected by Nonpoint Sources…

G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration…

H. Standard for the Restricted Classification of Growing Areas Affected by Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration…

@.03 Growing Area Classification.

A. General…
(1) Emergency Conditions…
(2) Classification of All Growing Areas…
(3) Boundaries…
(4) Revision of Classifications…
(5) Status of Growing Areas…
(a) Open Status…
(b) Closed Status...
(c) Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:
(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels. In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water; or
(ii) For emergency closures of harvest areas caused by the occurrence of raw untreated sewage discharged from a large community sewage collection system or wastewater treatment plant, the analytical sample results shall not exceed ~~background levels or~~ a level of fifty (50) male-specific coliphage per 100 grams or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions from shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted~~;~~ or until the event is over and 21 day have passed; or
(iii) The requirements for Biotoxins or conditional area management plans as established in Section .04 and Section .03, respectively, are met; and
(iv) Supporting information is documented by a written record in the central file.
(d) Inactive Status…
(e) Remote Status…

(f) Seasonally Remote/Approved Status…

B. Approved Classification…

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(1) Survey Required. The sanitary survey meets the following criteria:

(a) The area will be in the open status of the conditional classification for a reasonable period of time. The factors determining this period are known, are predictable, and are not so complex as to preclude a reasonable management approach;

(b) Each potential source of pollution that may adversely affect the growing area is evaluated;

(c) Microbiological water quality correlates with environmental conditions or other factors affecting the distribution of pollutants into the growing area; and

(d) For SSCAs utilizing MSC meat sample data, this data correlates with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.

(2) Management Plan Required. For each growing area, a written management plan shall be developed and shall include:

(a) For management plans based on wastewater treatment plant function, performance standards that include:

(i) Peak effluent flow, average flow, and infiltration flow;

(ii) Microbiological quality of the effluent;

(iii) Physical and chemical quality of the effluent;

(iv) Conditions which cause plant failure;

(v) Plant or collection system bypasses;

(vi) Design, construction, and maintenance to minimize mechanical failure, or overloading;

(vii) Provisions for monitoring and inspecting the waste water treatment plant; and

(viii) Establishment of an area in the prohibited classification adjacent to a wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;

(b) For management plans based on pollution sources other than waste water treatment plants:

(i) Performance standards that reliably predict when criteria for conditional classification are met; and

(ii) Discussion and data supporting the performance standards.

(c) For management plans based on waste water system discharge function or pollution sources other than waste water system discharge, criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum criteria

are:

(i) Performance standards of the plan are fully met;

(ii) Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;

(iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. The study may establish criteria for reopening based on coliform levels in the water;

(iv) For Conditional Management Plans based on waste water system discharge performance and for SSCAs utilizing MSC, sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. Analytical sample results shall not exceed ~~background levels or~~ a level of 50 MSC per 100 grams or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions. These studies~~y~~ may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed; and

(v) Shellstock feeding activity is sufficient to achieve microbial reduction.

(d) For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;

(e) For management systems based on marine Biotoxins, the procedures and criteria that reliably determine when the growing area may be placed in the open status;

(f) Procedures for immediate notification to the Authority when performance standards or criteria are not met;

(g) Provisions for patrol to prevent illegal harvest; and

(h) Procedures to immediately place the growing area in the closed status in 24 hours or less when the criteria established in the management plan are not met.

(3) Reevaluation of Conditional Classification…

(4) Understanding of and Agreement With the Purpose of the Conditional Classification and Conditions of Its Management Plan by All Parties Involved…

	(5) Conditional Area Types… (6) Conditionally Approved Classification… (7) Conditionally Restricted Classification... D. Restricted Classification… E. Prohibited Classification. (1) Exception… (2) General… (3) Sanitary Survey… (4) Risk Assessment… (5) Wastewater Discharges. (a) An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance. (b) The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria: (i) The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent; The SSCA may utilize MSC wastewater sample data in the determination of the performance of the sewage treatment plant; (ii) The decay rate of the contaminants of public health significance in the wastewater discharged; (iii) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and (iv) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries. NOTE: All references in Section II. Model Ordinance Chapter IV. Shellstock Growing Areas will be changed toWaste Water System Discharge (WWSD).
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-102 with referral to an appropriate committee as determined by the Conference Chair to develop a draft guidance document which will be presented to the ISSC Executive Board at the 2016 spring meeting for interim approval.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-102.

Proposal No. **15-109**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Alison Sirois and Jackie Knue
Affiliation	Department of marine Resources and Alaska State Environmental Health Laboratory
Address Line 1	194 McKown Point Road and 5251 Dr. MLK Jr., Avenue
Address Line 2	
City, State, Zip	West Boothbay Harbor, ME 04575 and Anchorage, AK 99507
Phone	207-633-9401 and 907-375-8229
Fax	207-633-9579 and 907-929-7335
Email	Alison.Sirois@maine.gov and Jacqueline.Knue@alaska.gov
Proposal Subject	PSP HPLC-PCOX Species Expansion
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	4. Approved Limited Use Methods for Marine Biotoxin Testing PCOX This submission presents data to support the use of PCOX method for Quahogs (M. mercenaria and A. icelandica), Surf Clams (S. solidissima), Geoducks (P. generosa), Butter Clams (S. giganteus), Little Neck Clams (P. stamineais), and Razor Clams (S. patula) for regulatory paralytic shellfish toxin (PST) testing. Results of the 2009 Interstate Shellfish Sanitation Conference (ISSC) proposal 09-104 concluded the PCOX method approved for official use as a Type IV method; subsequently after single laboratory validation (SLV) and collaborative studies, ISSC proposal 13-309 accepted PCOX method as an AOAC official method of analysis (OMA) in 2013. Currently PCOX is an "Approved for Limited Use" method for mussel, clam, oyster and scallop. SLV work will be presented for quahogs, surf clams, geoducks, butter clams, little neck clams, and razor clams that demonstrates comparable performance characteristics for these species as with mussels, clams, oysters, and scallops using the PCOX method. The cost and challenges associated with maintaining both the MBA and PCOX methods for these species are high; differing laboratory skill sets are required and state laboratories have limited budgets and staff resources. Additionally, the recent shortage of the NIST saxitoxin standard used for MBA proficiencies is of concern if laboratories are expected to maintain MBA for verification purposes for these species. The requested action is being made and data presented for the purpose of inclusion of quahogs, surf clams, geoducks, butter clams, little neck clams, and razor clams as approved species (by addition to the footnote that includes mussels, clams, oysters, and scallops or as the ISSC deems appropriate) within the NSSP Guide Section IV Guidance Documents Chapter II. Growing Areas .11 Laboratory Tests Methods Table, Methods for Marine Biotoxin Testing with Biotoxin Type: Paralytic Shellfish Poisoning (PSP), Application: Growing Area Survey & Classification Sample Type: Shellfish And Application: Controlled Relaying Sample Type: Shellfish.
Public Health Significance	The PCOX method was developed to provide a rapid, high throughput chemical assay that would eliminate the need to sacrifice animals, AOAC mouse bioassay

	(MBA), for toxin detection. There is a worldwide move to replace assays that use live animals as test subjects. Laboratories currently using PCOX for regulatory PST testing have found that the lower detection limits of the PCOX method allow for better early warning therefore better management of PST closures and significantly improved public health decision-making. The addition of the proposed species will allow regulatory laboratories to move away from the costliness of maintaining MBA and eliminate the need to sacrifice animals as well as improve management of species specific closure decision–making.
Cost Information	Total consumable costs for the analysis is estimated at $10/sample. A chemistry laboratory will usually be equipped with an LC system and a post column reactor to carry out the analysis. Total capital costs for the instrumentation required for the analysis is approximately $120,000. Although the upfront investment for instrumentation is high, the removal of care, maintenance, and cost of mice quickly offsets this expenditure.
Action by 2015 Laboratory Method Review Committee	Recommended referral of Proposal 15-109 to an appropriate committee as determined by the Conference Chair for evaluation of data and until additional data are received.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Method Review Committee recommendation on Proposal 15-109.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-109.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-109.

Proposal No. **15-110**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Executive Board
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Laboratory Method for *Vibrio parahaemolyticus (V.p.)* Enumeration and Detection through MPN and Real-Time PCR
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	This method was developed by William A. Glover (Washington State Public Health Laboratories) and is being submitted by the ISSC Executive Board. The Executive Board granted interim approval to this method on March 13, 2015. The Executive Board is submitting this proposal to comply with Article V. Section 1. of the ISSC Constitution, Bylaws, and Procedures. Submitted by method developer William A. Glover (Washington State Public Health Laboratories) 5. Approved Methods for Vibrio Enumeration

	Vibrio Indicator Type:	Application: PHP Sample Type: Shucked
EIA[1]	*Vibrio vulnificus (V.v.)*	X
MPN[2]	*Vibrio vulnificus (V.v.)*	X
SYBR Green 1 QPCR-MPN[5]	*Vibrio vulnificus (V.v.)*	X
MPN[3]	*Vibrio parahaemolyticus (V.p.)*	X
PCR[4]	*Vibrio parahaemolyticus (V.p.)*	X
MPN and PCR[6]	*Vibrio parahaemolyticus (V.p.)*	X

Footnotes:

[1] EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, 1992.

[2] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA -alkaline phosphatase labeled gene probe (vvhA).

[3] MPN format with confirmation by biochemical analysis, gene probe methodology as listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.

[4] PCR methods as they are listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.

	[5]*Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123. [6]William A. Glover, II, Ph.D. D9ABMM), MT(ASCP) Food and Shellfish Bacteriology Laboratory (FSBL) at the Washington State Public Health Laboratories (WAPHL)
Public Health Significance	The purpose of this method is to provide laboratories supporting the NSSP the ability to rapidly quantify *Vibrio parahaemolyticus (V.p.)* from oysters using a high throughput real-time PCR protocol. The Food and Shellfish Bacteriology Laboratory (FSBL) at the Washington State Public Health Laboratories (WAPHL) tests on average over 200 oyster samples per year for *Vibrio parahaemolyticus (V.p.)*Culture based assays for the enumeration of *V.p.* take four days or longer and require the Kanagawa test (media based) to detect pathogenicity. Due to the large number of samples and need for accurate and timely results, the FSBL at the WAPHL has tested Pacific oysters (Crassostrea gigas) for (*V.p.*) using a MPN based real-time PCR assay for over 10 years. The real-time PCR assay utilized by the FSBL at the WAPHL has gone through redesigns and improvements by various scientists at the WAPHL based on new published literature, clinical *V.p.* case data, experiences in WA State over the course of a season or seasons, and requests from the Office of Shellfish & Water Protection for enhanced detection of pathogenic *V.p.* strains and additional surveillance capabilities. The real-time PCR assay redesigned and implemented in 2009 and utilized through the 2013 *V.p.* monitoring season (June – September) was designed to detect *V.p.* using the species-specific thermolabile hemolysin gene (tlh) and virulent *V.p.* using the thermostable direct hemolysin gene (tdh). This assay was designed for high throughput in a 384-well based format. Additionally, the tlh and tdh targets were redesigned yielding amplicons between 50-150 base pairs. This is optimal for real-time PCR and is known to produce consistent results1. Validation of the assay and concept of a “molecular MPN” was conducted using FERN guidelines and was compared to the FDA BAM method. This assay served as the backbone for which further improvements and redesigns were made in 2013.
Cost Information	
Action by 2015 Laboratory Method Review Committee	Recommended referral of Proposal 15-110 to an appropriate committee as determined by the Conference Chair to await completed SLV data.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-110.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-110.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-110.

STATE OF WASHINGTON
DEPARTMENT OF HEALTH
Public Health Laboratories
1610 NE 150th Street • Shoreline, Washington 98155-9701

ISSC Vibrio Method Submission

January 9, 2015

Method: *Vibrio parahaemolyticus* enumeration and detection through MPN and real-time PCR

Developer/Submitter Contact

William A. Glover II, Ph.D., D(ABMM), MT(ASCP)

Microbiology Supervisor: Reference, Enterics, Food & Parasitology Labs

Washington State Public Health Laboratories

1610 NE 150th St

Shoreline, WA 98155-7224

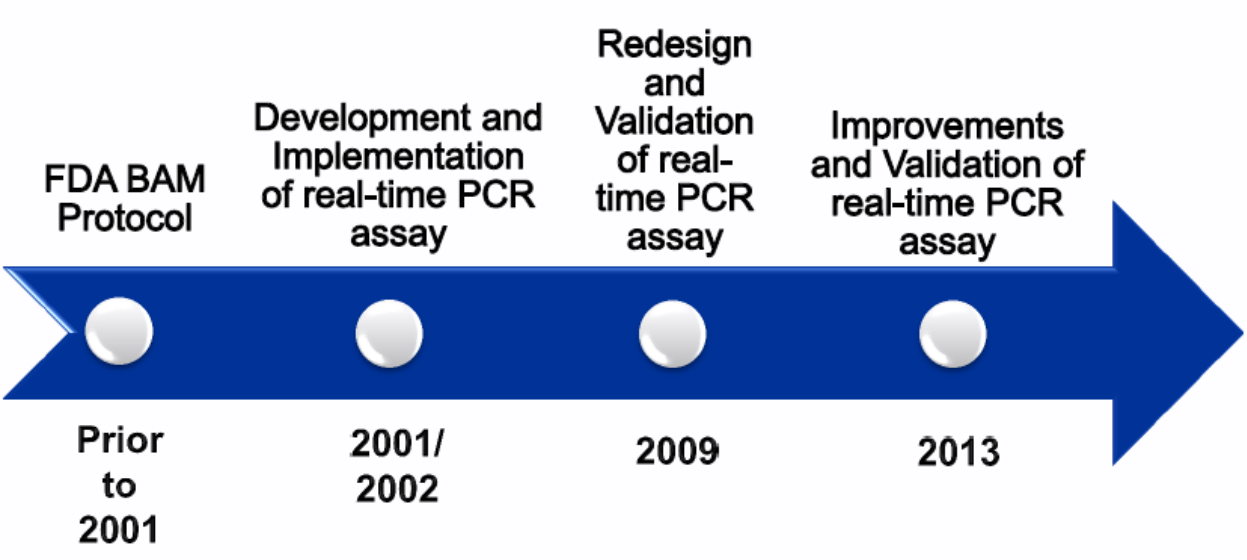
phone: 206 418-5422

email: william.glover@doh.wa.gov

Purpose of method for use in NSSP

The purpose of this method is to provide laboratories supporting the NSSP the ability to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) from oysters using a high throughput real-time PCR protocol.

History of Method



The Food and Shellfish Bacteriology Laboratory (FSBL) at the Washington State Public Health Laboratories (WAPHL) tests on average over 200 oyster samples per year for *Vibrio parahaemolyticus* (*Vp*). Culture based assays for the enumeration of *Vp* take four days or longer and require the Kanagawa test (media based) to detect pathogenicity. Due to the large number of samples and need for accurate and timely results, the FSBL at the WAPHL has tested Pacific oysters (*Crassostrea gigas*) for (*Vp*) using a MPN based real-time PCR assay for over 10 years. The real-time PCR assay utilized by the FSBL at the WAPHL has gone through redesigns and improvements by various scientists at the WAPHL based on new published literature, clinical *Vp* case data, experiences in WA State over the course of a season or seasons, and requests from the Office of Shellfish & Water Protection for enhanced detection of pathogenic *Vp* strains and additional surveillance capabilities.

The real-time PCR assay redesigned and implemented in 2009 and utilized through the 2013 *Vp* monitoring season (June – September) was designed to detect *Vp* using the species-specific thermolabile hemolysin gene (*tlh*) and virulent *Vp* using the thermostable direct hemolysin gene (*tdh*). This assay was designed for high throughput in a 384-well based format. Additionally, the *tlh* and *tdh* targets were redesigned yielding amplicons between 50-150 base pairs. This is optimal for real-time PCR and is known to produce consistent results[1]. Validation of the assay

and concept of a "molecular MPN" was conducted using FERN guidelines and was compared to the FDA BAM method. This assay served as the backbone for which further improvements and redesigns were made in 2013.

Current Assay Design & Improvements

(NOTE: Primer/ probe sequences are available upon request by the committee.)

This MPN based TaqMan probe real-time PCR assay was designed to provide quantitative results for total *Vibrio parahaemolyticus* (*Vp tlh*+), known pathogenic markers of *Vibrio parahaemolyticus* (*Vp tdh* + and *Vp trh*+), as well as some strains of potential pandemic Vibrio (*Vp* ORF8+). The assay is divided into 2 multiplex reactions utilizing FAM, VIC, NED dyes and run simultaneously under the same cycling parameters.

The assay continues using the *Vp* targets (*tlh* and *tdh)* designed for the 2009 assay. Multiple sequence alignments comparing target genes and existing published primers (i.e BAM, Nordstrom, Bej) were used along with ABI guidelines for primers and probes to aid in the design of the final primers and probes[2 3 4].

Final Amplicon Size ***tdh*-94bp | *tlh*-69bp**

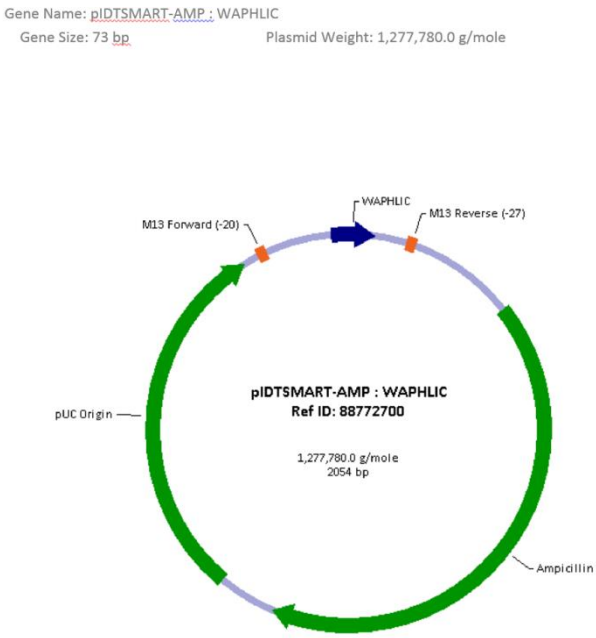
In addition, the current assay has two new *Vp* targets for the detection of the TDH-related hemolysin gene (*trh*) and ORF8 gene (*ORF8*). TRH has been shown to be an important virulence marker and is present in many of our clinical isolates [5 6]. Due to reported sequence variation in the *trh* gene, multiple sequence alignments comparing *trh* gene sequences were performed as well as the position of existing published primers [7 8]. Final *trh* primers for the assay consisted of: Degenerate forward primer redesigned based on the ward primer, two reverse primers, and the ward probe.

Strains of *Vibrio alginolyticus* have been described that contain a *trh* gene similar to that of *Vp* [9]. While these strains have not been previously described in WA State, during the course of the 2014 oyster season several strains of *Vibrio alginolyticus* were isolated from tubes that were negative for *tlh* using our assay. These strains were positive for urease and are currently under investigation to determine the presence of the *trh* gene. Please refer to our procedure manual for how we report these results.

ORF8 is present in pandemic *Vp* [10 11] and although not routinely detected in Washington State, pandemic *Vp* was responsible for 6 illnesses in 2011. To alert public health officials to the potential presence of pandemic *Vp* serovar O3:K6, primers were designed and are included in the assay.

Final Amplicon Size ***trh*-104bp | *ORF8*-150bp**

The new assay also includes an exogenous non-naturally occurring internal control plasmid (WA IC) which is added to the PCR mastermix for the detection of matrix inhibition or other assay failures. The 73bp fragment is synthesized and cloned into a pIDTSMART-AMP plasmid by Intergrated DNA Technologies (IDT). See figure below.



The final addition to the new assay is the *Vibrio vulnificus (Vv)* cytolysin-hemolysin A gene (*vvha*). Published primers and probe are utilized in this assay which yields a 79bp amplicon [12] . *Vibrio vulnificus* is an important addition after the detection of several *Vv* positive samples during the 2013 season. While it is present in the assay, it is currently for investigational use only and the reporting of *vvha* during the 2014 season was for surveillance purposes only. All *Vp* targets were validated and are included in the attached validation data. *Vv* data will be available to share with the committee after validation of the *vvha* gene target scheduled to be completed by the end of 2015.

Current Method Overview

Matrix- Pacific oyster (*Crassostrea gigas*)

- Oyster tissue enriched (18-24 hours) in Alkaline Peptone Water in a 3-tube MPN series

DNA Isolation- Roche© MagnaPure LC using Roche© DNA Isolation Kit III

Instrument Platform- Applied Biosystems™ ViiA™ 7 (384-well format)

Mastermix- Bioline SensiFAST™ Probe Hi-ROX Kit (BIO-82020)

Real-time PCR targets- 2 multiplex reactions run under the same cycling parameters

- Multiplex 1: *tlh*, *vvha,* internal control (IC)
- Multiplex 2: *tdh, trh, orf8*

Control Strains-

- Target organism one (1)- *V. parahaemolyticus* ATCC BAA-240 (contains *tlh, tdh,* and *orf8* genes)
- Target organism two (2)-*V. parahaemolyticus* WA4647 Washington State Department of Health Reference Lab clinical isolate (contains *tlh* and *trh* genes)

Final Background Summary

This method in total has been optimized and developed for environmental conditions, staffing, available instrumentation, and acquired experiences in WA State with *Vp* surveillance. Although this assay has not been published yet, there are plans to publish the method in total upon completion of the validation of the *Vv* portion of the assay which is planned to be completed in 2015. Every effort was made to validate the *Vp* portions of the assay according to ISSC guidelines, due to resource limitations some departures were required. We feel that the validation that was conducted takes into account the methodology and nuances of a molecular MPN. It is our hope that the committee finds this body of work acceptable and that we can work together to ensure adoption of the assay as an approved method for states who would like to use a molecular method for Vibrio surveillance in oysters. Due to the number of targets in this assay the data generated is substantial. We have summarized the data and organized it for review. All of the raw data files and primer/probe/internal control sequences are available to the committee upon request if needed. If the committee requests this information it will be submitted on CDs due to the size of the files. Many people have worked or have been a part of the development of this method in total and we are grateful for the opportunity to share this culmination of work with the ISSC.

References

[1] TaqMan® Universal PCR Master Mix Protocol

[2] Kaysner, C. A., and A. DePaola. "Bacteriological analytical manual chapter 9: Vibrio." 2009-06-06]. http://www. fda. gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalytical ManualBA M/ucm070830. htm (2004).

[3] Nordstrom, Jessica L., et al. "Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic Vibrio parahaemolyticus bacteria in oysters." Applied and Environmental Microbiology 73.18 (2007): 5840-5847.

[4] Bej, Asim K., et al. "Detection of total and hemolysin-producing Vibrio parahaemolyticus in shellfish using multiplex PCR amplification of tl, tdh and trh." Journal of Microbiological Methods 36.3 (1999): 215-225.

[5] Yeung, PS Marie, and Kathryn J. Boor. "Epidemiology, pathogenesis, and prevention of foodborne Vibrio parahaemolyticus infections." *Foodborne Pathogens & Disease* 1.2 (2004): 74-88.

[6] IIDA, TETSUYA, et al. "Evidence for genetic linkage between the ure and trh genes in Vibrio parahaemolyticus." *Journal of medical microbiology* 46.8 (1997): 639-645.

[7] Ward, Linda N., and Asim K. Bej. "Detection of Vibrio parahaemolyticus in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes." *Applied and environmental microbiology* 72.3 (2006): 2031-2042.

[8] Kishishita, Masamichi, et al. "Sequence variation in the thermostable direct hemolysin-related hemolysin (trh) gene of Vibrio parahaemolyticus." *Applied and environmental microbiology* 58.8 (1992): 2449-2457.

[9] González-Escalona, Narjol, George M. Blackstone, and Angelo DePaola. "Characterization of a Vibrio alginolyticus strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of Vibrio parahaemolyticus." Applied and environmental microbiology 72.12 (2006): 7925-7929.

[10] Myers, Michael L., Gitika Panicker, and Asim K. Bej. "PCR detection of a newly emerged pandemic Vibrio parahaemolyticus O3: K6 pathogen in pure cultures and seeded waters from the Gulf of Mexico." *Applied and environmental microbiology* 69.4 (2003): 2194-2200.

[11] Chen, Yuansha, et al. "Comparative genomic analysis of Vibrio parahaemolyticus: serotype conversion and virulence." *BMC genomics* 12.1 (2011): 294.

[12] Park, Jie Yeun, et al. "Multiplex Real-time Polymerase Chain Reaction Assays for Simultaneous Detection of< i> Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio vulnificus." *Osong Public Health and Research Perspectives* 4.3 (2013): 133-139.

2014 Validation Data

Linear Range, Limit of Detection, Sensitivity

Purpose
To determine the range where the results are proportional to the concentration of the analyte present in the sample. To identify the minimum concentration at which the analyte can be identified and determine at what concentration detection occurs with an acceptable level of precision and accuracy.

Method
In order to determine the Limit of Detection and Linear Range of the assay, 5 oyster matrices were spiked with *Vp ATCC BAA-240* and *Vp WA4647*. In addition, the dilution series for each target without oyster tissue was also tested.

For each matrix 18 APW tubes were seeded with 1g of oyster tissue. The tubes were enriched overnight at 35°C for 18-24 hours. A dilution series of the 16-20 hour Vibrio enrichment was made in APW, the enriched APW tubes were then spiked with each dilution of target organism. The spiking level for *Vp ATCC BAA-240* ranged from 3.55×10^{-1} to 3.55×10^{7} CFU/mL and for *Vp WA4647* it ranged from 7.64×10^{-1} to 7.64×10^{7} CFU/mL. The spiked APW tubes and dilutions were then immediately lysed. DNA extraction was performed and then PCR was run in duplicate. Instrument detection limit was determined without enrichment of the spiked APW tubes. The method detection limit was determined using APW tubes enriched after spiking for 18-24 hours.

Results
Vp ATCC BAA-240 was used to determine the Linear Range and Limit of Detection (LOD) for *tlh*, *tdh*, and *ORF8. WA4647* was used to determine the Linear Range and LOD for *trh.* The Internal Control (IC) Ct averaged around 25 cycles and was not affected by low or high *Vp* spiking levels.

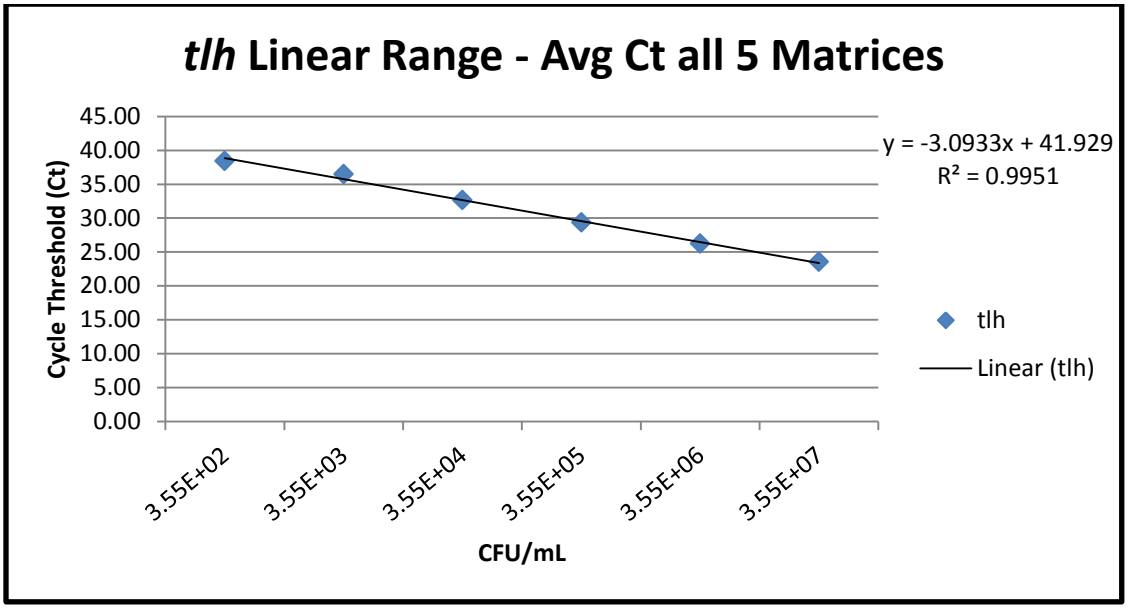
Instrument detection limit
The instrument detection limit for *tlh* and *tdh* is 100% at 3500 CFU/mL, 30% at 3.5 CFU/mL and 0% at .35 CFU/mL. *ORF8* has a detection limit of 90% at 3500 CFU/mL, 10% at 3.5 CFU/mL, and 0% at .35 CFU/mL. The instrument detection limit for *trh* is 100% at 7600 CFU/mL, 40% at 76 CFU/mL, and falls to 0% at 7.6 CFU/mL.

Method detection limit
The method detection limit for *tlh, tdh,* and *ORF8* is 100% at 5.8 cfu/g and falls to 33% at .58 cfu/g. The method detection limit for *trh* is 100% at .85 cfu/g and falls to 0% at .085 cfu/g.

Efficiency
See figures below
tlh = slope -3.09 = 111%
tdh = slope -3.23 = 104%
trh = slope -3.28 = 102%
ORF8 = slope -3.17 = 107%

Figure 1. Linear range graphs of (A) *tlh,(B) tdh, (C) trh,* and (D) *ORF8* along with slope equations used to calculate PCR efficiency and R^2 values. The data points were calculated by averaging all Ct values across all five spiked matrices.

A



B

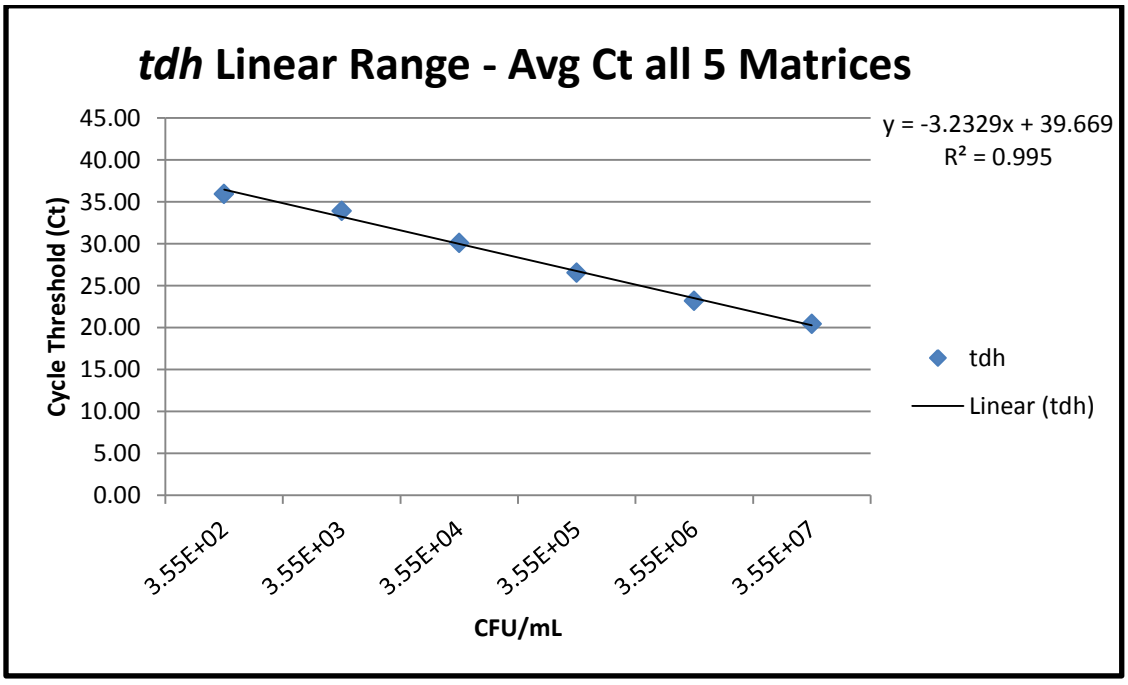
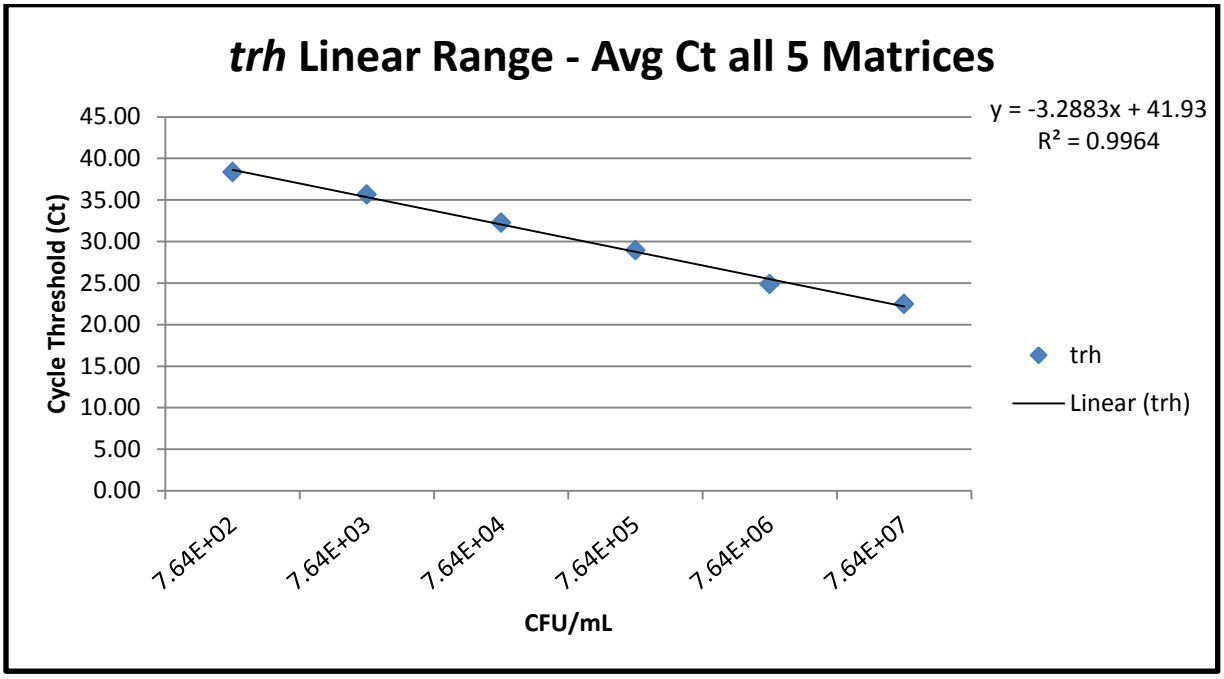
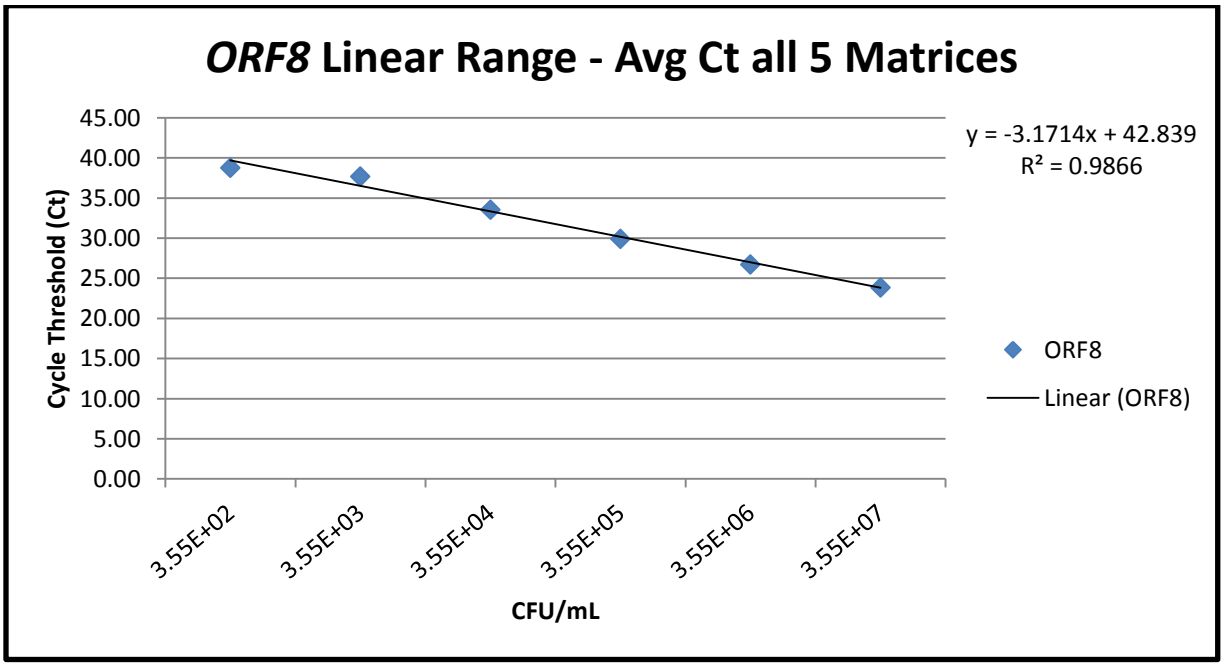


Figure 1. Continued

C

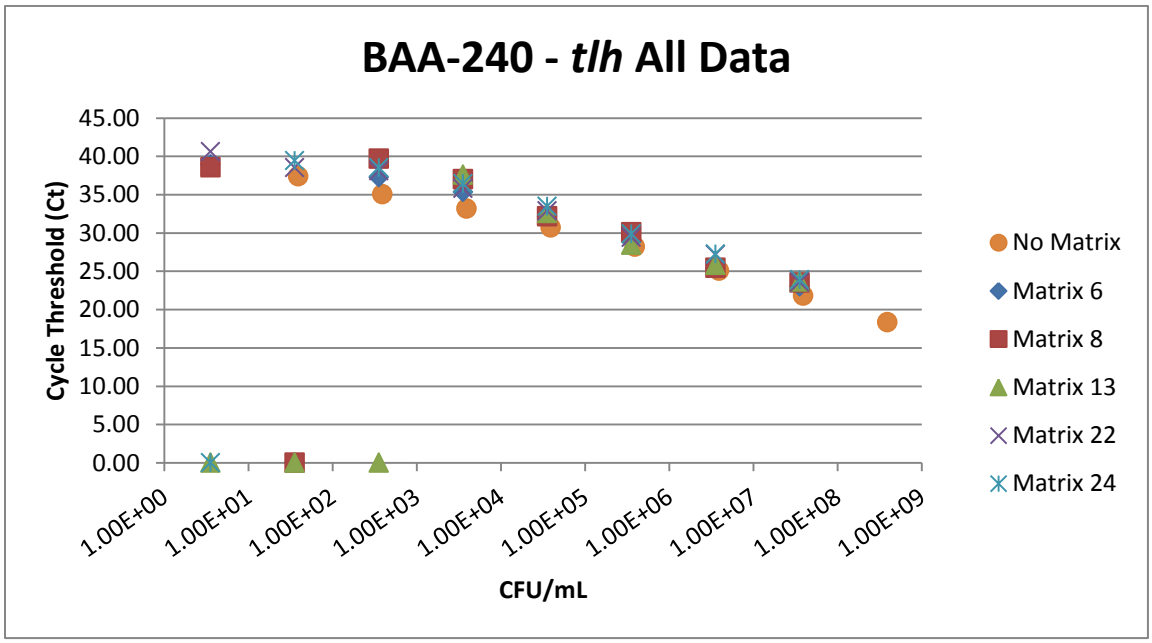


D



Figures 2. Graphs of the average Ct values for all data points within a matrix for (A) *tlh,(B) tdh,(C) trh,* and (D) *ORF8*.

A



B

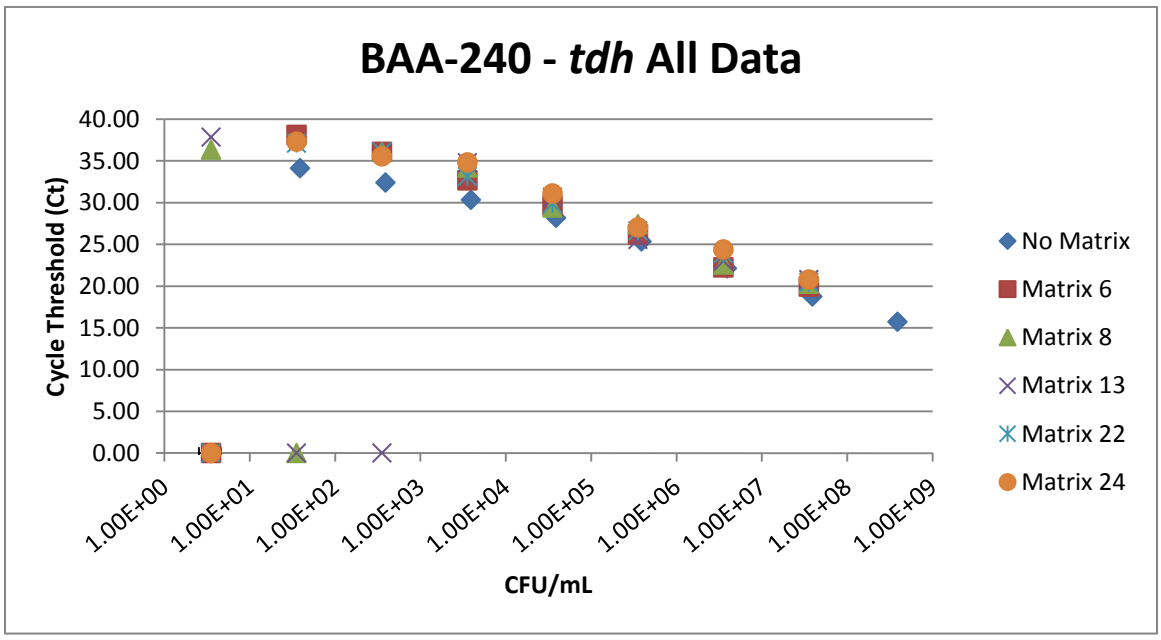
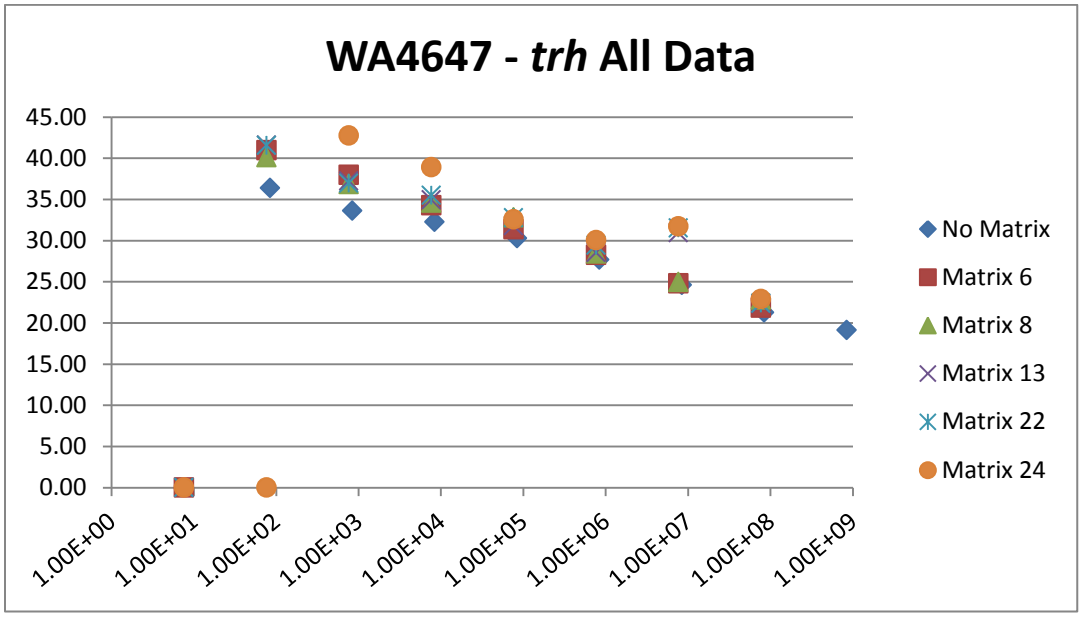


Figure 2. Continued

C



D

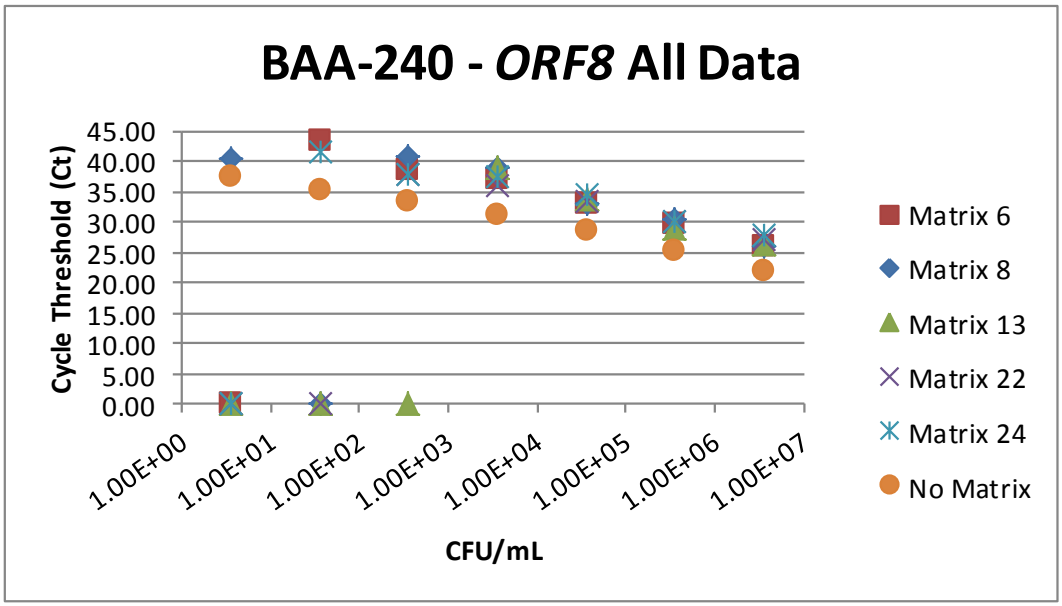
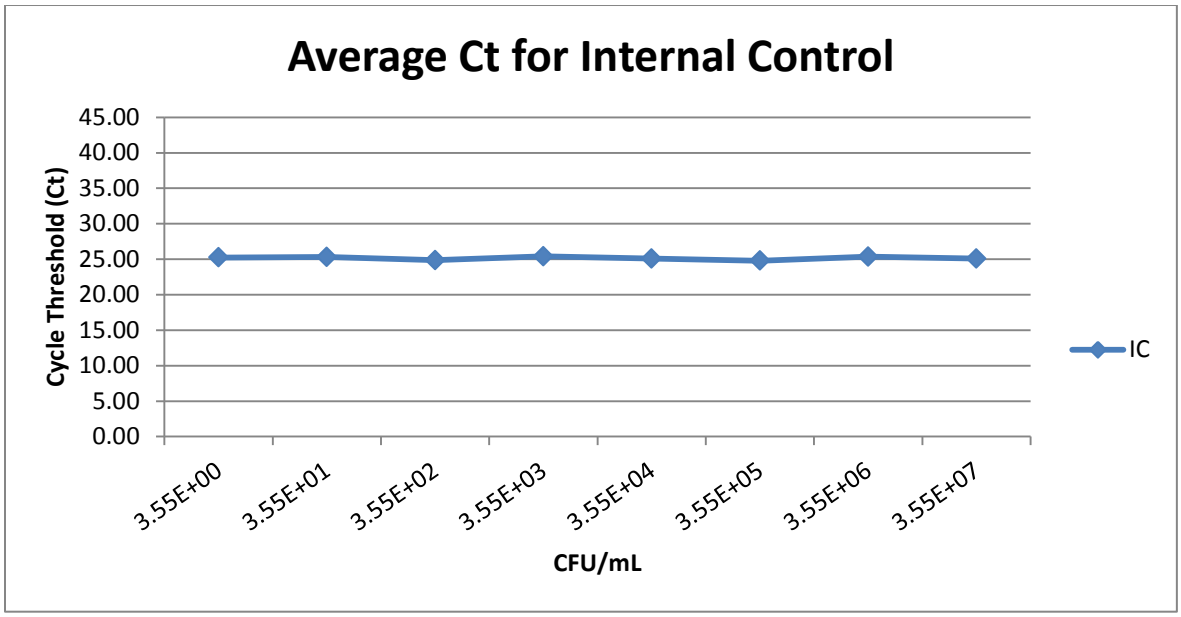


Figure 3. Graph of the average Ct for the Internal Control (IC) calculated from all matrices spiked with *Vp BAA-240.*



Inclusivity

Purpose
To assess the ability of the method to detect a wide range of target strains in various oyster tissues.

Method
Vibrio parahaemolyticus (*Vp*) strains were grown in APW for 18-24 hours. DNA was extracted using the MagNA Pure LC and was frozen at -20°C until PCR was performed in duplicate. The goal was at least 30 strains for each target. Confirmation was performed through a secondary assay. Since *tlh* is a species specific marker, a positive *Vp* identification through biochemicals or by ATCC paperwork was used as confirmation. For *tdh* some isolates were confirmed by NOAA and others were confirmed using *tdh* primers from Nordstrom et al. 2007. The *trh* marker was a little more problematic due to 2 variations of the *trh* gene. All isolates were confirmed using the the FDA BAM *trh* primer set and anything with discrepant results between our assay and the FDA assay we confirmed with a biochemical urease test. The ability of *Vp* to hydrolyze urea has been shown to be indicative of the presence of the *trh* gene (Iida et al paper 1997). The *ORF8* pandemic marker was confirmed using the primer set from Myers et al. 2003.

Results

See Table 1 for the breakdown of strains used and targets tested.

Primer / Probe Sensitivity
Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
tlh sensitivity = 78/78 = **100% *tlh* Sensitivity**
tdh sensitivity =38/38 = **100% *tdh* Sensitivity**
trh sensitivity = 33/33 = **100% *trh* Sensitivity**
ORF8 senstivity = 29/29 = **100% *ORF8* Sensitivity**

Table 1. Inclusivity panel with results by target. Targets not tested are blocked out.

Strain	Source	tlh	tdh	trh	orf8
F5828	CDC	+	+		+
F5835	CDC	+	+		+
F5847	CDC	+	+		+
F6820	CDC	+	+		+
F7630	CDC	+	+		+
F7635	CDC	+	+		+
F7636	CDC	+	+		+
F7680	CDC	+	+		+
F8701	CDC	+	+		+
F8949	CDC	+	+		+
F9083	CDC	+	+		+
K0071	CDC	+	+		+
K0456	CDC	+	-	+	-
K1533	CDC	+	+		+
17803	ATCC	+	-	+	
27519	ATCC	+	-		
27969	ATCC	+			
33844	ATCC	+	+		
33845	ATCC	+	+		
33846	ATCC	+	+		
33847	ATCC	+	+		
35117	ATCC	+	-		
35118	ATCC	+	+		
43996	ATCC	+	+		
49398	ATCC	+	-		
AP14861	NOAA - NWFSC	+	+		+
BAA-238	ATCC	+	+		+
BAA-239	ATCC	+	+		+
BAA-240	ATCC	+	+		+
BAA-241	ATCC	+	+		+
BAA-242	ATCC	+	+		+
BAC03255	NOAA - NWFSC	+	+		+
BE98-2029	NOAA - NWFSC	+	+		+
NWF 261	NOAA - NWFSC	+	-	-	-
NWF 512	NOAA - NWFSC	+	-	-	-
NWF 586	NOAA - NWFSC	+	+	-	+
NWF 605	NOAA - NWFSC	+	+	-	+
NWF 609	NOAA - NWFSC	+	+	-	+
NWF 735	NOAA - NWFSC	+	+		+
NWF 782	NOAA - NWFSC	+	+	-	+

Strain	Source	tlh	tdh	trh	orf8
NWF 797	NOAA - NWFSC	+	-	-	
NWF 800	NOAA - NWFSC	+	-		
NWF 805	NOAA - NWFSC	+	-		
NWF 843	NOAA - NWFSC	+	-		
NWF 846	NOAA - NWFSC	+	+	+	
NWF 864	NOAA - NWFSC	+	+		+
NWF 930	NOAA - NWFSC	+	+	-	+
VPHY145	NOAA - NWFSC	+	+		+
5412	WA PHL	+		+	
5419	WA PHL	+		+	
5423	WA PHL	+	+	+	
5424	WA PHL	+		+	
5425	WA PHL	+	+	+	
5426	WA PHL	+		+	
5429	WA PHL	+		+	
5430	WA PHL	+		+	
5434	WA PHL	+		+	
5436	WA PHL	+		+	
5437	WA PHL	+		+	
5442	WA PHL	+		+	
5444	WA PHL	+		+	
5454	WA PHL	+		+	
5456	WA PHL	+		+	
5463	WA PHL	+		+	
5468	WA PHL	+		+	
5469	WA PHL	+		+	
5470	WA PHL	+		+	
5471	WA PHL	+		+	
5473	WA PHL	+		+	
5474	WA PHL	+		+	
5475	WA PHL	+		+	
5487	WA PHL	+		+	
5488	WA PHL	+		+	
5492	WA PHL	+		+	
5501	WA PHL	+		+	
5508	WA PHL	+		+	
5518	WA PHL	+		+	
5519	WA PHL	+		+	
Total Confirmed Isolated		78	38	33	29

Exclusivity

Purpose
To demonstrate the ability of the method to distinguish the targeted analyte from other potentially cross-reactive non-target strains that could possibly contaminate shellfish.

Method
All organisms were inoculated into APW and incubated for 18-24 hours. DNA was extracted using the MagNA Pure LC and extract was frozen at -20°C until PCR was performed in duplicate.

The data used to determine the *G. hollisae* threshold cutoff was produced from two clinical strains of *G. hollisae* that were enriched in APW at 35°C for 18-24 hours in 15 replicates (total 30 tubes). Each replicate was processed as described above and PCR was performed.

Results
For the exclusivity panel 59 non-*Vibrio parahaemolyticus* organisms were used (Table 2). None of the exclusivity panel had detection of *tlh*, *trh*, or *ORF8*. *Grimontia hollisae*, ATCC 33564, did show amplification of *tdh*.

Primer / Probe Specificity
Specificity = (# of true negative/ (# of true negatives + # of false positives))
tlh specificity= 59/59= **100% *tlh* Specificity**
tdh specificity = 58/59= **98% *tdh* Specificity**
trh specificity = 59/59= **100% *trh* Specificity**
ORF8 specificity = 59/59= **100% *ORF8* Specificity**

Known limitations and interferences

The specificity of *tdh* is a known issue with *G. hollisae*, formerly known as *V. hollisae*, it has been shown to have greater than 93% homology with *Vp tdh* (Yamasaki et al. 1991).

Since the primers/probe for *tdh* have not been redesigned since the last method validation, the same principal to identify possible cross-reactivity with *G. hollisae* will be employed.
After analysis of the ΔRn values for all positive *Vp tdh* readings, we are able to identify a ΔRn threshold setting to exclude *G. hollisae tdh* amplification. An amplification curve will only be considered positive if it has a ΔRn above 104,000 in a linear analysis of the amplification plot (Figure 1). This threshold setting is 25% above the highest *G. hollisae tdh* amplification curve and 25% below the lowest *Vp tdh* amplification curve (Table 1).

Table 1. ΔRn values for *tdh* positive *Vp* and *tdh* positive *G. hollisae*.

Organism	Average ΔRn	Low ΔRn	High ΔRn
tdh Positive *Vp*	236,777	138,688	390,110
tdh Positive *G. hollisae*	64,769	39,711	83,130
tdh Negative wells	-12,766	-22,752	-3,331

Figure 1. Linear amplification plot showing true *Vp* positive *tdh* amplification and *G. hollisae tdh* amplification.

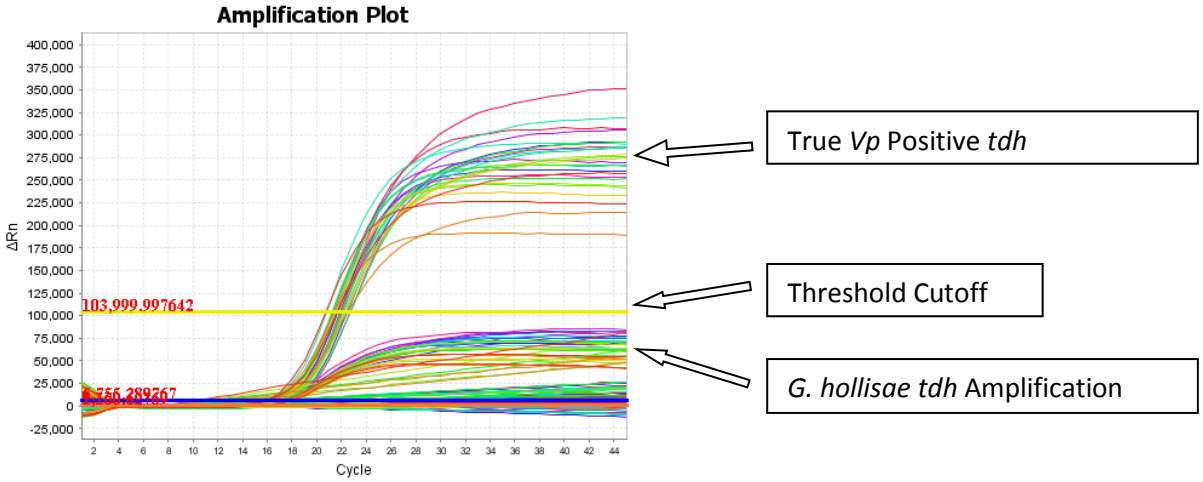


Table 2. All Exclusivity strains tested and results by target. All strains were purchased from ATCC, unless noted in parentheses.

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*
E. coli	25922	-	-	-	-
G. hollisae	33564	-	+	-	-
K. pneumoniae	33495	-	-	-	-
P. aeruginosa	33495	-	-	-	-
S. aureus	10145	-	-	-	-
S. sonnei	25925	-	-	-	-
S. typhimurium	9290	-	-	-	-
V. aestuarians	35048	-	-	-	-
V. alginolyticus	17749	-	-	-	-
V. algosus	14390	-	-	-	-
V. campbellii	25920	-	-	-	-
V. cholerae	39050	-	-	-	-
V. cinncinatiensis	35912	-	-	-	-
V. furnissii	33813	-	-	-	-
V. marinagilis	14398	-	-	-	-
V. marinofulvus	14395	-	-	-	-
V. marinovulgaris	14394	-	-	-	-
V. metschnikovii	700040	-	-	-	-
V. mimicus	33653	-	-	-	-
V. natriegens	14048	-	-	-	-
V. nereis	25917	-	-	-	-
V. nigripulchritudo	27043	-	-	-	-
V. ponticus	14391	-	-	-	-
V. proteolyticus	15338	-	-	-	-
V. splendidus	33789	-	-	-	-
V. tubiashii	19106	-	-	-	-

Table 1. Exclusivity Data (continued).

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*
V. vulnificus	06-2410 (CDC)	-	-	-	-
V. vulnificus	06-2450 (CDC)	-	-	-	-
V. vulnificus	07-2405 (CDC)	-	-	-	-
V. vulnificus	08-2468 (CDC)	-	-	-	-
V. vulnificus	08-2470 (CDC)	-	-	-	-
V. vulnificus	08-2472 (CDC)	-	-	-	-
V. vulnificus	08-2485 (CDC)	-	-	-	-
V. vulnificus	1831-81 (CDC)	-	-	-	-
V. vulnificus	2009V-1002 (CDC)	-	-	-	-
V. vulnificus	2009V-1055 (CDC)	-	-	-	-
V. vulnificus	2010V-1021 (CDC)	-	-	-	-
V. vulnificus	2011V-1065 (CDC)	-	-	-	-
V. vulnificus	2011V-1162 (CDC)	-	-	-	-
V. vulnificus	2012V-1089 (CDC)	-	-	-	-
V. vulnificus	2013V-1091 (CDC)	-	-	-	-
V. vulnificus	209V-1035 (CDC)	-	-	-	-
V. vulnificus	2409-05 (CDC)	-	-	-	-
V. vulnificus	2431-01 (CDC)	-	-	-	-
V. vulnificus	2431-04 (CDC)	-	-	-	-
V. vulnificus	2473-85 (CDC)	-	-	-	-
V. vulnificus	2492-88 (CDC)	-	-	-	-
V. vulnificus	2809-78 (CDC)	-	-	-	-
V. vulnificus	430-79 (CDC)	-	-	-	-
V. vulnificus	774-83 (CDC)	-	-	-	-
V. vulnificus	AM38622 (CDC)	-	-	-	-
V. vulnificus	AM38623 (CDC)	-	-	-	-
V. vulnificus	AM38625 (CDC)	-	-	-	-
V. vulnificus	27562	-	-	-	-
V. vulnificus	29307	-	-	-	-
A. trota	2013V-1197 (CDC)	-	-	-	-
A. punctata	N/A (CDC)	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-
Total Strains	59				

Accuracy/Trueness

Purpose
To assess the ability of the method to produce test results which are in agreement with the accepted reference value.

Method
Five (5) oyster samples were spiked with varying concentrations of *Vp ATCC BAA-240* and *Vp WA4647.* In PBS, serial dilutions were prepared from 18-24 hour growth of the target organisms. A 3-tube, 6-dilution MPN set was inoculated from the spiked oyster homogenate. The MPN set was incubated at 35°C for 18-24 hours, each individual tube was read for turbidity, DNA extraction was performed on the MagNA Pure LC, and PCR was performed on all selected tubes from positive turbidity readings.

For each matrix, 7 MPN sets (3 MPN replicates of *BAA-240*, 3 MPN replicates of *WA4647*, 1 MPN set uninoculated) were spiked with target organism. Concentrations of the target organism(s) were calculated by spread plate counts on T1N3 agar. Actual spiking levels achieved were 10^{-1}, 10^{0}, 10^{1}, 10^{2}, and 10^{4}.

Results
The actual spiking level was compared to the generated MPN value and the 95% confidence intervals associated with the MPN value. In all matrices the spiked CFU/g of *BAA-240* (*tlh*, *tdh*, and *ORF8*) and *WA4647* (*tlh*, *tdh*, *trh*) were evaluated to determine if the value was within the acceptable range of the lower and upper 95% confidence interval. For all matrices and targets there was only one instance of the generated MPN value falling outside the acceptable range. At the 10^0 spiking level (1.2 CFU/g), the *tdh* MPN of *BAA-240* Replicate 3 was 7.5 MPN/g. The positive pcr reaction was in the C1 tube and had a late tdh Ct of 37.15. During our routine surveillance a result such as this would be repeated twice to ensure it was not cross-contamination on the 384-well pcr plate. While this MPN is outside the confidence intervals the MPN generated for *tlh* was 4.3 MPN/g. Since the 4.3 MPN/g is acceptable and our reporting algorithm prevents reporting a positive *tdh* in the absence of *tlh* this data point was disregarded.

The matrix 24 blank did have late amplification of *tlh, tdh* and *ORF8* in the C2 tube, this matrix has previously been confirmed as negative and has been run several times resulting in no detection of *Vp*. We suspect a cross-contamination issue in the MagNA Pure cartridge; especially since it was *ORF8* positive it is very unlikely it was present in the oyster matrix prior to processing. This result was not used to adjust the overall MPN result of the spiked matrix 24 samples.

The FDA BAM 3-tube MPN chart has been copied as Figure 1.

Table 1. Tables display spiking level and calculated MPN along with the allowable range for associated MPN values. For each matrix 3 replicated were performed per target (R1, R2, and R3). (A) Matrix 6 – *ATCC BAA-240*, Matrix 7 – *WA4647* (B) Matrix 8 – *ATCC BAA-240* & *WA4647* (C) Matrix 13 - *ATCC BAA-240* & *WA4647* (D) Matrix 22 - *ATCC BAA-240* & *WA4647* (E) Matrix 24 - *ATCC BAA-240* & WA4647.

A

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^-1	ATCC BAA-240	0.17	tlh	0.36	0.92	0.36
			tdh	0.36	0.92	0.36
			orf8	0.36	0.92	0.36
	WA4647	0.63	tlh	0.36	0.92	0.92
			tdh	0.36	0.92	0.92
			trh	0.36	0.92	0.92

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
0.36	0.017	1.8
0.92	0.14	3.8

B

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^0	ATCC BAA-240	1.2	tlh	4.3	3.8	4.3
			tdh	4.3	3.8	7.5*
			orf8	2.3	3.8	4.3
	WA4647	6.3	tlh	9.3	23	21
			tdh	9.3	23	21
			trh	2.3	3.8	4.3

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
2.3	0.46	9.4
3.8	0.87	11
4.3	0.9	18
7.5	1.7	20
21	4	43
23	4.6	94

*late Ct

C

Spiking Level	Organism	Actual CFU/g	Target	MPN/g R1	MPN/g R2	MPN/g R3
10^1	ATCC BAA-240	14	tlh	43	43	43
			tdh	43	43	43
			orf8	43	43	43
	WA4647	84	tlh	75	93	120
			tdh	75	150	120
			trh	75	93	120

Allowable Range		
BAM MPN/g	Lower CI	Upper CI
43	9	180
75	17	200
93	18	420
120	37	420
150	37	420

Table 1. Continued

D				MPN/g			Allowable Range		
Spiking Level	Organism	Actual CFU/g	Target	R1	R2	R3	BAM MPN/g	Lower CI	Upper CI
10^2	ATCC BAA-240	460	tlh	430	240	930	240	42	1000
			tdh	430	240	2400	430	90	1800
			orf8	430	240	930	930	180	4200
	WA4647	630	tlh	430	930	430	2400	420	10000
			tdh	430	930	430			
			trh	430	930	430			

E				MPN/g			Allowable Range		
Spiking Level	Organism	Actual CFU/g	Target	R1	R2	R3	BAM MPN/g	Lower CI	Upper CI
10^4	ATCC BAA-240	58,000	tlh	46,000	46,000	46,000	24,000	4,200	100,000
			tdh	46,000	46,000	46,000	46,000	9,000	200,000
			orf8	46,000	46,000	46,000			
	WA4647	58,000	tlh	24,000	24,000	46,000			
			tdh	24,000	24,000	46,000			
			trh	24,000	24,000	46,000			

Figure 1. FDA BAM 3-tube MPN chart.

Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	–	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	–

Precision/Recovery

Purpose

To assess the closeness of agreement between independent rest results obtained under stipulated conditions and the percentage of an analyte recovered following sample analysis.

Method

The data generated in the accuracy/trueness phase was used to evaluate precision/recovery. The variation in the total number of tubes tested is determined by the turbidity in the MPN series and can vary between replicates without affecting overall results.

Results

The precision of the assay is difficult to demonstrate due to the nature of enumerating through MPN dilution sets. However, by showing the total number of positive tubes/target the precision of the assay is demonstrated between all targets tested at a specific spiking level.

Table 1. MPN results for all matrices along with the total number of positive tubes in the MPN dilution series/total number of tubes tested (determined by turbidity). (A) Matrix 6 – *ATCC BAA-240*, Matrix 7 – *WA4647* (B) Matrix 8 – *ATCC BAA-240* & *WA4647* (C) Matrix 13 - *ATCC BAA-240* & *WA4647* (D) Matrix 22 - *ATCC BAA-240* & *WA4647* (E) Matrix 24 - *ATCC BAA-240* & WA4647.

A

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#6&7				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^-1	ATCC BAA-240	0.17	tlh	0.36	1/12	0.92	2/12	0.36	1/12
			tdh	0.36	1/12	0.92	2/12	0.36	1/12
			orf8	0.36	1/12	0.92	2/12	0.36	1/12
	WA4647	0.63	tlh	0.36	1/12	0.92	2/15	0.92	2/12
			tdh	0.36	1/12	0.92	2/15	0.92	2/12
			trh	0.36	1/12	0.92	2/15	0.92	2/12

B

Spiking Level	Organism	Actual CFU/g	Target	Data					
				R1		R2		R3	
M#8				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^0	ATCC BAA-240	1.2	tlh	4.3	4/12	3.8	4/15	4.3	4/12
			tdh	4.3	4/12	3.8	4/15	7.5*	5/12
			orf8	2.3	3/12	3.8	4/15	4.3	4/12
	WA4647	6.3	tlh	9.3	5/12	23	6/12	21	7/12
			tdh	9.3	5/12	23	6/12	21	7/12
			trh	2.3	5/12	3.8	6/12	4.3	7/12

*late Ct – see Accuracy/Trueness data for explanation.

Table 1. Continued

C				Data					
Spiking Level	Organism	Actual CFU/g	Target	R1		R2		R3	
M#13				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^1	ATCC BAA-240	14	tlh	43	7/12	43	7/12	43	7/12
			tdh	43	7/12	43	7/12	43	7/12
			orf8	43	7/12	43	7/12	43	7/12
	WA4647	84	tlh	75	8/12	93	8/12	120	9/15
			tdh	75	8/12	150	9/12	120	9/15
			trh	75	8/12	93	8/12	120	9/15

D				Data					
Spiking Level	Organism	Actual CFU/g	Target	R1		R2		R3	
M#22				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^2	ATCC BAA-240	460	tlh	430	10/15	240	9/12	930	11/15
			tdh	430	10/15	240	9/12	2400	12/15
			orf8	430	10/15	240	9/12	930	11/15
	WA4647	630	tlh	430	10/15	930	11/15	430	10/15
			tdh	430	10/15	930	11/15	430	10/15
			trh	430	10/15	930	11/15	430	10/15

E				Data					
Spiking Level	Organism	Actual CFU/g	Target	R1		R2		R3	
M#24				MPN/g	tubes	MPN/g	tubes	MPN/g	tubes
10^4	ATCC BAA-240	58000	tlh	46000	16/18	46000	16/18	46000	16/18
			tdh	46000	16/18	46000	16/18	46000	16/18
			orf8	46000	16/18	46000	16/18	46000	16/18
	WA4647	58000	tlh	24000	15/18	24000	15/18	46000	16/18
			tdh	24000	15/18	24000	15/18	46000	16/18
			trh	24000	15/18	24000	15/18	46000	16/18

Specificity/Competitor Strain

Purpose
To assess the ability of the method to measure only what it is intended to measure.

Method
Two (2) oyster samples, both from Matrix #13, were spiked with 10^1 CFU/g of *Vp ATCC BAA-240* and *Vp WA4647.* The target organisms were prepared in PBS serial dilutions from 18-24 hour growth. Along with the target organisms the matrices were also spiked with *V. alginolyticus* at one log higher (10^2). A 3-tube, 6-dilution MPN set was inoculated from the spiked oyster homogenate. The MPN set was incubated at 35°C for 18-24 hours, each individual tube was read for turbidity, DNA extraction was performed on the MagNA Pure LC, and PCR was performed on all selected tubes from turbidity readings.

Concentrations of the target organism(s) were calculated by spread plate counts on T1N3 agar.

Results

There was no issue detecting the target organism in the presence of *V. alginolyticus*. All MPN values generated were within the acceptable ranges.

Table 1. *Vp WA4647* spiked at 84 cfu/g (2520 cells); Competitor *V. alginolyticus* spiked at 380 cfu/g (11400 cells).

	tlh	MPN/g	Lower CI	Upper CI	**tdh**	MPN/g	Lower CI	Upper CI	**trh**	MPN/g	Lower CI	Upper CI
R1	**3-2-0 x10**	**93**	18	420	**3-2-0 x10**	**93**	18	420	**3-2-0 x10**	**93**	18	420
R2	**3-2-0 x10**	**93**	18	420	**3-2-1* x10**	**150**	37	420	**3-2-0 x10**	**93**	18	420

*Late Ct

Table 2. *Vp ATCC BAA-240* spiked at 14 cfu/g (420 cells); Competitor *V. alginolyticus* spiked at 130 cfu/g (3900 cells)

	tlh	MPN/g	Lower CI	Upper CI	**tdh**	MPN/g	Lower CI	Upper CI	**orf8**	MPN/g	Lower CI	Upper CI
R1	**3-0-0 x10**	**23**	**4.6**	**94**	**3-0-0 x10**	**23**	**4.6**	**94**	**3-0-0 x10**	**23**	**4.6**	**94**
R2	**3-1-0 x10**	**43**	**9**	**180**	**3-1-0 x10**	**43**	**9**	**180**	**3-1-0 x10**	**43**	**9**	**180**

*The extra positive tube in the *tdh* MPN level for *Vp WA4647* R2 resulted from a very late tdh only amplification (Ct = 39.87). Usually, when this situation occurs it is due to cross-contamination on the 384-well PCR plate and the extract will be re-run twice to ensure proper reporting.

Ruggedness

Purpose
To assess the ability of the method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Method
Using 10 oyster samples, the ruggedness of the assay was challenged with different media/reagents, variability in oyster tissue concentration, and other environmental factors inherent in testing over a period of time.

For each matrix, 36 APW tubes (18 from each of the two lots of media) were spiked with oyster tissue (1g oyster to .000001g oyster) – 6 of each dilution. Tubes were designated as Lot A or Lot B and then processed through the entire assay with Lot A or Lot B media/reagents. Five different spiking levels were tested using *Vp ATCC BAA-240* and *Vp WA4647*; each level was spiked into two oyster matrices. The testing was performed over the course of two weeks on three separate days.

Results
While there were fluctuations between Ct values from Lot A to Lot B (Table 1), all samples were expected to show amplification and did so within a relatively small range. The average Ct value and standard deviation across all matrix dilutions and between Lot A and Lot B were calculated (Table 2 and Table 3). This is a qualitative assay and as such amplification at a certain Ct level is not necessary and there is no cycle cutoff to determine a positive or negative sample.

Table 1. Comparison between Lot A and Lot B of observed positive results and the expected positive results. The average Ct is calculated from all 10 matrices tested for each target.

		Lot A	Lot B
IC	Observed/ Expected	120/120	120/120
	Ave Ct	23.78	24.71
tlh	Observed*/ Expected	118/120	118/120
	Ave Ct	16.98	17.44
tdh	Observed*/ Expected	118/120	118/120
	Ave Ct	16.79	17.13
trh	Observed*/ Expected	58/60	58/60
	Ave Ct	17.82	18.21
ORF8	Observed/ Expected	60/60	60/60
	Ave Ct	20.56	22.02

***** Two dilutions of data points were negative in the WA4647 data set. Due to the consistency of the data in dilutions on either side, the most likely explanation is the tubes were inadvertently skipped during the spiking experiment.

Table 2. Low and High Ct values across all matrices and oyster concentrations.

		IC Low	IC High	*tlh* Low	*tlh* High	*tdh* Low	*tdh* High	*trh* Low	*trh* High	*ORF8* Low	*ORF8* High
ATCC BAA-240	Lot A	21.16	25.07	16.23	17.94	15.29	17.31	n/a	n/a	19.06	21.34
	Lot B	19.05	26.12	16.00	20.48	15.38	19.54	n/a	n/a	19.74	24.41
WA4647	Lot A	20.73	24.96	15.68	19.97	15.73	20.40	16.35	21.27	n/a	n/a
	Lot B	19.35	27.89	15.85	18.67	15.97	19.02	16.74	20.52	n/a	n/a

Table 3. Average Ct and Standard Deviation across all oyster concentrations and reagent/media lots for all samples spiked with *ATCC BAA-240*.

ATCC BAA-240										
Matrix Samples	Spiking Level (cfu/ml)	IC Avg Ct	IC StDev	*tlh* Avg Ct	*tlh* StDev	*tdh* Avg Ct	*tdh* StDev	*ORF8* Avg Ct	*ORF8* StDev	*trh*
2,5	6.50E+00	24.45	1.50	17.45	0.54	16.83	0.50	21.09	0.74	n/a
6,8	6.50E+01	24.47	1.67	17.32	0.44	16.71	0.40	20.96	0.66	n/a
13,15	4.40E+02	23.70	1.31	17.17	0.37	16.37	0.33	20.85	0.48	n/a
17,22	1.15E+04	24.23	1.49	18.00	0.89	17.30	0.80	21.80	1.19	n/a
24,27	1.15E+06	24.21	1.07	18.00	1.48	17.30	1.35	21.75	1.77	n/a

Table 4. Average Ct and Standard Deviation across all oyster concentrations and reagent/media lots for all samples spiked with *WA4647*

WA4647										
Matrix Samples	Spiking Level (cfu/ml)	IC Avg Ct	IC StDev	*tlh* Avg Ct	*tlh* StDev	*tdh* Avg Ct	*tdh* StDev	*trh* Avg Ct	*trh* StDev	*ORF8*
2,5	3.90E+00	24.60	1.94	17.07	0.91	17.12	1.00	18.13	0.99	n/a
6,8	3.90E+01	23.67	1.29	16.83	0.75	16.98	0.78	17.95	0.87	n/a
13,15	9.05E+02	23.97	1.72	16.67	0.55	16.88	0.60	17.87	0.74	n/a
17,22	9.90E+04	24.54	1.33	17.04	1.14	17.32	1.21	18.35	1.28	n/a
24,27	9.90E+06	24.61	1.46	16.60	0.56	16.84	0.60	17.81	0.79	n/a

Screenshots from 2014 Vibrio Season

Run from July 17, 2014

Sample Numbers: S14-095, S14-096, S14-097, S14-098, S14-099, S14-100

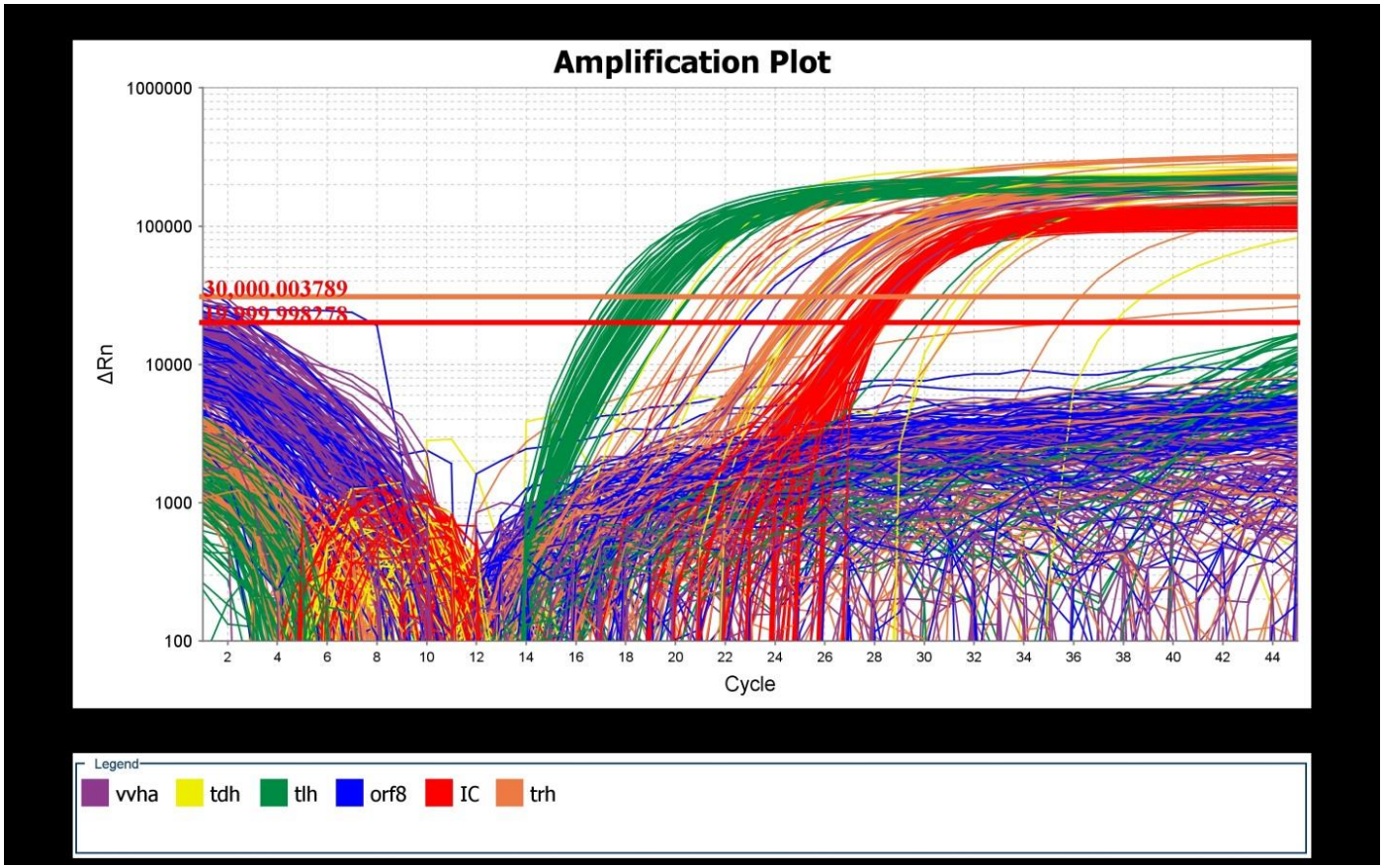


Figure 1. Screenshot showing all targets amplified over 45 cycles. Targets color-coded with legend at bottom of screenshot. For each multiplex 109 wells (including QC) were included. Samples ranged from 12 to 18 wells (per multiplex).

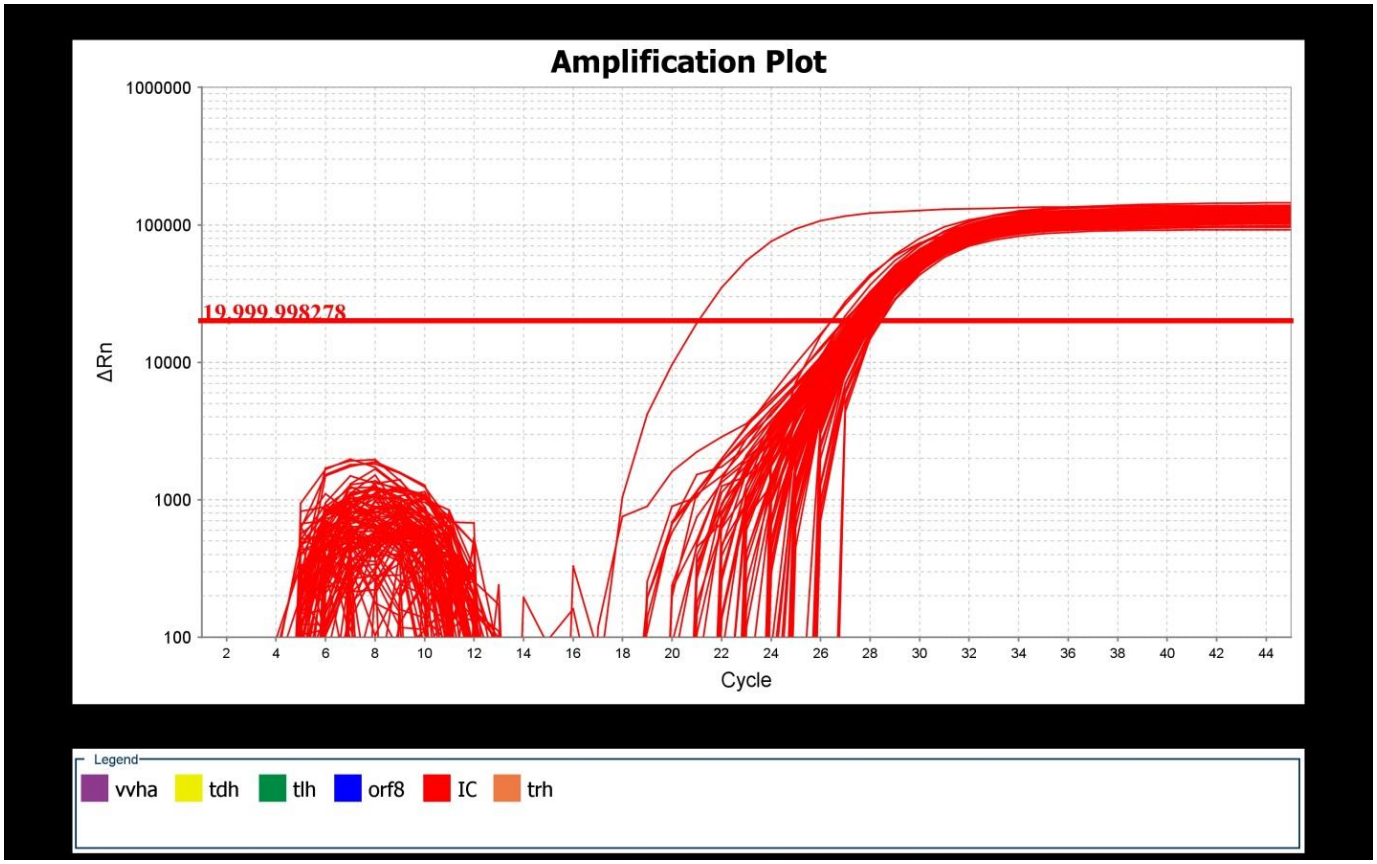


Figure 2. Internal control (IC) amplification plot. All wells in this run generated a positive result; however in the presence of a positive marker the IC may be negative. Most Ct values of our internal control range from 24 to 28, in this run a .00001g series MPN dilution tube generated an earlier Ct than usual. Since all quantitation is done with the MPN values generated from positive and negative tubes, PCR is presence/absence only so close scrutiny of Ct values is not necessary.

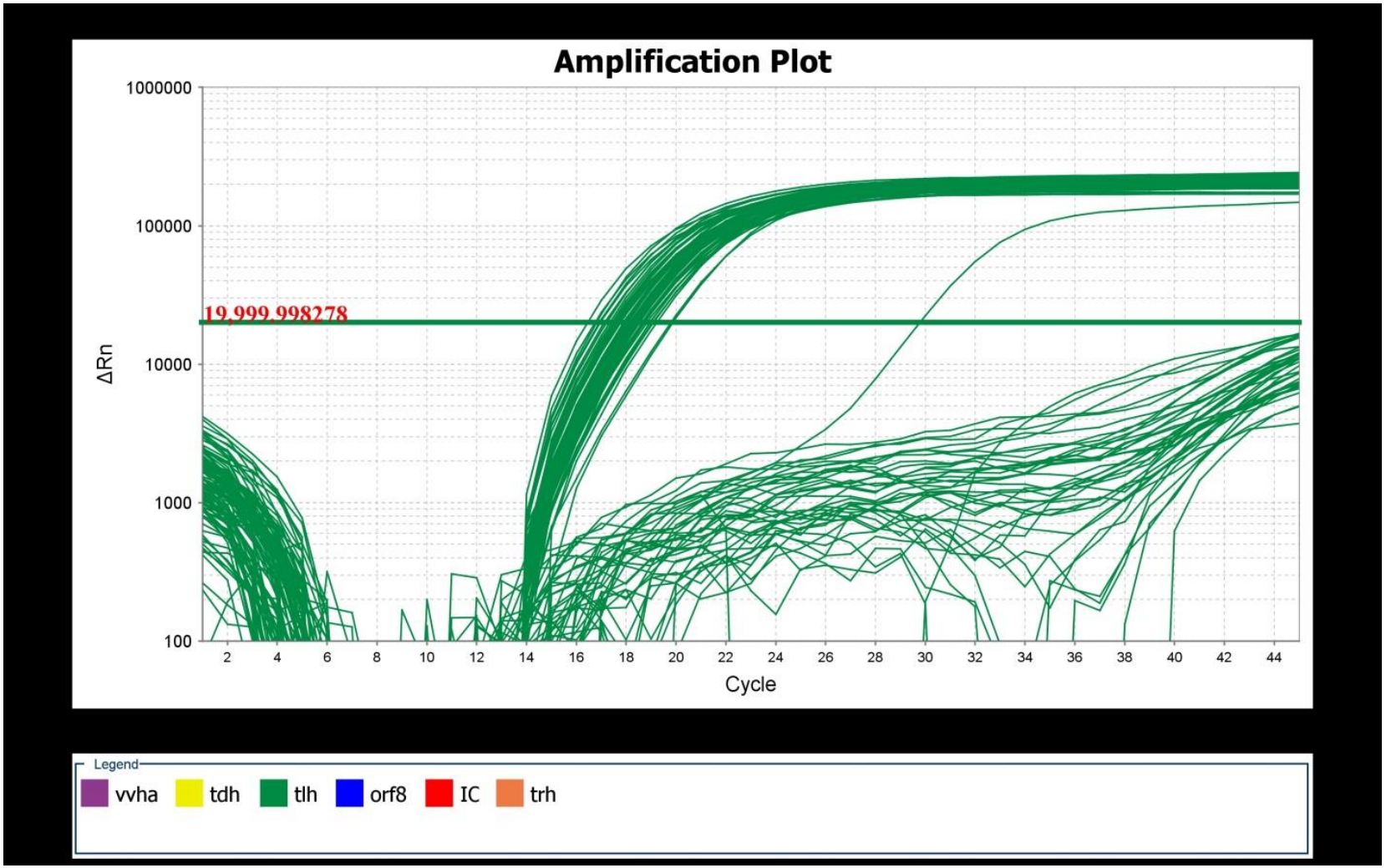


Figure 3. Thermolabile Hemolysin (*tlh*) amplification plot. Threshold set at approximately 20,000 ΔRn. One reaction with a significantly later Ct value is from a .01g dilution tube. No cutoffs have been assigned for this assay (other than *tdh*), this late amplification curve is considered positive.

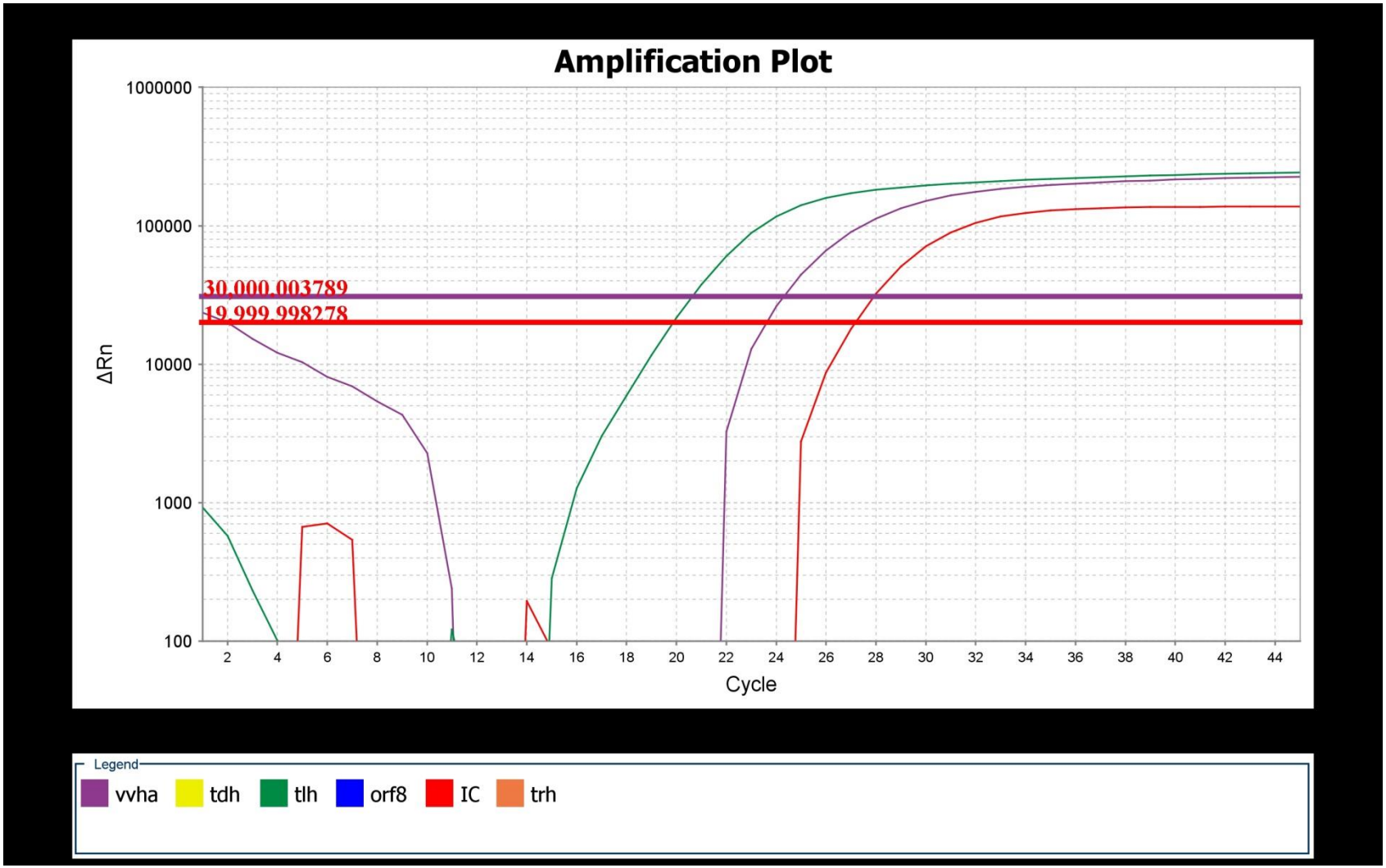


Figure 4. Positive QC for Multiplex 1. Shows amplification for IC (red), *tlh* (green), and *vvha* (purple).

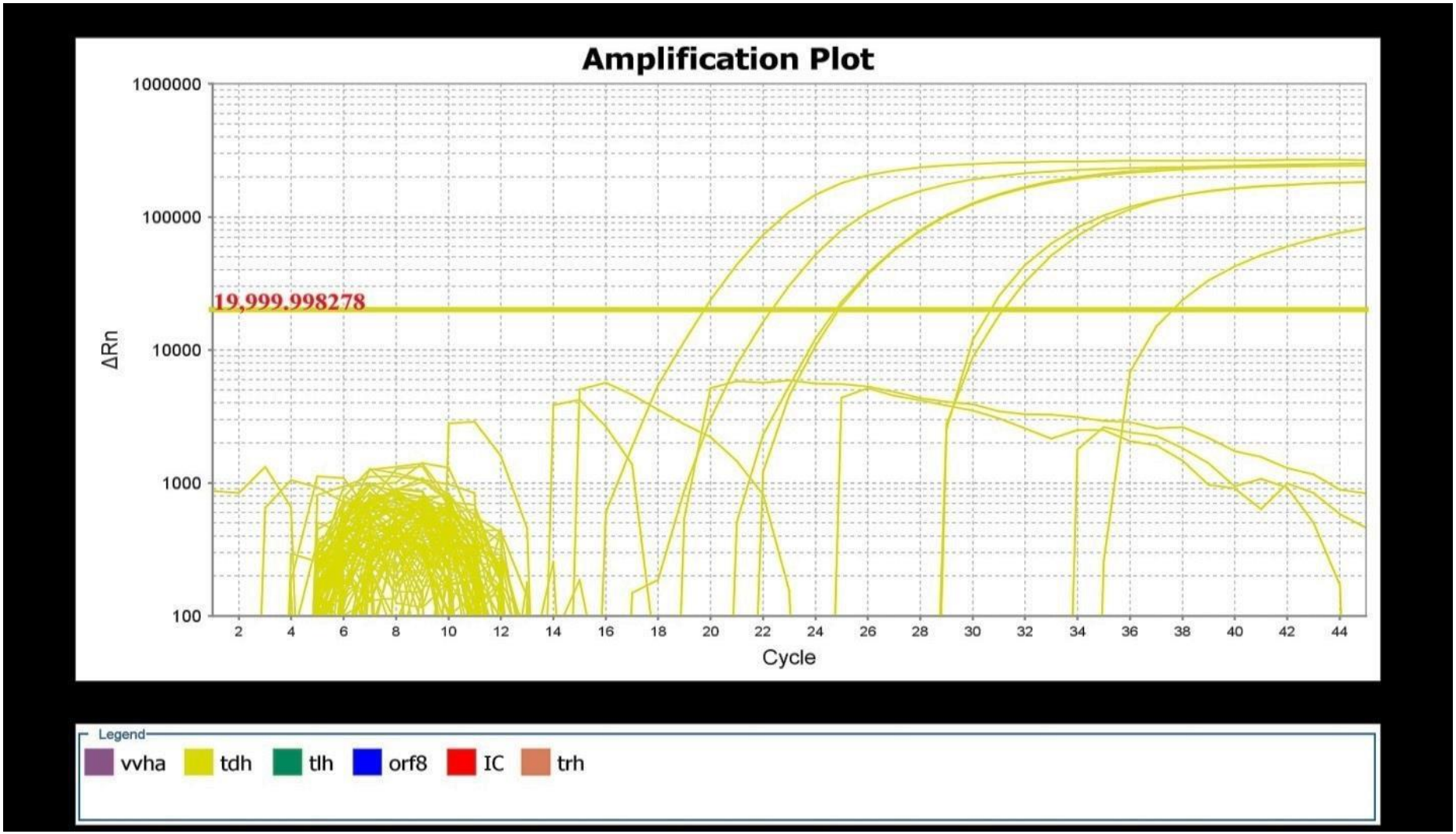


Figure 5. Thermostabile direct hemolysin (*tdh*) logarithmic amplification plot.

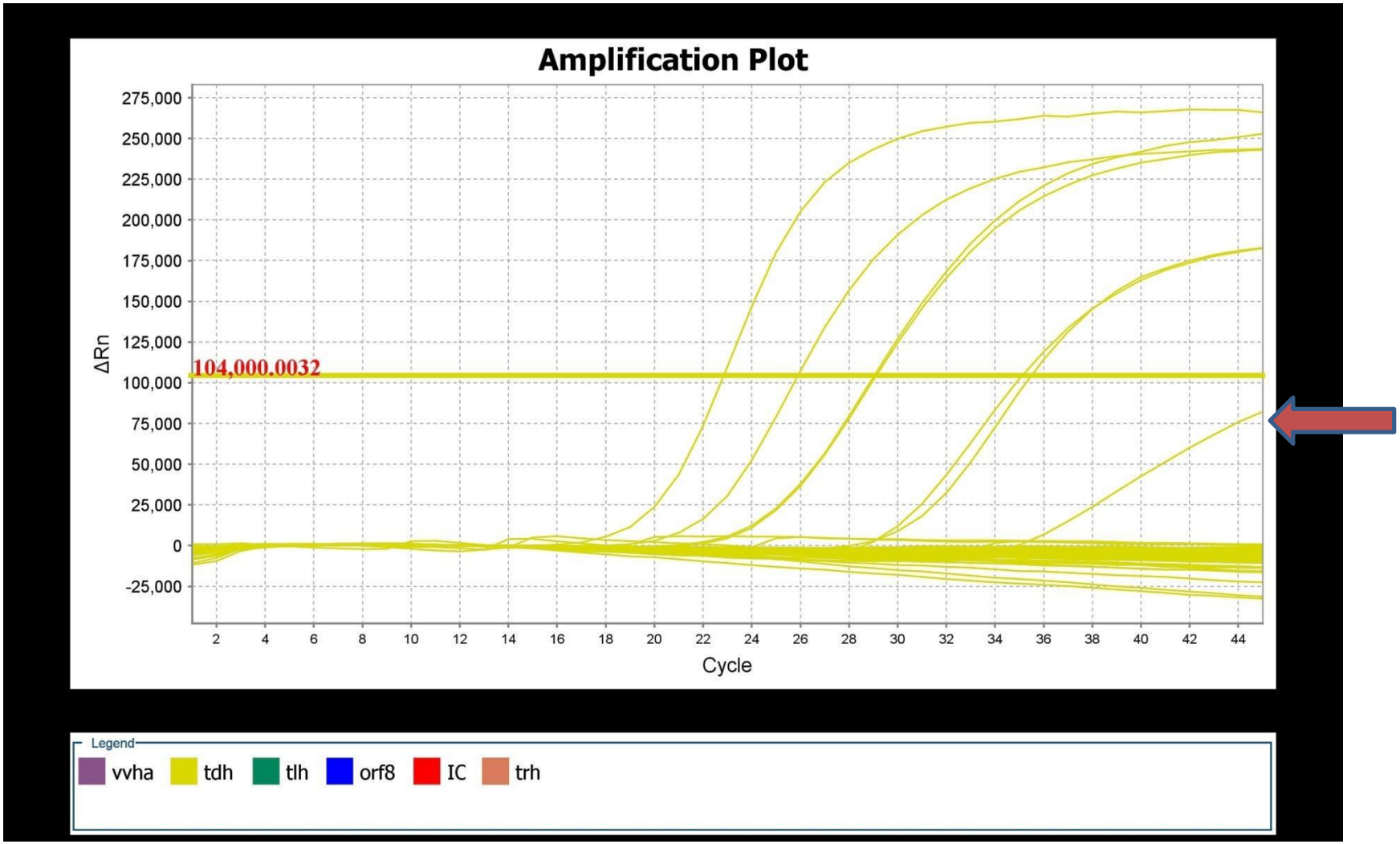


Figure 6. *tdh* linear amplification plot. Red arrow points to a reaction that was ruled negative due to 104,000 ΔRn cutoff.

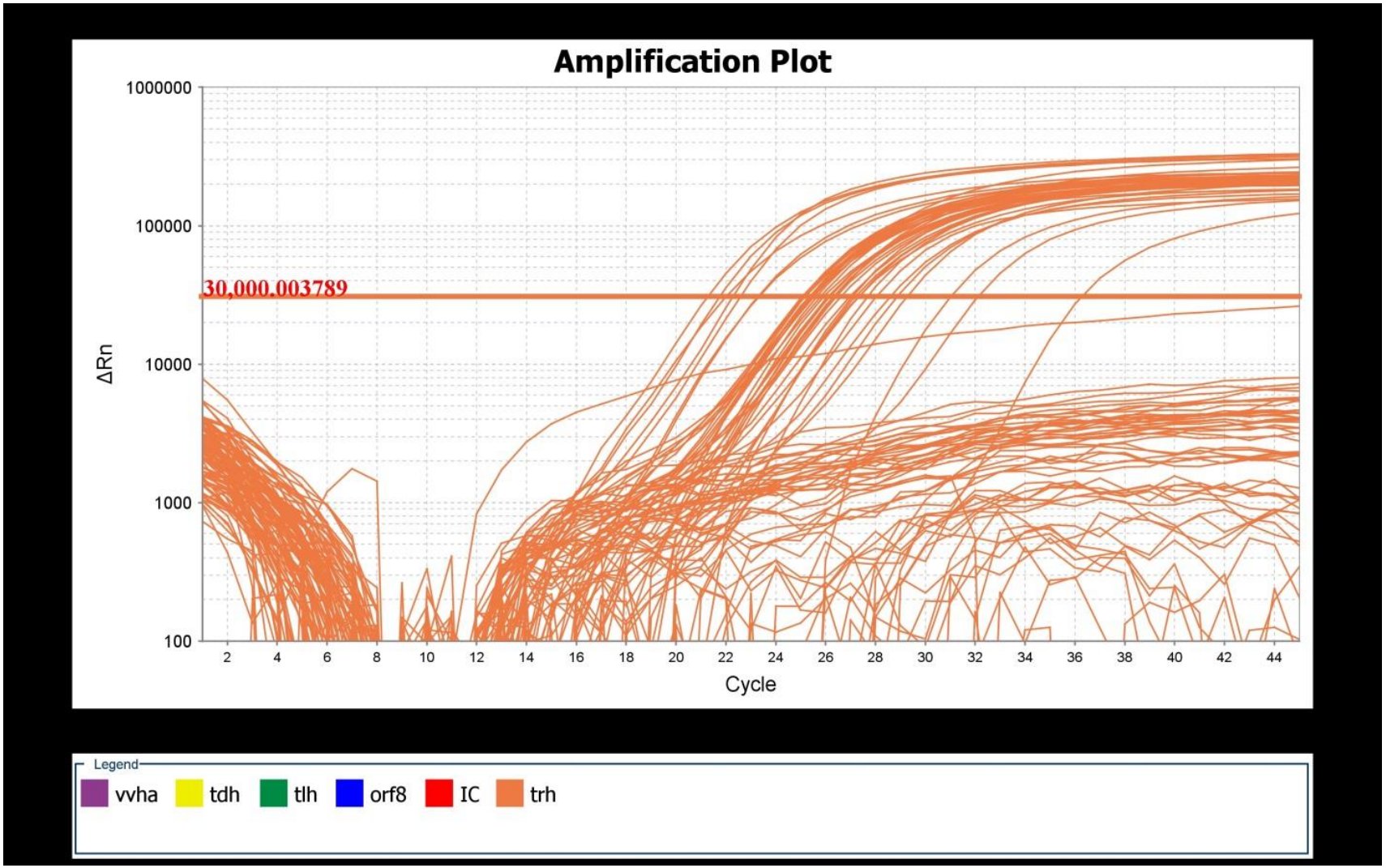


Figure 7. TDH-related hemolysin (*trh*) amplification plot. For a tube to be positive for *trh*, *tlh* must also been detected.

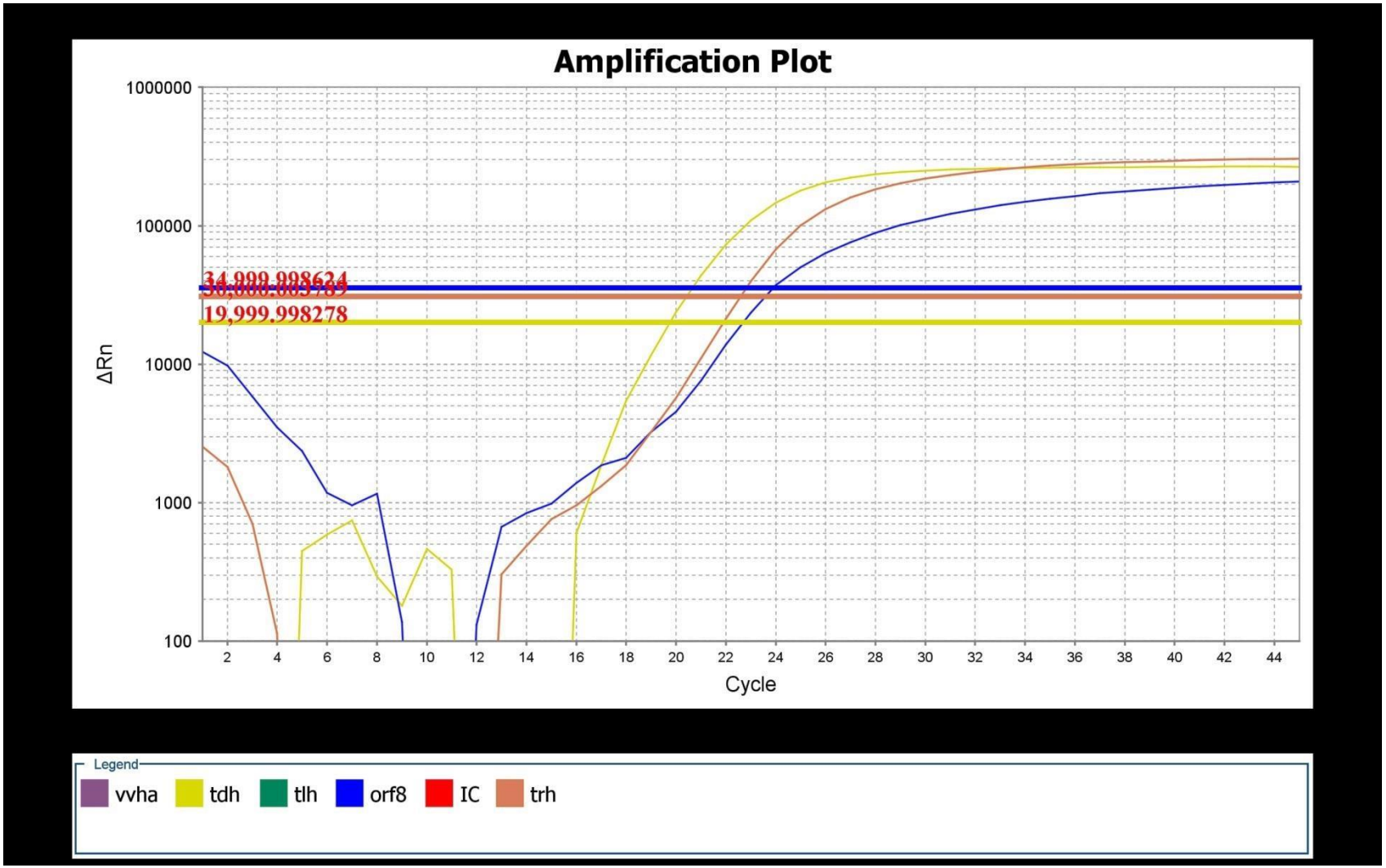


Figure 8. Positive QC for Multiplex 2. Shows amplification for *tdh* (yellow), *trh* (orange), *ORF8* (blue).

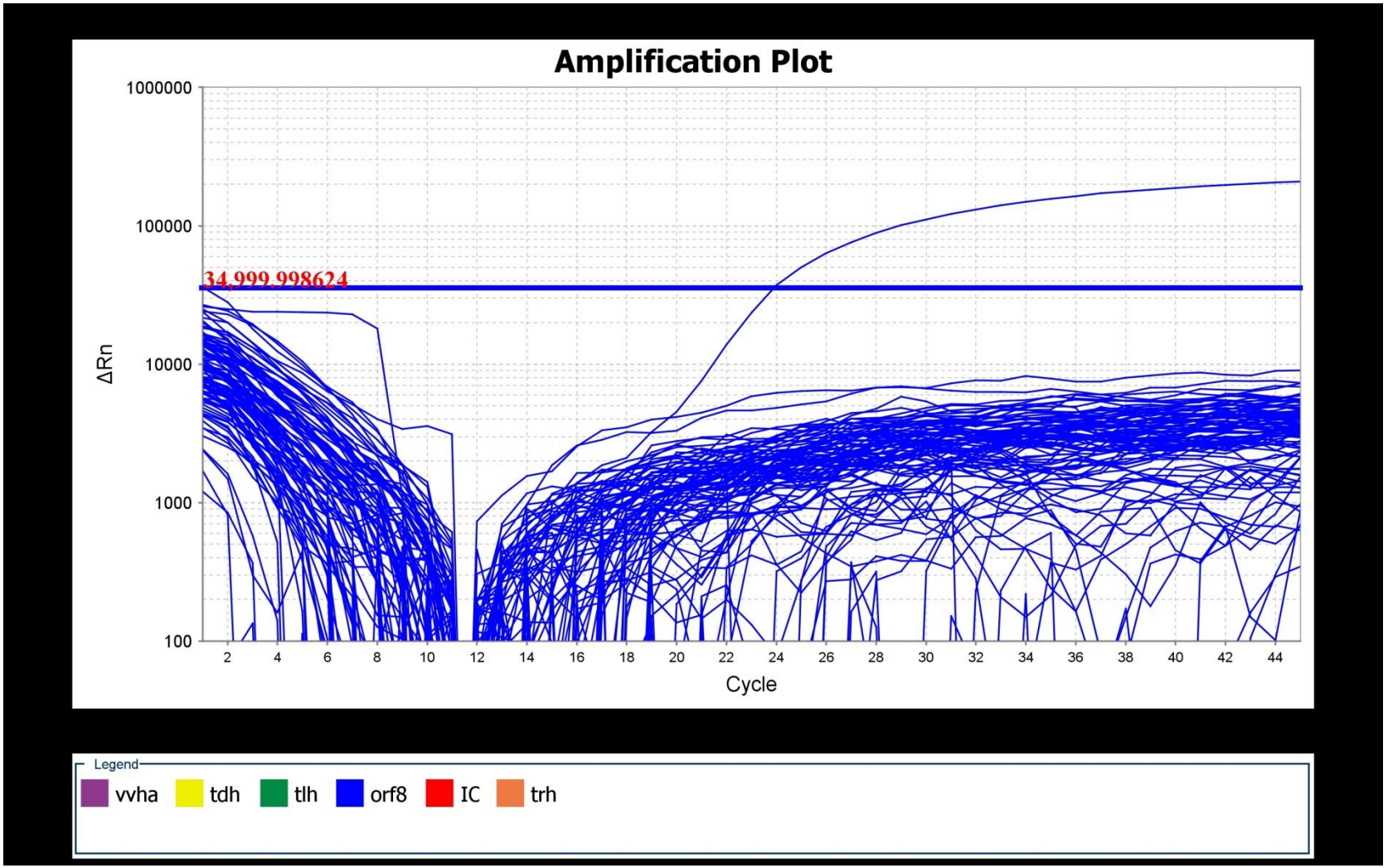


Figure 9. ORF8 gene (*ORF8*) amplification plot. This target was added to the assay to detect pandemic Vibrio, we did not have any positive wells in 2014.

State of Washington
Department of Health
Public Health Laboratories
1610 N.E. 150th Street
Shoreline, Washington 98155
Phone (206) 418-5400 FAX (206) 418-5445

Procedure:
Vibrio parahaemolyticus enumeration and detection through MPN and real-time PCR

StarLims StarDoc ID #: _____ StarLims Version #:

Approved By:

Section Lead: _____ _____ _____
Name Signature Date

Supervisor: _____ _____ _____
Name Signature Date

Office Director: _____ _____ _____
Name Signature Date

Laboratory Director: _____ _____ _____
(if necessary)* Name Signature Date

Supersedes Procedure of: _____
(Date)

* The *Laboratory Director* will sign all procedures that are new or where there has been major changes in the procedure.

Principle

The purpose of this test is to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) from oysters using a high throughput MPN based real-time PCR protocol. Culture based assays for the enumeration of *Vp* require a minimum of four days and require the Kanagawa test (media based) to detect pathogenicity. This assay was designed to provide quantitative results for total *Vibrio parahaemolyticus* (*Vp tlh+)*, known pathogenic markers of *Vibrio parahaemolyticus* (*Vp tdh*+ and *Vp trh*+), as well as some strains of potential pandemic *Vibrio* (*Vp* ORF8+). Additionally, the assay utilizes an exogenous internal control (WA IC) and an investigational marker (*vvhA*) for *Vibrio vulnificus* (*Vv*).

This test utilizes Taqman® probe real-time polymerase chain reactions to amplify 4 target genes from the *Vibrio parahaemolyticus (Vp)* genome as well as 1 target from the *Vibrio vulnificus* (*Vv*) genome.

Vp

1) The thermolabile hemolysin*, tlh* gene
2) The thermostable direct hemolysin*, tdh* gene
3) The thermostable direct related hemolysin*, trh* gene
4) The filamentous phage (f237) ORF8, gene

Vv

1) The cytolysin-hemolysin, *vvhA* gene

Pre-Analytic

1. Test Ordering Process

1.1 Test Ordering Process Flow Chart-N/A

2. Specimen Collection

2.1 Specimen Collection Process Flow Chart-N/A
2.2 Specimen Collection Procedures-N/A
2.3 Safety Considerations-N/A

3. Specimen Transport

3.1 Samples should be shipped in waterproof, puncture resistant containers. Samples should not come into direct contact with ice.

4. Specimen Receiving and Processing

4.1 Specimen Receiving and Processing Flow Chart-N/A
4.2 Specimen Receiving and Processing Procedures-N/A

5. Specimen

5.1 Criteria For Rejection of Sample
5.1.1 Samples received over 10°C are considered acceptable only under the following conditions:
5.1.1.1 The sample is shipped properly (adequate ice/cold packs) and was at an elevated temperature at collection and has had a short transit time (collected and received within 12 hours).

5.1.2 Sample processing at the lab must be initiated no more than 24 hours after collection. Samples received more than 24 hours after collection are considered unsatisfactory.

6. Other

6.1 Problems or Pitfalls-N/A

6.2 Computer Activities-N/A

Analytic

1. Reagents and Media

1.1 Materials

1.1.1 Phosphate Buffer Saline

1.1.2 Alkaline Peptone Water

1.1.3 Isopropanol, 70%

1.1.4 RNAse AWAY®

1.1.5 DNA Extraction Reagents – MagNA Pure LC

1.1.6 MagNAPure LC DNA Isolation Kit III (Bacteria, Fungi), (Roche, Cat. # 3264785)

1.1.7 PCR Reagents

1.1.7.1 Master Mix: Bioline SensiFAST™ Probe Hi-ROX Kit (BIO-82020)

1.1.7.2 Sterile Millipore water or other PCR grade water

1.1.7.3 TE buffer 1:10

1.1.7.4 Primers (See appendix A for sequences)

1.1.7.5 Probes (See appendix A for sequences)

1.2 Preparation

1.2.1 Media

1.2.1.1 Ingredients

1.2.1.1.1 Stock Buffer Solution

1.2.1.1.1.1 Potassium di-hydrogen Phosphate (KH_2PO_4) (J. T. Baker Cat. No. 3246-01 or equivalent)

1.2.1.1.1.2 Distilled Water

1.2.1.1.2 Phosphate Buffer Saline

1.2.1.1.2.1 Sodium Chloride (NaCl) (J.T. Baker Cat. No. 3264-05 or equivalent)

1.2.1.1.2.2 Stock Buffer Solution

1.2.1.1.2.3 Distilled Water

1.2.1.1.3 Alkaline Peptone Water

1.2.1.1.3.1 Peptone (BD Bacto Peptone Ref. No 211677)

1.2.1.1.3.2 Sodium Chloride (NaCl) (J.T. Baker Cat. No. 3264-05 or equivalent)

1.2.1.1.3.3 Distilled Water

1.2.1.2 Formulation and Prepartion

1.2.1.2.1 Stock Buffer Soulution

1.2.1.2.1.1 Mix 17.0 grams of KH_2PO_4 with 250mL of DI water. Adjust pH to 7.2 using 1N NaOH. Using distilled water and volumetric flask, bring volume to 500mL. Autoclave 15 minutes at 121° C. Store in refrigerator.

1.2.1.2.2 Phosphate Buffer Saline (PBS)

1.2.1.2.2.1 Mix 8.5 grams NaCl with 1.25mL of Stock Buffer Solution. Dilute to 1 liter with distilled water. Adjust pH to 7.4. Autoclave for 15 minutes @ 121° C. Final pH should be 7.2-7.5.

1.2.1.2.3 Alkaline Peptone Water (APW)

1.2.1.2.3.1 Mix 10 grams of Peptone and 10 grams of NaCl in 1 liter of distilled water. Adjust pH to 8.5 +/- .02. Autoclave 10 minutes at 121° C.

1.2.2 Reagents

1.2.2.1 Record the receipt and preparation of all reagents in the PCR reagent log book. All reagents must be labeled with a unique reagent number. The reagent number is sequentially recorded in the PCR reagent log where you can find, prep date, concentration, date received, date made, date opened, expiration date etc. The intent of the PCR reagent log book is to allow for complete reagent traceability. Therefore, the reagent numbers assigned to each item used must appear on the master mix prep sheet. Additionally the unique mastermix prep number assigned to each batch of mastermix must appear on the data sheet for all samples for which it was used. All sheets must be retained to allow traceback.

1.2.2.2 Use either filtered or sterilized MQ water from the Millipore filtration system or use commercially available PCR grade water. It is advisable to aliquot the water into smaller portions to minimize the potential for large scale contamination.

1.2.2.3 Prepare TE buffer by adding 10mL 1M Tris-HCl (pH 8.0) + 2mL 0.5M EDTA (pH 8.0). Dilute to a final volume of 1000mL MQ water. Autoclave. Store at room temperature.

1.2.2.4 Prepare supermixes containing primers and probes according to the worksheet. All sequences can be seen in appendix. Primers are diluted to 100µM stock concentrations with TE Buffer and stored at -20°C until use for supermix preparation.

1.2.2.5 Prepare exogenous control by adding 100µL TE Buffer to tube. Stock concentration will vary depending on volume synthesized. Create working concentration by diluting stock 1:10,000 in TE buffer. Freeze working concentration in 10µL aliquots (-20°C), before use add 990µL molecular grade water.

1.3 Performance Parameters-N/A

1.4 Storage Requirements

1.4.1 Container

1.4.1.1 Molecular grade water and TE Buffer can be stored in 50mL conical tubes

1.4.1.2 Primers, probes, and supermixes can be stored in 1.7mL Eppendorf tubes. Supermixes are to be stored in low light transmitting tubes.

1.4.2 Temperature

1.4.2.1 Store primers and probes at -20°C

1.4.3 Stability

1.4.3.1 Reagents free from contamination are good for 1 year unless otherwise stated by manufacturer. Stocks of primers and probes are good for 5 years in freezer (-20°C). Working concentrations of primers and probes are good for 6 months in freezer (-20°C) Thawed working concentrations (Supermix) of primers and probes are good for 2 weeks refrigerated (2-8°C).

1.4.4 Labeling
1.4.4.1 Labels should include: reagent name, date received, date prepared or reconstituted (if necessary), expiration date, and storage temperature

2. Equipment

2.1 Type of Equipment
2.1.1 Thermometer 0°C-10°C
2.1.2 Sink
2.1.3 Blender with sterile blender jars.
2.1.4 Timer
2.1.5 Vortexer
2.1.6 Bulb pipetter
2.1.7 Incubator (35°C±0.5)
2.1.8 Dry bath, 70°C
2.1.9 Refrigerator, 2-4°C
2.1.10 Freezer, -20 to -80°C (-80°C preferred)
2.1.11 Microcentrifuge
2.1.12 Autoclavable waste container
2.1.13 Biological safety cabinets (BSC) or Air Clean PCR stations
2.1.14 Rainin pipettes
2.1.14.1 P-2
2.1.14.2 P-10
2.1.14.3 P-20
2.1.14.4 P-200
2.1.14.5 P-1000
2.1.14.6 Multi-channel (8) 2µL-25µL
2.1.15 Applied Biosystems® ViiA™ 7 Real Time PCR station
2.1.16 Roche MagNaPure LC DNA purification system
2.1.17 Ice bucket/refrigerated block
2.1.18 Vortexer
2.1.19 Refrigerator, 4°C
2.1.20 Freezer, -20 to -80°C
2.1.21 Autoclavable waste container
2.1.22 Microcentrifuge
2.1.23 PCR plate centrifuge.
2.1.24 384-well microtiter-plate stand

2.2 Preparation-N/A

2.3 Performance Parameters
2.3.1 Equipment should perform within the manufacturer's specifications
2.3.2 Preventative maintenance is performed at least yearly or as needed
2.3.3 Refrigerators, incubators, and freezers are monitored daily for correct temperatures.
2.3.4 Maintenance and decontamination duties are performed on a regular basis to keep equipment in good working order and to reduce the chances of PCR contamination.

3. Supplies

3.1 Sterile scrub brushes
3.2 Sterile oyster knives

3.3 Clean specimen trays
3.4 Paper towels
3.5 Sterile wide mouth containers (1 liter capacity)
3.6 Oyster shucking block
3.7 Chain-mail glove
3.8 Dishwashing gloves
3.9 Glass or serological pipette tips
  3.9.1 25mL
  3.9.2 10mL
  3.9.3 5mL
  3.9.4 1mL (0.1mL hashes)
3.10 Rainin filtered pipette tips
  3.10.1 1000µL tips
  3.10.2 200µL tips
  3.10.3 10µL tips
3.11 Microcentrifuge tubes (1.7mL)
3.12 Reagent Tubs – Large
3.13 Reagent Tubs - Medium - 20mL
3.14 Reagent Tub Lids – Large
3.15 Reagent Tub Lids - S/M
3.16 MPLC Rxn Tips – Large
3.17 MPLC Rxn Tips – Small
3.18 MPLC Rxn Tips - L Refill
3.19 MPLC Rxn Tips - S Refill
3.20 MPLC Processing Cartridge
3.21 MPLC Sample Cartridge
3.22 Tip Stands
3.23 Waste Bags
3.24 Cartridge seals
3.25 Gloves, nitrile
3.26 384 well PCR plates
3.27 Optical covers
3.28 Cartridge seals
3.29 Sterile disposable reagent reservoirs (small volume 1-5mL)

4. Specimen
4.1 *Crassostrea gigas* (Pacific oyster)

5. Special Safety Precautions
5.1 *Vibrio* species are pathogenic and should be handled following PHL safety guidelines.
5.2 Dry baths can reach temperatures of 130° C; do not touch heated blocks. Tightly sealed tubes may burst if heated beyond safe levels
5.3 UV light can seriously burn skin and eyes. Keep safety shield lowered when UV light is on. Always keep skin covered by lab coat and gloves

6. Quality Control
6.1 Materials
  6.1.1 Pre-extracted *Vibrio parahaemolyticus* (ATCC BAA-240, WA4647) and *Vibrio vulnificus* (ATCC 27562) DNA is blended and aliquoted in 10µL aliquots and frozen at -20°C

6.1.2 Molecular grade water
6.1.3 Exogenous internal control plasmid containing a target fragment
6.2 Instructions
6.2.1 An extraction control is included in every extraction run. To prepare the extraction control material, enrich *V. parahaemolyticus* (ATCC 49398, *tlh*+, *tdh*-, *trh*-, ORF8-) overnight in APW. The material is lysed (MagNAPure protocol) and aliquoted into 1.7mL microcentrifuge tubes. Store the positive control lysate at -20°C to -80°C until needed.
6.3 Frequency
6.3.1 An NTC, exogenous internal control and positive control are included with every run
6.4 Acceptable Limits
6.4.1 A successful run should meet the following conditions:
6.4.1.1 No amplification should be present in any of the negative controls. Creeping curves without a logarithmic increase are not considered true amplification. If amplification is present in a negative control, determine the source of contamination, thoroughly clean BSC and pipettes, and discard contaminated reagents and disposables.
6.4.1.2 The positive control should show clear amplification. If NO amplification is present in the positive control for one or both of the sets, determine the problem and re-run the sample (see troubleshooting).
6.4.1.3 Wells negative for all targets should show amplification for the exogenous control (IC). The IC may be negative in positive samples.
6.5 Corrective Action
6.5.1 Runs for which the NTC is positive or the positive control and/or internal control is negative should be repeated
6.5.2 The Lead Microbiologist should be notified if any run fails QC
6.6 Recording QC Data
6.6.1 Record results for each well and record a + or – on the *Vp* worksheet

7. Calibration
7.1 Standard Prep
7.1.1 List of Standards Used
7.1.2 Directions for Preparation
7.1.3 Special Instructions
7.1.4 Degree of Accuracy
7.1.5 Storage Requirements
7.1.6 Container
7.1.7 Temperature
7.1.8 Stability
7.1.9 Labeling
7.2 Calibration Procedure

Testing Procedure

8. Quantitative Testing Procedure
8.1 Accessioning, Sample Preparation and Centrifugation
8.1.1 Samples are processed in accordance with recommended procedures described by the American Public Health Association (4). Oyster samples are removed from the shipping container and the sample submission form is located.

A laboratory worksheet is generated for each sample. Both the sample submission form and the lab worksheet are stamped in with the appropriate lab number. One oyster from each bag is opened to take tissue temperature. The temperature is recorded on the sample submission form. The bag of oysters is labeled with the sample number and placed into a 2-4°C refrigerator until they are ready to be processed.

8.2 Detailed Stepwise Procedure

8.2.1 The intent of the assay is to determine the concentration of *Vp* in the oyster tissue and liquor. Any material on the outside of the oyster that gets introduced into the interior of the animal during shucking can alter the concentration.

8.2.1.1 Clean the sink before beginning to scrub the oyster sample. The sink must be clean at this stage but sterility is not required.

8.2.1.2 Before beginning the scrubbing process, wash gloves with soap and water. Using sterile scrub brushes, each oyster is cleaned under cold running water. All barnacles, mud, vegetation and debris should be removed. Pay close attention to the hinge and shell seam. A sterile brush should only be used for one sample. Do not re-use brushes when scrubbing multiple samples.

- Any oyster that does not tightly close during handling is likely dead and should be discarded.
- In addition any oyster whose shell is broken to expose tissue should be discarded.

8.2.1.3 After cleaning each oyster place the animal upside down on a clean, labeled, paper towel lined tray. Laying the oysters upside down will prevent the liquor (fluid inside a closed oyster) from draining out of the oysters while waiting to be shucked. Clean trays must be used for each sample.

8.2.1.4 Once cleaned, return the oysters to the refrigerator to dry or towel dry them for immediate shucking.

8.2.2 In order to accurately quantify *Vp* in oyster tissue it is very important to avoid introduction of bacteria (*Vp* or other) into the oyster tissue.

8.2.2.1 The sink must be sterile before shucking can begin. Wash the sink with water or soap and water. Completely dry the sink. Wipe the sink down with 70% isopropanol and allow it to air dry.

8.2.2.2 Place a sterile pre-weighed (remove rubberband prior to weighing) tissue collection container on the sink counter.

8.2.2.3 Wipe a shucking block down with 70% isopropanol and place on the sink counter to air dry.

8.2.2.4 Place the oyster sample to be shucked on the sink counter.

8.2.2.5 Put on clean nitrile gloves. Over one of the gloves put on a chain-mail glove. The chain-mail glove should be on the hand that will not be holding the knife. Put another nitrile glove on over the chain-mail glove. Cover both hands in 70% isopropanol and allow them to air dry.

8.2.2.6 Grab and hold each oyster with the chain-mail hand and use the other hand and a sterile oyster knife to shuck each oyster.

8.2.2.7 Use the sterilized shucking block while shucking to minimize knife accidents and to protect the counter surface.

8.2.2.8 Collect all tissue and liquor (fluid) in the sterile pre-weighed container.

8.2.2.9 A fresh knife, shucking container and gloves must be used for each specimen.

8.2.2.10 The shucking block and counter must be washed and sterilized between specimens.

8.2.3 Enumeration in this assay is achieved by conducting an MPN (most probable number) analysis.

8.2.3.1 Weigh the container and shellfish tissue. Subtract the container weight from the total weight to determine the amount of oyster tissue and liquor. Record the weight of tissue on the sample worksheet.

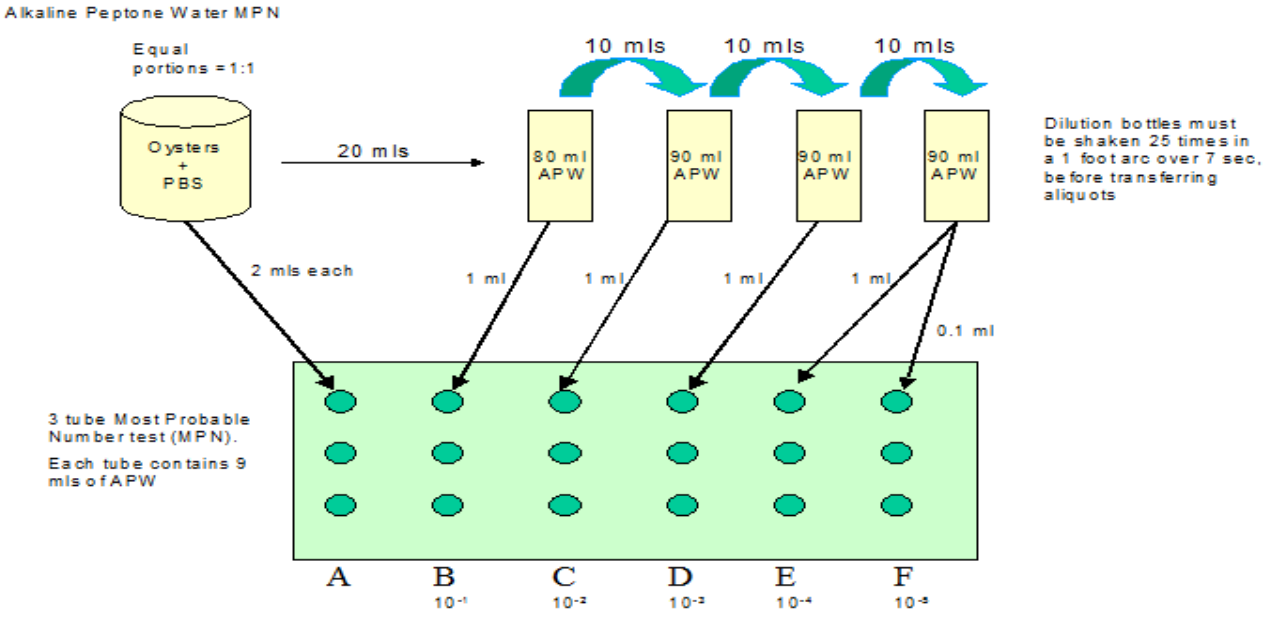
8.2.3.2 Transfer the oyster tissue and liquor to a sterile blender jar.

8.2.3.3 Add an equal weight of PBS to the sample container (the PBS can be used to rinse any residual tissue from the container). Transfer the PBS to the blender jar. Record the weight of PBS used on the sample worksheet.

8.2.3.4 Blend the shellfish sample with PBS (now a 1:2 dilution) at high speed for 90 seconds.

8.2.3.5 The resulting homogenate should be relatively smooth. If the blender isn't generating a smooth homogenate, it is advisable to service the blender (sharpen/replace blades).

8.2.3.6 From this homogenized sample, set up a 3-tube most probable number (MPN) serial dilution series. Use PBS for making dilutions and alkaline peptone water (APW) as the enrichment broth in each of the MPN tubes.



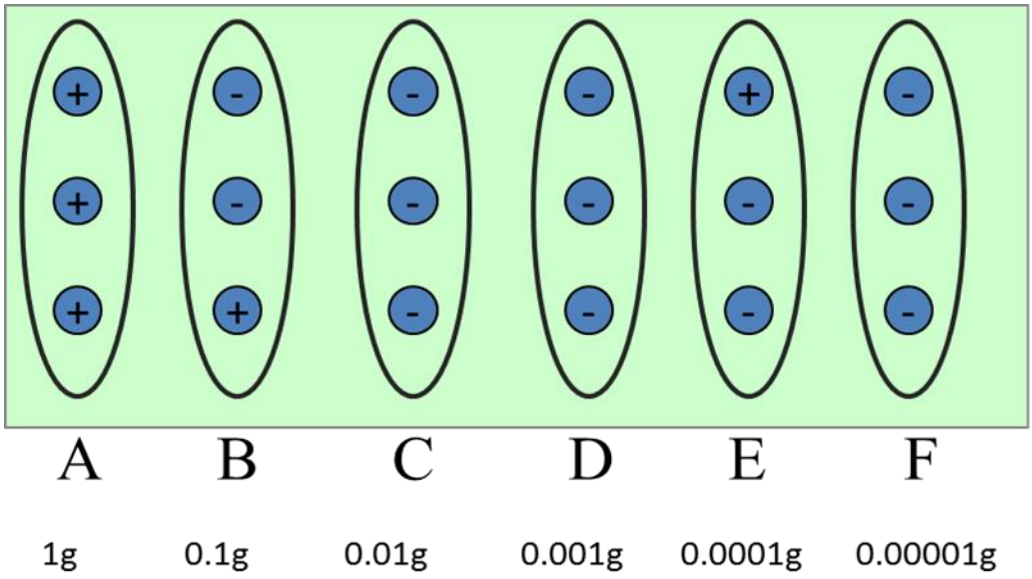
8.2.3.7 Incubate at 35°C± for 18 to 24 hours. Write sample number, date, and time placed in incubator on tube A1.

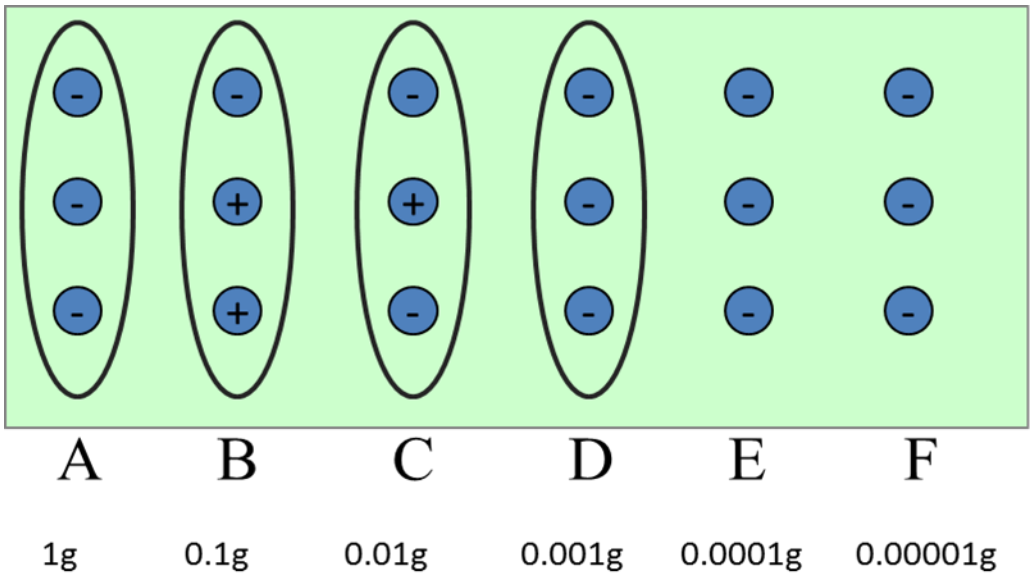
8.2.3.8 Following the 18-24 hour incubation step, each APW tube must be checked for growth. Use the following criteria to select tubes for further testing.

8.2.3.9 Examine all tubes for turbidity. Examine each tube with a light source shining through the tube.

8.2.3.10 Record all positive and negative results on the lab worksheet.

8.2.3.11 The following examples illustrate the selection process. Each tube is labeled as +/- for turbidity. The dilutions circled should be selected for further testing.





8.2.3.12 If one tube in a given three tube dilution series is positive for turbidity all tubes in that series must be tested (you will still record the actual positive or negative values).

8.2.3.13 In addition, test one complete dilution series beyond the last series that contained any growth and all tubes of higher concentration.

8.2.4 DNA Extraction

8.2.4.1 DNA extraction must be initiated within the 18-24 hour incubation window.

8.2.4.2 Reagent prep should be carried out in the Pre-PCR Lab in order to minimize the potential for contamination. The lysis procedure itself may be carried out in the DNA extraction room or in the main lab on the bench. Once complete, the material is considered stable and may be stored at 2-4°C for 1-

2 days or frozen at -20 to -80°C for extended periods. NOTE: Multiple freeze thaw cycles should be avoided due to potential DNA degredation.

8.2.4.3 Each tube that is selected for additional testing will require one 1.7mL microcentrifuge tube that was pre-loaded with lysis buffer and proteinase K (proK). (130µL lysis buffer + 20µL proK provided in MagNA Pure kit)

8.2.4.4 Label each tube with the sample number and tube ID.

8.2.4.5 Vortex each APW tube that is to be extracted briefly.

8.2.4.6 Pipette 1mL from each APW tube to its labeled 1.7mL tube.

8.2.4.7 Briefly vortex each 1.7mL tube.

8.2.4.8 Place the 1.7mL tubes in the 65°C dry bath for 10 minutes.

8.2.4.9 The lysed tubes are now ready for DNA extraction. **NOTE:** Due to the high number of DNA extractions that are required as part of this assay, the Roche MagNA Pure was chosen for initial validation.

8.2.4.10 Lysed material from each 1.7mL tube should be manipulated in either the DNA extraction room or the template addition lab.

8.2.4.11 At this point due to the high number of lysates it is necessary to create a document to track the location of each lysate. This document is referred to as the “MagNA Pure Plate Map”.

8.2.4.12 After the MagNA Pure Plate Map is created, load the MagNA Pure cartridge accordingly. Loading should take place in a BSC or an AirClean hood. 200µL of each lysate should be added to the plate. Include 200 ul of pre-lysed *Vp* culture and 200µL of sterile PCR grade water. These will act as controls for the assay.

8.2.4.13 Once loaded, seal the MagNA Pure cartridge with an adhesive cartridge seal.

8.2.4.14 Label the plate with the sample number, date, initials and the label “Pre-Ext”. The specimen can now be loaded onto the MagNA Pure.

8.2.4.15 Turn on both the computer and the MagNA Pure.

8.2.4.16 Ensure that the correct protocol is selected “DNA III Bacteria”.

8.2.4.17 Enter in your sample order. Be sure that the correct cartridge wells are highlighted on the screen. Incorrect set up here will lead to a failed extraction.

8.2.4.18 Sample volume should be entered as 200µL.

8.2.4.19 Elution volume should be entered as 100µL.

8.2.4.20 Click the “Stage Set-up” button.

8.2.4.21 Begin adding in the appropriate plastics. Click the items on the screen as you add them to the MagNA Pure. Make sure that the discard bag is not too full before you begin your extraction.

8.2.4.22 Once all plastics are added and the discard bag is replaced remove the reagent tray from the machine.

8.2.4.23 Add the amount requested of each color coded reagent to the appropriate size of tub and apply a lid (with holes for tip access). Using the wrong tub will likely lead to a failed extraction.

8.2.4.24 Before adding the Magnetic Glass Particles (MGPs), vortex them for 15-20 seconds to completely suspend them. Once the particles are completely re-suspended in solution, rapidly deliver the appropriate volume to the correct tub and cover. It is important to get the extraction started soon after the beads are pipetted as they will rapidly settle out of suspension.

8.2.4.25 Once the reagent tray is completely loaded, place the tray in the machine.
8.2.4.26 Remove the cartridge seal from the lysate cartridge and add it to the machine. Discard the seal into an autoclavable container.
8.2.4.27 Ensure that all plastics, reagents and sample cartridges are in place and accounted for on the computer screen.
8.2.4.28 Close the door and press the start extraction button.
8.2.4.29 Note the time that the run will be completed. The final extracted template DNA will be refrigerated on-board the MagNA Pure until it is removed. It is however not advisable to leave the extract uncovered for any length of time.
8.2.4.30 Once completed, open the door, remove the extracted DNA, immediately seal the cartridge with a new cartridge seal, and refrigerate at 2-4°C until ready for PCR (if PCR is to be completed in 1-2 days). If PCR will not be complete in the next two days freeze the DNA at -20 to -80°C.
8.2.4.31 Remove all soiled plastics, replace used tips and run the decontamination protocol from the MagNA Pure main screen.

8.2.5 PCR Mastermix Preparation

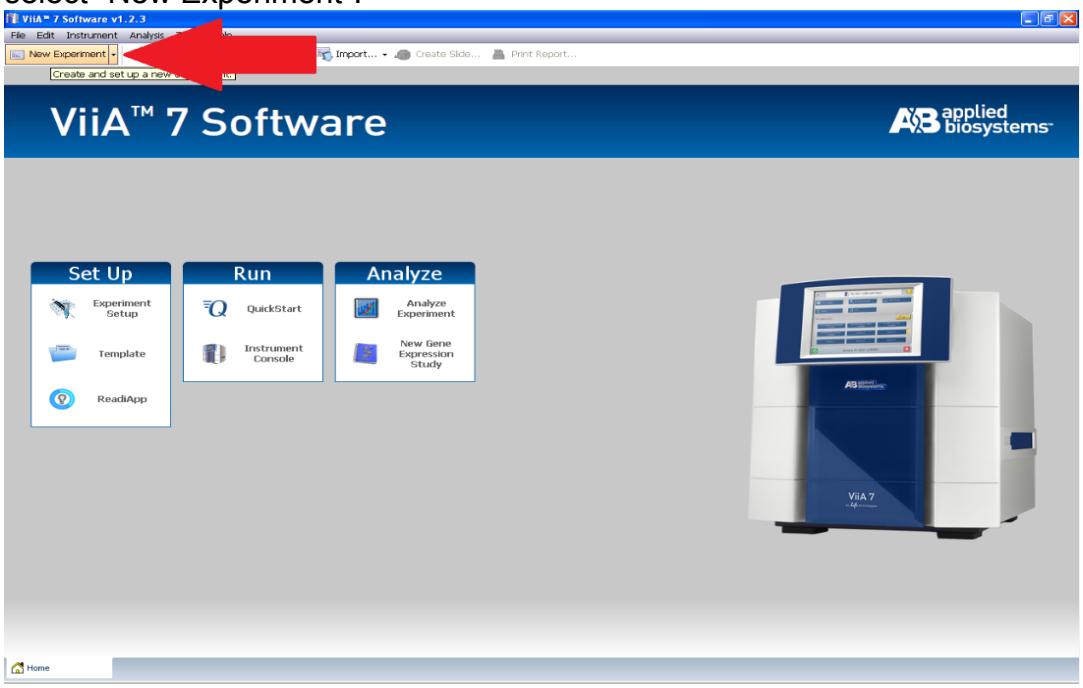
8.2.5.1 Mastermix preparation is performed in the Pre-PCR Lab biological safety cabinet or Airclean hood. This includes primer and probe manipulations and mastermix loading into the PCR plate. Thorough decontamination before and after use of the room is advisable. **NOTE**: A person who has previously in the same day worked in the Template addition lab should not re-enter the Pre-PCR lab.
8.2.5.2 Turn on the ABI® ViiA™7 machine and the computer.
8.2.5.3 Prepare a PCR platemap. Always include a positive and a negative control for each MagNA Pure plate that was extracted.
- Negative control: No DNA, milli-Q water (from MagNA Pure cartridge)
- Positive control: *tlh*+ *V. parahaemolyticus* ATCC 49398 (from MagNA Pure cartridge)

8.2.5.4 Prepare the mastermix according to the mastermix prep sheet (Appendix C). Determine how many reactions you will need and calculate how much of each component you must use. It is advisable to prepare several reactions more than are needed to account for pipetting variability.
8.2.5.5 Once prepared, briefly vortex to completely mix the components.
8.2.5.6 Add the mastermix to a sterile disposable reagent reservoir.
8.2.5.7 Using a pipetter (multichannel advisable), add 18µL of mastermix to each appropriate well (384 well plate) according to the PCR platemap.
8.2.5.8 Once the plate is loaded with mastermix, place the plate in a biological transport container (sealed box) and take the plate into the Template addition lab. It is important to quickly add the template, seal the plate and initiate PCR. Therefore make sure that the extracted DNA is ready before setting up your PCR reactions.
8.2.5.9 Add the extracted DNA template to the appropriate wells according to your PCR platemap. Use 2µL of DNA for a total reaction volume of 20µL.
8.2.5.10 Once all wells are loaded including the positive and negative controls, seal the plate with an optical seal. Avoid touching either side of the seal as the adhesive side will come into contact with your reactions (poses a

contamination risk) and the outside must be clear of smudges to allow accurate readings. Apply the seal using the plastic applicator supplied with the instrument. Take care to completely seal each well. Any unsealed well will rapidly evaporate during PCR and lead to inaccurate results.

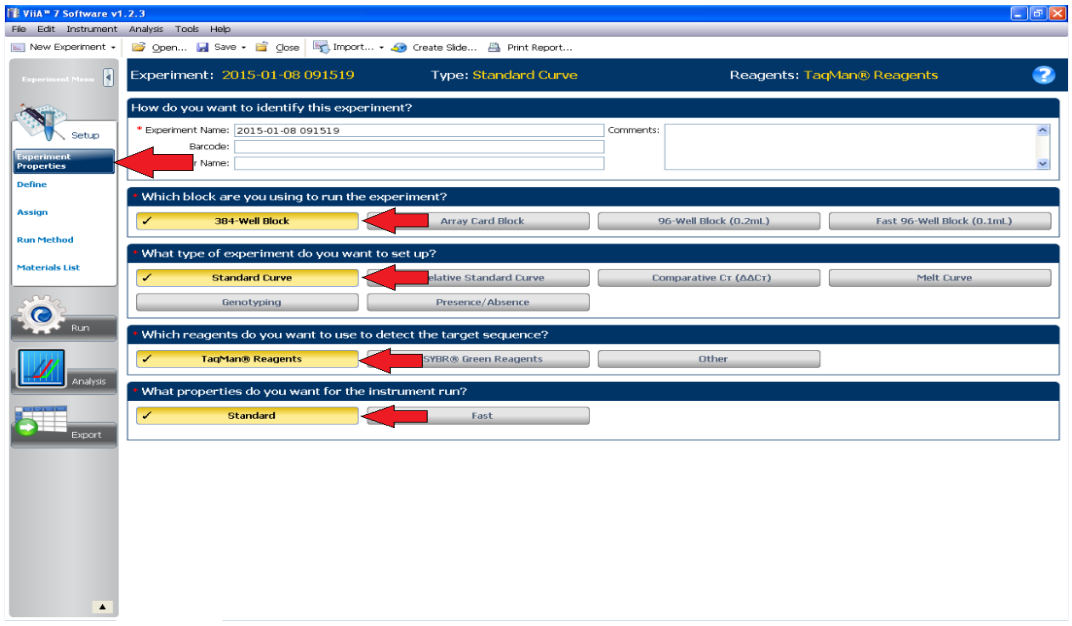
8.2.5.11 Once the plate is sealed, centrifuge the plate briefly to remove bubbles from the wells and ensure that the template is in contact with the reaction mix.

8.2.5.12 Return to the ABI ViiA™7. Open the SDS software, under the File menu select “New Experiment”.

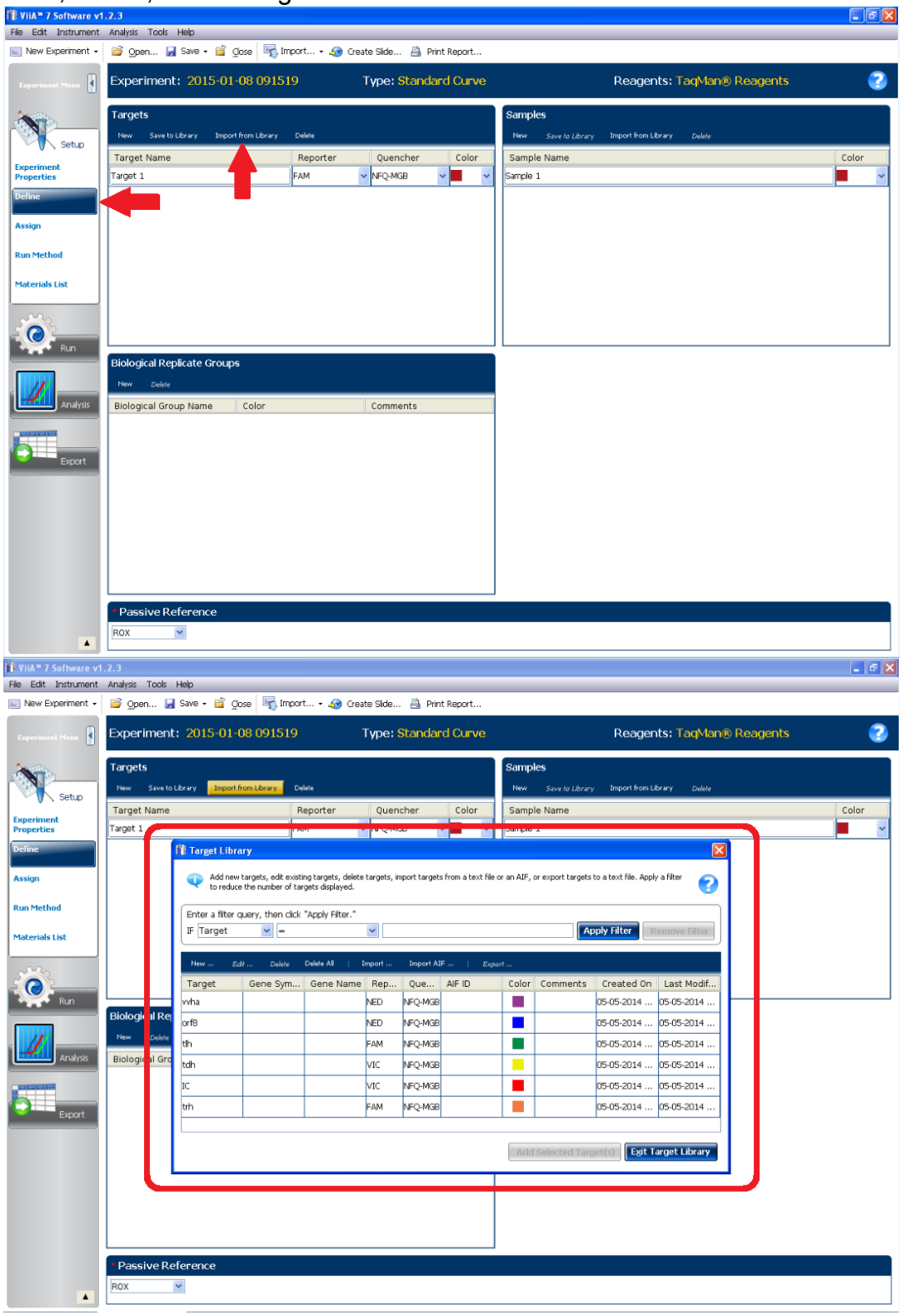


8.2.5.13 Rename experiment with the appropriate sample numbers and date of run.

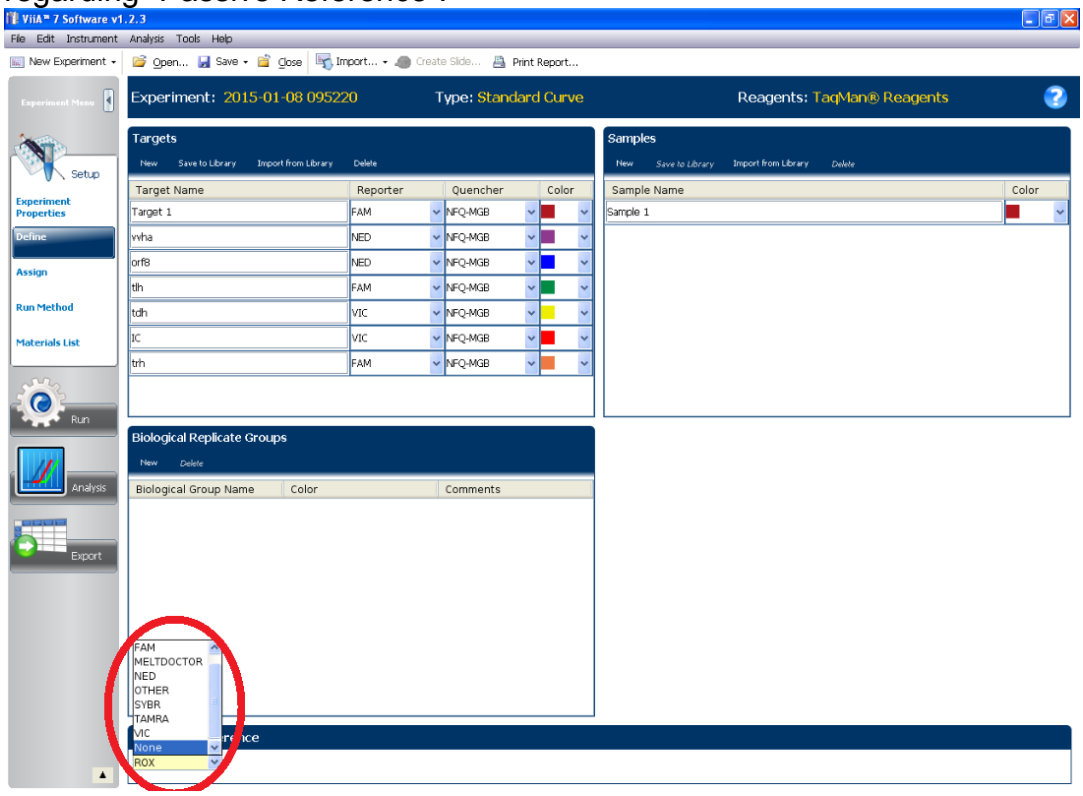
8.2.5.14 Under the tab “Experiment Properties” ensure “384-Well Block”, “Standard Curve”, TaqMan® Reagents”, and “Standard” (for run mode) are selected.



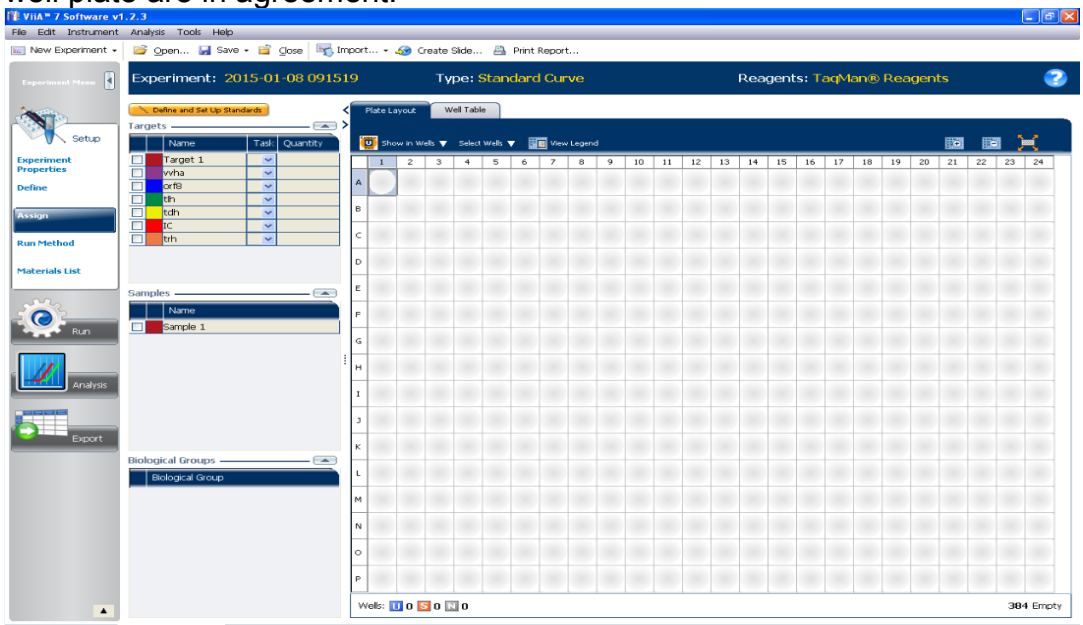
8.2.5.15 The "Define" tab is used to select the targets of detection. The targets are saved to the library. Import targets from library. Select the *tlh, tdh, trh, vvha,* ORF8, and IC targets.



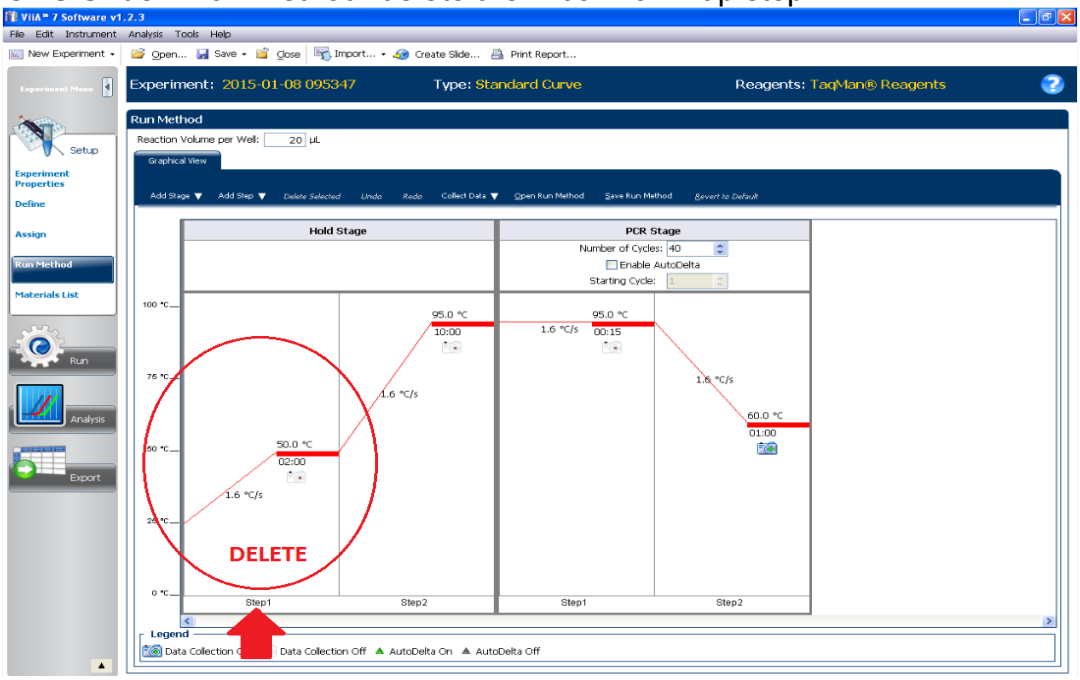
8.2.5.16 At the bottom of the screen, select “NONE” from the drop down menu regarding “Passive Reference”.



8.2.5.17 Select the “Assign” tab and assign the appropriate wells with the corresponding targets of interest. Be sure to double check the map and 384-well plate are in agreement.

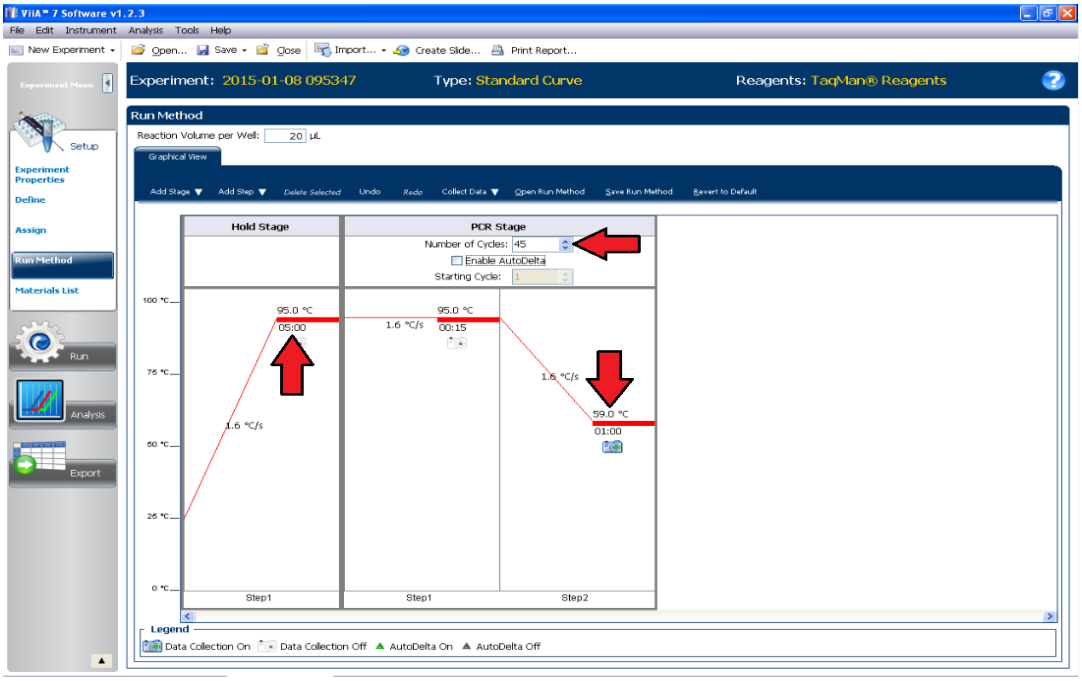


8.2.5.18 Under “Run Method” delete the initial warm-up step.



After doing so the parameters are the following:

- 95° C for 5 minutes
- 45 cycles of
  - 95° C for 15 seconds
  - 59° C for 60 seconds

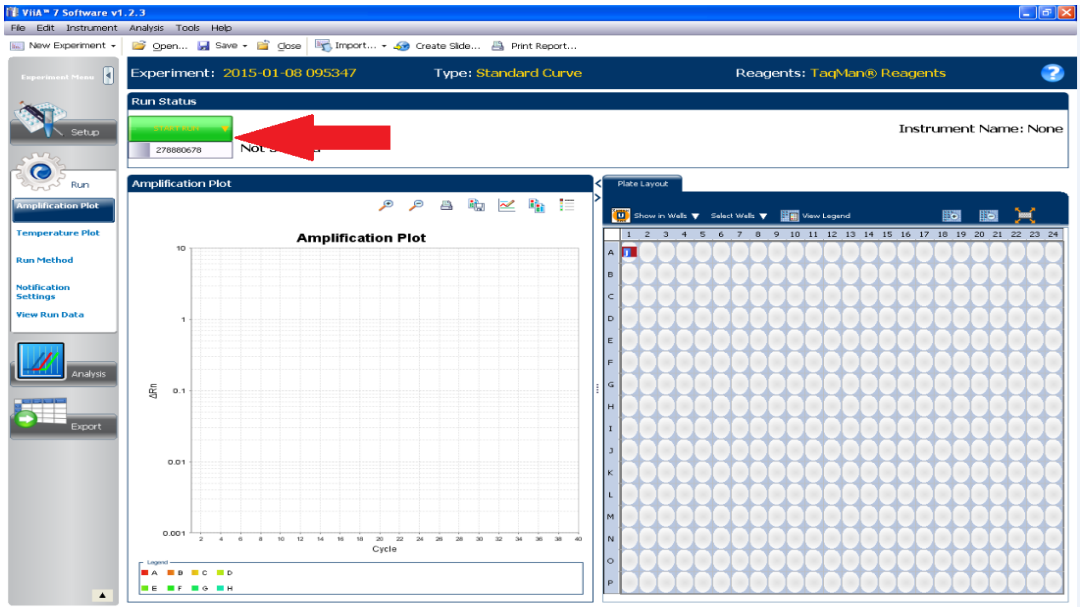


8.2.5.19 Turn on the machine user interface but touching the touchscreen. To open loading tray, touch the eject button.

8.2.5.20 Load the plate and close tray.

8.2.5.21 From the PC, click the “Run” tab and find the green “START RUN” button. Select the appropriate machine from the drop down menu and click

the “START RUN” button.



8.2.5.22 Save run file using sample numbers and date.

8.3 Measurements from Instruments

8.3.1 Once run is complete, remove plate from instrument and discard in appropriate waste receptacle.

8.3.2 Select “Analysis Settings” on the Amplification Plot screen.

8.3.3 Change all thresholds and baseline settings to “manual” and set all thresholds to 20,000 and leave baseline settings at 3 to 15.

8.3.4 Apply analysis settings and exit to Amplification Plot screen.

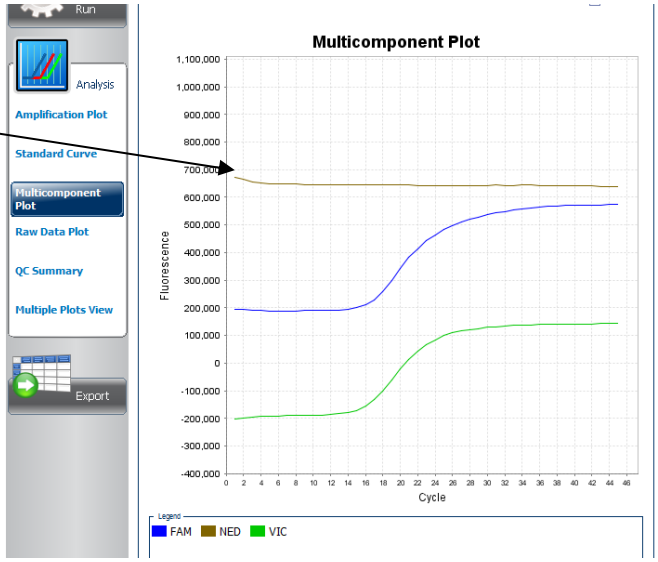
8.3.5 Select all wells in the plate by clicking in the upper left box of the plate layout.

8.3.6 View each target individually and make necessary changes to the threshold and baseline. The threshold should be set above background levels. It may be necessary to change baseline settings to lower background levels.

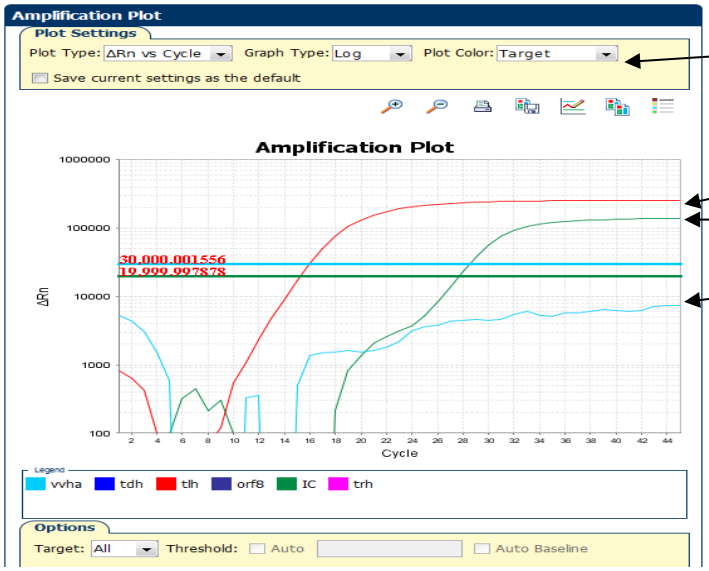
8.3.7 Acceptable threshold settings range from 10,000 to 50,000, if setting the threshold outside these values please consult with the lead or supervisor.

8.3.8 If baseline changes are necessary - view individual wells in the Multicomponent Plot screen. Change baseline settings as needed to exclude early background noise.

Exclude early background noise when setting baseline



8.3.9 Once threshold and baseline are set at appropriate levels, record results from each well for every target. Targets within the amplification plot may have high background in the early stages of the run (i.e. <10 cycles). Disregard background that crosses the threshold before cycle 10. Change the Plot color to "Target" to help read results.

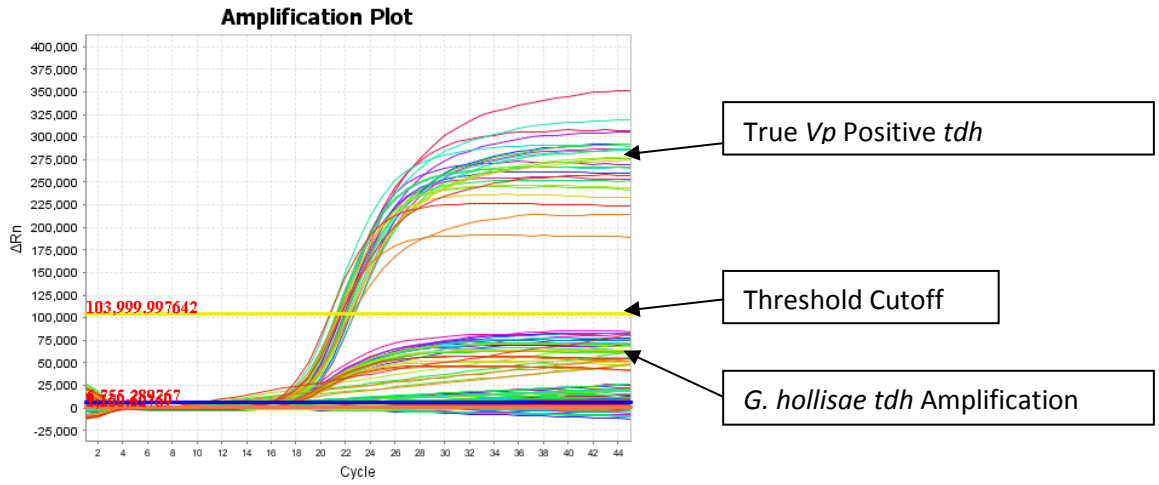


Plot Color = Target

Positive = amplification above threshold

Negative = No amplification

8.3.10 When reading *tdh* amplification results a ΔRn of greater than 104,000 (read in Linear setting) is necessary for a positive result. This cutoff will exclude any *G. hollisae tdh* amplification.



Post Analytic

1. Interpretation & Reporting of Results

1.1 Procedure for Abnormal Results

1.1.1 Abnormal MPN Index

1.1.1.1 If abnormal results appear to be caused by cross contamination (i.e. late CT value) rerun real-time PCR in duplicate of suspected contaminated wells

1.1.1.2 If duplicate results are in agreement, report these results. If the duplicates differ, report the results in agreement with ORIGINAL rtPCR run.

1.1.2 Positive pathogenic markers (*tdh, trh,* ORF8) in absence of *Vibrio parahaemolyticus* marker (*tlh)*

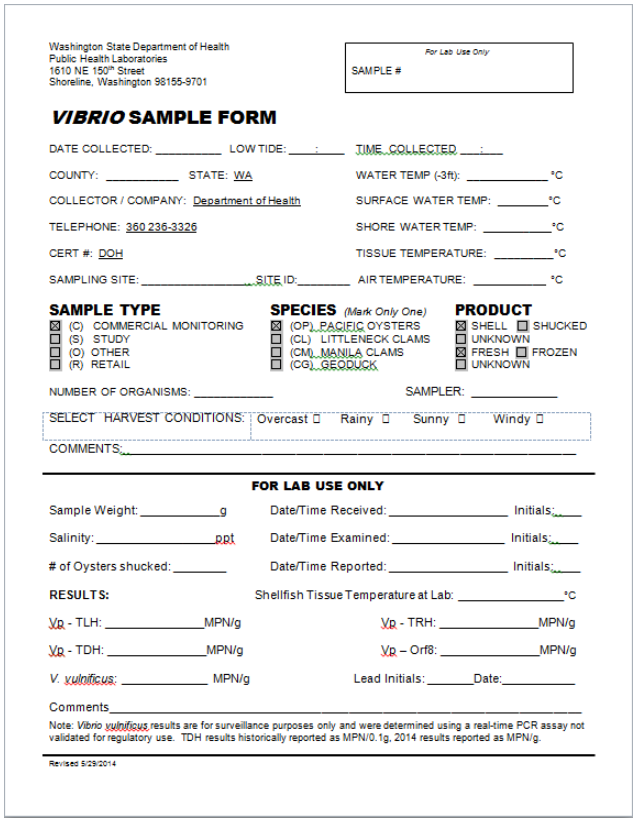
1.1.2.1 Current findings do not support pathogenic markers being present without *Vp* being present. Real-time PCR reactions resulting in this situation should be re-run upon Lead Microbiologist discretion.

1.1.2.2 The presence of the *trh* gene in the absence of the *tlh* gene has been documented. This is due to the *Vibrio parahaemolyticus trh* gene having 98% homology with the *trh* gene of *Vibrio alginolyticus*[i]. Any *trh* positive wells must be *tlh* positive as well.

1.2 Reporting Format

1.2.1 Real-time PCR results should be reported in MPN per gram according to FDA BAM 3 tube MPN chart

1.2.1.1 Sample Submission Form



Washington State Department of Health
Public Health Laboratories
1610 NE 150th Street
Shoreline, Washington 98155-9701

For Lab Use Only
SAMPLE #

VIBRIO SAMPLE FORM

DATE COLLECTED: _____ LOW TIDE: ___:___ TIME COLLECTED ___:___

COUNTY: _____ STATE: WA WATER TEMP (-3ft): _____ °C

COLLECTOR / COMPANY: Department of Health SURFACE WATER TEMP: _____ °C

TELEPHONE: 360.236-3326 SHORE WATER TEMP: _____ °C

CERT #: DOH TISSUE TEMPERATURE: _____ °C

SAMPLING SITE: _____ SITE ID:_____ AIR TEMPERATURE: _____ °C

SAMPLE TYPE
☒ (C) COMMERCIAL MONITORING
☐ (S) STUDY
☐ (O) OTHER
☐ (R) RETAIL

SPECIES *(Mark Only One)*
☒ (OP) PACIFIC OYSTERS
☐ (CL) LITTLENECK CLAMS
☐ (CM) MANILA CLAMS
☐ (CG) GEODUCK

PRODUCT
☒ SHELL ☐ SHUCKED
☐ UNKNOWN
☒ FRESH ☐ FROZEN
☐ UNKNOWN

NUMBER OF ORGANISMS: _____ SAMPLER: _____

SELECT HARVEST CONDITIONS: Overcast ☐ Rainy ☐ Sunny ☐ Windy ☐

COMMENTS:_____

FOR LAB USE ONLY

Sample Weight: _____g Date/Time Received: _____ Initials:____

Salinity: _____ppt Date/Time Examined: _____ Initials:____

# of Oysters shucked: _____ Date/Time Reported: _____ Initials:____

RESULTS: Shellfish Tissue Temperature at Lab: _____°C

Vp - TLH: _____MPN/g Vp - TRH: _____MPN/g

Vp - TDH: _____MPN/g Vp – Orf8: _____MPN/g

V. vulnificus: _____ MPN/g Lead Initials: _____Date:_____

Comments_____

Note: *Vibrio vulnificus* results are for surveillance purposes only and were determined using a real-time PCR assay not validated for regulatory use. TDH results historically reported as MPN/0.1g, 2014 results reported as MPN/g.

Revised 5/29/2014

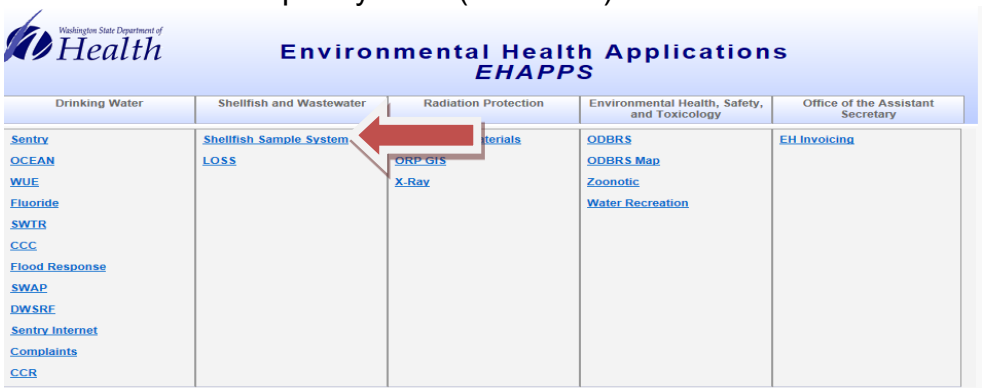
1.3 Prioritizing Results-N/A

1.4 Entering Laboratory Results in Reporting System

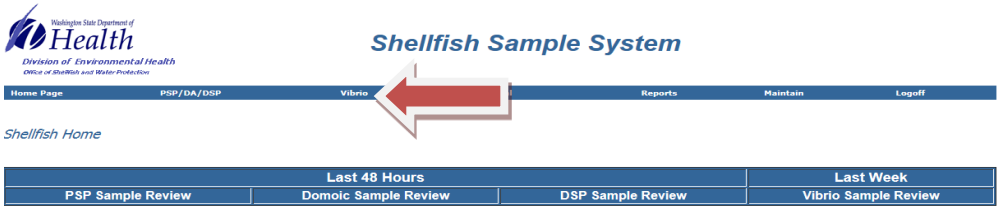
1.4.1 Office of Shellfish and Water Protection database

1.4.1.1 Access to the database must be authorized. Lead Microbiologist will facilitate the authorization process.

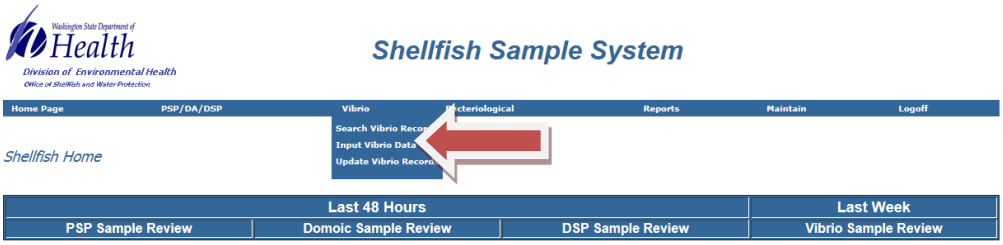
1.4.1.2 After entering web address, find column “Shellfish and Wastewater” and click “Shellfish Sample System” (red arrow)



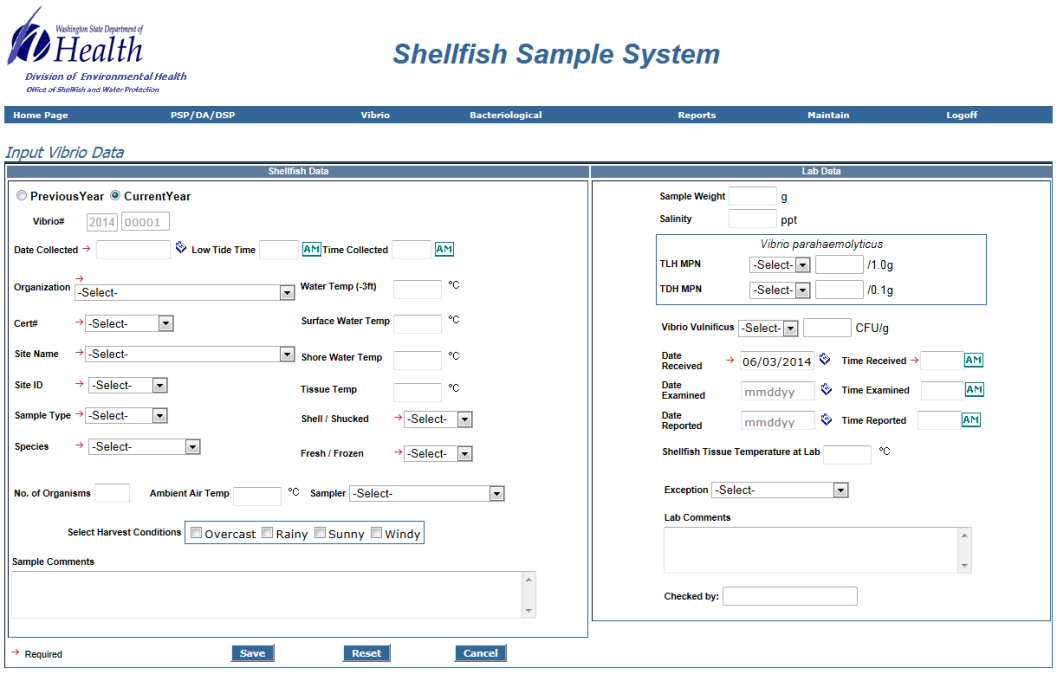
1.4.1.3 Along the top, hover mouse over “Vibrio” (red arrow)



1.4.1.4 Click “Input Vibrio Data” (red arrow)



1.4.1.5 The sample number will auto-populate. Enter all information and data provided from Vibrio Sample Submission form. Enter final results if available.



1.5 Notification of Test Results

1.5.1 Fax

1.5.1.1 Test results are faxed to the Office of Shellfish and Water Protection after Lead Microbiologist approval and signature.

1.5.1.2 Fax cover sheet and *Vibrio* sample submission form(s) to X2257. Completely fill out cover sheet and note which sample numbers are contained in fax (i.e. *Vibrio* S14-001 through S14-003).

1.5.2 Phone

1.5.2.1 For STAT results (per request of OSWP), the Lead Microbiologist will contact the appropriate personnel at OSWP

1.6 Archiving Results & Retention

1.6.1 Filing Results

1.6.1.1 Results are to be filed in filing cabinet located in Food and Shellfish Bacteriology Laboratory.

1.6.1.2 File approved samples that have NOT been entered into the Office of Shellfish and Water Protection (OSWP) database in the folder titled “Results NOT Entered in Database”

1.6.1.3 File approved samples that have been entered into the OSWP database into the folder titled “Entered in Database-Need Lead Approval”. The lead microbiologist will then file completed samples into appropriate folder.

1.6.2 Retention

1.6.2.1 Sample results and all paperwork pertaining to samples will be kept in Food and Shellfish Bacteriology Laboratory file cabinet for a minimum of seven (7) years.

1.6.2.2 OSWP database is maintained by OSWP

2. Calculations

2.1 Instructions

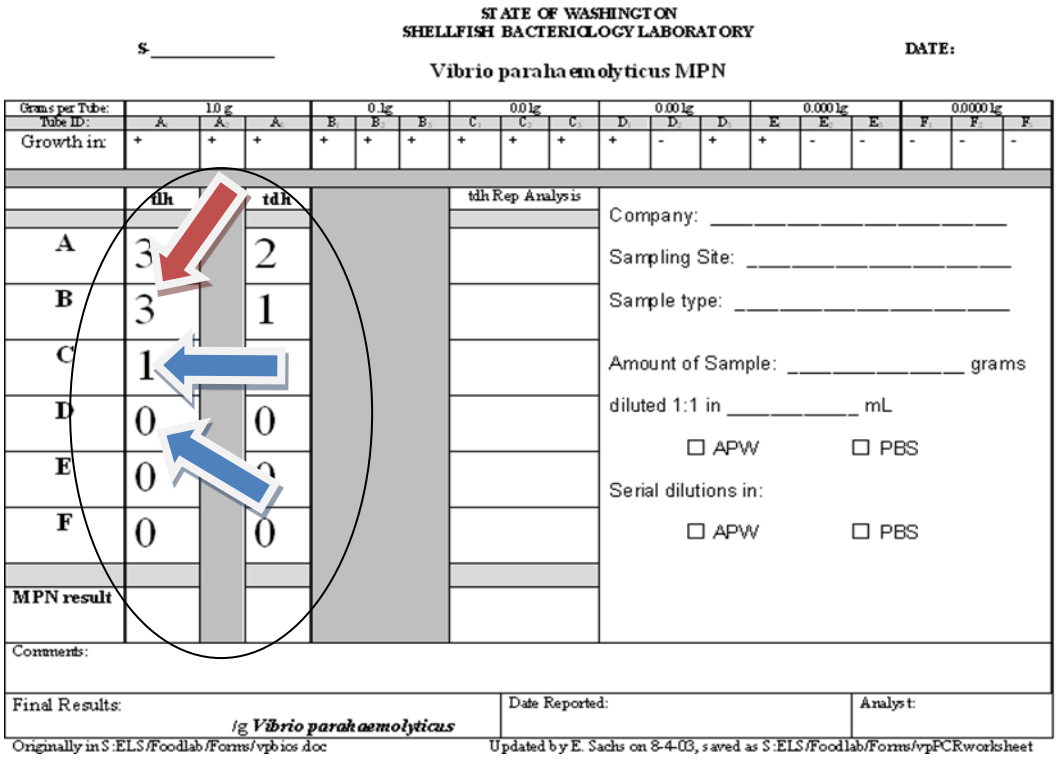
2.1.1 To calculate the concentration we must first determine the MPN index for all targets (*tlh*, *tdh, trh*, *ORF8*, and *vvhA*).

2.1.2 The MPN index is a 3 digit number where each digit represents the number of positive tubes in a given dilution

2.1.3 The first dilution set used in the MPN index is always the most dilute set of three tubes that are all positive for a given target. The next two digits represent the number of positive tubes in the next two dilution series.

2.1.3.1 Examples

2.1.3.1.1 The circled columns represent the number of positive (by PCR) tubes in each dilution set. The MPN index for TLH in the example below is 3-1-0. The first number is selected by finding the most dilute set of tubes that are all positive (red arrow). The next two numbers in the index are the numbers of positive tubes in the next two dilution sets (blue arrows).


STATE OF WASHINGTON
SHELLFISH BACTERIOLOGY LABORATORY

S-_____ DATE:

Vibrio parahaemolyticus MPN

Grams per Tube:	1.0 g			0.1g			0.01g			0.001g			0.0001g			0.00001g		
Tube ID:	A	A	A	B	B	B	C	C	C	D	D	D	E	E	E	F	F	F
Growth in:	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-

	tlh	tdh	tdh Rep Analysis
A	3	2	
B	3	1	
C	1		
D	0	0	
E	0	0	
F	0	0	
MPN result			

Company: _____

Sampling Site: _____

Sample type: _____

Amount of Sample: _____ grams

diluted 1:1 in _____ mL

☐ APW ☐ PBS

Serial dilutions in:

☐ APW ☐ PBS

Comments:

Final Results: /g *Vibrio parahaemolyticus*

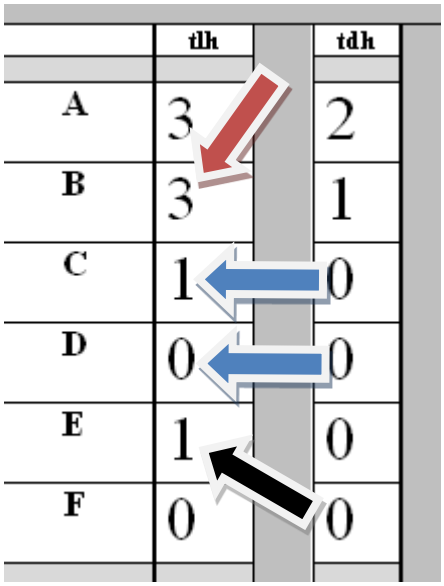
Date Reported:

Analyst:

Originally in S:ELS/Foodlab/Forms/vpbios.doc Updated by E. Sachs on 8-4-03, saved as S:ELS/Foodlab/Forms/vpPCRworksheet

2.1.3.1.2 The following sample has an MPN index of 3-1-1. The first number is selected by finding the most dilute set of tubes that are all positive (red arrow). Typically, the next two numbers in the index are the numbers of positive tubes in the next two dilution sets (blue arrows). However, when there is a positive tube that exists beyond the selected dilution sets (black

arrow), that tube is added to the positive total of the last selected dilution set.



	tlh	tdh
A	3	2
B	3	1
C	1	0
D	0	0
E	1	0
F	0	0

2.1.3.1.3 The following results are unable to generate an MPN index. Unusual MPN indexes are typically due to some sort of contamination. It may be necessary to re-extract and/or re-run PCR. If this does not resolve the issue, further investigation is required to determine the source of contamination.

	tlh	tdh
A	3	2
B	2	1
C	1	0
D	3	0
E	0	0
F	0	0

2.1.4 Once the MPN index has been determined, calculate the concentration by using the MPN chart. According to the 3 tube MPN chart from the FDA Bacteriological Analytical Manual, an index of 3-1-1 corresponds to a concentration of 75 MPN/g of shellfish tissue. Always ensure that you have accounted for any dilutions when determining final concentrations. Since the amount of tissue in our MPN index matches the column headings on the MPN chart, in this case all dilutions are accounted for

2.1.4.1 MPN Chart from FDA BAM

Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	–	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	–

2.2 Equations

2.3 Computer Validation Protocol

2.4 Examples

3. Expected Values-N/A

4. Method Limitations

4.1 Reportable Range

4.1.1 *tlh*: <0.3 MPN/g to >110,000 MPN/g

4.1.2 *tdh*: <0.3 MPN/g to >110,000 MPN/g

4.1.3 *trh*: <0.3 MPN/g to >110,000 MPN/g

4.1.4 *ORF8*: <0.3 MPN/g to >110,000 MPN/g

4.2 Interfering Substances

4.2.1 Upon validation, *Grimontia hollisae* (formerly *Vibrio hollisae*) has been found to have cross reactivity with the real-time PCR assay. *G. hollisae* shows slight amplification for the *tdh* marker. See discussion in Analytic 8.3.10 on how to determine true *Vp tdh* amplification vs. *G. hollisae* amplification.

4.2.2 As noted in the Post Analytic 1.1.2.2, a strain of *Vibrio alginolyticus* possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*. Most probable number values for *trh* should be reported only if *tlh* is present in the

corresponding tube. Tubes only positive for *trh* should not be accounted for when generating the MPN value.

5. Procedure Notes

5.1 Reason for Special Precaution
5.2 Possible Sources of Errors
5.3 Helpful Hints
5.4 Situations That May Influence Test
5.5 Applications
5.6 Turn Around Time
5.6.1 Samples results from samples that meet all criteria of acceptable submission should be reported to the Office of Shellfish and Water Protection within 3 days
5.7 Alternative Back up Procedures
5.8
5.9 Definitions

6. References

6.1 Manufacturer Product Literature
6.2 Textbooks
6.3 Standards Publications
6.4 Written Personal Communications
6.5 Research

7. Supplemental Materials

7.1 Manufacturer Product Instructions
7.2 Flow Diagrams
7.3 Method Evaluation Assessment Sheet
7.4 Proficiency Provider

[i] González-Escalona, Narjol, George M. Blackstone, and Angelo DePaola. "Characterization of a Vibrio alginolyticus strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of Vibrio parahaemolyticus." Applied and environmental microbiology 72.12 (2006): 7925-7929.

Proposal No. **15-112**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Executive Board
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Direct Plating Method for trh
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	This method was developed by Jessica Jones (FDA Gulf Coast Seafood Laboratory) and is being submitted by the ISSC Executive Board. The Executive Board granted interim approval to this method on March 13, 2015. The Executive Board is submitting this proposal to comply with Article V. Section 1. of the ISSC Constitution, Bylaws, and Procedures.

Submitted by method developer Jessica Jones (FDA Gulf Coast Seafood Laboratory)

5. Approved Methods for Vibrio Enumeration

	Vibrio Indicator Type:	Application: PHP Sample Type: Shucked	Applicatio Reopenin
EIA[1]	*Vibrio vulnificus (V.v.)*	X	
MPN[2]	*Vibrio vulnificus (V.v.)*	X	
SYBR Green 1 QPCR-MPN[5]	*Vibrio vulnificus (V.v.)*	X	
MPN[3]	*Vibrio parahaemolyticus (V.p.)*	X	
PCR[4]	*Vibrio parahaemolyticus (V.p.)*	X	
Direct Plating[6]	*trh+ Vibrio parahaemolyticus (V.p.)*	X	X

Footnotes:

[1] EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, 1992.

[2] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA -alkaline phosphatase labeled gene probe (vvhA).

[3] MPN format with confirmation by biochemical analysis, gene probe methodology as listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.

[4] PCR methods as they are listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State

	can demonstrate is equivalent. [5]*Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123. [6]Direct plating method for *trh* as described in Nordstrom et al., 2006.
Public Health Significance	Scientific evidence suggests that the presence of the *trh* gene in *V. parahaemolyticus (V.p.)* is correlated with higher virulence. Additionally, at the 2013 conference, proposal 13-202 was adopted which requires testing for the presence of trh prior to reopening of growing areas closed as a result of *V.p.* illnesses [Chapter II @.01.F(5)]. Currently, there are no NSSP approved methods for enumeration of *trh*. This method is a needed option for testing following *V.p.* illness closures.
Cost Information	This method costs ~$5 per test for laboratory consumables, supplies, and reagents. Most equipment needed for testing is standard microbiology equipment, but purchase of a specialized water bath or environmental chamber may be necessary at a cost of ~$3,000-$5,000. Additional costs for a laboratory would vary based on their operational overhead and labor.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 15-112 to an appropriate committee as determined by the Conference Chair to further review the data submitted.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-112.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-112
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-112.

Validation Data for Direct plating method for trh

Name of Method Submitter: Jessica L. Jones, Ph.D.

Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as appropriate for PHP validation and verification testing, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked with a *tdh+/trh+ Vibrio parahaemolyticus* (unless otherwise noted), while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilution of the culture in triplicate onto TSA+2% NaCl. Appropriate aliquots of spiked samples were spread plated onto T1N3 agar and colony lifts hybridized with an alkaline phosphatase-labeled probe specific for *trh*.

1. Accuracy/Trueness: Using the data from Table 1, the average of plate counts was 3.80 log and the average from DNA probe was 3.62 log. The *Accuracy/Trueness of the method is 95%.*

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty.

Sample	Plate Count (log CFU)	Probe Result (log CFU/g)
1-2X	5.18	4.76
2-2X	5.18	4.65
3-4X	3.15	2.90
4-4X	3.15	2.85
5-6X	1.23	1.48
6-6X	1.23	1.00
7-2X	5.76	5.59
8-2X	5.76	5.64
9-4X	3.68	3.59
10-4X	3.68	3.72

2. Measurement Uncertainty: Using the data from Table 1 above, *measurement uncertainty is 0.11*.

3. Precision: Using the data from Table 2, there *was no significant difference between the plate counts and the values generated with DNA probe* (p=0.58). The difference in variance is not significant (p=0.48) for any platform/gene target combination.

4. Recovery: The average of plate counts was 3.40 log the average (adjusted for sample blanks) from DNA probe was 3.65 log. Using this data, the *Recovery of the methods was determined to be 107% on both platforms for both gene targets.*

Table 2. Data for determination of Precision and Recovery			
Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**Blank**	N/A	<1.00
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
3	**Blank**	N/A	<1.00
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
5	**Blank**	N/A	1.85
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48
5	**6Z**	1.23	1.30
7	**Blank**	N/A	1.00
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.7
9	**Blank**	N/A	<1.00
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48

5. Specificity: Samples were prepared as above and the interfering organism was spiked at an ~4 log higher concentration than *Vibrio parahaemolyticus*. Using the data from Table 3, the average *Specificity of the method is 1.38,* which is within the 95% confidence interval of the method (0.44) from 1.

Table 3. Data for determination of Specificity.	
	Probe Result

	(log CFU/g)	
Sample	**Spiked with Vp only**	**Spiked with Vp and Vv**
6-Blank	<1.00	---
6-6T	1.60	1.30
6-6U	1.30	<1.00
6-6W	1.48	1.48
6-6X	1.00	1.00
6-6Z	1.48	1.48

6. Working and Linear Range: Based on the data presented in Table 4, there is a significant correlation between the plate counts and CFU values by DNA probe ($p<0.001$). The *correlation coefficient is 0.96*, demonstrating the linearity of the method.

Table 4. Data for determination of Working and Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity

Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**1X**	6.18	5.36
1	**1Z**	6.18	6.18
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
1	**7X**	0.18	<1.00
1	**7X**	0.18	<1.00
3	**1X**	6.15	6.29
3	**1Z**	6.15	6.09
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
3	**7X**	0.15	1.00
3	**7Z**	0.15	<1.00
5	**1X**	6.23	5.57
5	**1Z**	6.23	5.64
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48

5	**6Z**	1.23	1.30
5	**7X**	0.23	1.30
5	**7Z**	0.23	1.48
7	**1X**	6.76	6.68
7	**1Z**	6.76	6.37
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.70
7	**7X**	0.76	1.00
7	**7Z**	0.76	<1.00
9	**1X**	6.68	6.44
9	**1Z**	6.68	4.70
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48
9	**7X**	0.68	<1.00
9	**7Z**	0.68	<1.00

7. Limit of Detection: The *Limit of Detection of the method is 10 CFU/g*. This is reliant upon the amount of sample (0.1g) that can be tested by the spread plate method.

8. Limit of Quantification/ Sensitivity: The limit of quantification/sensitivity is also reliant upon the amount of sample that can be tested.

9. Ruggednes: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 5, there was *no significant difference (p=0.94) between batches/lots* of media and reagents.

Table 5. Data for determination of Ruggedness.

Sample	**Probe Result (log CFU/g)**	
	Replicate 1 (X)	**Replicate 2 (Z)**
2	4.78	4.88
4	2.85	2.78

6	1.00	1.48
8	5.64	5.73
10	3.72	3.57

10. ***Matrix Effects:*** Effects of oyster matrix on the performance of the method was taken into consideration in testing all of the above criteria by using the sample blank.

11. Additional Data: *Inclusivity/Exclusivity*. Control filters with the isolates listed below were prepared and tested as outlined above. All isolates. All isolates gave the expected reaction, demonstrating 100% *Inclusivity/Exclusivity*.

Species	**Number of Strains Tested**	**Number *trh*-positive**
V. parahaemolyticus[†]	43	43
*V. parahaemolyticus**	39	0
V. cholerae	25	0
V. vulnificus	13	0
V. metschnikovii	12	0
V. fluvialis	6	0
V. hollisae	5	0
V. algenolyticus	2	0
Salmonella spp.	20	0
Listeria spp.	20	0
Other non-*Vibrio* species	15	0

[†] *V. parahaemolyticus* strains previously determined to be *trh*-positive.

***** *V. parahaemolyticus* strains previously determined to be *trh*-negative.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   1.1. Special Equipment and Materials Required
      1.1.1.Shaking water bath(s) (42°C and 54°C)
      1.1.2.Orbital shaker

1.1.3. Microwave
1.1.4. Plastic tubs with lids (300-500 ml capacity)
1.1.5. Whatman 541 filters, 85mm
1.1.6. Sterile spread rods
1.1.7. Sterile inoculating loops
1.1.8. Sterile toothpicks
1.1.9. Whirl-Pak bags (4.5”x9”)

1.2. Media and Reagents
1.2.1. Alkaline peptone water (APW)
1.2.2. Phosphate buffered saline (PBS)
1.2.3. Thiosulfate citrate bile salts sucrose (TCBS) agar
1.2.4. T_1N_3 agar
1.2.5. Lysis solution
1.2.6. 2M ammonium acetate
1.2.7. 20X SSC and 1X SSC
1.2.8. 1X SSC/SDS
1.2.9. Proteinase K
1.2.10. Hybridization solution
1.2.11. NBT/BCIP tablets
1.2.12. AP-labeled DNA probes (DNA Technology)

2. Outlined Procedure
2.1. Preparation of shellfish
2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.
2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.
2.1.3. Place clean shellstock on clean towels or absorbent paper.
2.1.4. Change gloves and brushes between samples.
2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.
2.1.6. Tare a sterile blender.
2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
2.1.9. The upper shell can then be pried loose at hinge and discarded.
2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
2.1.11. A minimum of 12 animals or 200g is required.
2.1.12. Blend without adding diluent for 60-120 sec at 14,000 rpm.

2.2. Preparation of spread plates
2.2.1. Prepare 10-fold serial dilutions of shellfish homogenate in PBS
2.2.2. Inoculate 100µl of appropriate dilutions onto pre-dried T_1N_3 agar plates
2.2.3. Spread inoculum gently into agar until completely absorbed
2.2.4. Invert plates and incubate at 30-37°C overnight.

2.2.5. Alternately, this method can be utilized with suspect isolates replicated to T_1N_3 agar from 96 well plates obtained from a standard MPN method

2.3. DNA Probe Colony Hybridization

2.4. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh*, AP-*tdh*, and AP-*trh*) can be stored in the refrigerator (4°C) for 1-2 years; do not freeze.

2.5. Filter Preparation

2.5.1. Label #541 Whatman filters with sample number, date, analyst initials, and probe to be hybridized with (*tlh, tdh,* or *trh*). Make sure orientation of filter is noted so that positive spots can be correlated to the appropriate well in the microtiter plate. A dot near the A1 well is sufficient.

2.5.2. Place each filter label-side down on appropriate T_1N_3 plate; apply gentle pressure to ensure contact with each colony. Allow labeled filter to sit at RT for 1-30 min. Transfer each filter with colony-side up to a plastic or glass petri dish lid containing 1 ml of lysis solution.

2.5.3. Microwave filters in petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown.

2.5.4. Transfer filters to a plastic wash container (up to 30 filters can be combined in one container) and neutralize with ammonium acetate (4 ml/filter) for 5 min on shaker at RT.

2.5.5. Decant ammonium acetate and rinse filters 2 times with 1X SSC buffer (10 ml/filter), for 2 min each time. (Filters can be air dried and stored at this point.)

2.6. Proteinase K (proK) treatment

2.6.1. Prepare proK solution (this is made by adding 10 ml/filter of 1X SSC and 20 µl/filter of proK stock solution) for the appropriate number of filters. Place filters (up to 30) in plastic wash container of proK solution. Incubate for 30 min in a 42°C water bath with shaking (50 rpm) to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

2.6.2. Decant proK solution. Rinse filter 3 times in 1X SSC (10 ml/filter) for 10 min at RT with shaking at 50 rpm. (Filters can be air dried by placing on paper towels and stored when completely dry.)

2.7. Hybridization

2.7.1. Place up to 5 proK-treated filters (either dried or straight from treatment) in a Whirl-Pak bag. Add 10 ml of pre-warmed hybridization buffer and close bag to exclude air. Avoid trapping air bubbles. Incubate filters for 30 min at 54°C in a shaking (50 rpm) water bath.

2.7.2. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/bag. Add probe (final conc. is 0.5 pmol/ml) to bag with filters. Reseal bag, excluding air, and incubate 1 h in a 54°C water bath with shaking. The temperature is critical for hybridization and washing steps.

2.7.3. Remove filters from hybridization bags and place in plastic wash container(s).

2.7.4. Add 10 ml/filter 1X SSC/1% SDS . Incubate in a 54°C water bath with shaking for 10 min. Repeat wash a second time.

2.7.5. Rinse filter 5 times for 5 min each in 1X SSC at RT on an orbital shaker, 100 rpm.

2.8. Color development

2.8.1. In petri dish, add 20 ml of NBT/BCIP solution. Add filters (5 or fewer) to dish and incubate with gentle shaking at 35-37°C; cover to omit light. Check development of positive control every 30 min.

2.8.2. Transfer filters to a plastic wash container and add tap water (10 ml/filter). Rinse filters at RT with shaking for 10 min. Repeat rinse 2 additional times to stop color development. Do not expose filters to light as they will continue to develop. Consider purple or brown spots positive.

Validation Data for Direct Plating Method for *trh*, Proposal 15-112

Name of Method Submitter: Jessica L. Jones, Ph.D.

Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as appropriate for PHP validation and verification testing of oysters, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked with a *tdh+/trh+ Vibrio parahaemolyticus* culture (unless otherwise noted), while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilutions of the culture in triplicate onto TSA+2% NaCl. Appropriate aliquots of spiked samples were spread plated onto T1N3 agar and colony lifts hybridized with an alkaline phosphatase-labeled probe specific for *trh* as detailed in the step-by-step procedure. Data were analyzed as described in the "SLV Documents for Marine Biotoxin and Non-MPN Based Microbiological Methods" on the ISSC website.

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty

Sample	Plate Count (log CFU/g)	Sample Blank Probe Result (log CFU/g)	Spiked Sample Probe Result (log CFU/g)
1-2X	5.18	<1.00	4.76
2-2X	5.18	<1.00	4.78
3-4X	3.15	<1.00	2.90
4-4X	3.15	<1.00	2.85
5-6X	1.15	1.85	1.48
6-6X	1.15	<1.00	1.00
7-2X	5.76	1.00	5.59
8-2X	5.76	<1.00	5.64
9-4X	3.68	<1.00	3.59
10-4X	3.68	<1.00	3.72
11-6X	1.60	<1.00	1.70
12-6X	1.60	<1.00	1.85
13-2X	5.72	<1.00	5.70
14-2X	5.72	<1.00	5.44
15-4X	3.62	<1.00	3.49
16-4X	3.62	<1.00	3.53
17-6X	1.74	1.00	1.70
18-6X	1.62	1.00	2.04
19-2X	5.74	<1.00	5.45
20-6X	1.62	1.00	1.78

1. Accuracy/Trueness: Using the data from Table 1, the average of plate counts was 3.52 log and the average from DNA probe (after adjustment with sample blank results) was 3.48 log. The *Accuracy/Trueness of the method is 99%.*

Measurement Uncertainty: Using the data from Table 1 above, *measurement uncertainty is 0.13* log CFU/g.

Table 2. Data for determination of Ruggedness		
Sample	**Probe Result (log CFU/g)**	
	Replicate 1 (X)	**Replicate 2 (Z)**
2	4.78	4.88
4	2.85	2.78
6	1.00	1.48
8	5.64	5.73
10	3.72	3.57
12	1.85	1.85
14	5.44	5.29
16	3.53	3.71
18	2.04	2.08
20	1.78	1.78

2. Ruggedness: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 2, a left skew of both sets of data was observed, with a variance ratio of 1.09 (not significant, p=0.91), so a paired t-test was used to compare the results. There was *no significant difference (p=0.94) between batches/lots* of media and reagents.

Table 3. Data for determination of Precision and Recovery				
Sample	**Aliquot**	**Plate Count (log CFU/g)**	**Sample Blank Probe Result (log CFU/g)**	**Spiked Sample Probe Result (log CFU/g)**
1	**2x**	5.18	<1.00	4.76
1	**2z**	5.18	<1.00	5.38
1	**4x**	3.18	<1.00	2.48
1	**4z**	3.18	<1.00	2.85
1	**6x**	1.18	<1.00	<1.00
1	**6z**	1.18	<1.00	1.00
3	**2x**	5.15	<1.00	4.65
3	**2z**	5.15	<1.00	4.76
3	**4x**	3.15	<1.00	2.90
3	**4z**	3.15	<1.00	3.04
3	**6x**	1.15	<1.00	<1.00
3	**6z**	1.15	<1.00	1.78
5	**2x**	5.16	1.85	3.54
5	**2z**	5.16	1.85	3.84
5	**4x**	3.16	1.85	2.70
5	**4z**	3.16	1.85	3.00

5	**6x**	1.15	1.85	1.48
5	**6z**	1.15	1.85	1.30
7	**2x**	5.76	1.00	5.59
7	**2z**	5.76	1.00	5.23
7	**4x**	3.76	1.00	3.57
7	**4z**	3.76	1.00	3.64
7	**5x**	2.76	1.00	2.58
7	**5z**	2.76	1.00	2.57
7	**6x**	1.76	1.00	1.78
7	**6z**	1.76	1.00	1.70
9	**2x**	5.68	<1.00	5.25
9	**2z**	5.68	<1.00	5.11
9	**4x**	3.68	<1.00	3.59
9	**4z**	3.68	<1.00	3.54
9	**6x**	1.68	<1.00	2.94
9	**6z**	1.68	<1.00	1.48
11	**2x**	5.60	<1.00	5.54
11	**2z**	5.60	<1.00	5.46
11	**4x**	3.60	<1.00	3.48
11	**4z**	3.60	<1.00	3.71
11	**6x**	1.60	<1.00	1.70
11	**6z**	1.60	<1.00	1.00
13	**2x**	5.72	<1.00	5.70
13	**2z**	5.72	<1.00	5.08
13	**4x**	3.72	<1.00	3.36
13	**4z**	3.72	<1.00	3.36
13	**6x**	1.72	<1.00	1.48
13	**6z**	1.72	<1.00	1.30
15	**2x**	5.62	<1.00	5.53
15	**2z**	5.62	<1.00	4.98
15	**4x**	3.62	<1.00	3.49
15	**4z**	3.62	<1.00	3.48
15	**6x**	1.62	<1.00	1.30
15	**6z**	1.62	<1.00	1.00
17	**2x**	5.74	1.00	5.51
17	**2z**	5.74	1.00	5.42
17	**4x**	3.74	1.00	3.57
17	**4z**	3.74	1.00	3.64
17	**6x**	1.74	1.00	1.70
17	**6z**	1.74	1.00	1.90
19	**2x**	5.74	<1.00	5.45

19	**2z**	5.74	<1.00	5.64
19	**4x**	3.74	<1.00	3.51
19	**4z**	3.74	<1.00	3.45
19	**6x**	1.74	<1.00	1.78
19	**6z**	1.74	<1.00	1.70

3. Precision: Using the data from Table 3, there was no significant difference (p>0.05) in the variance ratio across the range of concentrations, with a coefficient of variance of 45% for the method. Additionally, there *was no significant difference between the plate counts and the values generated with DNA probe* (p=0.68).

Recovery: The average of plate counts was 3.51 log CFU/g the average (adjusted for sample blanks) from DNA probe was 3.28 log CFU/g. Using this data, the *Recovery of the method was determined to be 93%.*

Table 4. Data for determination of Specificity				
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vv)**	**Log CFU/g**
6	**6T**	1.60	**VVT**	1.30
6	**6U**	1.30	**VVU**	<1.00
6	**6W**	1.48	**VVW**	1.48
6	**6X**	1.00	**VVX**	1.00
6	**6Z**	1.48	**VVZ**	1.48
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vc)**	**Log CFU/g**
12	**6T**	1.60	**VCT**	1.48
12	**6U**	2.00	**VCU**	<1.00
12	**6W**	1.70	**VCW**	1.60
12	**6X**	1.85	**VCX**	1.48
12	**6Z**	1.85	**VCZ**	1.00
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vf)**	**Log CFU/g**
18	**6T**	1.95	**VFT**	<10.00
18	**6U**	2.11	**VFU**	<10.00
18	**6W**	2.11	**VFW**	<10.00
18	**6X**	2.04	**VFX**	<10.00
18	**6Z**	2.08	**VFZ**	<10.00
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Va)**	**Log CFU/g**
20	**6T**	1.70	**VAT**	<1.00
20	**6U**	1.48	**VAU**	<1.00
20	**6W**	1.78	**VAW**	<1.00
20	**6X**	1.78	**VAX**	<1.00
20	**6Z**	1.78	**VAZ**	<1.00

4. Specificity: Interfering organisms tested were *V. vulnificus* (Vv)*, V. cholerae* (Vc)*, V. fluvialis* (Vf)*, and V. alginolyticus* (Va). Using the data from Table 4, the overall average *Specificity Index of the method is 1.43,* which is within the 95% confidence interval of the method (0.44) from 1.

However, significant differences between the average specificity indices and 1 were observed when examining the data from each interfering organism. Differences were significant for *V. fluvialis* ($p<0.001$) and *V. alginolyticus* ($p<0.001$), as well as *V. cholerae* ($p=0.05$). This significance is likely due to the differences in spike levels, rather than the specific organism as the ratio of *V. parahaemolyticus* to interfering organism was 1:13000, 1:6000, 1:1500, and 1:240 for *V. fluvialis, V. alginolyticus, V. cholerae,* and *V. vulnificus*, respectively. Additionally, it should be noted that filters were lifted from plates with more colonies than recommended from the samples with the interfering organisms to give the best likelihood of enumerating *V. parahaemolyticus*. Together, this indicates that the method specificity is dependent on the ratio of target to interfering organism, where ratios of 1:1000 may cause interference, but lower ratios do not. In real world samples, this ratio of *trh+ V. parahaemolyticus* to other *Vibrio* species, is unlikely, supporting the fit-for-purpose of this method.

Table 5. Data for Working/Linear Range

Sample	Plate Count	Replicate 1 (X)	Replicate 2 (Z)
1-1	6.18	5.36	6.18
1-2	5.18	4.76	5.38
1-4	3.18	2.48	2.85
1-5	2.18	2.00	1.85
1-6	1.18	<1.00	1.00
1-7	0.18	<1.00	<1.00
3-1	6.15	6.29	6.09
3-2	5.15	4.65	4.76
3-4	3.15	2.90	3.04
3-5	2.15	2.23	2.68
3-6	1.15	<1.00	1.78
3-7	0.15	1.00	<1.00
5-1	6.16	5.57	5.64
5-2	5.16	3.54	3.84
5-4	3.16	2.70	3.00
5-5	2.16	2.20	2.38
5-6	1.15	1.48	1.30
5-7	0.15	1.30	1.48
7-1	6.76	6.68	6.37
7-2	5.76	5.59	5.23
7-4	3.76	3.57	3.64
7-5	2.76	2.58	2.57
7-6	1.76	1.78	1.70

7-7	0.76	1.00	<1.00
9-1	6.68	6.44	4.70
9-2	5.68	5.25	5.11
9-4	3.68	3.59	3.54
9-5	2.68	2.49	2.79
9-6	1.68	2.94	1.48
9-7	0.68	<1.00	<1.00
11-1	6.60	6.40	6.44
11-2	5.60	5.54	5.46
11-4	3.60	3.48	3.71
11-5	2.60	3.06	2.84
11-6	1.60	1.70	1.00
11-7	0.60	<1.00	<1.00
13-1	6.72	6.71	5.55
13-2	5.72	5.70	5.08
13-4	3.72	3.36	3.36
13-5	2.72	2.48	2.38
13-6	1.72	1.48	1.30
13-7	0.72	<1.00	<1.00
15-1	6.62	6.22	6.40
15-2	5.62	5.53	4.98
15-4	3.62	3.49	3.48
15-5	2.62	1.85	1.60
15-6	1.62	1.30	1.00
15-7	0.62	<1.00	<1.00
17-1	6.74	6.33	6.39
17-2	5.74	5.51	5.42
17-4	3.74	3.57	3.64
17-5	2.74	2.60	2.72
17-6	1.74	1.70	1.90
17-7	0.74	1.30	<1.00
19-1	6.74	6.36	6.32
19-2	5.74	5.45	5.64
19-4	3.74	3.51	3.45
19-5	2.74	2.72	2.53
19-6	1.74	1.78	1.70
19-7	0.74	1.30	<1.00

5. Working and Linear Range: Based on the data presented in Table 4, the linear range of the method is 50 to 100,000 CFU/g. There is a significant correlation between the plate counts and CFU values by DNA probe ($p<0.001$), with a *correlation coefficient is 0.93*.

Limit of Detection: The theoretical LOD based on the data above is 4.7 CFU/g. However, this is reliant upon the amount of sample (0.1g) that can be tested by the spread plate method. Therefore, the *Limit of Detection of the method is 10 CFU/g*.

Limit of Quantitation/ Sensitivity: The limit of quantification/sensitivity is also reliant upon the amount of sample that can be tested. As such, the *Limit of Quantitation of the method is 10 CFU/g*.

Additional Data: *Inclusivity/Exclusivity*. Control filters with the isolates listed below were prepared and tested as outlined above. All isolates. All isolates gave the expected reaction, demonstrating 100% *Inclusivity/Exclusivity*.

Species	**Number of Strains Tested**	**Number *trh*-positive**
V. parahaemolyticus†	43	43
*V. parahaemolyticus**	39	0
V. cholerae	25	0
V. vulnificus	13	0
V. metschnikovii	12	0
V. fluvialis	6	0
Grimontia hollisae	5	0
V. alginolyticus	2	0
Salmonella spp.	20	0
Listeria spp.	20	0
Other non-*Vibrio* species	15	0

† *V. parahaemolyticus* strains previously determined to be *trh*-positive.

***** *V. parahaemolyticus* strains previously determined to be *trh*-negative.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   1.1. Special Equipment and Materials Required
      1.1.1.Shaking water bath(s) (42°C and 54°C)

1.1.2. Orbital shaker
1.1.3. Microwave
1.1.4. Plastic tubs with lids (300-500 ml capacity)
1.1.5. Whatman 541 filters, 85mm
1.1.6. Sterile spread rods
1.1.7. Sterile inoculating loops
1.1.8. Sterile toothpicks
1.1.9. Whirl-Pak bags (4.5”x9”)

1.2. Media and Reagents
1.2.1. Alkaline peptone water (APW)
1.2.2. Phosphate buffered saline (PBS)
1.2.3. Thiosulfate citrate bile salts sucrose (TCBS) agar
1.2.4. T_1N_3 agar
1.2.5. Lysis solution
1.2.6. 2M ammonium acetate
1.2.7. 20X SSC and 1X SSC
1.2.8. 1X SSC/SDS
1.2.9. Proteinase K
1.2.10. Hybridization solution
1.2.11. NBT/BCIP tablets
1.2.12. AP-labeled DNA probes (DNA Technology)

2. Outlined Procedure
2.1. Preparation of shellfish
2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.
2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.
2.1.3. Place clean shellstock on clean towels or absorbent paper.
2.1.4. Change gloves and brushes between samples.
2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.
2.1.6. Tare a sterile blender.
2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
2.1.9. The upper shell can then be pried loose at hinge and discarded.
2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
2.1.11. A minimum of 12 animals or 200g is required.
2.1.12. Blend without adding diluent or with equal weight of diluent (APW or PBS) for 60-120 sec at 14,000 rpm.

2.2. Preparation of spread plates
2.2.1. Prepare 10-fold serial dilutions of shellfish homogenate in PBS
2.2.2. Inoculate 100µl of appropriate dilutions onto pre-dried T_1N_3 agar plates
2.2.3. Spread inoculum gently into agar until completely absorbed

2.2.4. Invert plates and incubate at 30-37°C overnight.

2.2.5. Alternately, this method can be utilized with suspect isolates replicated to T_1N_3 agar from 96 well plates obtained from a standard MPN method

2.3. DNA Probe Colony Hybridization

2.4. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh*, AP-*tdh*, and AP-*trh*) can be stored in the refrigerator (4°C) for 1-2 years; do not freeze.

2.5. Filter Preparation

2.5.1. Label #541 Whatman filters with sample number, date, analyst initials, and probe to be hybridized with (*tlh, tdh,* or *trh*). Make sure orientation of filter is noted so that positive spots can be correlated to the appropriate well in the microtiter plate. A dot near the A1 well is sufficient.

2.5.2. Place each filter label-side down on appropriate T_1N_3 plate; apply gentle pressure to ensure contact with each colony. Allow labeled filter to sit at RT for 1-30 min. Transfer each filter with colony-side up to a plastic or glass petri dish lid containing 1 ml of lysis solution. Alternatively, to avoid overheating, a Whatman #3 filter pad can be saturated with 4ml of lysis buffer in the bottom of the petri dish onto which the #541 filter is transferred.

2.5.3. Microwave filters in petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown.

2.5.4. Transfer filters to a plastic wash container (up to 30 filters can be combined in one container) and neutralize with ammonium acetate (4 ml/filter) for 5 min on shaker at RT.

2.5.5. Decant ammonium acetate and rinse filters 2 times with 1X SSC buffer (10 ml/filter), for 1-2 min each time. (Filters can be air dried and stored at this point.)

2.6. Proteinase K (proK) treatment

2.6.1. Prepare proK solution (this is made by adding 10 ml/filter of 1X SSC and 20 µl/filter of proK stock solution) for the appropriate number of filters. Place filters (up to 30) in plastic wash container of proK solution. Incubate for 30 min in a 42°C water bath with shaking (50 rpm) to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

2.6.2. Decant proK solution. Rinse filter 3 times in 1X SSC (10 ml/filter) for 10 min at RT with shaking at 50 rpm. (Filters can be air dried by placing on paper towels and stored when completely dry.)

2.7. Hybridization

2.7.1. Place up to 5 proK-treated filters (either dried or straight from treatment) in a Whirl-Pak bag. Add 10 ml of pre-warmed hybridization buffer and close bag to exclude air. Avoid trapping air bubbles. Incubate filters for 30 min at 54°C in a shaking (50 rpm) water bath.

2.7.2. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/bag. Add probe (final conc. is 0.5 pmol/ml) to bag with filters. Reseal bag, excluding air, and incubate 1 h in a 54°C water bath with shaking. The temperature is critical for hybridization and washing steps.

2.7.3. Remove filters from hybridization bags and place in plastic wash container(s).

2.7.4. Add 10 ml/filter 1X SSC/1% SDS . Incubate in a 54°C water bath with shaking for 10 min. Repeat wash a second time.

2.7.5. Rinse filter 5 times for 5 min each in 1X SSC (10ml/ filter) at RT on an orbital shaker, 100 rpm.

2.8. Color development

2.8.1. In petri dish, add 20 ml of NBT/BCIP solution. Add filters (5 or fewer) to dish and incubate with gentle shaking; cover to omit light. Incubation temperatures from room temperature up to 40˚C can be used; color development will be quicker at higher temperatures. Check development of positive control every hour.

2.8.2. Transfer filters to a plastic wash container and add tap water (10 ml/filter). Rinse filters at RT with shaking for 10 min. Repeat rinse 2 additional times to stop color development. Do not expose filters to light as they will continue to develop. Consider purple or brown spots positive.

Proposal No. **15-114**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Executive Board
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Pre-Proposal for Male-Specific Coliphage Enumeration in Wastewater by Direct Double-Agar Overlay Method
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	The submitter of the pre-proposal requests approval to submit a full proposal to the ISSC for approval of the analytical method for use in the NSSP. Submitted by the developer Kevin Calci (FDA Gulf Coast Seafood Laboratory) Proposed Use of the Method: This method is applicable for the enumeration of MSC wastewater influent, effluent and sewage contaminated surface waters. The method will directly determine the quantity of MSC in wastewater to provide information of the viral reduction efficiencies of wastewater treatment plants. Method is also applicable for the analysis of surface source waters as part of a shoreline survey. Description of Method: This method employs E. coli HS (pFamp) RR as a male-specific coliphage host in a direct double agar overlay for the quantification of plaque forming units. All sample volumes are plated in triplicate. Briefly, 2.5ml of sample is mixed with 2.5ml of soft agar and 0.2ml of Famp host and then poured onto bottom agar petri plate. One ml of the sample is serially diluted down to 1:10 and 1:100. Those two dilutions are then plated by placing 2.5ml of sample is mixed with 2.5ml of soft agar and 0.2ml of Famp host and then poured onto bottom agar petri plate. The plates are incubated at 35-37°C for 16-20 h. Under indirect light the plaque forming units are counted. The working range of the 9 plate method would be 14pfu/1OOml to 1.0 x 106 pfu/1 OOml.
Public Health Significance	Scientific consensus at the MSC informational meeting supported the use of MSC to evaluated wastewater treatment plant viral reduction efficiency to better inform the SSCA's conditional management plans impacted by wastewater treatment plant operations. This method would identify a consistent and accurate measure of MSC load in wastewater influent, effluent and surface waters.
Cost Information	
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 15-114 to an appropriate committee as determined by the Conference Chair to await SLV data.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-114.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-114.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-114.

Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the ISSC LMR Committee for acceptance will require at a minimum 6 months for review from the date of submission.

Name of the New Method	Male-specific Coliphage for Wastewater
Name of the Method Developer	USFDA Gulf Coast Seafood Laboratory
Developer Contact Information	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	
What is the intended purpose of the method?	Y	
Is there an acknowledged need for this method in the NSSP?	Y	
What type of method? i.e. chemical, molecular, culture, etc.	Y	Culture method for Male-specific Coliphage in Wastewater Influent, Mid-process Samples, and Effluent

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any proprietary aspects	N	
Equipment required	Y	
Reagents required	Y	
Sample collection, preservation and storage requirements	Y	

Safety requirements	Y	
Clear and easy to follow step-by-step procedure	Y	
Quality control steps specific for this method	Y	

C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement uncertainty	Y	
3. Precision characteristics (repeatability)	Y	
4. Recovery	Y	
5. Specificity	NA	
6. Working and Linear ranges	Y	
7. Limit of detection	Y	
8. Limit of quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix effects	N	
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	NA	

D. Other Information		
1. Cost of the method	Y	
2. Special technical skills required to perform the method	Y	
3. Special equipment required and associated cost	Y	
4. Abbreviations and acronyms defined	Y	
5. Details of turn around times (time involved to complete the method)	Y	
6. Provide brief overview of the quality systems used in the lab	Y	

Submitters Signature	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:
Recommendations for further work	Date:

Comments:

Single Laboratory Validation (SLV) Protocol For Submission to the ISSC For Method Approval

Name of the New Method -	A Culture Method/Double Agar Overlay Method for the Determination of Male-specific Coliphage (MSC) for Wastewater
Name of Method Developer -	Kevin Calci, USFDA-GCSL
Developer Contact Information -	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov
Date of Interim Submission -	May 15, 2017

Section A. Need for the New Method

FDA has long been using Male-Specific Coliphage (MSC) to evaluate the potential viral contamination of shellfish growing water by wastewater treatement plant (WTP) outfalls. Methods using MSC as an indicator of viral contamination have been successful in evaluation of viral persistence in molluscan shellfish impacted by WTP outfalls (Daskin et al, 2008)(ISSC MSC Workshop). Studies continue to show a significant inverse relationship between decreasing MSC levels in shellfish and increasing wastewater dilution, which is in turn strongly associated with increasing distance from the WTP disharges (Goblic et al, 2011). The relationship between the level of viral contamination in shellfish and dilution of treated wastewater is really contingent on the viral reduction efficientcy of the WTP impacting the area.

The purpose of this method is to assess the $\log_{10}$ reduction of MSC, as a process indicator for enteric viruses,namely Human Norovirus, in wastewater samples including raw influent, pre-disinfected effluent and final effluent. By comparing $\log_{10}$ values of these results, the viral reduction performance of a WTP can be assessed under different environmental and operational conditions (Amarasiri et al, 2017) (Pouillot et al, 2015). Understanding the viral reduction performance at different stages in a wastewater treatment process is a valuable assessment tool to determine growing area classsification and management options for shellfish growing areas adjacent to and downstream from the WTP outfall. This newly configured FDA method for the determination of MSC in wastewater samples has been adapted from previous methods so that it may be more readily implimented at NSSP Laboratories.

The recognized need for an alternative viral indicator is addressed in detail in the newly accepted 2015 Revision of the NSSP Guide for the Control of Molluscan Shellfish, Section IV Guidance Documents, Chapter II, @ .19, Determining Appropriately Sized Prohibited Areas Associated with Wastewater Treatment Plants, page 292. The need and utility for this method was likewise address at the MSC Informational Meeting of the Growing Area Committee (MSC Summit) in

Charlotte, NC in August 2014. A pre-proposal was reveiwed at the 2015 ISSC Meeting and given the Proposal Number of 15-114. The LMRC agreed that the pre-proposal was sufficient and that there is a need for the method. The LMRC recommended to Task Force I that Proposal 15-114 be referred to an appropriate committee as determined by the Conference Chair and await the SLV data.

Section B. Method Documentation

Modified Double Agar Overlay Method for Determination of Male-specific Coliphage in Wastewater

May 2017 Revision

This method for determining levels of male-specific coliphage in wastewater is based on the method described by Cabelli in work on the Narragansett Bay Project. (Cabelli, 1998) The development of an *Escherichia coli* host cell that consitutively expresses the F plasmid and is resistent to 95% of the somatic phage in wastewater was described in a subsequent paper. (DeBartolomeis and Cabelli,1991) FDA refined the method for oyster and hard clam meats as described in the workshop instructions, *Male-specific Bacteriophage (MSB) Workshop*, conducted in Gloucester, Massachusetts on March 9-12, 2004. (US Food and Drug Administration, 2004) This original FDA (2004) method was submitted as ISSC Proposal 05-114. Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clams and American Oysters in work funded by the Maine Technology Institute in 2006 with the assistance of Mercuria Cumbo of the Maine Department of Marine Resources. This method was approved for limited use by the 2009 ISSC in Manchester, NH. In work funded by UNH Sea Grant, SLV work continued for species extension to quahogs, which was approved for limited use by the 2013 ISSC in San Antonio, Texas. Method development and preliminary SLV trials were conducted in 2015 by Kevin Calci and Ashley Cooper at USFDA-GCSL. Additional SLV trials were conducted at the Spinney Creek Shellfish Laboratory in collaboration with Kevin Calci, the method developer in work supported by UNH Sea Grant.

A. Apparatus and Materials.

Equipment and Materials for Collection and Transport of Wastewater Samples:
250 or 500 ml Sterile Sample Containers
Sealable Bio-hazard Bags (used when shipping)
Labels
Cooler
Gel Packs
Sampling Device
10% Sodium Thiosulfate Solution (for effluent samples)

Laboratory Equipment:
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile
Mini vortexer
Autoclave, 119°C - 121°C
Refrigerator, 0–4° C
Freezer, -20°C

pH meter
Erlenmeyer flasks, 2L and 4L
Graduated cylinders, 1000 ml
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 □L volume)
Bacti-cinerator or flame
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45µm pore size
Syringes, sterile disposable; 5ml
Serological Pipets- 1 ml, 2 ml, 5 ml, 10 ml
Pipet-aid, or
(Micropipette option; 100 µL and 1000 µL (marked with red tape for positive controls), 200 µL (for aliquots of host cells), 2500 µL (for sample aliquots),
Micropipette tips, sterile 100 µL, 1000 µL, 2500 µL
Micropipette Stand)
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Dilution tubes, 16 x 150 mm, sterile with screw caps
50ml conical tubes, sterile with screw caps
Test tube racks--sizes to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil
Counter-pen, digital

Reagents:
Reagent water
Glycerol- sterile
Ethanol, 70% or laboratory disinfectant
Calcium chloride, 1M
Mineral oil
Sodium Thiosulfate (for effluent sample bottles to eliminate chlorine residual)

Antibiotic stocks:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)
Streptomycin and Ampicillin stock solutions (50 µg/ml each).
Note: Antibiotics must always be added to liquids and media after these have been autoclaved and cooled.

Media Components:
Agar, Granulated
Dextrose
NaCL

$CaCl_2$
DI water

Media:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli F_{amp} □ *E. coli* HS(pF_{amp})RR (ATCC # 700891).

MSC (Coliphage) Stock:
Type Strain - MS2, ATCC # 15597

B. Media Composition.

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
Final pH	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components, except antibiotics, to 990ml of dH2O in a 1000ml flask (increase flask size to make larger volumes). Dissolve, heat until clear, bringing to a boil.

2. Sterilize at 121°C ± 2°C for 15 minutes.

3. Temper to 50°C in the water bath.

4. Add 5 ml of Streptomycin sulfate/Ampicillin solution, aseptically to the flask (50 µg/ml each in final) and mix. Transfer to 2 – 500ml sterile jars (easier to pour plates from jars).

5. Pour 15-17 ml aliquots aseptically into sterile 100 x 15 mm Petri dishes and allow the agar to harden. Tip Petri dish lids off slightly to reduce condensation.

6. Store bottom agar plates inverted at 4°C and warm to room temperature for 1 hour before use.

7. Plates stored sealed at 4°C can be used up to 3 months.

Streptomycin sulfate/Ampicillin Solution:

1. Dissolve 0.5g of streptomycin sulfate and 0.5g of ampicillin in 50 ml of dH_2O with a sterile 100 ml graduated cylinder in sterile 600 ml beaker with sterile stir bar.

2. Stir for 2 to 3 minutes, no heat.

3. Filter by injecting through a sterile 0.22 µm filter.

4. Store in 5 ml aliquots in sterile 30 ml capped freezer vials at -20°C for up to one year. Label and date.

5. Allow to come to room temperature before adding and mixing in tempered bottom agar at 50°C.

DS Soft Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
1M $CaCl_2$	0.5 ml
Agar	7.0 g
DI water	500 ml
Final pH	6.7 ± 0.2

1. With gentle mixing, add all the components to 500 ml of dH_2O in a 1000 ml flask.

2. Bring flask contents to a boil.

3. Dispense in 2.5 ml aliquots into 16 x 100 ml tubes, cover and freeze (-20°C)

4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C in a water bath set to 50 ºC ± 2 ºC for no longer than 2 hours.

5. Store up to 3 months at -20 °C.

1M $CaCl_2$ Solution:

1. Add 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) to 100 ml dH_2O in a screw top bottle and dissolve or use prepared from VWR.

2. Sterilize by autoclaving at 121°C for 15 minutes.

3. Store up to three months at 4°C.

Growth Broth:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
DI water	1000 ml

1. With gentle mixing, add all the components to 1000 ml of dH_2O water in a 2000 ml flask.

2. Dissolve and dispense into sterile screw top containers.

3. Sterilize at 121°C ± 2°C for 15 minutes.

4. Store for up to three months at 4°C.

C. Storage and Propagation of Host Strain, E. coli F_{amp}:

Storage:

1. Lab stock culture – Frozen at – 80°C indefinitely (most desirable method) in broth culture containing 10% glycerol under no selective pressure. Selective pressure is reapplied when the culture is retrieved, by streaking onto Bottom Agar plates containing the two antibiotics.

2. Long-term working stock culture – Grown tryptic soy agar slant with sterile mineral oil overlay under no selective pressure and stored at room temperature in the dark for up to 2 years.

3 Long-term working stock – 6-hour grown tryptic soy agar slant and deep stab with sterile mineral oil overlay containing the two antibiotics, Ampicillin and Streptomycin (least desirable method).

4. Short-term working stock culture - Grown Bottom Agar streak plate stored at 4°C up to 3 weeks.

Glycerol Solution, 10%:

1. Add 9 ml of distilled water to 1 ml of undiluted glycerol.

2. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature.

3. For storage, add 1/5th volume of 10% glycerol solution, let stand for 30 minutes, dispense 1 ml aliquots in 2 ml cryo-vials and store at -70 to –80°C (best) or at –20°C.

D. Control Plates.

1. Negative Control - Add 2.5 ml of Growth Broth and 0.2 ml host to the 2.5 ml DS Soft Agar tube.

2. Positive Control - Make serial dilutions using growth broth of the concentrated MS2 control (to grow approximately 50-100 PFU per 2.5 ml), and add 2.5 ml of appropriate MS2 dilution and 0.2 ml of host to 2.5 ml DS Soft agar.

E. MSC Density Determinations in wastewater Treatment Plant (WWTP) Samples.

Sample Requirements:

1. Sterile 250 or 500 ml Nalgene bottles (or comparable bottle) with a permanent fill mark at the approximate 200 or 400 ml level are recommended for wastewater samples including influent, pre-treated effluent, and effluent.

2. Sample collection bottles must be properly labeled with sample number, location, sample type, date and time.

3. Sample bottles are filled to the 200 or 400 ml line. Effluent sample bottles must contain 1.0 ml of 10% sodium thiosulfate solution for 200 ml or 2.0 ml of 10% sodium thiosulfate solution to for 400 ml to inactivate any residual chlorine.

4. Wastewater samples are held under refrigerated conditions at 1-4ºC.

Note - A sealed bio-hazard bag is recommended for the samples bottles containing sewage samples going into an insulated shipping box when using overnight carriers.

Propagation of Host Cells:

1. Allow grown Bottom Agar streak plate and Growth Broth to temper to room temperature.

2. Vortex to aerate 20 ml of Growth Broth in a 16 x 150 mm tube, with screw cap.

3. Transfer host strain to Growth Broth using sterile swab to collect material from three colonies off grown Bottom Agar streak plate.

4. Gently shake to mix, then incubate at 35–37°C for 4-6 hours.

5. Once turbidity is observed, use of the host strain broth culture (log-phased growth) may commence.

Note - Following initial inoculation and mixing, do not shake or mix the host strain broth culture (to avoid mixing of cell debris at bottom with log-phase E. coli with pili)

Preparation of Wastewater samples for Analysis:

1. Analyst must wear gloves during handling of stir bars and sample bottle.

2. Water samples are removed from 1 - 4º C.

3. Sample bottle is shaken vigorously for 20 seconds (ensure cap is tightened), and a sterile magnetic stir bar is aseptically transferred to bottle.

4. Sample bottle is placed on stir plate set to medium for five minutes prior to analysis.

5. For the **high range** of this method a 10^{-2} decimal dilution is prepared by transferring 1ml of sample with a sterile 2 ml pipette (using a pipette aid) to a sterile 16x150mm screw cap tube containing 9 ml of growth broth. Sample tube is then vortexed for 10 seconds. For the second decimal dilution, 2ml are transferred from the first tube to a sterile 50ml conical tube with cap containing 18ml of growth broth using a second sterile 2ml pipette. The appropriately labeled 50 ml conical tube is then vortexed for 10 seconds.

6. For the **low range** of this method, 30ml of sample is transferred to a sterile 50 mm conical tube with cap using a sterile 10 ml pipette. The appropriately labeled 50 ml conical tube is vortexed for 10 seconds.

7. Prepped samples in labeled 50ml conical tube are stored in a test tube rack which can be stored short term at 0-4°C.

8. Return sample bottles to refrigeration and clean the work surface with disinfectant.

Note: The samples bottles containing wastewater samples should be autoclaved prior to disposal. Sample bottles must be washed and sterilized for re-use.

Direct Analytical Technique for WWTP samples:

This MSC method for wastewater has both a **high range** and a **low range** routine. Combined, the working range is from 5 to 1,200,000 PFU/100ml. The **high range** routine is adequate for enumeration of MSC in WWTP influent and has a working range from 1,000 to 1,200,000 PFU/100ml. The **low range** routine is generally adequate for enumeration of MSC in final effluent and has a working range from 5 to 12,000 PFU/100ml. When testing for pre-treatment effluent (before disinfection) or at times when the effluent is questionable, both high and low ranges routines should be used together.

1. In the morning, propagate host cells as described above.

2. Tubes may be inoculated on a staggered time schedule:

Tubes in incubator at 7:00am	Ready at 11:00am
Tubes in incubator at 8:00am	Ready at 12:00pm
Tubes in incubator at 10:00am	Ready at 2:00pm
Tubes in incubator at 11:00am	Ready at 3:00pm

3. Before experimentation, prepare the wastewater samples for analysis as described above.

4. One hour before experimentation (at 3 hours of host growth), autoclave required number of soft agar tubes at 121ºC for 15min. Temper soft agar tubes in water bath set to 50-52ºC.

High Range Routine:

For each high range (influent) sample, four (4) Bottom Agar plates and four (4) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 4 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 4 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar

mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **High Range** Routine Results;

N = Total number of PFUs counted on 4 the plates,

The maximum readable limit on PFUs count is 1000 on the four plate,

PFU count exceeding 1,000/4 plate is considered TNTC or >1,000,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{.1 \text{ ml}} = \text{N} * 1{,}000 \text{ PFU/100ml}$$

Example: High range version plate counts - 13, 23, 12, and 16 PFUs

Result = (64)(1000) = 64,000 PFU/100ml*

Low Range Routine:

For each low range (effluent) sample, eight (8) Bottom Agar plates and eight (8) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 8 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 8 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **Low Range** Routine Results;

N = Total number of Plaque forming units (PFUs) counted on 8 the plates,

The maximum readable limit on PFUs count is 2000 on the eight plate,

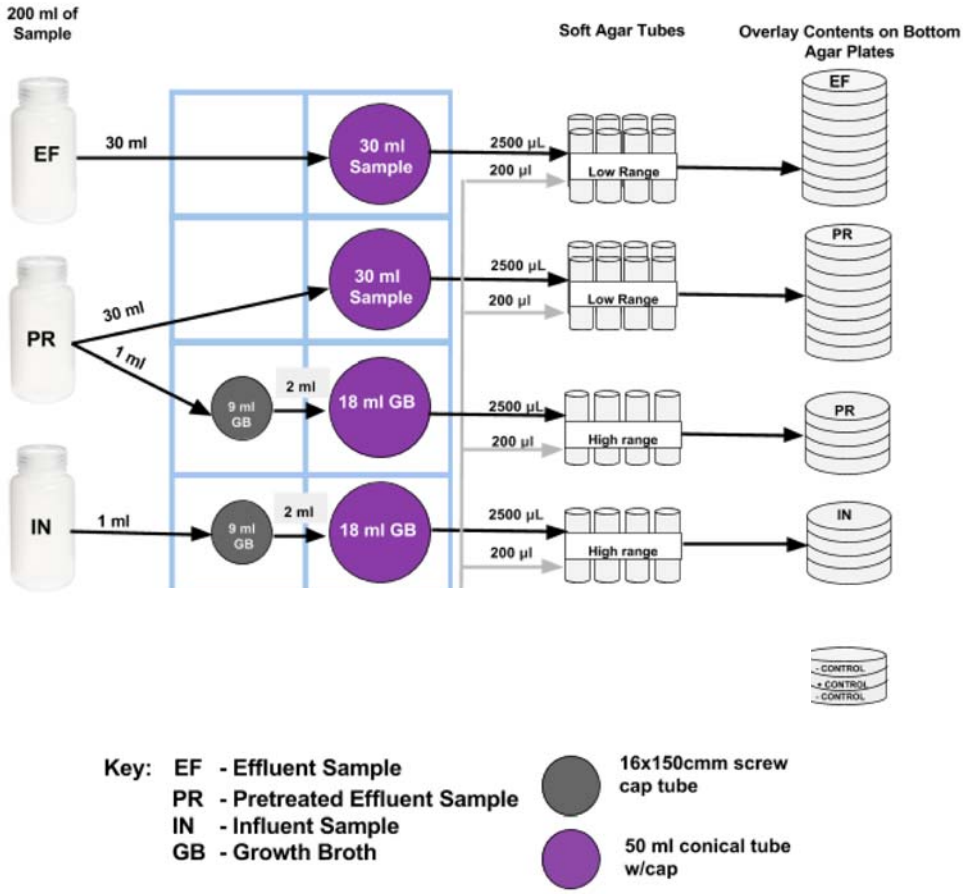
PFU count exceeding 2,000/8 plates is considered TNTC or >10,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{20 \text{ ml}} = \text{N} * 5 \text{ PFU/100ml}$$

Example: High range version plate counts - 21, 17, 20, 19, 13, 23, 12, and 16 PFUs

Result = (141)(5) = 702 PFU/100ml. Rounding off result to nearest 10s... Result = 700PFU/100gm*

MSC Method for Wastewater Schematic:



Samples Bench Sheet:

Sample Bench Sheet
For use with the NSSP Method for Determination of MSC in Wastewater
2016 Edition

Initiation Analysts: Jerry Jone Date: 10/10/16 Time: 1:00PM
Completing Analysts: Bill Bellicheck Date: 10/11/16 Time: 8:00AM

Low Range Routine

Sample#:1EF	WWTP: Hopetown, NE				Type: Effluent					Date/Time: 10/9/16
PFU Counts	3	2	0	1	0	2	0	1	= 9	45 PFU/100ml

Sample#:3Pre	WWTP: Hopetown, NE				Type: Pre-treatment					Date/Time: 10/9/16
PFU Counts	199	215	203	170	233	210	206	188	= 1,624	8,120 PFU/100ml

Sample#:	WWTP:				Type:					Date/Time:
PFU Counts									=	

Hign Range Routine

Sample#:2In	WWTP: Hopetown, NE				Type: Influent		Date/Time: 10/9/16
PFU Counts	171	193	201	177	= 742		177,000 PFU/100ml

Sample#:	WWTP: Hopetown, NE				Type: Pre-treatment		Date/Time: 10/9/16
PFU Counts	3	1	2	0	= 6		6,000 PFU/100ml

Sample#:	WWTP:				Type:		Date/Time:
PFU Counts					=		

For unknown or mid-range sample, run both rountines above on the sample

Controls:	Start Neg Contol:	count	End Pos Control:	count
		0	End Neg Control:	43 / 0

F. Sample Collection and Storage.

1. Record all pertinent information on the collection form.

2. During transportation store samples in a cooler at 0 to 10°C

3. At laboratory, store samples in a refrigerator at 0 to 4 °C.

4. Maximum holding times for wastewater samples is up to 72 hours.

G. Quality Assurance.

1. Positive and negative control plates are run with MSC analyses each day.

2. Media sterility checks are made per batch and records are maintained.

3. Media log book is maintained (pH, volume, weights of each components, lot numbers, etc.).

4. An intra- and inter-laboratory performance program is developed.

5. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16- 20 hours of incubation are counted as plaques. (Count the number of plaques on each plate.)

6. MSC determinations are reported as plaque forming unit (PFU) per 100 grams.

7. The desired range for counting is 0 to 300 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC) or >12,000 PFU/100ml for **Low Range** Routine and >1,200,000 PFU/100ml for **High Range** Routine.

8. Temperatures incubators are checked twice daily (at least 4 hours apart) to ensure operation within the stated limits of the method, and results are recorded in a logbook.

9. Check thermometers at least annually against a NIST-certified thermometer.

10. Calibrate the balance monthly using ASTM-certified Class 1 or 2 or NIST Class S reference weights.

11. Laboratory analysts adhere to all applicable quality control requirements set forth in the most recent version of FDA's *Shellfish Laboratory Evaluation Checklist*.

12. Calibration of micropipettes needs to be checked quarterly and records kept. Micropipettes used for handling MSC control and transferring host cells need to have a barrier tip or be dedicated to the specific use to prevent contamination

H. Safety.

Samples, reference materials, and equipment known or suspected to have sewage, coliphage and/or *E.coli* attached or contained must be sterilized prior to disposal.

I. Technical Terms.

°C	-	degrees Celsius
µL	-	microliter
g	-	gram
L	-	liter
M	-	molar
ml	-	milliliter
Ave.	-	average
MSC	-	Male-specific Coliphage, Male-specific Bacteriophage, F+ Bacteriophage
NIST	-	National Institute of Standards and Technology
PFU	-	plaque forming units
RT	-	room temperature
TNTC	-	too numerous to count

Host Strain:	*E.coli* F_{amp} bacteria (*E.coli* HS(pFamp)RR)
Male-specific Coliphage:	Viruses that infect coliform bacteria only via the F-pili.
Plaque :	Clear circular zones (typically 1 to 10 mm in diameter) in lawn of host cells after incubation.

References:

Amarasiri, M., M. Kitajima, T.H. Nguyen, S. Okabe, and D. Sano. Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater raclamation: Review. Water Research 121 (2017) 258-269.

Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. Report to the Narragansett Bay Project, Providence, RI.

Daskin, J. H., K.R. Calci, W. Burkhardt III, and R.H. Carmichael. Use of N stable isotope and microbial analyses to define wastewater influence in Mobile Bay, AL. Marine Pollution Bullentin 56 (2008) 860-868.

DeBartolomeis, J. and V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific Coliphages. Appl. Environ. Microbiol. 57(4):1201-1205.

Goblic, G.N., J.M. Anbarcian, J. Woods, W. Burkhardt III, and K.R. Calci. Evaluating the dilution of wastewater treatment plant effluent and viral impacts on shellfish growing areas in Mobile Bay, Alabama. Journal of Shellfish Research. 30:3, 1-9, 2011.

Pouillot, R., J.M. van Doren, , J. Woods, , D. Plante, M. Smith, G. Goblick, C. Roberts, A. Locas, W. Hajen, J. Stobo, J. White, J. Holtzman, E. Buenaventura, W. Burkhardt III, A. Catford, R. Edwards, A. DePaola, and K.R. Calci, 2015. Meta-analysis of the reduction of norovirus and male-specific coliphage concentrations in wastewater treatment plants. Appl. Environ. Microbiol. 81, 4669-4681.

U.S. Food and Drug Administration. 2004. Male-specific Coliphage (MSC) Workshop, conducted in Gloucester, Massachusetts on March 9-12, 2004.

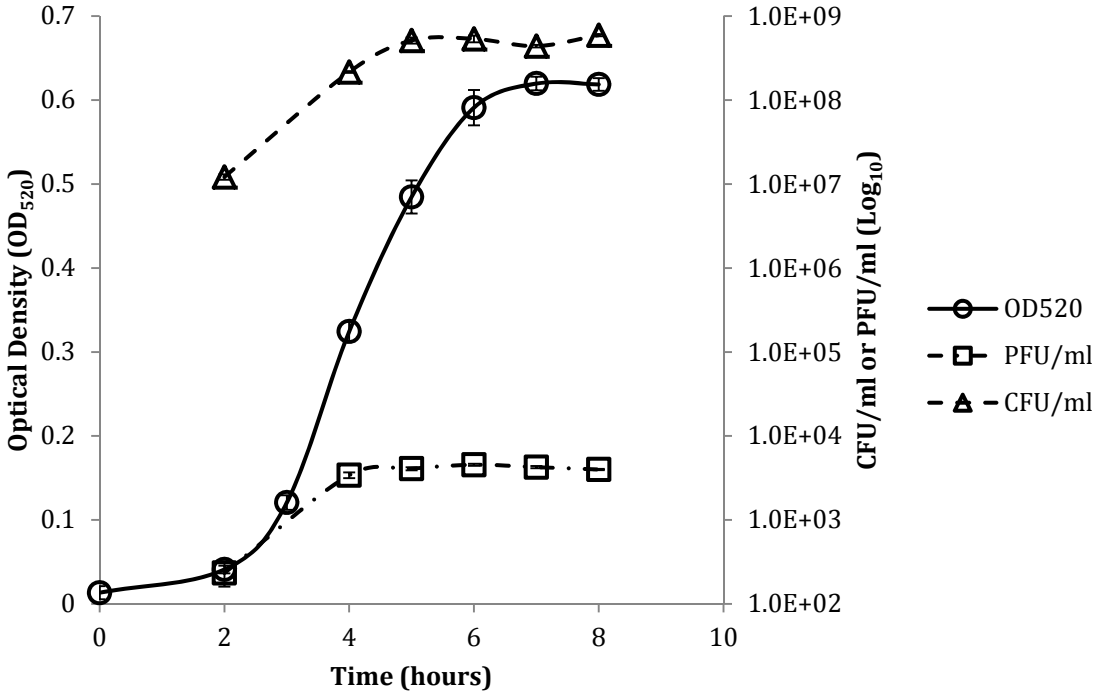
Other Information:

This method for the enumeration of male-specific coliphage in wastewater samples is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $18 per series of samples (an influent, a pre-disinfection effluent, and a final effluent sample). In a well set-up laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

To standardize these assessments, an index of viral performance for use in the NSSP to determine classification options adjacent to WWTP outfall can be estimated. Subtracting the log value of final effluent score from the log value of the corresponding raw influent score will yield an index of viral performance ranging from 0 to 5. A viral index of 4 to 5 indicates 99.99% to 99.999% reduction of enteric viruses and would be consider high performance. A viral index of 1 to 2 indicates 90% to 95% reduction of enteric viruses through the treatment process and would be considered poor performance. A viral index <1 would be considered ineffective and should lead the SSCA to consider 10,000:1 rather than 1000:1 for the determination of the size of the prohibited zone adjacent to the outfall.

C. Validation Criteria

Determination of Optimal Optical Density of Famp Host (OD)
Procedures for enumeration of double-agar overlay method for male-specific coliphage provide different ranges for OD of host growth. Effort was taken to determine the working range of the host *E. coli* at 520nm, which is the current EPA measurement. Graph 1 shows that at approximately 4 to 6 hours growth the OD520 of host is in the range of 0.35 to 0.6, during which time the MS2 plaquing efficiency of the host *E. coli* is optimal and consistent. Therefore, we conclude that a host OD520 of 0.35 to 0.7, or approximately 4 to 6 hours of growth, is ideal for MSC enumeration.



Graph 1. Optical Density (OD520) of *E. coli* HS(pFamp)RR in tryptone broth compared to plaque forming units (PFU) of MS2 coliphage. 10 ml of tryptone broth was inoculated with *E. coli* and incubated at 35ºC. OD was measured every hour starting at t = 2 hours. At these intervals 100 µL of host was serially diluted and 100 µL of pre-determined dilutions were plated to determine CFU/mL. 200 µL of the same host sample was used to determine PFU/ml of stock MS2 controls.

The Determination of LOD, LOQ, and Linear Range using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

The SOP for the determination of LOQ, LOD, and the Linear Range is the most robust of the SOPs and yields a database from which subsets of data can be use to generate other validation criteria. For this database ten trials were run using clean effluent samples. Great effort was taken to find and verify clean effluent. The Dover, NH WWTP and the Hampton, NH WWTP were identified as high performing plants of different design capable of consistently producing clean effluent. Table 1 list the metadata for the effluent samples collected for these trials

Table 1. Effluent Samples used for the Determination of LOD, LOQ, Linear Range

Trial #	Date Sampled	WWTP	Treatment Process
1	4/11/17	Dover	Tertiary, UV Dis-infection
2	4/11/17	Hampton	Secondary, Chlorine
3	4/18/17	Dover	Tertiary, UV Dis-infection
4	4/18/17	Hampton	Secondary, Chlorine
5	4/24/17	Dover	Tertiary, UV Dis-infection
6	4/24/17	Hampton	Secondary, Chlorine
7	5/1/17	Dover	Tertiary, UV Dis-infection
8	5/1/17	Hampton	Secondary, Chlorine
9	5/8/17	Dover	Tertiary, UV Dis-infection
10	5/8/17	Hampton	Secondary, Chlorine

For each of the 10 validation trials, 150 ml of clean effluent sample was aseptically transferred into 5-200ml sterile dilution bottles. A master spike solution was prepared in growth broth and was varied in concentration during the trials. The master spike solution was on the order of 10^3 MSC/ml. Four subsequent serial dilutions were made for each trial from the master spike at a 3:1 dilutions. This represented different spike concentrations over the working range of the method. The 5 dilution bottles were asceptically spiked with 5 ml of spike concentration 1 through 5, shaken vigourously and then 4 aliquots of 30 ml were transferred into 4-50 ml sterile conical tubes for each spike concentration (3 conical tubes for the replicates and a 4th tube for spike determination). In this way, 3 true replicates were generated at each of the 5 spike concentrations. This methodology was consistently applied throughout the ten trials. The 5 sets of 3 aliquots were processed and plated according to the method description above. Clean effluent was used instead of growth broth for the spike determination. Spike determinations using growth broth were underestimating the sample results. This problem was solved by using the same clean effluent similiarly spiked. This strategy was employed as there is no standard method available for a truly independant spike determinations.

Table 2 below shows the Spiking Study Database for MSC Method in Wastewater SLV. Spike Concentrations and MSC replicate plate count results are in units of PFU of MSC/100ml.

Table 2. Spiking Study Database for MSC Method in Wastewater SLV

WW Spiking Database 8 plate -

Date	X Value Measured Spike (PFU/100ml)	Repicate Plates (PFU/100ml)	Log of plates	Y Value RSD	Log RSD	X Value log of spike
	3400	4105	3.613	0.0025	-2.603	3.531
		4140	3.617			
		3980	3.600			
	1050	1025	3.011	0.0134	-1.874	3.021
		1225	3.088			
		1180	3.072			
Trial 1						
Dover	315	245	2.389	0.0352	-1.453	2.498
4/11/17		330	2.519			
		360	2.556			
	60	75	1.875	0.0629	-1.202	1.778
		60	1.778			
		45	1.653			
	10	30	1.477	0.2090	-0.680	1.000
		10	1.000			
		30	1.477			

	5950	5490	3.740	0.0046	-2.340	3.775
		5110	3.708			
		5155	3.712			
	1515	1355	3.132	0.0051	-2.289	3.180
		1365	3.135			
		1450	3.161			
Trial 2						
Hampton	410	225	2.352	0.0304	-1.517	2.613
4/11/17		225	2.352			
		170	2.230			
	70	65	1.813	0.0204	-1.690	1.845
		55	1.740			
		60	1.778			
	25	25	1.398	0.0321	-1.494	1.398
		30	1.477			
		25	1.398			

	5135	4315	3.635	0.0063	-2.199	3.711
		4800	3.681			
		4550	3.658			
	980	1085	3.035	0.0139	-1.856	2.991
		1005	3.002			
Trial 3		1220	3.086			
Dover						
4/18/17	405	385	2.585	0.0241	-1.618	2.607
		315	2.498			
		415	2.618			
	75	65	1.813	0.0379	-1.422	1.875
		90	1.954			
		80	1.903			
	25	20	1.301	0.1072	-0.970	1.398
		25	1.398			
		40	1.602			

	5175	4925	3.692	0.0065	-2.189	3.714
		5300	3.724			
		5490	3.740			
	1130	1280	3.107	0.0103	-1.986	3.053
		1160	3.064			
Trial 4		1340	3.127			
Hampton						
4/18/17	355	280	2.447	0.0317	-1.499	2.550
		335	2.525			
		405	2.607			
	40	60	1.778	0.0590	-1.229	1.602
		100	2.000			
		75	1.875			
	20	25	1.398	0.0634	-1.198	1.653
		30	1.477			
		20	1.301			

	11575	10655	4.028	0.0128	-1.891	4.064
		12800	4.107			
		10220	4.009			
	2080	2025	3.306	0.0212	-1.674	3.318
		2650	3.423			
		2735	3.437			
Trial 5						
Dover	525	680	2.833	0.0360	-1.444	2.720
4/24/17		705	2.848			
		465	2.667			
	190	205	2.312	0.0179	-1.746	2.279
		185	2.267			
		170	2.230			
	20	45	1.653	0.2069	-0.684	1.301
		60	1.778			
		15	1.176			

	12210	11140	4.047	0.0076	-2.121	4.087
		12165	4.085			
		10580	4.024			
	2555	2720	3.435	0.0058	-2.239	3.407
		2510	3.400			
		2520	3.401			
Trial 6						
Hampton	495	555	2.744	0.0395	-1.403	2.695
4/24/17		350	2.544			
		395	2.597			
	85	90	1.954	0.1082	-0.966	1.929
		110	2.041			
		45	1.653			
	20	20	1.301	0.1396	-0.855	1.301
		35	1.544			
		15	1.176			

	4430	3530	3.548	0.0131	-1.882	3.646
		4370	3.640			
		4075	3.610			
	1035	1100	3.041	0.0256	-1.592	3.015
		780	2.892			
		880	2.944			
Trial 7	240	275	2.439	0.0188	-1.727	2.380
Dover		230	2.362			
5/1/17		230	2.362			
	65	80	1.903	0.1126	-0.949	1.813
		75	1.875			
		35	1.544			
	10	20	1.301	0.1305	-0.884	1.000
		15	1.176			
		10	1.000			

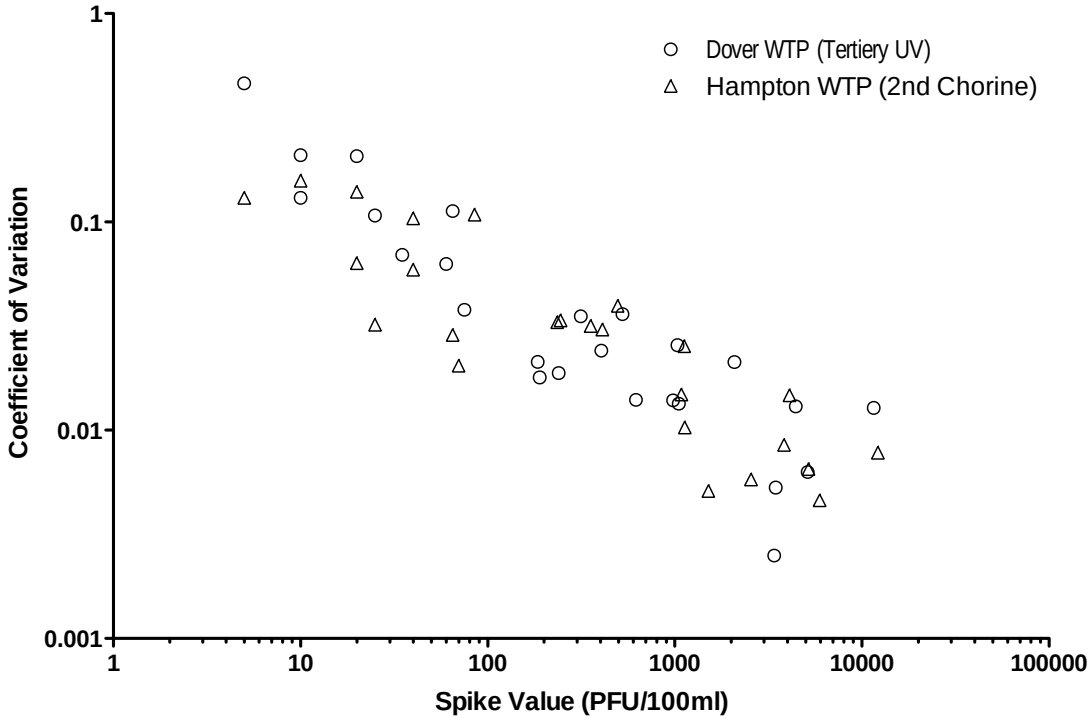
	4110	4415	3.645	0.0147	-1.833	3.614
		5630	3.751			
		5260	3.721			
	1125	955	2.980	0.0253	-1.596	3.051
		1060	3.025			
		1350	3.130			
Trial 8	245	315	2.498	0.0336	-1.474	2.389
Hampton		450	2.653			
5/1/17		325	2.512			
	40	35	1.544	0.1040	-0.983	1.602
		55	1.740			
		80	1.903			
	10	10	1.000	0.1580	-0.801	1.000
		10	1.000			
		20	1.301			

	3460	2765	3.442	0.0053	-2.273	3.539
		2940	3.468			
		3000	3.477			
	620	605	2.782	0.0140	-1.853	2.792
		725	2.860			
		650	2.813			
Trial 9						
Dover	185	210	2.322	0.0213	-1.672	2.267
5/8/17		175	2.243			
		215	2.332			
	35	35	1.544	0.0694	-1.158	1.544
		25	1.398			
		40	1.602			
	5	10	1.000	0.3618	-0.442	0.699
		5	0.699			
		3	0.477			

	3840	3490	3.543	0.0085	-2.073	3.584
		3675	3.565			
		4005	3.603			
	1085	825	2.916	0.0148	-1.831	3.035
		710	2.851			
Trial 10		855	2.932			
Hampton						
5/8/17	235	175	2.243	0.0330	-1.482	2.371
		135	2.130			
		185	2.267			
	65	60	1.778	0.0386	-1.413	1.813
		55	1.740			
		75	1.875			
	5	10	1.000	0.1305	-0.884	0.699
		15	1.176			
		20	1.301			

The replicate plate count results were log transformed and the relative standard deviation (RSD) were calculated. The RSD or coefficient of variation was plotted against the spike concentration and appears in Graph 2 below.

Graph 2. Coefficient of Variation verses Spike Concentration for Clean Effluent



To accurately determine the LOD and LOQ graphically, it was necessary to take the Coefficient of Variation and the Spike Determinations and to re-plot these as log values. Graph 3 below show the linear regression of the log transformed replicate and spiking data. Graphically, the LOQ/sensitivity of the method may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –1.0 (or its antilog, 10%). The LOD may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –0.477 (or its antilog of, 33%). Taking the antilog of the spike concentrations at these points of intersection gives the LOQ and LOD, respectively. Graph 3 indicates the LOQ and LOD for clean effluent to be 5.8 PFU/100gm and 0.9 PFU/100ml, respectively. The biostatistics program Prism 5.0 for Mac OS was used linear regression analysis and plots. The statistical summary of the linear regression from the log coefficient of variation verses log spike for the clean effluent data is presented in Table 3.

Graph 3. The LOD and LOQ/Sensitivity for Clean Effluent Samples

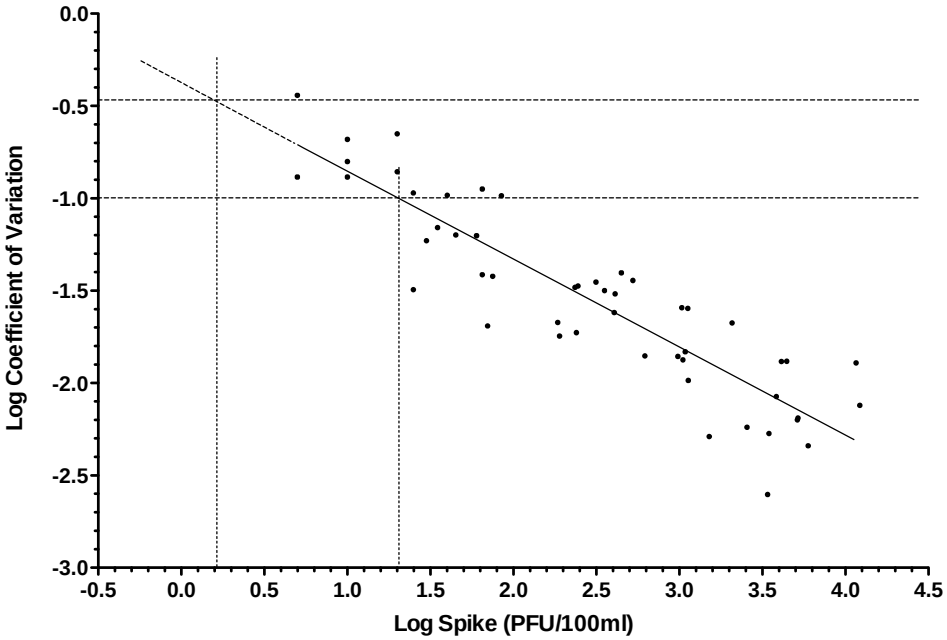


Table 3. Linear Regression Statistics for the Effluent Spiking Trials

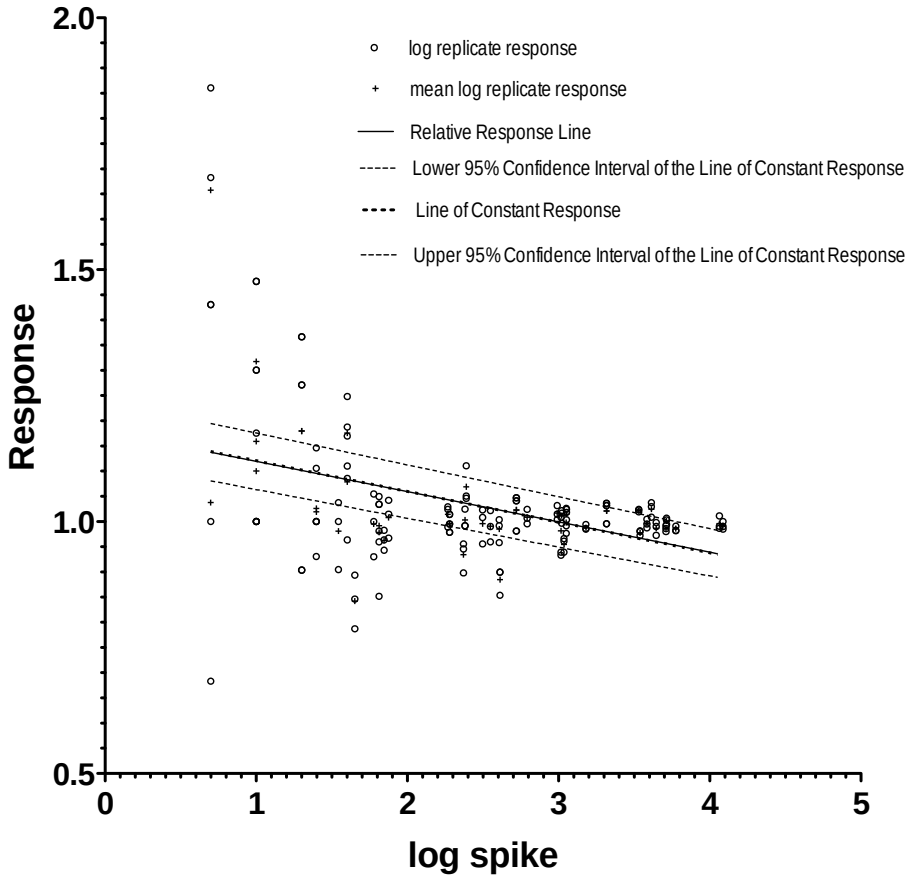
Best-fit values	
Slope	-0.4767 ± 0.03387
Y-intercept when X=0.0	-0.3746 ± 0.08890
X-intercept when Y=0.0	-0.7859
1/slope	-2.098
95% Confidence Intervals	
Slope	-0.5449 to -0.4085
Y-intercept when X=0.0	-0.5536 to -0.1957
X-intercept when Y=0.0	-1.345 to -0.3618
Goodness of Fit	
R square	0.8049
Sy.x	0.2244
Is slope significantly non-zero?	
F	198.1
DFn, DFd	1.000, 48.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	50
Maximum number of Y replicates	1
Total number of values	50
Number of missing values	0

LOQ = Antilog [-2.098 (-1.0 + 0.375)] = 20.42 PFU/100ml
LOD = Antilog [-2.098 (-0.477 + 0.375)] = 1.63 PFU/100ml

The correlation coefficient (R square value) of this linear regression is 0.8049 which is above the threshold level of 0.64 and indicates a good fit. The LOD and LOQ as determined by the spiking trials shows LOD and LOQ of 1.63 PFU/100ml and 20.42 PFU/100ml, respectively. Rounding up, the LOD and LOQ are 2 PFU/100ml and 21 PFU/100ml, respectively.

To determine the **Linear Range**, data from Table 2 was manipulated to construct the relative response line, the line of constant response and the upper and lower 95% confidence interval bracketing the line of constant response as instructed in the SOP. Graph 4 below show that the upper (1.05) and the lower (.95) 95% confidence interval estimates are essentially parallel to the Relative Response line. This suggests that the method is linear through the working range of 5 PFU/100ml to 12,000 PFU/100ml.

Graph 4. Linear Range Determination



Data Summary:

Linear range of the method as implemented 5 to 12,000 PFU/100ml
The limit of detection of the method as implemented 2 PFU/100ml
The limit of quantitation/sensitivity of the method as implemented 21 PFU/100ml
Indeterminates <2 PFU/100ml to >12,000 PFU/100ml

The Determination of Accuracy/Trueness and Measurement Uncertainty is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust databases acquired from the determination of the LOQ/LOD/Linear Range. The Accuracy/Trueness is calculated by dividing the log average of the plates by the log average of the spike concentrations, then multiplying the result by 100 to get a percent value. Table 4 shows the results for the Accuracy/Trueness of the method.

Table 4. Calculation of the Accuracy/Trueness of the MSC Method for Wastewater (Low Range Routine).

Average log of plates (2.473 PFU/100ml)/Average log of spike (2.455 PFU/100ml)
= Accuracy/Trueness of 100.7 %.

The Measurement Uncertainty is determined by subtracting the log mean replicate plate values from the reference or log spike values, then calculating the 95% confidence limits of the mean difference. Table 5 show the results of statistical analyis for Method Uncertainty.

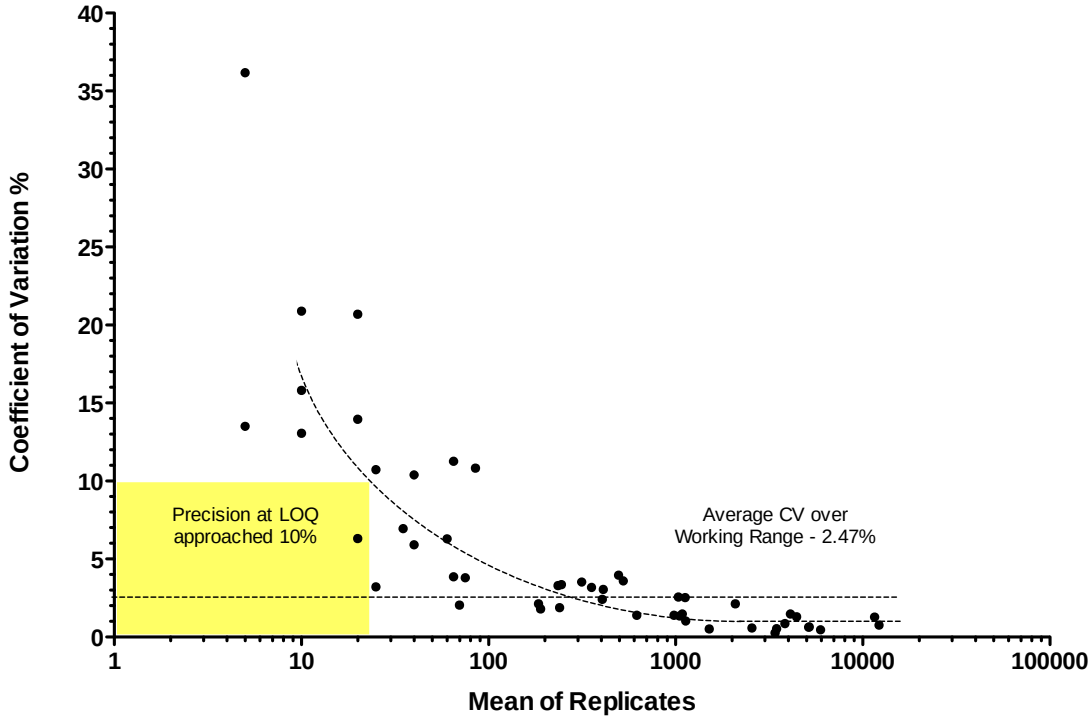
Table 5 – Measurement Uncertainty in wastewater using low range routine.

		Antilog
Number of values	150	
Mean	-0.01787	**0.960**
Std. Deviation	0.1566	
Std. Error	0.01278	
Lower 95% CI of mean	-0.04314	**0.905**
Upper 95% CI of mean	0.00739	**1.017**

Data Summary: Wastewater using Low Range Routine
Calculated % accuracy/trueness 100.7 %
Calculated measurement uncertainty 0.905 to 1.017

The Determination of the Precision and Recovery is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust data set acquired from the determination of the LOQ/LOD/Linear Range. To examine the precision over the working range of the method, a simple graphical approach was followed. The coefficients of variation were determined from the log transformed replicate data (50 sets of three true replicates) and were plotted verses the mean of the triplicate results (non log transformed data). The results are shown in Graph 5 for effluent using the low range routine.

Graph 5 - Coefficient of Variability (%) of Replicate verses Mean of Replicate for Effluent Samples using the Low Range Routine of the Method.



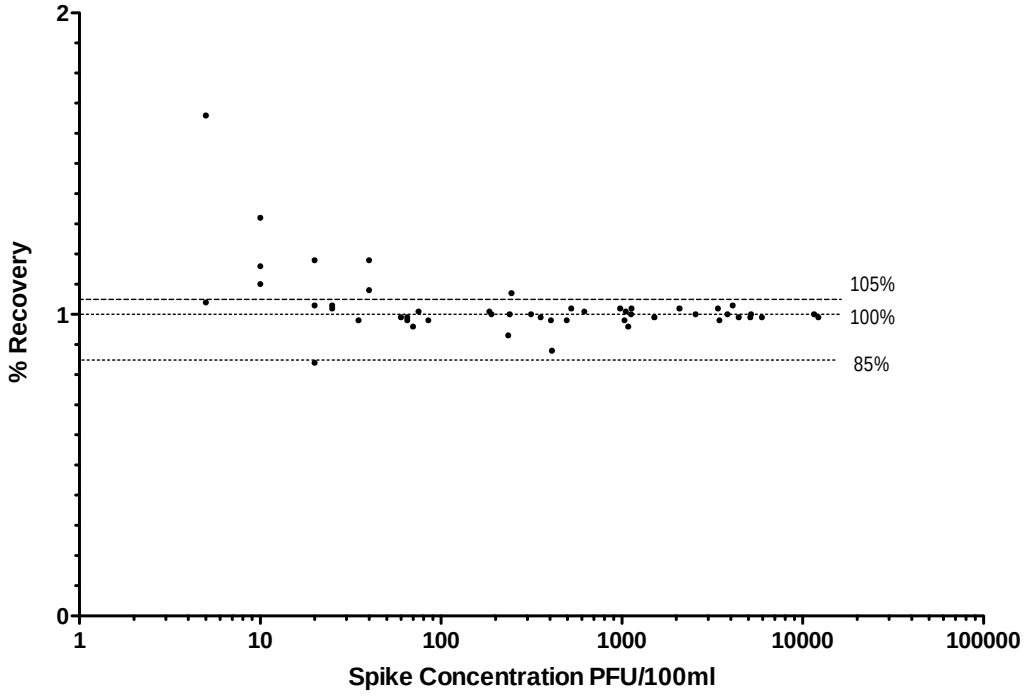
In Graph 4 above, the precision decreases as the LOQ and LOD are approached. The mean, minimum, and maximum coefficient of variations as determined over the working range for effluent samples appear in Table 6 below.

Table 6 – Mean, Minimum, and Maximum Coefficient of Variation over the Working Ranges.

Average Coefficient of Variation = 2.47%
Minimum Coefficient of Variation = 0.25%
Maximum Coefficient of Variation = 36.2%

To examine the **Recovery** over the working range of the method, a simple graphical approach was followed. The data from the LOD/LOQ/Linear Range was used for this determination. The mean of replicates was divided by the spike concentration. The percent recovery was then plotted against the spike concentrations. Graph 6 show these recovery plots with the recoveries bracketed at 85% and 105% for clean wastewater samples using the low range method routine. Recovery by the method is highly variable due to the problems associated with spike determinations. However, recovery for the method over all is high at 98.8%. (see Table 7)

Graph 6 - Percent Recovery verse the Spike Concentration for effluent using the low range method routine



As indicated above, the percent recovery of the method as implemented by this laboratory was calculated by dividing the log average of the replicates by the log spike concentration and multiplying by 100 to get a percent. Table 6 below shows this calculation from effluent trials for the low range method routine

Table 7 – Method Recovery from effluent trials.

Average Log of Spike	Average Log Replicates	% Recovery
2.455	2.473	100.7%

Data Summary:

- Is the precision of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- The coefficient of variation of the test method as implemented is **2.5%**
- Is the recovery of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- What is the overall percent recovery of the method under study? **100.7%**

Ruggedness was determined using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

Two batches of bottom plates, soft agar tubes, and growth browths were prepare using two different lots of granulate agar (Media A and Media B), well in advance of the trials. Ten samples of clean effluent from the Dover and Hampton WTPs were similarly spiked and plated using media A and media B batches. The spike level was varied throughout the experiment. Table 8 show the data, data analysis, and the results of the paired t-test for effluent

Table 8 - Determination of the Method Ruggedness for Effluent

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
3000	3575	3.48	3.55
5130	5055	3.71	3.70
5335	5465	3.73	3.74
14055	14980	4.15	4.18
13370	15955	4.13	4.20
12275	12200	4.09	4.09
5545	4845	3.74	3.69
5340	4495	3.73	3.65
210	235	2.32	2.37
190	175	2.28	2.24
	Skew	-1.35	-1.29
	Variance	0.47	0.48
	Ratio of Larger Var to Lower Var	0.01	

skew between -2 and 2 indicates symmetry
Ratio of Varieances < 2 indicates homogeneity of variance

Paired t-test (Media A verses Media B)

P value	0.0.7648
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3084 df=9
Number of pairs	10

Data Summary:

Value for the test of symmetry of the distribution of Media A data -1.35
Value for the test of symmetry of the distribution of Media B data -1.29
Variance of Media A data .0.47
Variance of Media B data .0.48
Ratio of the larger to the smaller of the variances of Media A and Media B 0.01
Is there a significant difference between Media A and Media N

Proposal No. **17-100**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	J. Michael Hickey
Affiliation	Massachusetts Division of Marine Fisheries
Address Line 1	1213 Purchase Street
Address Line 2	
City, State, Zip	New Bedford, MA 02740
Phone	508-965-2273
Fax	508-990-0449
Email	Michael.hickey@state.ma.us
Proposal Subject	Marina Definition
Specific NSSP Guide Reference	Section I Purposes and Definitions B. Definition of Terms (71) Marina
Text of Proposal/ Requested Action	**(71) Marina** means any water area with a structure (docks, basin, floating docks, etc.) which is: (a) Used for docking or otherwise mooring vessels to a dock or pier; and (b) Constructed to provide temporary or permanent docking space for more than ten boats.
Public Health Significance	There has been ever increasing pressure to include mooring areas which are not defined in the Model Ordinance into the Marina Proper; Section II- Chapter IV **@ .05 Marinas**. When the criteria were developed to deal with the classification of Marinas as defined, and the determination of a buffer zone in adjacent waters; mooring areas were purposely not included. It was left to the discretion of the SSCA to determine, classification criteria that could be different from the marina calculations depending on local circumstances and local knowledge. FDA is now interpreting anchors, chains and mooring blocks as "structures "and as such is requiring that mooring areas be treated as Marinas. Structure in the Marina definition means "(docks, basin, floating docks, etc.)" not anchors and chains. There are many different kinds of marinas, some essentially parking lots with no overnight occupancy and others that are destination mooring areas. Some states have outstanding boat pump out programs and large areas, if not the entire state, that are federal No Discharge Areas, in addition to local well enforced no discharge and occupancy regulations or by-laws. SSCAs should be allowed to assess the pollution impact of mooring areas based on actual circumstances and data not just an assumed risk.
Cost Information	NONE, Possible savings to SSCAs.

Proposal No. **17-101**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Debra Barnes
Affiliation	New York State Department of Environmental Conservation
Address Line 1	205 North Belle Mead Road, Suite 1
Address Line 2	
City, State, Zip	East Setauket, NY 11733
Phone	631-444-0477
Fax	631-444-0472
Email	debra.barnes@dec.ny.gov
Proposal Subject	Parking lot mooring/anchoring areas in EPA-approved vessel no discharge zones
Specific NSSP Guide Reference	Section I Purposes and Definitions B. Definition of Terms (72) Marinas
Text of Proposal/ Requested Action	**(72) Marina** means any water area with a structure (docks, basin, floating docks, etc.) which is: (a) Used for docking or otherwise mooring vessels; and (b) Constructed to provide temporary or permanent docking space for more than ten boats Exemption: Mooring areas located within EPA-approved "vessel no discharge zones" are excluded from this definition where the requirement that a vessel's capacity to discharge is disabled by locking or wiring shut the discharge valve of a vessel's marine sanitation device and is enforced by the SSCA's law enforcement/patrol program or by uniformed local/municipal law enforcement (bay constables, harbormasters, marine police, etc.)
Public Health Significance	Boat mooring/anchoring areas located within EPA-approved vessel no discharge zones that are enforced by the SSCA's patrol program or other state or municipal uniformed local law enforcement officials present no significant threat to public health. Having such areas designated as closed to harvest, seasonally or year-round, requires the SSCA to patrol those areas to enforce the closures. This requirement also draws enforcement resources away from other closed areas with actual water quality problems of public health significance.
Cost Information	$ 0.00

Proposal No. **17-102**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Update definition of "replicate"
Specific NSSP Guide Reference	Section I Purposes and Definitions B. Definition of Terms (101) Replicate
Text of Proposal/ Requested Action	**(101) Replicate** is defined as two (2) laboratory analyses conducted from the same sample ~~filters for thermostable direct hemolysin (tdh) analysis from the same homogenate~~ at the same dilution.
Public Health Significance	The current definition of "replicate" is specific for one type of laboratory analysis conducted infrequently in the NSSP. The proposed change provides the same intent for the definition of "replicate", but makes it more broadly applicable.
Cost Information	None.

Proposal No. **17-103**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Specific NSSP Guide Reference	Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Biotoxin Testing) and Table 4 (Approved Limited Use Methods for Marine Biotoxin Testing)
Text of Proposal/ Requested Action	The intention is for this method to be an Approved Method for Marine Biotoxin Testing for clams and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the new heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP), and the applications should be (1) Growing Area Survey and Classification and (2) Controlled Relaying with the sample type of Shellfish for both. In addition, the method should also be included in Table 4 (Approved Limited Use Methods for Biotoxin Testing) for mussels and oysters. Additional validation will be submitted later in order to move mussels and oysters also to Table 2.
Public Health Significance	Method will be used to control hazard from Diarrhetic Shellfish Poisoning (DSP) in shellfish. No methods for DSP are currently listed in the NSSP yet shellfish harvesting closures have occurred due to these toxins in Texas since 2008, in the Pacific Northwest since 2011, and in the New England region since 2015. Regulatory laboratories in these regions are currently using best available science of LC-MS/MS according to the EU reference SOP for LC-MS/MS determination of lipophilic shellfish toxins.
Cost Information	Capital equipment purchases: $500,000. Consumable cost per sample: $10.00
Research Needs Information	
a. Proposed specific research need/ problem to be addressed	No methods are currently approved for use to control DSP hazard under the NSSP. The EU has adopted LC-MS/MS as the reference method for all of the lipophilic shellfish toxins, including DSP. This method is a modified version of the EU LC-MS/MS method optimized specifically for DSP.
b. Explain the relationship between proposed research need and program change recommended in the proposal	The proposal will provide full SLV data for the detection of DSP toxins in clams. Therefore it would be considered an Approved Method for clams (Table 2). Based on the immediate need for this method, it was felt that the submission should be made with the available data for clam with the intention of subsequent validation for mussels and oysters, for which only preliminary data is provided here. Therefore, the method should be considered for Approved Limited Use at this time for mussel and oyster and be included in Table 4 for these matrices.
c. Estimated cost	$10,000
d. Proposed sources of funding	FDA internal funding
e. Time frame anticipated	Submission of all materials in order to be reviewed prior to the 2017 bi-annual ISSC meeting.

For Research Guidance Committee Use Only	Relative priority rank in terms of resolving research need ☐ Immediate ☐ Required ☐ Valuable ☐ Important ☐ Other

Single Laboratory Validation (SLV) Protocol for Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Name of the New Method:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Name of Method Developer:

Whitney Stutts, Ph.D. and Jonathan Deeds, Ph.D.

Developer Contact Information:

FDA Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

Phone: 240-402-1474 (Deeds) or 240-402-1895 (Stutts)

whitney.stutts@fda.hhs.gov; jonathan.deeds@fda.hhs.gov

A. Need for the New Method

1. Need for Which the Method has been Developed:

Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). Shellfish hazards for domestic products are managed under the National Shellfish Sanitation Program (NSSP), but at present approved and approved limited use methods are only available for ASP, NSP, and PSP. Shellfish harvesting closures have been required due to DSP toxins in excess of the established regulatory guidance level of 16 µg OA eq./100 g shellfish on the Texas Gulf Coast since 2008, in the Puget Sound region since 2011, and in the New England region since 2015. This report describes the validation of an LC-MS/MS method for DSP toxins for use in the NSSP for the control of this hazard in clams. Once found to be fit for purpose for clams, the method will be fully validated for the additional matrices of mussel and oyster. Due to the immediate need for approved methods for this toxin group it was felt that submitting this proposal now, with the available full SLV data for clam, was important. Preliminary data is available for mussel and oyster such that the method can be used for these matrices in an approved limited use capacity.

2. Purpose and Intended Use of the Method: The intention is for this method to be an Approved Method for Biotoxin testing for DSP toxins under the NSSP (for clams) and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the new heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP), and the applications should be (1) Growing Area Survey and Classification and (2) Controlled Relaying with the sample type of Shellfish for both. Preliminary data is also provided for the additional matrices of mussel and oyster such that the method should be included in Table 4 (Approved Limited Use Methods for Biotoxin Testing) for these matrices while additional SLV data is generated.

3. Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:

The regulatory guidance level in the U.S. for diarrhetic shellfish poisoning toxins (DSTs) is 16 µg total (free plus esterified) toxins/100 g shellfish. The European Union (EU) recently adopted LC-MS/MS as the reference method for lipophilic shellfish toxins, which include DSTs.[1] However, because the EU harmonized protocol also measures a number of additional lipophilic toxins, including pectenotoxins, yessotoxins, and azapriracids, the protocol contains multiple method modifications and variations to account for this, depending on the needs of individual laboratories. This work optimized the EU lipophilic toxin reference method specifically for the quantitation of DSTs in clams for use in the NSSP. Some labs in the U.S. are already using best available science based on the EU LC-MS/MS reference method; thus, an NSSP-validated method for use in the U.S. is urgently needed. This LC-MS/MS method would be complimentary to other available testing methods such as the *in-vitro* protein phosphatase inhibition assay (PPIA), which has also been submitted for approval to the ISSC under a previous proposal, and comparative data is provided between these two methods in the comparability section.

4. Type of Method (Chemical, Molecular, or Culture):

Chemical Confirmatory Method: Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) specifically measures okadaic acid, dinophysistoxin 1, and dinophysistoxin 2.

B. Method Documentation

Method Title:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Method Scope:

This method is fully validated for the determination of diarrhetic shellfish poisoning toxins in clams. Preliminary and comparative data only is provided for mussels and oysters.

References:

1. European Union Reference Laboratory for Marine Biotoxins. Harmonized Standard Operating Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS. Version 5, January 2015.
2. Gerssen, A.; McElhinney, M.A.; Mulder, P.P.J.; Bire, R.; de Boer, J. Liquid chromatograph-tandem mass spectrometry method for the detection of marine lyophilic toxins under alkaline conditions. Journal of Chromatography A, 1216 (2009) 1421-1430.
3. ICH Q2B, Validation of Analytical Procedures: Methodology, Fed. Regist. 1997, 62 (96), 27463-27467.
4. McNabb, P.; Selwood, A. I.; Holland, P. T. Multi-residue method for determination of algal toxins in shellfish: single-laboratory validation and inter-laboratory study. Journal of AOAC International, 88 (2005) 761.
5. AOAC International "Appendix K: Guidelines for Dietary Supplements and Botanicals, Part 1 AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals", 2013.
6. Smienk et al. 2012 Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins, 5, 339-352.
7. Smienk et al. 2013. Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal of AOAC International Vol. 96, No. 1.

Principle:

Liquid chromatography tandem mass spectrometry is an analytical technique that provides both physical separation (liquid chromatography) and mass analysis (mass spectrometry) of sample components. In this work, a Waters ACQUITY® Ultra Performance Liquid Chromatography system was coupled with an AB Sciex 5500 QTrap® triple quadrupole mass spectrometer by electrospray ionization. Pre-validation studies were performed to assess the impacts of acidic versus basic chromatography and the use of neat versus matrix matched standard curves on overall method performance (data included). For targeted quantitation of DSTs, structurally informative transitions were specified for multiple reaction monitoring (MRM). In MRM mode, an ion of interest (precursor ion) is preselected in the first quadrupole and fragmented in the second quadruple. Multiple product ions resulting from fragmentation of the precursor ion are then mass analyzed in the third quadrupole. Two analyte specific transitions, one for quantitation and one for confirmation, are specified for each analyte and monitored in both the calibration standard solutions and in the extracted shellfish matrices. A linear fit is applied to the peak area data for the quantitation ion collected for the calibration standards, and the equation for this line is utilized to calculate the concentration of each analyte in the spiked matrix samples. The enhanced resolving power afforded by LC and the selectivity gained by tandem mass spectrometry permitted the accurate detection and quantitation of DSTs in complex shellfish matrices. Individual toxin values are converted to a single integrated okadaic acid equivalents value through the use of established

toxicity equivalency factors (OA: 1, DTX1: 1, DTX2: 0.6). Only OA and DTX1 have been found to date in the U.S.

Analytes of Interest:

Diarrhetic shellfish poisoning toxins: Okadaic Acid (OA), Dinophysistoxin-1 (DTX1), and Dinophysistoxin-2 (DTX2). Shellfish metabolites (fatty acid acyl-esters for all 3 toxins, collectively referred to as DTX3) are included through the use of a required alkaline hydrolysis step.

Proprietary Aspects: None

Cost of the Method:

Capital equipment purchases:

1. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [example: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount)]

2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)]

Cost per sample (approx. for consumables): $10/sample

Sample Turn Around Time: Analysis of 10 samples, including extraction, hydrolysis, sample analysis, and quantitation can be accomplished in approx. 6 -7 hours. Analysis time increases by 2.5 hours for every 10 additional samples.

Equipment Required:

1. Instrumentation for sample preparation: knives for shucking, stainless steel laboratory spatulas, sieve for draining
2. 4 oz. plastic screw top specimen cups (if a sample of unextracted homogenate is to be saved) (e.g., Covidien #17099)
3. Balance, accuracy to the nearest 0.01 g
4. Blender or food processor
5. Laboratory homogenizer (e.g., Polytron, Ultraturax, etc.) equipped with a generator appropriate for shellfish (e.g. 20 mm saw toothed)
6. Vortex mixer (either single or multi-tube)
7. Centrifuge, capable of 2000 x *g*, with adaptors for 50 mL and 15 mL centrifuge tubes
8. Heat block or water bath capable of maintaining 76 ± 2 °C
9. Volumetric flasks or graduated cylinders capable of accurately measuring 20, 100, 500, and 1000 mL

10. Disposable 50 mL centrifuge tubes
11. Disposable glass Pasteur pipettes with bulbs
12. 20 mL glass scintillation vials with coned polyethylene lined caps (if a sample of non-hydrolyzed methanolic extract is to be saved) (e.g., Wheaton #986560)
13. Disposable 16 × 100 mm glass screw cap tubes with phenolic PTFE lined caps (e.g., Fisher #14-959 35AA (tubes), Corning #9998-15 (caps))
14. 0.01, 0.05, 0.1, 0.5, and 1 mL positive displacement microdispensers or syringes (e.g., Drummond #3-000-510, 3-000-575, 3-000-590 or Hamilton #80530, 80630, 81330, 81365)
15. 1 mL disposable syringes (e.g., Becton Dickinson #309602)
16. PTFE syringe tip membrane filters, 13 mm, pore size 0.2 µm (e.g., Pall #PN4542)
17. LC-MS autosampler vials with pre-slit caps (e.g., Agilent #5182-0715)
18. Reversed Phase HPLC Column (e.g., Acquity UPLC BEH C18 1.7 µm particle size, 1.0 × 150 mm)
19. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode
20. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities

Reagents Required:

1. Certified Reference Standards

Okadaic acid: e.g. (NRC-CRM-OAc) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-1: e.g. (NRC-CRM-DTX1) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-2: e.g. (NRC-CRM-DTX2) NRC-CNRC Institute for Marine Biosciences, Canada

2. Chemicals

Acetonitrile, HPLC Optima Grade
Methanol, HPLC Optima Grade
Water, HPLC Optima Grade
Ammonium formate (≥98% purity)
Formic Acid (≥98% purity)
Hydrochloric acid (37%)
Sodium hydroxide (≥98% purity)
Hexanes (Certified ACS, ≥98.5%)

Solution Preparation

1. Extraction solvent: 100% methanol

2. Hydrochloric Acid 2.5 M: Add 60 mL of water to a 100 mL volumetric flask or graduated cylinder. To the water, add 20 mL of hydrochloric acid and then dilute with water to 100 mL. Place in an appropriate glass jar for storage marked with the date of creation. This solution may be stored at room temperature and can be used for up to three months.

3. Sodium hydroxide 2.5 M: Dissolve 10 grams of sodium hydroxide in 75 mL of water in a glass beaker and add to a 100 mL volumetric flask or graduated cylinder. Make up to 100 mL with water and transfer to an appropriate storage vessel marked with the date of creation. This solution may be stored at room temperature and used for up to 3 months.

Matrix or Matrices of Interest:

Clam, mussel, and oyster. Full SLV validation data is provided here for clam but pre-validation and method comparability studies (data provided) indicate that the method will also be applicable to additional shellfish matrices such as mussel and oyster. Data for additional matrices will be provided upon approval of the method for clam.

Sample Collection, Preservation, and Storage requirements:

CRM-DTX1, CRM-DTX2, CRM-OA stock solutions (in MeOH in sealed ampules) were purchased from the National Research Council Canada and stored at −20 °C according to manufacturer's recommendations.

For each bivalve type, animals were collected from four different growing areas (Table 1). Shellfish were shucked, rinsed and drained to remove salt water. Ten to twelve animals were combined and homogenized using a blender/food processor and/or a Polytron homogenizer. Homogenized tissues were stored in plastic screw top specimen containers at −20 °C until used.

Table 1. Sources of blank shellfish matrices for spiking experiments

Source	Clam (*Mercenaria mercenaria*)	Mussel (*Mytilus edulis*)	Oyster (*Crassostrea virginica*)
A	Provided by Maryland Department of Natural Resources	Provided by MD Department of Natural Resources (used for pre-validation studies)	Provided by Maryland Department of Natural Resources (used for pre-validation studies)
B	Purchased live directly from harvester in Virginia	Washington Department of Health (used for method comparability data)	Provided by Texas Department of State Health Services (used for method comparability data)
C	Purchased live from retail. Harvest location Northport, Maine	Not performed yet	Not performed yet
D	Provided by Florida Wildlife Research Institute. Collection location Cedar Key, Florida.	Not performed yet	Not performed yet

Safety Requirements:

Proper precautions should be taken to avoid inhalation of harmful reagents or contact with skin or eyes. Analyst should wear a lab coat, gloves and safety glasses when working with chemicals. Chemical reagents that are flammable and/or toxic should be used within a chemical fume hood to protect laboratory workers.

Other Information:

Technical Skills Required: General laboratory skills (i.e., ability to accurately pipette small volumes, etc.). Experience with operation and general maintenance of liquid chromatography mass spectrometry equipment is required.

Abbreviation and acronyms:

DTX1: Dinophysistoxin-1
DTX2: Dinophysistoxin-2
OA: Okadaic acid
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

Step by Step Test Procedure:

Shellfish Preparation:

1. Clean outside of the shellfish with fresh water.
2. Cut the adductor muscles to open and rinse the inside with fresh water to remove any debris.
3. Remove the meat from the shell and drain tissue using a sieve to remove salt water.
4. Combine 10–12 animals and homogenize using a blender/food processor or a laboratory homogenizer.

Extraction Procedure:

1. Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a 50 mL disposable centrifuge tube.
2. Add 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min.
3. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
4. Transfer the supernatant to a clean 20 mL graduated cylinder, volumetric flask, or glass scintillation vial.
5. Re-extract the residual tissue pellet with an additional 9.0 mL of methanol and homogenize using a laboratory stick homogenizer (e.g. Polytron, Ultraturax).
6. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
7. Transfer the supernatant to a 20 mL volumetric flask or graduated cylinder and combine with the first extract. Adjust total extract volume to 20 mL with 100% methanol.
8. Transfer the 20 mL of total extract back into the 20 mL scintillation vial for storage (if desired).

Hydrolysis:

Note: *For this procedure, total DSP toxins (i.e. free toxin plus toxin fatty acid esters) must be quantified. To do this, all samples must go through an alkaline hydrolysis step prior to analysis.*

Accurately transfer a 2 mL aliquot of the 20 mL methanolic extract to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement microdispenser or syringe. Add 250 µL of 2.5 M NaOH to the 2 mL extract aliquot. Homogenize with a vortex mixer for 30 seconds and record the total weight of the tube. Make sure caps are securely fastened and heat the mixture at 76 °C for 40 minutes. [Note: 76 °C is above the boiling point for methanol, therefore sample loss will occur if tube caps do not fit well and are not securely fastened.] Dry the water from the outside of the tubes, allow the tubes to cool to room temperature (approx. 5-10 minutes), and then re-weigh each tube. If the weight has dropped by more than 0.1 g, replace lost weight using 100% MeOH. Finally, neutralize samples with 250 µL of 2.5 M HCL, mix by vortexing for 30 seconds.

Sample Clean-up:

Partitioning of the methanolic shellfish extracts with hexane is performed to remove nonpolar lipids such as triglycerides, which could contaminate the source region of the instrument and/or suppress the responses of the DSTs.[4] Add 5 mL of hexanes directly to the 2.5 mL hydrolyzed methanolic extract. Mix by vortexing for 1 minute. Partition by centrifuging at 2,000 *g* for 10 min. Using a disposable glass pasture pipette, collect the hexanes (top layer) and discard to an appropriate waste container. Transfer approximately 1 mL of the methanolic extract (bottom layer) into a 1 mL disposable syringe equipped with a 0.2 µm PTFE syringe tip filter. Filter directly into an LC-MS certified glass vial and cap.

Quality Control Steps:

1. If available, a divert valve should be used to divert LC flow at the beginning and end of each chromatographic run. If a divert valve is not used, the ion source region and curtain plate will need to be cleaned between each batch (approximately every 24 hours) to maintain adequate sensitivity.
2. Use a new calibration curve each day of analyses. The calibration curve should be analyzed before and after each set of samples, and the data from both curves should be fitted with a line using least squares linear regression. Each calibration curve should be derived from at least six calibration points and the linear regression should yield a correlation coefficient (R^2) greater than or equal to 0.98. Analysts should also visually inspect the plot of the calibration to confirm linearity. If a calibration curve yields a correlation coefficient less than 0.98 or if non-linearity is visually observed, a new calibration curve should be prepared and samples should be reanalyzed.
3. The variation in the calibration curve slopes between the first and second set of calibration standards should not exceed 25%.
4. Reagent Blanks (methanol solvent) should be analyzed after the high calibration standard and periodically after fortified samples to insure that analyte carryover is not occurring; toxins

should not be detected above 10% of the lowest calibration point or should be below LOD. If carryover is observed, the LC gradient should be extended to allow for a longer wash at high organic (99% B).

5. Procedural Blanks (methanol carried through sample preparation process at the same time as the samples) should be analyzed before and after extracted samples.
6. One mid-scale calibration standard (e.g., 10 ng/mL) should be analyzed bracketing at least every 10 samples to assure that no retention time shifts (possibly due to column failure) or loss in signal intensity (due to fouling of the column or mass spectrometer) has occurred.
7. The retention time of analytes in all matrix solution should be within 3% that of the neat toxin standards.
8. Each chromatographic peak must be defined by at least 10 data points.
9. To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio(S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3.
10. Ion ratios between the quantitative and qualitative ion transitions should be within ±20% that of the relative ion abundance of the neat toxin standards.
11. Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (R_s) of OA/DTX2 should be calculated using the equation below and must be ≥ 1 for correct identification).

$$R_s = \frac{2x(RT_2 - RT_1)}{W_1 - W_2}$$

LC-MS/MS Method

Instrumentation Used for Validation: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) and a Waters Corporation Acquity UPLC system (Manchester, UK).

LC Parameters:

UPLC column used for validation: Waters BEH C18 (1.7 µm, 1.0 mm × 150 mm)
Column Temperature: 40 °C
Autosampler Temperature: 10 °C
Injection Volume: 5µL

LC Gradient:

Mobile phase A: 2mM ammonium formate and 50 mM formic acid in 100% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with water and add 1.9 mL of formic acid. Fill to mark with water.

Mobile phase B: 2mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in 48.1 mL water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with acetonitrile and add 1.9 mL of formic acid. Fill to mark with acetonitrile.

Weak needle* and strong needle* wash solvent composition matched that of mobile phase A and B, respectively (*specific to Waters Acquity UPLC).

For the gradient in Table 2, LC flow should be diverted to waste from time 0.0–3.5 min and from 9.0–15.0 min.

Table 2. LC Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0.0	0.120	50	50
2.0	0.120	50	50
6.0	0.120	30	70
8.0	0.120	1.0	99
10.0	0.120	1.0	99
10.5	0.120	50	50
15.0	0.120	50	50

Analyte retention times for this gradient and column can be seen in Figure 1.

MS Ion Source Parameters:

Turbo V ion source parameters were optimized in negative ionization mode for all analytes under the acidic chromatographic conditions listed above. These parameters will vary between different instrument platforms or ionization sources.

Table 3. Turbo V ion source parameters

Source Temperature	550 °C
Ion Spray Voltage	−4500 V
Curtain Gas	25 au
Gas 1	40 au
Gas 2	40 au

MRM Parameters:

Manual or automatic compound optimization must be performed by every laboratory to determine the optimal settings for the MRM parameters as these may vary between different instrument platforms. Ruggedness studies (detailed later in the document) found that compound re-optimization was required even for detector replacement with the same make and model. Instrument must be correctly calibrated for negative ionization mode. OA, DTX1, DTX2 should be monitored in negative ionization mode. The precursor and product ion mass-to-charge (*m/z*) should be confirmed by acquiring full scan MS1 and MS/MS spectra for each toxin. At least two product ions must be monitored for each toxin, one for quantitation and one for confirmation. Dwell times for transitions should be set such that at least 10 data points are acquired across each peak. Declustering Potential (DP), Entrance Potential (EP), Collision gas (CAD), Collison Energy (CE), and Collision Cell Exit Potential (CXP) should be optimized for each MRM transition monitored. Table 4 lists the compound-dependent parameters optimized for two different AB Sciex QTRAP 5500 detectors. For method validation, two confirmatory ions were monitored; however, for routine analysis monitoring the product ion at *m/z* 151 is not required.

Table 4. MRM Parameters used for Method Validation on an AB Sciex QTrap 5500 Mass Spectrometer

Compound	Polarity	Q1 (*m/z*)	Q3 (*m/z*)	Dwell Time (ms)	DP† (V)	EP (V)	CAD Gas	CE (V)	CXP (V)
OA	Negative	803.5	255.2	100	−110	−10	High	−70	−15
OA	Negative	803.5	113.1	100	−110	−10	High	−100	−19
OA*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	255.2	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	113.1	100	−110	−10	High	−100	−19
DTX2*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	255.2	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	113.1	100	−110	−10	High	−100	−19
DTX1*	Negative	817.5	151.1	100	−110	−10	High	−70	−15

*One additional confirmatory ion transition was monitored for method validation purposes.

† Compound re-optimization for ruggedness testing using a second AB Sciex QTRAP 5500 detector found that the declustering potential had to be changed to -5 V for optimum method performance.

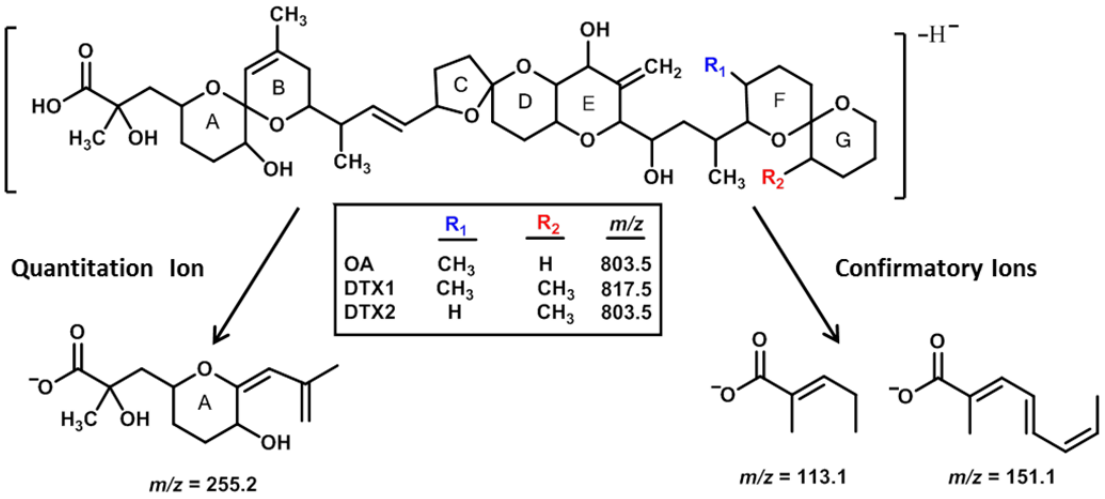


Figure 1. Structure of precursor ions and proposed product ion structures for DSTs.

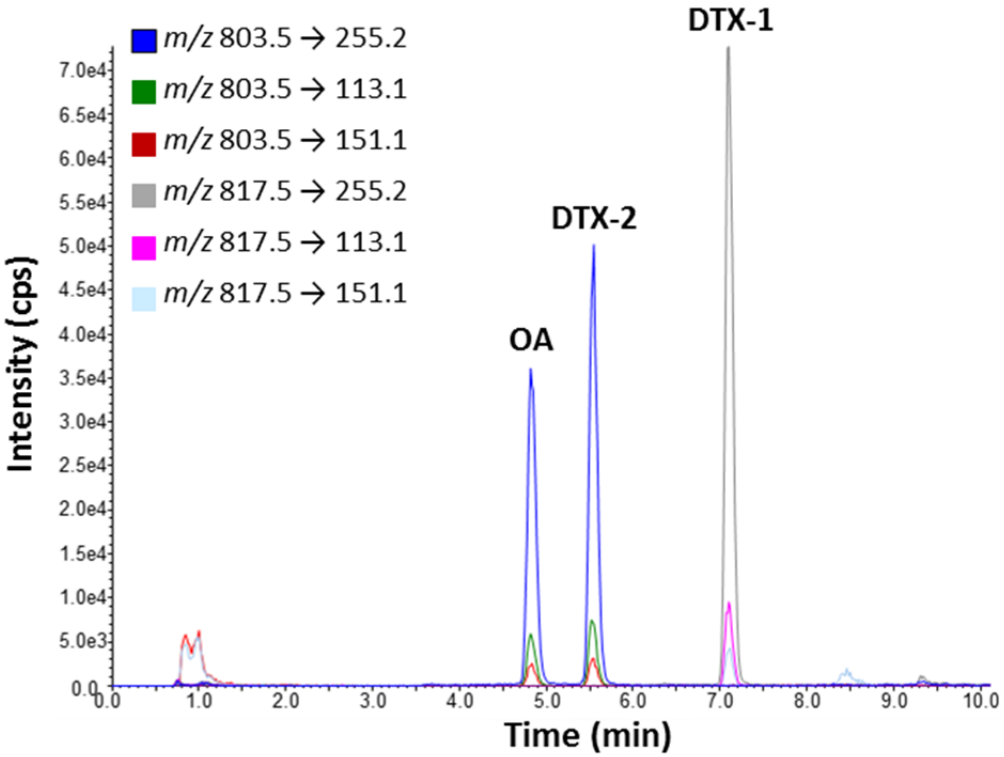


Figure 2. MRM chromatogram for the 12.5 ng/mL (regulatory level = 12.8 ng/mL) spike of OA, DTX1, and DTX2 into blank clam matrix.

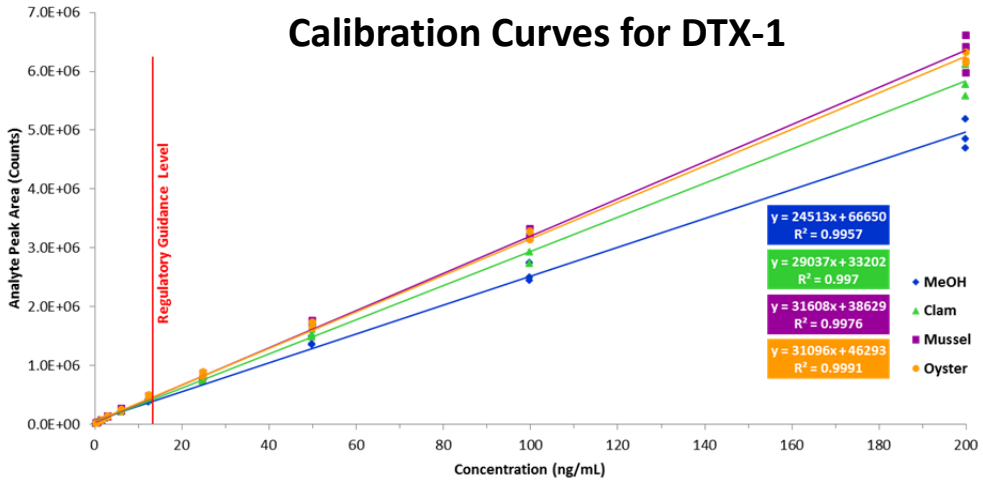
Pre-Validation Study Results (Testing for potential shellfish matrix effects for clam, mussel, and oyster, and ruggedness testing for acidic versus basic chromatography)

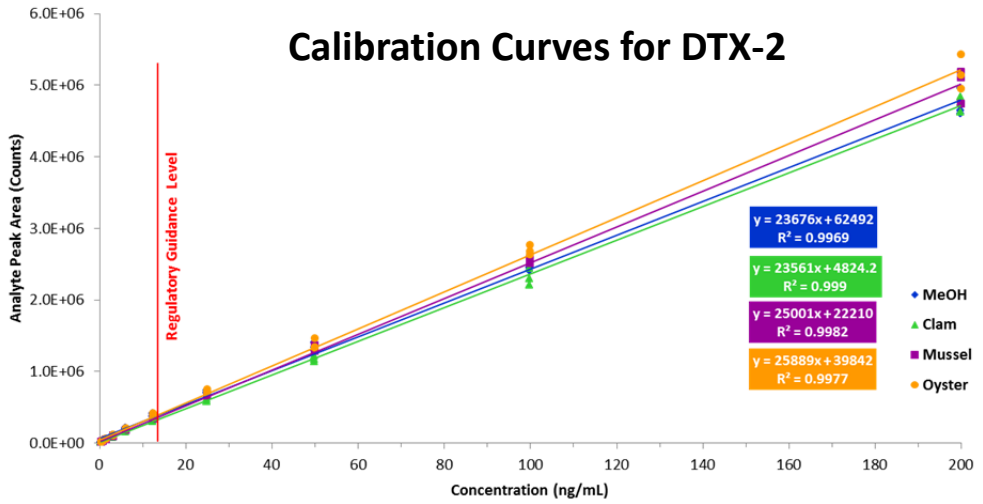
Initial pre-validation testing investigated the impact of mobile phase pH on method performance, and potential matrix effects (i.e. MS signal suppression or enhancement) for clam (*Mercinaria mercinaria*), mussel (*Mytilus edulus*), and oyster (*Crassostrea virginica*) in neat versus matrix matched spiked standard curves. Various laboratories in the United States and abroad are using different chromatographic mobile phase conditions, based on individual lab preference and need. Specifically, some labs are utilizing acidic chromatography (pH 2.4) while others are using basic chromatography (pH 11). Acidic chromatography is routinely used for quantifying total DSP toxin following hydrolysis. However, for methods that require polarity switching to investigate many classes of lipophilic toxins, basic chromatography is advantageous in that analytes that are preferentially ionized in positive mode are sufficiently separated from those that are preferentially ionized in negative mode.[2] Basic chromatography has also been reported to enhance sensitivity for certain lipophilic toxins, but due to matrix effects often requires the use of matrix matched standard curves, which adds additional cost and time to the method.

Figures 3 and 4 illustrate calibration curves from neat standard solutions in methanol compared to spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic and basic chromatographic conditions, respectively. For the preparation of blank shellfish extracts, homogenates from 5 previously tested composite shellfish samples (10-12 animals each) for each species, found to have no detectable DSTs, were re-extracted, hydrolyzed, and hexane washed following the procedure described above. These 5 extracts were then pooled per species and used for the preparation of matrix-matched standard curves. Ten fortification concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin (from 2 µg/mL stocks) were used to construct each calibration curve. Fortification with DSTs was done post extraction to evaluate potential matrix interferences without having to account for analyte recovery. Each curve was analyzed in triplicate. All calibrations curves were shown to be linear ($R^2 \geq 0.99$) within the range of 0.391–200 ng/mL using both acidic and basic chromatography.

Our results indicate that under acidic chromatographic conditions over an extended working range, the slope of the calibration curves for OA and DTX2 in the presence of matrix is within 10% that of the neat toxin standards in methanol for all three matrices. DTX1 response, on the other hand, showed signal enhancement in matrix compared to neat standards, especially at high toxin concentrations (>50 ng/mL in the hydrolyzed shellfish extract). The percent difference in the slope of the solvent-only calibration curve and the matrix-matched calibration curve for DTX1 was 19% in clam, 29% in mussel, and 27% in oyster. However, at the regulatory guidance level (16 µg/100g shellfish, 12.8 ng/mL on column, in the hydrolyzed shellfish extract), the difference in peak areas for DTX1 in matrix versus solvent is <20% for all three matrices. In comparison, under basic chromatographic conditions OA and DTX2 signals were suppressed in the presence of matrix, resulting in significantly lower calibration curve slopes (up to 19% lower) for matrix-matched calibration curves. At the regulatory guidance level, matrix suppression resulted in response differences as high as 40% for OA and 37% for DTX2. LODs and LOQs were comparable for both acidic and basic chromatography, and both were well below the level of concern for these toxins (additional information below). Based on this information, acidic chromatographic conditions were chosen for the SLV study because they would allow the use of neat standard curves in methanol, saving both time and expense. In addition, the greatest risk from the use of acidic chromatography with non-matrix-matched standard curves is potentially overestimating DTX1 concentrations (increased chance of false positive result of exceeding guidance level), while the greatest

risk from basic chromatography with non-matrix matched standard curves is underestimating OA or DTX2 concentrations (increased chance of false negative result of exceeding guidance level). From a public health perspective, overestimation is more protective than underestimation and it was felt that this slight risk was acceptable when weighted against the additional burden in both time and expense in requiring the use of matrix-matched standard curves. Furthermore, additional validation including the use of matrix matched standard curves from multiple matrix sources would likely be required for accurate quantitation of DSTs when using mobile phases at high (basic) pH.





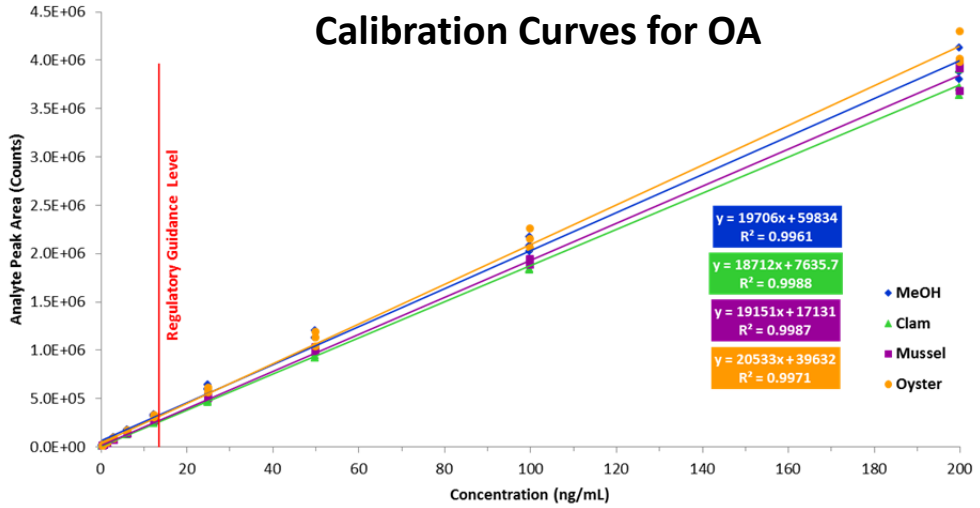
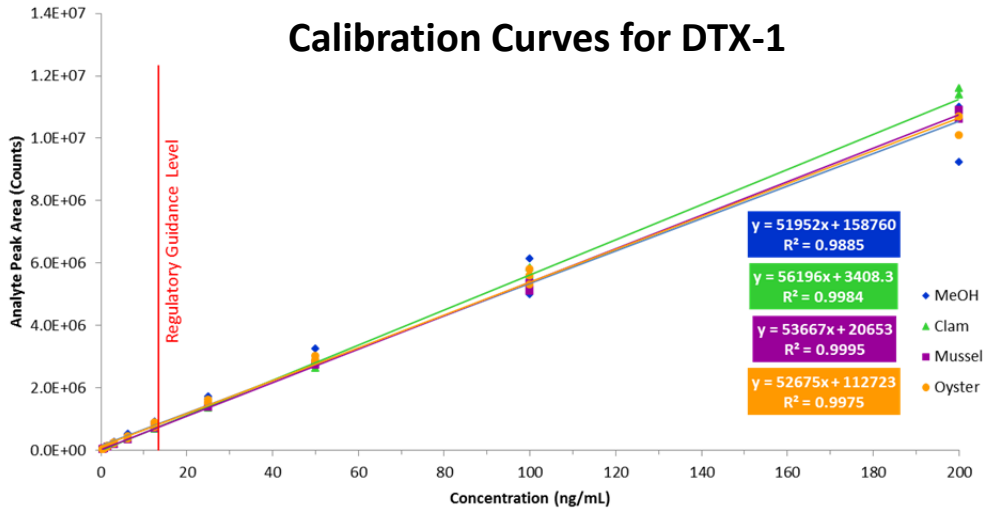


Figure 3. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.



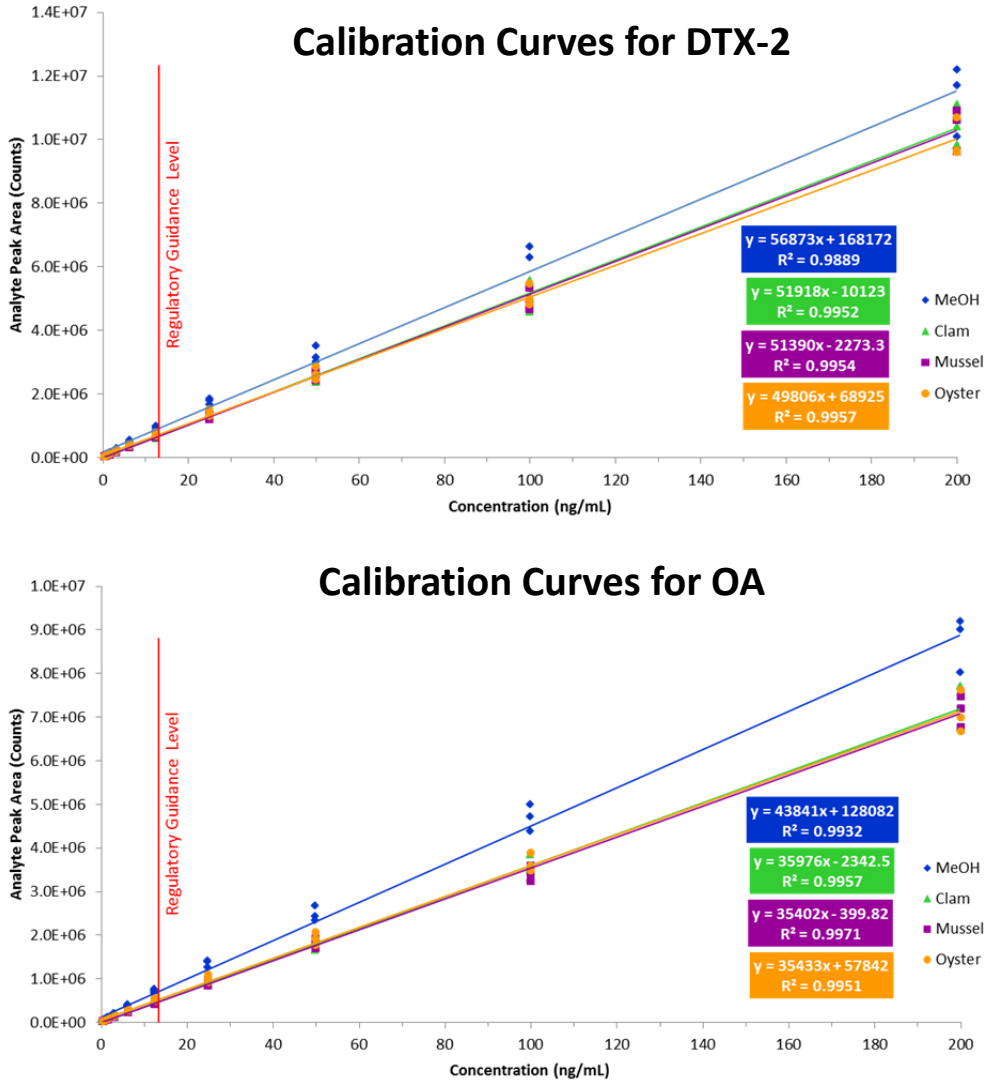


Figure 4. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under basic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.

Pre-validation Limits of Detection (LODs) and Quantitation (LOQs):

LODs and LOQs are based on the standard deviation of the response and the slope.[3] The equations for each are expressed as:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Where σ = the standard deviation of the response from five blank matrix samples
S = the slope of the calibration curve

	LOD (ng/mL)			**LOQ (ng/mL)**		
	OA	DTX-2	DTX-1	OA	DTX-2	DTX-1
	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic
Clam	0.055 / **0.040**	0.010 / **0.003**	**0.032** / 0.043	0.166 / **0.120**	0.031 / **0.009**	**0.096** / 0.129
Mussel	0.019 / **0.007**	0.007 / **0.006**	0.018 / **0.008**	0.057 / **0.020**	0.022 / **0.018**	0.053 / **0.023**
Oyster	0.017 / **0.015**	**0.008** / 0.011	0.018 / **0.016**	0.050 / **0.046**	**0.025** / 0.034	0.054 / **0.049**

Method Validation

Preparation of Stock Solutions for Validation:

Certified reference materials from the National Research Council Canada are supplied in sealed glass ampoules containing at least 0.5 mL of MeOH containing each toxin at a certified concentration that differs between toxins and lots. Thus, different volumes of each toxin standard, in 0.5 mL aliquots transferred using a 0.5 mL positive displacement Hamilton syringe, were transferred to a 20 mL glass scintillation vial and diluted with methanol to achieve stock standard solutions for each toxin at a concentration of 2 µg/mL. Stock solutions were stored at −20 °C.

Table 4. Toxin Stock Solution Preparation for Clam Validation Study

Certified Reference Material	Certified Concentration (µg/mL)	Lot #	Volume (mL)	Solvent (ml)	Total Volume	Final Concentration (µg/mL)
CRM-DTX1	15.1 ± 1.1	20071024	2	13.10	15.10	2
CRM-DTX2	7.8± 0.4	20071121	4	11.60	15.60	
CRM-OA-c	13.7 ± 0.6	20070328	2.5	14.625	17.125	

Validation Criteria:

Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

Accuracy/Trueness

Each shellfish sample used for this study was screened for DSTs using the described LC-MS/MS method to assure that each matrix did not contain any naturally accumulated DSP toxins. Once these samples were established to be free of contamination, twenty sample homogenates (five each from matrix sources A through D from Table 1) were spiked at five concentrations ranging from 8 µg/100 g to 32 µ/100 g. Concentrations for spiking were selected to cover one half to two times the regulatory limit for DSTs in shellfish which is 16 µg of toxin per 100 g of shellfish tissue. Detailed procedures are described below.

1. For each sample, ten-twelve animals were rinsed, shucked, drained, and homogenized in a commercial food processor.
2. 2.0 ± 0.05 g of each homogenate was weighted into a 50 mL disposable centrifuge tube using a stainless steel laboratory spatula.
3. 9.0 mL of 100% methanol was added to the centrifuge tube

4. Individual DSP toxin standards were added into the tube, using positive displacement pipettes, so that the final concentration of each toxin was 8, 12, 16, 24, or 32 µg/100 g
5. Each sample was mixed for 3 min using a vortex mixer
6. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
7. The supernatant was transferred to a 20 mL glass scintillation vial.
8. The residual tissue pellet was re-extracted with 9.0 mL of methanol and homogenized using a Polytron homogenizer, followed by vortexing for 3 min.
9. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
10. The supernatant was transferred to a clean 25 mL graduated cylinder, combined with the first extract, and the total extract volume was adjusted to 20 mL by adding 100% methanol. The adjusted extract was then transferred back into the original 20 mL glass scintillation vial.
11. A 2 mL aliquot of the 20 mL extract was transferred to a 16 × 100 mm glass tube using a 1 mL positive displacement Hamilton syringe and 250 µL of 2.5 M NaOH was added. Each tube was sealed with a phenolic PTFE lined screw cap and vortexed to mix for 30 seconds. Tube weights were recorded, then placed in a 76 ± 2 °C water bath for 40 minutes.
12. Sample tubes were dried, allowed to cool to room temperature for 5–10 minutes, and re-weighted to assess any evaporative sample loss. No samples required volume adjustment during these experiments. Samples were then neutralized with 2.5 M HCL, followed by vortex mixing for 30 seconds.
13. Approx. 5 mL of hexane was added to each 2.5 mL hydrolyzed methanolic extract and vortexed for 30 seconds to mix. Samples were partitioned by centrifuging at 2,000 *g* for 10 min at 20 °C. Using a disposable glass Pasteur pipette, the upper hexane layer was removed and discarded into an appropriate waste container, and approximately 1 mL of the remaining methanolic extract (bottom layer) was transferred into a 1 mL disposable syringe equipped with a 13 mm, 0.2 µm syringe tip filter using a clean disposable glass Pasteur pipette. Each filtrate was collected directly into an LC-MS certified glass vial for analysis.

Data for OA, DTX1, and DTX2 in clam are reported in tables 4, 5, and 6 respectively. These data were collected on four different days over the course of nine days.

Precision and Recovery

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of the ten samples (five samples each from sources A and B from Table 1) was fortified at concentrations of 8, 16, and 32 µg/100 g. Even number samples, comprised of clams from two source locations, were prepared on the same day and ran within 24 h. Odd number samples also consisting of clams from the two sources were prepared and analyzed nine days later than the even number samples. Data for OA, DTX1, and DTX2 are reported in tables 7, 8, and 9, respectively. Precision is reported as percent relative standard deviation (%RSD).

Specificity

All three DSTs were analyzed in the presence of the potentially co-occurring lipophilic toxins azaspiracids (NRC-CRM-AZA 1, NRC-CRM-AZA 2, NRC-CRM-AZA 3), pectenotoxin (NRC-CRM-PTX2) and yessotoxin (NRC-CRM -YTX). For each sample three aliquots of blank tissue matrix were used. One aliquot served as a control blank, one sample contained a ½ action level spike (8 µg/100 g) of each DSTs, and one

contained the same concentration of DSTs and also a moderate to high concentration of each potential interfering compound. Five technical replicates of each aliquot, excluding the negative control blank, were analyzed. The specificity index is reported for each DST.

Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of ten replicate clam samples (five each from source locations A and B from Table 1) were fortified at five concentrations spanning 50-150% of the range of interest (4, 8, 16, and 32, 48 µg/100 g). Two replicate injections of each fortified sample were analyzed. The linear range for OA, DTX1, and DTX2 extracted from fortified clam is shown in figures 5, 6, and 7. These data were collected on four different days over the course of ten days.

Ruggedness

In addition to pre-validation ruggedness testing of acidic versus basic chromatography and neat verses matrix matched calibration curves (data presented earlier in this report), two additional factors were assessed: 1. Effect of the hexane washing step on accuracy/trueness, and 2. Effect of using a different mass spectrometer of the same make and model on method performance.

To assess the effect of the hexane washing step on accuracy/trueness, two sub-samples from each of 10 extracts from previously spiked samples, representing two different matrix sources (5 samples each from matrix sources A and B), each spiked at 5 different concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were hydrolyzed following the procedure above. For each sample, one hydrolyzed extract was put through the above described hexane washing step, while the other was filtered straight into an LC vial for analysis. This entire procedure was repeated on different days so that in total 20 samples were tested both with and without the hexane washing step. The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

To assess the effect of using a different mass spectrometer of the same make and model on method performance (i.e. method transferability), ten samples (spiked at either 8, 16, or 32 µg/100 g) were analyzed using two different AB Sciex QTrap 5500 detectors equipped with a Turbo V ionization source (same make and model but purchased several years apart). The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

Matrix Effects

See Pre-Validation Study Results above.

Comparability

Method comparability**,** typically accomplished through comparison to a reference method, was not directly possible due to the fact that no reference method has been established under the NSSP. LC-MS/MS is the established reference method for the measurement of lipophilic shellfish toxins in the EU. The method described here is a version of the EU reference method optimized specifically for DSP toxins. One of the biggest differences between the method described here and the EU reference method is that the EU reference method is designed to detect multiple groups of lipophilic toxins in addition to DSP such as pectenotoxins, azaspiracids, and yessotoxins. But the analysis of these additional toxin groups in a single chromatographic run requires switching between positive and negative ion modes and initial analysis without sample hydrolysis as hydrolysis destroys several of these toxin groups. Analysis of total DSP toxins (free plus esterified) requires a second sample injection after alkaline hydrolysis of a sub-sample of the shellfish extract. Pectenotoxins and yessotoxins are not required to be analyzed for under the NSSP as they have not been proven to cause human illness, and while azaspiracids are required to be monitored, an optimized LC-MS/MS method for azaspiracids would be performed in positive ion mode and without sample hydrolysis. Therefore we are treating the LC-MS/MS method for azaspiracids as a separate method, even though the same extract, mobile phase, and equipment can be used for the analysis of both toxins. Different labs in the EU do run different versions of the "reference method" including some analyzing with acidic chromatographic conditions and some using basic, but pre-validation studies performed here found that analysis under basic chromatographic conditions would require the use of matrix matched standards. Furthermore, the two regulatory laboratories in the U.S. currently running a version of the EU reference LC-MS/MS method for lipophilic shellfish toxins as best available science are both using acidic chromatographic conditions. Therefore, running a different version of the EU reference LC-MS/MS method, such as basic chromatography or analysis without hydrolysis did not seem relevant for the method comparability requirement.

Analysis of certified reference materials would be another way to assess method performance as these naturally contaminated materials are certified to contain a known amount of all three of the target compounds. Until recently the only source of CRMs for DSP toxins, NRC Canada, only certified these materials for free toxins (i.e. toxins present pre-hydrolysis not taking into account the potential presence of fatty acid acyl ester shellfish metabolites (DTX3), which are known to be present in naturally contaminated shellfish samples. But new materials produced by NRC, both a frozen shellfish homogenate and a lyophilized material, are now provided with informational concentrations for total toxins (free plus esterified). Five aliquots of the frozen CRM-DSP-Mus-c (at $185.00 each) and one aliquot of the lyophilized NRC-FDMT1 (at $1,175 each) were purchased from the NRC Canada Certified References Materials Program. The frozen CRM contains 4 g ± 0.5 g of homogenized material therefore can only be reliably tested once using the required 2 g extraction method. The lyophilized material reportedly contains enough material for approximately 8 extractions. This material was extracted and tested 5 times, for a total of 10 CRM replicate tests (5 frozen and 5 lyophilized). Each extract was hydrolyzed and tested twice on separate days (for a total of 20 analyses) to assess the methods performance.

Although LC-MS/MS is the only reference method currently accepted in the EU for the analysis of lipophilic shellfish toxins, EU regulations do allow for the use of supplementary methods if they are shown to be equally protective (Commission Regulations (EC) No.853/2004 and No.15/2011). One such supplementary method that has been both single[6] and multi-laboratory[7] validated and is recognized as equally protective for DSP toxins in the EU is the OkaTest, produced by ZEU Inmunotec in Spain. The colorimetric protein phosphatase inhibition assay (PPIA), OkaTest, complies with the criteria stipulated by the European Reference Laboratory on Marine Toxins and Commission Regulation 15/2012 for determination of OA-group toxins in molluscs, according to the European Commission (DG-SANCO). But since this method specifically detects DSP toxins while the EU reference LC-MS/MS method detects a number of different lipophilic shellfish toxins, the OkaTest is considered a supplementary method for the detection of DSP and not a full alternative to LC-MS/MS for the lipophilic shellfish toxins by the EU. The OkaTest is now sold in the United States by Abraxis LLC as the Protein Phosphatase Inhibition Assay (PPIA) for DSP toxins. Since the PPIA OkaTest provides a composite toxicity score for all DSP toxins present, it was not possible to test all of the spiked samples generated during this validation directly by both methods as all samples in this study were spiked with all three of the target compounds. Furthermore, the linear range of the OkaTest is 6.3 – 35.4 µg OA eq./100 g. Only two of the spiking levels used in this study were within the linear range of the OkaTest, the 4 and 8 µg/100 g spiking levels (n=10 each; 20 samples total) from the liner range determination, which equated to 12 and 24 µg/100 g in total DSP toxins, closely bracketing the regulatory level of 16 µg OA eq./100 g. In addition, all shellfish homogenates (n = 10 each; 30 samples total) from the three species of shellfish used in the pre-validation matrix effect studies (clams, *Mercinaria mercinaria*; mussels, *Mytilus edulus*; and oysters, *Crassostrea virginica*), that had been previously tested and found to be <LOD for DSP toxins by LC-MS/MS were also tested using the OkaTest to show that they were also negative by this alternative method. Lastly, naturally contaminated shellfish from a variety of species and geographic locations including softshell clams (*Mya arenaria*) from New York (n=9) and containing both OA and DTX1 (range <LOD – 37.3 µg OA eq. /100 g), blue mussels (*Mytilus edulus*) from Washington (n=12) containing DTX1 only (<LOD – 52.5 µg/100 g), and eastern oysters (*Crassostrea virginica*) from Texas (n=11) containing OA only (1.6 – 56.3 µg/100g) were tested by both methods to assess comparability of the LC-MS/MS method with PPIA.

Results

To be provided to the LMRC with sufficient time to be reviewed prior to the 2017 ISSC meeting.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Name of the Method Developer	Jonathan Deeds
Developer Contact Information	Jonathan.deeds@fda.hhs.gov; 240-402-1474 US FDA, 5001 Campus Drive, College Park, MD 20740

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	
2. What is the intended purpose of the method?	Y	
3. Is there an acknowledged need for this method in the NSSP?	Y	
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	
B. Method Documentation		
1. Method documentation includes the following information:	Y	
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	Y	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	

8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	Y	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	
Submitters Signature Jonathan R. Deeds -S Digitally signed by Jonathan R. Deeds -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, 0.9.2342.19200300.100.1.1=1300218767, cn=Jonathan R. Deeds -S Date: 2017.05.31 12:46:30 -04'00'	Date: 5/31/2017	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Proposal No. **17-104**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Guidance for verifying the performance of a quantitative single laboratory validated (SLV) method of analysis being transferred from the originating laboratory/submitter to the implementing laboratory before being placed in service by the implementing laboratory.
Specific NSSP Guide Reference	Section IV Guidance Documents – Chapter II. Growing Areas
Text of Proposal/ Requested Action	Section IV Guidance Documents – Chapter II. Growing Areas .20 Quantitative Analytical Method Verification This guidance is provided to verify the performance of a quantitative single laboratory validated (SLV) method of analysis being transferred from the originating laboratory/submitter to the implementing laboratory before being placed in service by the implementing laboratory. The following performance criteria are to be verified: recovery, precision (repeatability or intermediate precision), linear range, limit of detection (LOD), limit of quantitation (LOQ), measurement uncertainty and comparability when applicable to a new or modified method used as a substitute/alternative to an established (NSSP) method. **Recovery** is the fraction or percentage of an analyte(s)/measurand(s)/organism(s) of interest recovered after sample analysis. **Precision** is the closeness of agreement between independent test results obtained under the stipulated conditions of repeatability (same laboratory, same analyst) or intermediate precision (same laboratory, different/multiple analysts). **Linear Range** is the range within the working range where the results are proportional to the concentration of the analyte(s)/measurand(s)/organism(s) of interest present in the sample. **Limit of Detection** (LOD) is the minimum concentration at which the analyte(s)/measurand(s)/organism(s) of interest can be identified under the conditions of the test. **Limit of Quantitation** (LOQ) is the minimum concentration of analyte(s)/measurand(s)/ organism(s) of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test. **Measurement Uncertainty** is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of

probability. It takes into account all recognized effects operating on the result including overall precision of the complete method, the method and laboratory bias and matrix effects.

Comparability is the acceptability of a new or modified method as a substitute/alternative for an established (NSSP) method.

Suggested Test Procedure: Shellfish
Use samples free of the target analyte(s)/measurand(s)/organism(s) of interest. For each shellfish type of interest use a minimum of 12 shellfish per sample and prepare as a homogenate. For each sample take a minimum of six aliquots of the homogenate appropriately sized for the work and spike five of the six aliquots with five different concentrations of the target analyte(s)/measurand(s)/organism(s) of interest spanning 50-150% of the working range/range of interest for the method under study. Do not spike the sixth aliquot of each sample as this is the sample blank. Process each aliquot including the sample blank to determine the concentration of the target analyte(s)/measurand(s)/organism(s) of interest. Do three replicates for each aliquot excluding the sample blank. Do only one blank per sample. Repeat this process with a minimum of three samples for each shellfish type of interest collected from different growing areas, the same growing area harvested on different days or from different process lots. Use the same spike level for each sample analyzed.

Suggested Test Procedure: Comparability Testing of Shellfish for Methods Used as a Substitute/Alternative for an Established (NSSP) Method
For each shellfish type of interest use a minimum of 12 shellfish per sample and prepare as a homogenate. For each sample take two aliquots and analyze one by the established (NSSP) method and the other by the substitute/alternative method. Naturally contaminated (incurred) samples having a variety of concentrations spanning the range of the intended application of the method should be used in the comparison. Analyze a minimum of eight paired samples from different growing areas, the same growing area harvested on different days, from different process lots and covering different seasons as necessary. In case the target analyte(s)/measurand(s)/organism(s) of interest are intermittently present, spiked samples may be used as described above.

Suggested Test Procedure: Water (growing water, wastewater, etc.)
Use samples free of the target analyte(s)/measurand(s)/organism(s) of interest. For each sample take a minimum of six aliquots of the sample appropriately sized for the work and spike five of the six aliquots with five different concentrations of the target analyte(s)/measurand(s)/organism(s) of interest spanning 50-150% of the working range/range of interest for the method under study. Do not spike the sixth aliquot of each sample as this is the sample blank. Process each aliquot including the sample blank to determine the concentration of the target analyte(s)/measurand(s)/organism(s) of interest. Do three replicates for each aliquot excluding the sample blank. Do only one blank per sample. Repeat this process with a minimum of three samples choosing samples from different growing areas/wastewater plants, etc. Use the same spike level for each sample analyzed.

Suggested Test Procedure: Comparability Testing of Water for Methods Used as a Substitute/Alternative for an Established (NSSP) Method
For each sample take two aliquots and analyze for the target

analyte(s)/measurand(s)/ organism(s) of interest by both the established (NSSP) method and the substitute/alternative method. Naturally contaminated (incurred) samples having a variety of concentrations spanning the range of the intended application of the method should be used in the comparison. Analyze a minimum of eight paired samples from different growing areas/wastewater plants, etc. covering different seasons as necessary. In case the target analyte(s)/measurand(s)/organism(s) of interest are intermittently present, spiked samples may be used as described above.

Suggested Data Handling; For microbiological methods use log transformed data.

Calculate the percent recovery by comparing the average recovery of the method to the average spike concentration.

Calculate the precision (repeatability, same laboratory, same analyst or intermediate precision, same laboratory, multiple/different analysts) by determining the coefficient of variation of the test data.

Calculate the linear range by plotting the test data versus the spike concentration and determining the correlation coefficient.

Calculate the limit of quantitation (LOQ) by plotting the coefficient of variation for the triplicates of each of five concentrations used per sample versus the spike concentration. There will be fifteen data points to be plotted. Using the equation of the line ($y = mx + b$) where m is the slope and b is the y-intercept, calculate the LOQ by setting $y = 10\%$ (0.1) and solving the equation for x (the LOQ).

Calculate the limit of detection (LOD) by dividing the limit of quantitation (LOQ) by 3.3 or by using the equation of the line and setting $y = 33\%$ (0.33) and solving the equation for x (the LOD).

Calculate the measurement uncertainty by subtracting the test results from the spike concentration that produced the result and determining the two-sided 95% confidence interval of these differences. This range represents the measurement uncertainty of the test data.

Calculate the two-sided 95% confidence interval estimate for the regression line (as a whole) relating the established (NSSP) method and the substitute/alternative method.

Suggested Method Acceptance: Compare the performance criteria calculated in the method verification study with the values obtained in the original single laboratory validation (SLV) submission by calculating the two-sided 95% confidence interval for the laboratory's mean recovery, estimated LOD and LOQ. If the ranges calculated for the recovery, LOD, LOQ and measurement uncertainty encompass (intersect) the values for the mean recovery, LOD, LOQ and measurement uncertainty obtained from the original SLV and the data is linear over the working range/range of interest with a precision/coefficient of variation which does not exceed that obtained in the original SLV, then it can be concluded that the method (which does not also require comparability testing) has been successfully transferred. For methods that also require comparability testing, the two-sided 95% confidence interval of the regression line relating the established (NSSP) method and the substitute/alternative method should encompass the slope of the regression

	line relating the two methods in the original SLV. This requirement in addition to the substitute/alternative method meeting the requirements for recovery, LOD, LOQ, measurement uncertainty, precision and linearity are necessary in order to conclude that the method has been successfully transferred.
Public Health Significance	With the number of new analytical methods being adopted for use in the NSSP, it is necessary to have a standardized approach to verify the successful transfer of the method from the originating laboratory/SLV submitter to the implementing laboratory before the method is placed in service.
Cost Information	Not Available

Proposal No. **17-105**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Blaine N. Rhodes
Affiliation	Washington State Department of Health
Address Line 1	1610 NE 150th Street
Address Line 2	
City, State, Zip	Shoreline, WA 98155
Phone	206-418-5520
Fax	206-418-5465
Email	blaine.rhodes@doh.wa.gov
Proposal Subject	High Pressure Liquid Chromatography (HPLC) test method for Domoic Acid (Amnesic Shellfish Poison)
Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter II. Growing Areas, 4. Approved Limited Use Methods for Marine Biotoxin Testing, HPLC entry for Biotoxin Type: Amnesic Shellfish Poisoning (ASP), p. 263 The method reference is in the footnote of the Approved Limited Use Methods for Marine Biotoxin Testing table that includes use of HPLC to detect ASP in shellfish references the method used by M.A. Quilliam, et al, to publish the Technical Report, "Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Samples" in 1991. At the time of publication, however, the Report did not include a full operating procedure.
Text of Proposal/ Requested Action	The Washington State Shellfish Biotoxins Laboratory proposes to perform a Single Laboratory Validation (SLV) for the detection of ASP by the HPLC method that was developed at the WA Public Health Laboratories (WAPHL) in 1991, modified in 1996 and which is currently used in the Laboratory, running the CFSAN recommended method (Quilliam et. al 1991) in tandem with the WAPHL method.
Public Health Significance	Marine biotoxins are poisons that are produced by certain kinds of microscopic algae (a type of phytoplankton) that are naturally present in marine waters, normally in amounts too small to be harmful. Molluscan shellfish (shellfish with hinged shells such as oysters, clams, and mussels) are filter feeders and ingest any particles, both good and bad, that's in the surrounding water. Algae is a food source for them, and HABs create a plentiful food supply. When shellfish eat toxin producing algae, the toxin remains in their system; large amounts of algae means more toxin can concentrate in their tissue. Biotoxins don't harm shellfish, but they can accumulate in shellfish to levels that can cause illness or death in humans and other mammals that eat them. Domoic Acid, the agent responsible for Amnesic Shellfish Poisoning, is a naturally occurring shellfish biotoxin. It is one of several potent neurotoxins that acts as agonists to glutamate, a neurotransmitter in our central nervous systems. It is imperative that modern, rapid and accurate laboratory testing methods be developed or refined to assure that adequate monitoring programs are in place to protect public health.
Cost Information	There is no significant difference in cost between the two methods.
Research Needs Information	
a. Proposed specific research need/ problem to be addressed	Between the 1991 time of publication and adoption of the CFSAN procedural interpretation of this particular method by the ISSC in 2014 most state laboratories that needed to screen for Amnesic shellfish Poisoning have developed their own in house HPLC methods, which were roughly based on the Quilliam report. Over

	time, the methods have been updated with minor changes and modernizations in the technology which has increased sensitivity and throughput of the method. Because of the increased speed and accuracy of the WAPHL method, protection of public health will be increased as compared with the CFSAN recommended method. The FDA is now insisting that all laboratories standardize on the CFSAN Procedure, which has demonstrated lower sensitivity and longer sample cycle times than the current method used by the proposing laboratory. Changing to the CFSAN method at this time, while there are increased ASP concentrations on the Pacific Coast and therefore higher sample loads at the laboratory is viewed as detrimental to public health in Washington State. CFSAN needs to be satisfied that the methods in place at the labs testing for ASP are robust and may not need reversion to 25-year old technology and the ISSC SLV is the proper mechanism for this demonstration. Unfortunately there is currently no Proficiency Testing program offered by CFSAN for biotoxins which would also lend itself to demonstrating the comparability of the different methods.
b. Explain the relationship between proposed research need and program change recommended in the proposal	The SLV is the mechanism by which the laboratories of the ISSC can demonstrate new methodology and technologies. The Washington State Shellfish Biotoxins Laboratory feels the method they have used since 1996 is superior to the CFSAN procedural interpretation of Quilliam's 1991 work. Furthermore, the CFSAN recommended procedure has not undergone a published ISSC SLV and its adoption by the FDA seems premature.
c. Estimated cost	The cost of this study will be borne by the Washington State Public Health Laboratories.
d. Proposed sources of funding	N/A
e. Time frame anticipated	2 years
For Research Guidance Committee Use Only	Relative priority rank in terms of resolving research need ☐ Immediate ☐ Required ☐ Valuable ☐ Important ☐ Other

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Pacific Rim Shellfish Sanitation Association
Affiliation	Sitka Tribe of Alaska
Address Line 1	456 Katlian St
Address Line 2	
City, State, Zip	Sitka, AK 99835
Phone	907-747-7356
Fax	907-747-4915
Email	michael,jamros@sitkatribe-nsn.gov
Proposal Subject	Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck
Specific NSSP Guide Reference	Section IV, Chapter II.14 -- NSSP Approved Laboratory Tests (p. 261 Table 2. Approved Methods for Marine Biotoxin Testing -- footnote 2, and/or p. 263 Table 4. Limited Use Methods for Marine Biotoxin Testing -- footnote 5)
Text of Proposal/ Requested Action	This submission presents the 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' for consideration as an NSSP Approved Method for Marine Biotoxin Testing for PSP in Geoduck. The RBA is a competition-based assay that employs radiolabeled saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity. The RBA offers a high-throughput, sensitive, and quantitative alternative to the mouse bioassay (MBA), which has been the long-standing reference method for PSP toxicity. Further, the RBA eliminates the use of live animals for detection of these toxins. While the RBA still uses receptors prepared from animals, the number of animals required for analysis is significantly reduced. Using native receptors as the analytical recognition elements for the assay allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods that require conversion factors of equivalent toxicity to calculate the overall toxicity. The RBA has undergone AOAC single and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27). The RBA is currently an NSSP Approved Method for Marine Biotoxin Testing for PSP in mussels as well as a NSSP approved for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP (ISSC 2015 Summary of Actions Proposal 13-114). Here we provided results from a single laboratory validation study for use of RBA with the matrix geoduck (*Panopea*) viscera for submission for the RBA to be considered for approval as an NSSP Approved Method for Marine Biotoxin Testing for PSP.
Public Health Significance	Paralytic shellfish poisoning intoxications result from the consumption of seafood (primarily bivalve molluscs) contaminated with neurotoxins known as paralytic shellfish toxins (PSTs). This suite of toxins binds to voltage-gated sodium channels and may result in paralysis if enough toxin is consumed. In extreme cases when

	respiratory support is not available to the patient, the intoxication may prove fatal. Since the toxins cannot be destroyed during cooking and there is no way to remove the toxins from seafood, the best control strategy is to ensure that contaminated product never reaches the market. To protect public health, harvesting closures are implemented when toxicity exceeds the guidance level of 80 micrograms saxitoxin equivalents per 100 grams of shellfish tissue. As such, accurate analytical methods are needed to monitor shellfish toxicity for making decisions regarding opening and closing shellfish growing areas accordingly. Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck (*Panopea*) would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA, thereby providing an early warning system for monitoring programs as toxin levels begin to rise.
Cost Information	For the assay: The estimated cost per 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample[ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. (Van Dolah 2013) For proposal: The cost of RBA work for geoduck matrix expansion is covered by and existing grant awarded to the Sitka Tribe of Alaska. Naturally contaminated samples from Washington and Alaska are pulled from regular samples tested by the respective state agencies that are part of routine shellfish testing. Therefore, there is no additional cost or funding necessary for the proposal.
Research Needs Information	
a. Proposed specific research need/ problem to be addressed	Paralytic shellfish poisoning (PSP) is a foodborne illness caused by ingestion of contaminated shellfish. The paralytic shellfish toxin, saxitoxin (STX), and its analogs are potent neurotoxins responsible for PSP. Marine dinoflagellates and freshwater cyanobacteria produce STX. The STX can accumulate in filter-feeding bivalve mollusks to levels that are toxic to humans. Symptoms of PSP include: tingling and numbness of the perioral area and extremities, drowsiness, incoherence, loss of motor control, and following high dose consumption, respiratory paralysis. In 1965 the mouse bioassay (MBA) was adopted as an official AOAC method for STX determination. The MBA has been the only method available for PSP testing for the last five decades. Both North American and European regulatory agencies have expressed the desire to transition to a more humane PSP testing method that does not require the use of live animals and is not subject to the matrix effects documented for the MBA (Turner 2012). Recently, the NSSP approved a post-column oxidation liquid chromatographic (PCOX) method and a receptor binding assay (RBA) as alternatives to the MBA. The PCOX method is approved for full use; whereas, the RBA is approved for limited use (the RBA is only approved for shellfish matrices evaluated in the single lab and multi-lab validation studies). Both the PCOX and RBA are sensitive quantitative assays for STX detection, and they do not require the use of live animals. The RBA is approved for regulatory testing of mussels as an alternative to the MBA and is approved for limited use as a screening tool for clams and scallops, but

	is not yet approved for use with geoduck (*Panopea*) due to a lack of data. Geoduck are a major commercial product, with large dive fisheries in Southeast Alaska and the Puget Sound that require STX testing. This proposal requests consideration for the NSSP RBA approval to be expanded to include geoduck. The proposal provides data from a single laboratory validation (SLV) of the RBA for geoduck testing as support for this request.
b. Explain the relationship between proposed research need and program change recommended in the proposal	This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish. The RBA serves as an alternative to the MBA in these applications, offering a measure of composite toxicity with high throughput and the elimination of live animal testing. (Van Dolah 2013) This application is for the addition of geoduck to the list of matrices approved for use with the RBA. There is an acknowledged need for this method in NSSP. A significant portion of the Washington and Alaska state shellfish industries are comprised of the harvest of geoduck. Approval of the RBA for use with geoduck would provide an alternative to (1) the MBA, which uses live animals, and (2) the PCOX HPLC method, which requires costly equipment and skilled personnel and offers low throughput. Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA. **References**: Van Dolah 2013. ISSC application: Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP)Toxicity Determination. Van Dolah et al. 2012. Determination of paralytic shellfish toxins in shellfish by receptor binding assay: collaborative study. J AOAC Int. May-Jun;95(3):795-812. Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. Nov-Dec;92(6):1705-13. Ruberu et al. 2012. Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins. Food Addit Contam Part A Chem Anal Control Expo Risk Assess.29(11):1770-9. Turner et al. 2012. Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. Toxicon : official journal of the International Society on Toxicology 59, 215-230. OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org.
c. Estimated cost	
d. Proposed sources of funding	This research was performed by the Sitka Tribe of Alaska using funds from an ANA ERE grant
e. Time frame	

anticipated	
For Research Guidance Committee Use Only	Relative priority rank in terms of resolving research need ☐ Immediate ☐ Required ☐ Valuable ☐ Important ☐ Other

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method
Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck

Name of the Method Developer
Michael Jamros, Chris Whitehead

Developer Contact Information
Sitka Tribe of Alaska, 456 Katlian St, Sitka, AK 99835
907-747-7356 phone
907-747-4915 fax
michael.jamros@sitkatribe-nsn.gov
chris.whitehead@sitkatribe-nsn.gov

Checklist		

A. Need for the New Method

1. Clearly define the need for which the method has been developed.
Paralytic shellfish poisoning (PSP) is a food born illness caused by ingestion of contaminated shellfish. The paralytic shellfish toxin, saxitoxin (STX), and its analogs are potent neurotoxins responsible for PSP. Marine dinoflagellates and freshwater cyanobacteria produce STX. The STX can accumulate in filter-feeding bivalve mollusks to levels that are toxic to humans. Symptoms of PSP include: tingling and numbness of the perioral area and extremities, drowsiness, incoherence, loss of motor control, and following high dose consumption, respiratory paralysis.

In 1965 the mouse bioassay (MBA) was adopted as an official AOAC method for STX determination. The MBA has served as the primary method available for PSP testing for the last five decades. Both North American and European regulatory agencies have expressed the desire to transition to a more humane PSP testing method that does not require the use of live animals and is not subject to the matrix effects documented for the MBA (Turner 2012). Recently, the NSSP approved a post-column oxidation liquid chromatographic (PCOX HPLC) method and a receptor binding assay (RBA) as alternatives to the MBA. The PCOX HPLC method is approved for full use; whereas, the RBA is approved for limited use (the RBA is only approved for shellfish matrices evaluated in the single lab and multi-lab validation studies, which does not include geoduck (*Panopea*). Both the PCOX and RBA are sensitive quantitative assays for STX detection, and they do not require the use of live animals. The PCOX HPLC requires skilled personnel and offers low throughput in comparison to the RBA.

2. What is the intended purpose of the method?
The RBA is approved for regulatory testing of mussels as an alternative to the MBA and is approved for limited use as a screening tool for clams and scallops, but is not yet approved for use with geoduck (*Panopea*) due to a lack of data. Geoduck are a major commercial product that requires PSP testing. This proposal requests consideration for the NSSP RBA approval to be expanded to include geoduck. The proposal provides data from a single laboratory validation (SLV) of the RBA for geoduck testing as support for this request.

This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish, specifically geoducks. The RBA serves as an alternative to the MBA in these applications, offering a measure of integrated toxicity with high throughput and the elimination of live animal testing (Van Dolah 2013). This application is for the addition of geoduck to the list of matrices approved for use with the RBA.

3. Is there an acknowledged need for this method in the NSSP?

There is an acknowledged need for this method extension in the NSSP. A significant portion of the Washington and Alaska state shellfish industries are comprised of the harvest of geoduck. Approval of the RBA for use with geoduck would provide an alternative to (1) the MBA, which uses live animals, and (2) the PCOX HPLC method, which requires costly equipment and skilled personnel and offers low throughput.

Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA.

4. What type of method? i.e. chemical, molecular, culture, etc.

Molecular. The RBA is a functional assay, whereby toxins present in the standard/sample bind to sodium channel preparations in the assay. Radiolabeled toxins (3H-STX) compete with toxins present in the standard or sample for sodium channel binding sites in a microplate format. Thus a decrease in signal from radiolabeled toxins represents an increase in standard/sample toxicity. This competitive RBA allows for quantitation that directly relates to the composite toxicity of the sample.

B. Method Documentation

1. Method documentation includes the following information:

Method Title

Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck

Method Scope

This submission presents the 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' for consideration as an NSSP Approved Method for Marine Biotoxin Testing for PSP in Geoduck.

The RBA offers a high-throughput, sensitive, and quantitative alternative to the mouse bioassay (MBA), which has been the long-standing reference method for PSP toxicity. Further, the RBA eliminates the use of live animals for detection of these toxins. While the RBA still uses receptors prepared from animals, the number of animals required for analysis is significantly reduced. Using native receptors as the analytical recognition elements for the assay allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods that require conversion factors of equivalent toxicity to calculate the overall toxicity.

The RBA has undergone AOAC single- and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27). The RBA is currently an NSSP Approved Method for Marine Biotoxin Testing for PSP in mussels as well as a NSSP approved for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP (ISSC 2015 Summary of Actions Proposal 13-114). Here we provided results from a single laboratory validation study for use of RBA with the matrix geoduck viscera for submission for the RBA to be considered for approval as an NSSP Approved Method for Marine Biotoxin Testing for PSP.

References

Van Dolah 2013. ISSC application: Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP)Toxicity Determination.

Van Dolah et al. 2012. Determination of paralytic shellfish toxins in shellfish by receptor binding assay: collaborative study. J AOAC Int. May-Jun;95(3):795-812.

Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. Nov-Dec;92(6):1705-13.

Ruberu et al. 2012. Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins. Food AdditContam Part A Chem Anal Control Expo Risk Assess.29(11):1770-9.

Turner et al. 2012. Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. Toxicon: official journal of the International Society on Toxicology 59, 215-230.

OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org.

Principle

The RBA is a competition-based assay that employs radiolabeled Saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity.

Any Proprietary Aspects

None

Equipment Required

The following list identifies the equipment and supplies needed for conducting the RBA.
For the assay:
(a) Scintillation counter (traditional or microplate)
(b) An 8-channel pipettor (5-200 ul variable volume and disposable tips)
(c) Micropipettors (1-1000 ul variable volumes and disposable tips)
(d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 um pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50)
(e) MultiScreen vacuum manifold (Millipore; Cat. No. NSVMHTS00)
(f) Vacuum pump
(g) Centrifuge tubes (15 and 50 ml, conical, plastic)
(h) Mini dilution tubes in 96-tube array
(i) Reagent reservoirs
(j) Ice bucket and ice
(k) Vortex mixer
(l) Sealing tape (Millipore; Cat. No. MATA HCL00)
(m) Volumetric flask or graduated beaker (1 L)
(n) -80 °C freezer
(o) Refrigerator

For sample extraction:
(p) Blender or homogenizer for sample homogenization
(q) Pipets
(r) Centrifuge tubes (15 ml, conical, plastic)
(s) pH meter or pH paper
(t) Hot plate or water bath
(u) Graduated centrifuge tubes (15 ml)
(v) Centrifuge and rotor for 15 ml tubes

Reagents Required

For the assay:
a) STX diHCl standards (NIST RM 8642; available through the National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA] or (CRM-STX; National Research Council of Canada;

www.nrc-cnrc.gc.ca/eng/solutions/advisory/crm/list_product.html#B-PSP)
(b) 3H-STX (0.1 mCi per ml, ≥10 Ci per mmol; available through American Radiolabeled Chemicals, St. Louis, MO [or equivalent])
(c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent])
(d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent])
(e) Ultima Gold liquid scintillation cocktail (PerkinElmer Inc.; Waltham, MA; Cat. No. 6013321 [or equivalent])

For the sample extraction:
(f) Hydrochloric acid (HCl; 1.0 and 0.1 M)
(g) Sodium hydroxide (0.1 M)
(h) Water (distilled or deionized [18 μΩ])

Sample Collection, Preservation and Storage Requirements
Samples should be kept cool until meat is removed from shell, meat should be removed from shell within 48 hours of collection and either frozen or extracted.

Safety Requirements
General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Even though low levels of radiation are used for this assay, users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of isotopes.

Clear and Easy to Follow Step-by-Step Procedure
Please see the detailed protocol AOAC OMA 2011.27 (Appendix 1)

Quality Control Steps Specific for this Method
Only data falling within the linear part of the curve (0.2-0.7 B/B0) is used for quantitation. Binding curve data shown here is from 14 RBA plates run on separate days. All analysis was performed using GraphPad Prism version 7.02.

The following parameters are required for quality control and acceptance of RBA results and were met by all assays included in this study:

(a) Slope must be between -0.8 and -1.2 (theoretical slope is -1). In this study, the average slope was -0.98 +/- 0.08.
(b) IC50 (inhibitory concentration at which CPM is 50% maximum) is in the acceptable range (2.0 nM ± 30%), between 1.4 and 2.6 nM. In this study, the average IC50 was 1.7 nM +/- 0.1 nM.
(c) A QC sample (1.8 x 10 M-8 STX concentration, 3 nM STX in-well concentration) should be within 30% (2.1 nM to 3.9 nM in-well concentration). In this study, the measured QC had an average value of 3.1 nM +/- 0.4 nM.
(d) The RSDs of triplicate counts per minute must be less than 30%. All standards, QC samples, and geoduck samples in this study met these criteria.

C. Validation Criteria

1. Accuracy / Trueness
Accuracy was evaluated based on recovery of known amounts of saxitoxin added as a QC check sample. A QC check sample is included in every receptor binding assay. Recovery of the QC check sample (3nM in-well solution) was 105% +/- 13% (Table 1).

Table 1: Calibration curve and QC check parameters in receptor binding assays

RBA ID	Slope	R^2	IC_{50} (nM)	IC_{70} (nM)	LOQ (ug STX eq/ 100g tissue)	QC (nM)
17-001	-0.86	0.99	1.6	0.59	2.6	2.9
17-002	-0.88	0.99	1.8	0.68	3.0	2.8
17-003	-0.94	0.96	1.6	0.65	2.9	2.5
17-004	-0.99	0.96	1.7	0.71	3.2	2.6
17-005	-0.92	0.98	1.5	0.60	2.7	3.1
17-006	-0.98	0.98	1.8	0.78	3.5	3.1
17-009	-0.95	0.94	1.5	0.62	2.8	3.6
17-010	-1.00	0.96	1.5	0.66	2.9	3.0
17-011	-1.15	0.96	1.9	0.92	4.1	3.7
17-012	-1.08	0.97	1.7	0.77	3.4	3.3
17-013	-1.04	0.97	1.8	0.81	3.6	3.1
17-014	-0.99	0.95	1.7	0.70	3.1	3.1
17-015	-0.95	0.99	1.5	0.62	2.8	3.7
17-016	-1.04	0.96	1.8	0.77	3.4	3.4
Average	-0.98	0.97	1.7	0.71	3.2	3.1
+/-	0.08	0.02	0.1	0.09	0.4	0.4

2. Measurement Uncertainty

3. Precision Characteristics (repeatability and reproducibility)

Repeatability was determined by analyzing each sample in three assays performed on independent days. The average RSD was 14.6%, with a range of 5.4% to 25.6% (Table 2). These results are consistent with the mean RSD of 17.7% (Van Dolah 2009), used to demonstrate repeatability in ISSC 2015 Proposal 13-114.

Table 2: Receptor binding assay results, summary statistics, and comparison to MBA results

Sample ID	RBA (ug/100g)			RBA mean (ug/100g)	MBA (ug/100g)	% MBA value	SD	RSD (%)
1823	29	32	38	33	42	79	5	13.9
2095	22	37	34	31	45	69	8	25.6
1594	45	74	58	59	58	102	15	24.6
2094	51	56	48	52	59	88	4	7.8
1607	60	43	47	52	67	78	12	23.3
1865	88	111	86	95	75	127	14	14.6
1933	88	85	74	82	88	93	7	9
1830	121	108	83	104	116	90	19	18.6
2315	93	97	82	91	128	71	8	8.6
2420	103	98	111	104	129	81	7	6.3
2071	129	141	163	144	140	103	17	11.9
2072	169	152	158	160	142	113	9	5.4
2138	406	344	332	361	447	81	40	11
1595	25	31	19	25	<38	-	6	24
1674	3	9	6	6	NTD	-	3	50*
Average						90	12	14.6

*RSD value omitted due to value below LOQ

4. Recovery

The average recovery of the QC check sample (3 nM in-well solution) was 105% +/- 13%.

5. Specificity

The RBA is specific to toxins that bind to site 1 of voltage-gated sodium channels. This includes all PSP congeners, whereby binding affinity is proportional to potency. Tetrodotoxin also binds to site 1 of the sodium channels, yet the typical combinations of sources, vectors, and geographical regions of tetrodotoxin and the saxitoxins differ.

6. Working and Linear Ranges

The dynamic range of the RBA is 1.2-10.0 nM in-well concentration (Van Dolah 2012). When necessary, samples must be diluted prior to analysis so that they are within the dynamic range of the RBA. Sigmoidal dose response with variable slope analysis is used to generate a binding curve from standard STX concentrations evaluated on each plate.

7. Limit of Detection

See Table 3 in the next section for a description of the limit of detection (LOD) for this method

8. Limit of Quantitation / Sensitivity

The limit of quantitation (LOQ) was determined from the average IC70 of all assays ran in the study, which was 0.71 nM +/- 0.09 nM.Using an adaptation of Eurachem Guide definitions for limit of detection (LOD) and LOQ by Van Dolah et. al. (2012), where B/B0 = 0.7 (average IC70 value) is used as the cutoff for quantitation, we obtain the below values for LOD and LOQ (Table 1). The numbers are for a sample diluted 1/10 (the established minimum dilution to avoid matrix effects) and extraction according to the AOAC protocol.

Table 3: LOD and LOQ for RBA matrix expansion of geoduck SLV

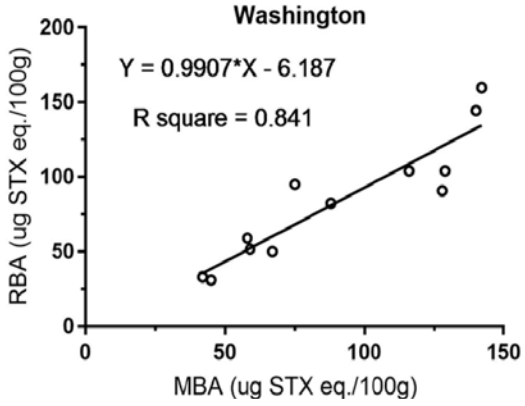
	Equation	SLV Results
LOD	IC_{70} + 3 x SD	4.4 ug STX eq/100 g
LOQ	IC_{70} +10 x SD	7.2 ug STX eq/100 g

9. Ruggedness Previous work has been done to identify critical steps to ensure accuracy and ruggedness (Ruberu et al. 2012, Van Dolah et al. 2012, VanDolah et al. 2009). It was deemed important to clarify the shellfish extracts by centrifugation prior to performing the assay, particularly if the sample was refrigerated or frozen. The rat brain preparations should be vortexed frequently to ensure the synaptosomes are in suspension, and the buffer should be ice cold to ensure that toxins are not released from the receptor. Assay plate filtration should be at a rate of 2-5 seconds for well clearance. Lastly, a minimum of 30 minutes should be allowed before reading the plates after scintillation liquid is added such that scintillant can penetrate the filters (Van Dolah 2013).
10. Matrix Effects No matrix effects were reported. Minimum dilutions of shellfish extracts were 10-fold and were found to be sufficient to eliminate matrix effects. (Van Dolah 2013)

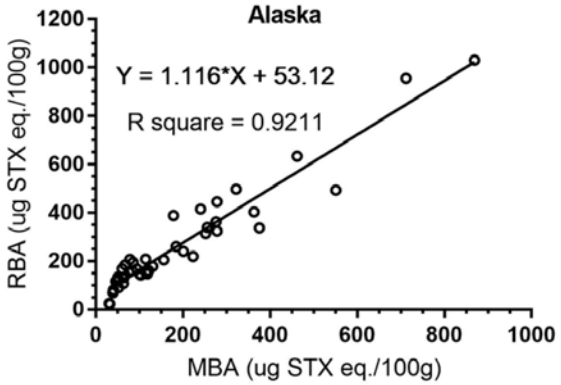
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)

Comparability to MBA

A comparison of STX concentration assayed in naturally contaminated samples by the MBA and the RBA was performed using linear regression analysis (GraphPad Prism, version 7.02). MBA results for samples from Washington were analyzed by the Washington Department of Health Shellfish Biotoxins & Water Bacteriology Laboratories and samples from Alaska were analyzed by the Alaska Department of Environmental Conservation Environmental Health Lab. All RBA results are from analysis by the Sitka Tribe of Alaska Environmental Research Laboratory. 57 total samples were compared, with the RBA yielding no false negatives relative to the regulatory limit of 80 ug/100g. Overall there were 12 false positives relative to the MBA.



	RBA +	RBA -
MBA +	7	0
MBA -	1	7



	RBA +	RBA -
MBA +	28	0
MBA -	11	3

Comparability to Previous RBA Validation Work

Previous work by (Van Dolah et al. 2012, Van Dolah et al. 2009) was submitted to the ISSC as ISSC 2015 Proposal 13-114, resulting in approval of the RBA as a NSSP Approved Method for PSP in mussels and as a NSSP Approved Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP. The results from this SLV for matrix expansion of RBA for geoduck matrix is consistent with the data from the previous validation studies.

A comparison of this SLV to previous validation work for the RBA demonstrates the ability of the RBA to withstand minor changes in analytical technique, reagents, and environmental factors (Table 4).

Table 4: Comparison of SLV results to previous RBA validation studies

	Accuracy (recovery of QC)	Repeatability (Average RSD)	Linear Range (slope, R^2)	IC_{50} (nM)	LOQ (mean IC_{70} - nM)	Comparison to MBA (R^2 from linear regression analysis)
STA Geoduck	104.5%	14.6%	-0.98, 0.97	1.7 +/- 0.1	0.7	0.84, 0.92
Van Dolah et. al. 2009 - SLV	99.3%	17.1%	-0.98, 0.97	2.3 +/- 0.3	1.1	0.98, 0.88
Van Dolah et. al. 2012 - MLV	106.9%	17.1%	-1.03, ND*	1.9 +/- 0.5	0.8	0.84

*No data available

D. Other Information

1. Cost of the Method

The estimated cost per 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample[ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced.
(Van Dolah 2013)

2. Special Technical Skills Required to Perform the Method

General laboratory training is necessary (this would include being able to prepare reagent solutions, pipetting, centrifugation, and simple calculations). Additional training for working with low levels of radioactive material is required.

3. Special Equipment Required and Associated Cost

A microplate scintillation counter is needed and the cost is ~$50-120K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$15K.

4. Abbreviations and Acronyms Defined

3H-STX Tritiated saxitoxin
AOAC, Association of Analytical Communities
ARC, American Radiolabeled Chemicals
B, Bound CPM
Bo, Maximum bound CPM
CFSAN, Center for Food Safety & Applied Nutrition
CPM, Counts per minute
diHCl,Dihydrochloride
Eq, Equivalents
HCl, Hydrochloric acid
IC50, Inhibitory concentration at which CPMs are at 50% of maximum
LC-FD, Liquid chromatography with fluorescence detection
LOD, Limit of detection
LOQ, Limit of quantitation
MBA, Mouse bioassay
MOPS, 3-Morpholinopropanesulfonic acid
NaOH, Sodium hydroxide
NIST, National Institute of Standards and Technology
NSSP, National Shellfish Sanitation Program
OMA, Official method of analysis
PCOX, Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX, Pre-column oxidation liquid chromatography with fluorescence detection
PSP, Paralytic shellfish poisoning
PSTs, Paralytic shellfish toxins
QC, Quality control

QS, Quality System
RBA, Receptor binding assay
RSD, Relative standard deviation
SLV, Single laboratory validation
STX, Saxitoxin

5. Details of Turn Around Times (time involved to complete the method)

Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turn around time for data in approximately 3 hours. Up to six plates per analyst are possible in one day, yielding a throughput of 42 samples per day. If the assay is run in screening mode where only a single dilution (1/10) is run, then through-puts of >120 samples per day can be achieved.

6. Provide Brief Overview of the Quality Systems Used in the Lab

The Center for Food Safety and Applied Nutrition (CFSAN) Quality System (QS) provides guidance to (1) design and develop processes, products, and services related to CFSAN's mission, the FDA's regulatory mission, and critical management and administrative support services, and (2) continually improve and strengthen product and service quality. The Laboratory Quality Assurance program serves as CFSAN's logical application of QS to Center laboratories and lab-based activities. The third edition (October 2009) of the Laboratory Quality Manual was followed. Standard reference materials for saxitoxin are obtained through the National Institute of Standards and Technology (NIST) and are accompanied by a Report of Investigation. The standard reference saxitoxin used in the RBA is the same as that employed with the MBA. The 3H-STX is obtained through American Radiolabeled Chemicals, Inc., and is accompanied by a Technical Data Sheet with lot specifications.

Appendix 1

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish

Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration. All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA, USA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, USA, or International Isotopes Clearinghouse, Leawood, KS, USA).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO, USA; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—*Appendix 1* [*J. AOAC Int*. (future issue)].

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA, USA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL, USA; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk [*Appendix 1*; *J. AOAC Int.* (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Laboratory	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter*.—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)\,\text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	-0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

$$(\text{nM STX equiv.}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv. in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

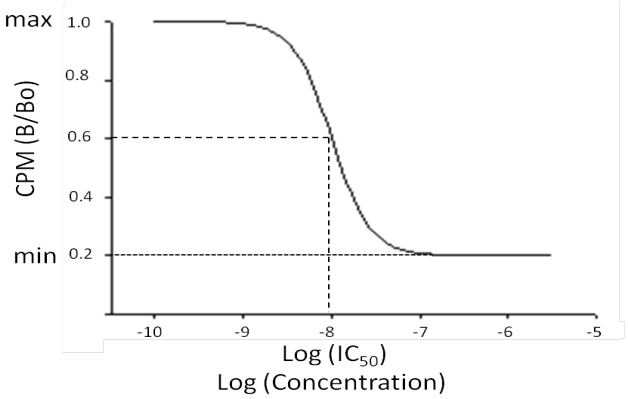


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int*. (future issue)

Appendix 2

FOOD CHEMICAL CONTAMINANTS

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

FRANCES M. VAN DOLAH, TOD A. LEIGHFIELD, and GREGORY J. DOUCETTE
NOAA National Ocean Service, Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412
LAURIE BEAN
Maine Department of Natural Resources, McKown Point, W. Boothbay Harbor, ME 04575
BARBARA NIEDZWIADEK and DOROTHEA F.K. RAWN
Health Canada, Food Research Division, Sir Frederick Banting Research Centre, Ottawa, ON, Canada K1A 0L2

A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX $\pm$ 0.3 (RSD = 10.8%) with a slope of 0.96 $\pm$ 0.06 (RSD = 6.3%) and a dynamic range of 1.2–10.0 nM. The LOQ was 5.3 μg STX equivalents/100 g shellfish. Linearity, established by quantification of three levels of purified STX (1.5, 3, and 6 nM), yielded an r^2 of 0.97. Recovery from mussels spiked with three levels (40, 80, and 120 μg STX/100 g) averaged 121%. Repeatability (RSD_r), determined on six naturally contaminated shellfish samples on 5 independent days, was 17.7%. A method comparison with the AOAC mouse bioassay yielded r^2 = 0.98 (slope = 1.29) in the SLV study. The effects of the extraction method on RBA-based toxicity values were assessed on shellfish extracted for PSP toxins using the AOAC mouse bioassay method (0.1 M HCl) compared to that for the precolumn oxidation HPLC method (0.1% acetic acid). The two extraction methods showed linear correlation (r^2 = 0.99), with the HCl extraction method yielding slightly higher toxicity values (slope = 1.23). A similar relationship was observed between HPLC quantification of the HCl- and acetic acid-extracted samples (r^2 = 0.98, slope 1.19). The RBA also had excellent linear correlation with HPLC analyses (r^2 = 0.98 for HCl, r^2 = 0.99 for acetic acid), but gave somewhat higher values than HPLC using either extraction method (slope = 1.39 for HCl extracts, slope = 1.32 for acetic acid). Overall, the excellent linear correlations with the both mouse bioassay and HPLC method and sufficient interassay repeatability suggest that the RBA can be effective as a high throughput screen for estimating PSP toxicity in shellfish.

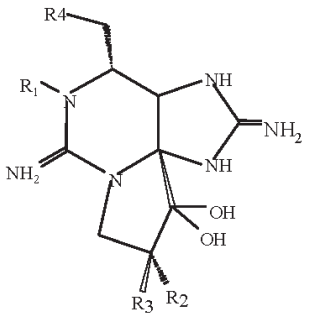
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fran.vandolah@noaa.gov

Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 μg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/μmol
Carbamate	STX	H	H	H	$OCONH_2$	2483
	Neo STX	OH	H	H	$OCONH_2$	2295
	GTX1	OH	OSO3-	H	$OCONH_2$	2468
	GTX2	H	OSO3-	H	$OCONH_2$	892
	GTX3	H	H	OSO3-	$OCONH_2$	1584
	GTX4	OH	H	OSO3-	$OCONH_2$	1803
Sulfocarbamoyl	GTX5 (B1)	H	H	H	OCONHSO3-	160
	GTX6 (B2)	OH	H	H	OCONHSO3-	-
	C1	H	OSO3-	H	OCONHSO3-	15
	C2	H	H	OSO3-	OCONHSO3-	239
	C3	OH	OSO3-	H	OCONHSO3-	33
	C4	OH	H	OSO3-	OCONHSO3-	143
Decarbamoyl	dcSTX	H	H	H	OH	1274
	dcNeoSTX	OH	H	H	OH	-
	dcGTX1	OH	OSO3-	H	OH	-
	dcGTX2	H	OSO3-	H	OH	1617
	dcGTX3	H	H	OSO3-	OH	1872
	dcGTX4	OH	H	OSO3-	OH	-
Deoxydecarbamoyl	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO3-	H	-
	doGTX3	H	OSO3-	H	H	-

Figure 1. Structures and toxic potency of 21 saxitoxin congeners. Toxic potency is listed as mouse units (MU)/μmole, where a mouse unit is defined as the minimum amount required to kill a 20 g mouse in 15 min when administered by IP injection. The table is modified from ref. 15.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200
B	10^{-7}	10^{-7}	10^{-7}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
C	3 x 10^{-8}	3 x 10^{-8}	3 x 10^{-8}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
D	10^{-8}	10^{-8}	10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
E	3 x 10^{-9}	3 x 10^{-9}	3 x 10^{-9}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
F	10^{-9}	10^{-9}	10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
G	10^{-10}	10^{-10}	10^{-10}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
H	10^{-11}	10^{-11}	10^{-11}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			

U = unknown sample

Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(**a**) *Microplate scintillation counter*.—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(**b**) *Microplate filtration manifold*.—Millipore (Bedford, MA).

(**c**) *Hot plate*.—Fisher Scientific (Suwannee, GA).

(**d**) *Countertop centrifuge*.—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(**e**) *Microtiter filter plates (96 well) with 1.0* μ*m pore size type FB glass fiber filter/0.65* μ*m pore size Duropore support membrane*.—Cat. No. MSFB N6B 50 (Millipore Corp., Billerica, MA).

(**f**) *Microplate sealing tape*.—Cat. No. MATA HCL00 (Millipore Corp.).

(**g**) *Vortex mixer*.—Daigger Vortex Genie II (Daigger Scientific, Vernon Hills, IL).

(**h**) *Teflon/glass tissue homogenizer*.—Wheaton (Millville, NJ).

(**i**) *Polytron homogenizer*.—Brinkmann Instruments (Westbury, NY).

Reagents

(**a**) *Hydrochloric acid (HCl)*.—0.1 M.

(**b**) *[^{3}H]STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (International Isotopes Clearinghouse, Leawood, KS).

(**c**) *STX diHCl*.—FDA reference standard (Office of Seafood, Laurel, MD) or National Research Council (NRC) of Canada Institute of Marine Biosciences (Halifax, NS, Canada).

(**d**) *Assay buffer*.—75 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat. No. H9136]/140 mM NaCl, pH 7.5 (Sigma, St. Louis, MO).

(**e**) *Liquid scintillation cocktail*.—Optiphase (PerkinElmer Life Sciences, Downers Grove, IL).

Preparation of Samples (0.1 M HCl Extraction)

Shellfish samples were shucked and homogenized according to the AOAC mouse bioassay protocol (1). For the HCl extraction method, 5.0 (±0.1) g of tissue homogenate was transferred to a tared 15 mL conical polypropylene centrifuge tube. A 5.0 mL volume of 0.1 M HCl was added, and the sample was mixed on a Vortex mixer. The pH was checked to

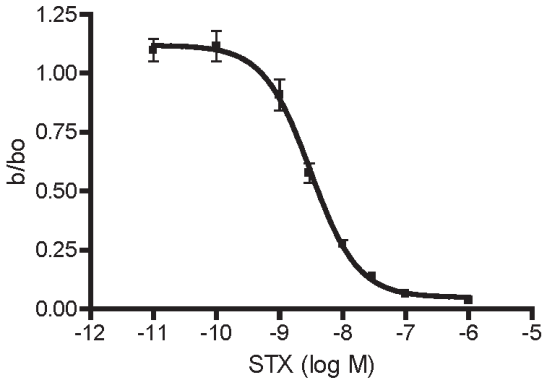


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

confirm it was between 3.0 and 4.0 in order to avoid alkalinization and destruction of the toxin, and adjusted with 1 M HCl or 0.1 M NaOH as needed. Tubes were placed in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Following removal from the boiling water bath, samples were allowed to cool to room temperature, and the pH was again confirmed to be between 3.0 and 4.0. The entire contents were then transferred to a graduated cylinder, diluted volumetrically to 10 mL, and centrifuged for 5 min at 1000 × *g*. The supernatant was transferred to a clean tube.

Preparation of Samples (Acetic Acid Extraction Method)

In a 50 mL plastic centrifuge tube, 5.0 ± 0.1 g homogenate was mixed with 3.0 mL 1% acetic acid on a vortex mixer. Tubes were capped loosely to avoid pressure buildup and placed in a boiling water bath for 5 min. Following removal from the water bath, samples were cooled in a beaker of cold water for 5 min, and then centrifuged for 10 min at 3000 × *g*. The supernatant was transferred to a 15 mL graduated conical test tube. A 3 mL amount of 1% acetic acid was added to the original tube with solid residue, mixed well on a vortex mixer, and centrifuged again for 10 min at 3000 × *g*. The second supernatant was combined with the first and diluted to 10 mL with water.

Preparation of Stock Solutions, Standards, and Reagents for Assay

(**a**) *Radioligand solution.*—[^{3}H]STX stock is provided in 50 μCi ampules, 24 Ci/mmol, 0.1 mCi/mL (4.17 μM). A 15 nM working stock of [^{3}H] STX was prepared fresh daily in 75 mM HEPES/140 mM NaCl (for 2.5 nM final in-well concentration).

(**b**) *STX standard curve.*—FDA STX dihydrochloride reference standard (100 μg/mL or 268.8 μM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6×10^{-6}, 6×10^{-7}, 1.8×10^{-7}, 6×10^{-8}, 1.8×10^{-8}, 6×10^{-9}, 6×10^{-10}, 6×10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total [^{3}H]STX binding in the absence of unlabeled STX.

Table 1. RBA measurements of calibration standards for assay linearity assessment (nM STX; *n* = 5)

Nominal	Mean	SD	RSD
1.5	1.7	0.16	10
3.0	3.0	0.52	17
6.0	6.0	0.34	6

(**c**) *Calibration standard (QC check).*—A reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquotted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 μL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor.

(**d**) *Rat brain membrane homogenate.*—Cerebral cortices from 6-week-old male Holzman rats (Harlan Bioproducts, Indianapolis, IN) were homogenized on ice in a glass/Teflon tissue homogenizer in 75 mM HEPES/140 mM NaCl, pH 7.5, containing 0.1 mM PMSF (phenylmethanesulfonylfluoride;12.5 mL/brain) at 385 rpm for 10 strokes. Pooled homogenates were centrifuged at 20 000 × *g* for 15 min at 4°C and the pellet was resuspended in HEPES buffer (12.5 mL/brain) and rehomogenized on ice using a Polytron homogenizer set at 70% power for 20 s to ensure a fine suspension. The brain homogenate was aliquotted 2 mL/tube in cryovials and stored at –80°C. The protein concentration of the brain homogenate was determined using the Micro bicinchoninic acid (BCA) Assay (Pierce, Rockford, IL). For each assay, an aliquot of brain homogenate was thawed on ice and diluted with ice cold 75 nM HEPES/150 mM NaCl, pH 7.5, to yield a final protein concentration of 0.5 mg/mL in the assay.

Table 2. Recovery of analyte from spiked samples (μg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD_r	Recovery, %
0	<dl[a]			
40	47	8.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

[a] <dl = Less than LOQ (5 μg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; *n* = 5) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	65	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

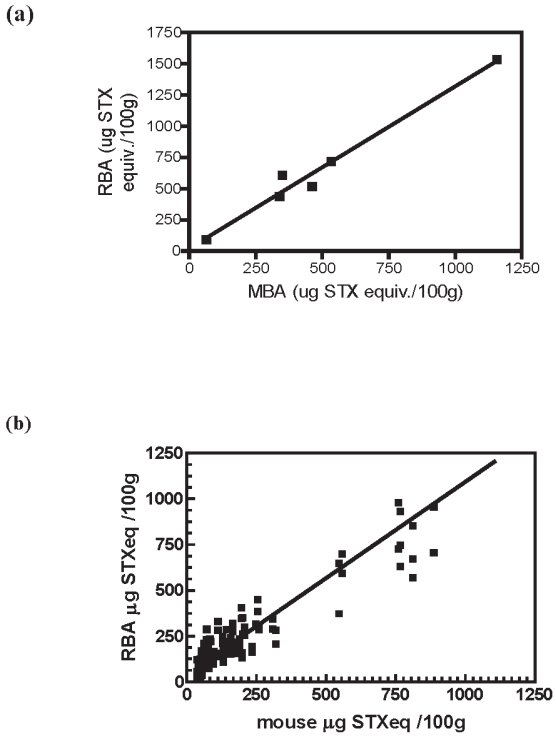


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

Assay Procedure

(**a**) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(**b**) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equialents/100 g shellfish using the following formulas:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM equiv. STX in extract}$$

$$(\text{nm equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, $b/b_o < 0.2$), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., $b/b_o > 0.7$) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD_r = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

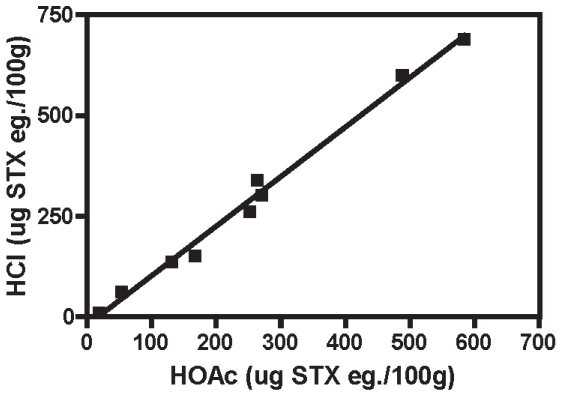


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.6	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Plactopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

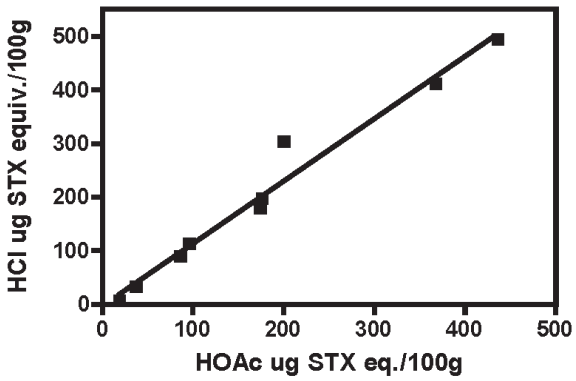


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

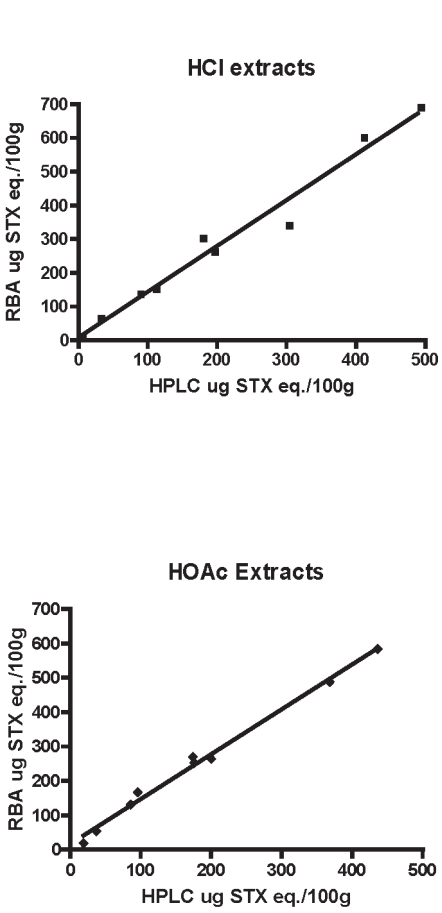


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

Acknowledgments

We thank Karen Mao and Steven Eaker for excellent technical support in the conduct of this study.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2006) *J. AOAC Int.* **88**, 1714–1732

(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(4) Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009) *J. AOAC Int.* **92**, 190–207

(5) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(6) Hall, S., Strichartz, G., Moczydlowshi, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(7) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636

(8) Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(9) Powell, C.L., & Doucette, G.J. (1999) *Nat. Toxins* **7**, 393–400

(10) Suarez-Isla, B.A., & Valez, P. (2000) in *Seafood and Freshwater Toxins,* L.M. Botana (Ed.), Marcel Dekker Inc., New York, NY, pp 187–202

(11) Llewellyn, L.E., Doyle, J., Jellett, J., Barrett, R., Alison, C., Bentz, C., & Quilliam, M. (2001) *Food Addit. Contam.* **18**, 970–980

(12) Ruberu, S.R., Liu, Y.-G., Wong, C.T., & Perera, S.K. (2003) *J. AOAC Int.* **86**, 1–9

(13) Usup, G., Leaw, C.-P., Cheah, M.-Y., Ahmad, A., & Ng, B.-K. (2004) *Toxicon* **44**, 37–43

(14) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667

(15) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

FOOD CHEMICAL CONTAMINANTS

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

FRANCES M. VAN DOLAH, SPENCER E. FIRE, TOD A. LEIGHFIELD, CHRISTINA M. MIKULSKI, and GREGORY J. DOUCETTE
Center for Coastal Environmental Health and Biomolecular Research, NOAA Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412

Collaborators: Å. Andersson; L. Bean; D. Couture; S. DeGrasse; A. DeLeon; V. Dell'Ovo; L. Flewelling; P. Holland; G. Langlois; R. Lewis; M. Masuda; P. McNabb; C. Mikulski; B. Niedzwiadek; B. Porntepkasemsan; D. Rawn; E. Sombrito; K. Srisuksawad; B. Suarez; S. Subsinserm; A. Tubaro

A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

Submitted for publication December 3, 2011.

The recommendation was approved by the Method Committee on Paralytic Shellfish Toxins as First Action. *See* "Methods News," (2011) *Inside Laboratory Management*, January/February issue.

Corresponding author's e-mail: Fran.vandolah@noaa.gov

DOI: 10.5740/jaoacint.CS2011_27

Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
2	MLV06	California mussel (*Mytilus californianus*) from the U.S. west coast	x
3	MLV08	Green mussel (*Perna canaliculus*) from New Zealand	
4	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
5	MLV12	Blue mussel (*M. edulis*) east coast U.S., spiked with 200 µg/kg STX diHCl	
6	MLV14	Blue mussel (*M. edulis*) east coast U.S., spiked with 1200 µg/kg STX diHCl	
7	MLV16	Almeja clam (*Venus antique*) from Chile	
8	MLV01	Surf clam (*Spisula solidissima*) from the U.S. east coast	
9	MLV02	Chorito mussel (*M. chiliensis*) from Chile	
10	MLV04	Scallop (*Placopecten magellanicus*) from the U.S. east coast	
11	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
12	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
13	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
14	MLV13	Blue mussel (*M. edulis*) east coast U.S., spiked with 500 µg/kg STX diHCl	
15	MLV03	Chorito mussel (*M. chiliensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
17	MLV06	California mussel (*M. californianus*) from the U.S. west coast	x
18	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
19	MLV10	Butterclam (*Saxidomus gigantea*) from the U.S. west coast	
20	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
21	MLV15	Blue mussel (*M. edulis*) negative control, east coast U.S.	

[a] Sample number identifies the individual samples analyzed in the assays, with 1–7 analyzed in assay 1, 8–14 in assay 2, and 15–21 in assay 3. Sample identification (MLV for multilaboratory validation) describes the 16 unique samples, among which five were assayed as blind duplicates, to make a total of 21 samples. Blind duplicates, run in different assays, are identified by an "x."

metabolized in shellfish that accumulate them, making analytical determination of paralytic shellfish toxins (PST) in shellfish complex. The long-standing regulatory method for PST is the AOAC mouse bioassay (1; AOAC Method **959.08**), with a regulatory limit of 800 µg STX di HCl equiv./kg shellfish generally applied, but established at 400 µg STX diHCl equiv./kg in certain countries (e.g., the Philippines). However, at concentrations near the regulatory limit, the mouse bioassay can significantly underestimate PST in shellfish (2). This, in addition to increasing resistance to live animal testing in both the United States and the European Union (EU), has increased the need to develop alternative methods suitable for use in a high-throughput monitoring or regulatory setting.

In the past decade, several alternatives to the mouse bioassay have been developed. In the EU, the mouse bioassay remains the reference method for PST in shellfish, but European Commission (EC) Regulation 1664/2006 specifies that other internationally recognized methods may be used. Two HPLC methods, a precolumn oxidation method (3, 4; AOAC Method **2005.06**) and a postcolumn oxidation method (5; AOAC Method **2011.02**), have been approved by AOAC as *Official Methods*SM for PSP toxin analysis. The EC directive recognizes the precolumn oxidation HPLC method (AOAC Method **2005.06**) as an alternative to the mouse bioassay, but retains the mouse bioassay as the reference method in instances where results are challenged. HPLC methods separate and quantify individual STX congeners, which are then recombined according to their toxic equivalencies to yield a composite PST toxicity value. Although the HPLC methods perform well quantitatively, a high-throughput screening method capable of reporting toxic potency directly is still desirable for monitoring programs that often screen large numbers of negative samples. A qualitative lateral flow antibody test for PST with a reported detection limit of 400 µg STX equiv./kg was developed by Jellett Rapid Testing Ltd (Chester Basin, NS, Canada) and approved by the U.S. Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration as a screening method in specific circumstances. This method performed well in a comparison study with the mouse bioassay (6), but is not fully quantitative and has not been subjected to a full AOAC collaborative trial. To date, a suitable quantitative, high-throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The receptor binding assay (RBA) for PST is an excellent candidate for fulfilling the requirements of a high-throughput, quantitative assay that directly reports a composite toxic potency.

The basis of the RBA is the interaction between the toxins and their pharmacological target. All STX congeners bind to site 1 on the alpha subunit of the voltage-gated sodium channel with binding affinities proportional to their toxic potency (7). Therefore, an RBA can quantitatively measure the combined toxic potency of mixtures of STX congeners in a sample,

independent of the toxin congeners present (8). In the RBA for PST, tritiated STX ([^{3}H] STX) competes with unlabeled STX and/or its congeners for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H] STX is removed by filtration and receptor bound [^{3}H] STX quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unlabeled toxin present. A standard curve is generated using increasing concentrations of nonradiolabeled STX standard from 10^{-10} to 10^{-6} M STX. The concentration of toxin in samples is determined in reference to the standard curve.

The assay being tested in this collaborative trial is a modification of the method of Doucette et al. (9) to incorporate a 96-well microtiter plate format, which increases sample throughput and minimizes error by reducing sample handling and pipetting steps. This microplate PST RBA was evaluated in a single-laboratory validation (SLV) study (10), which established an interassay repeatability (RSD_r) of 17.7% and good correlation with the mouse bioassay and precolumn oxidation HPLC methods. The toxin concentrations in shellfish tested in the SLV study ranged from near to well above the regulatory limit (approximately 900–15 000 μg STX diHCl equiv./kg). The current study focuses more specifically on the performance of the RBA in the critical range of shellfish toxicities below, near, and slightly above the regulatory limit (approximately 150–2400 μg STX diHCl equiv./kg).

The results of the collaborative study suggest that the RBA for PST is a suitable high-throughput screen for PST in shellfish. Although HPLC methods offer quantitative information on congener composition of samples, often the desired information is composite toxic potency, which requires the summation of individual congeners, corrected for their individual toxic equivalencies. The RBA provides a single integrated toxic potency value that reflects activity of all known and potential unknown congeners present in the sample. Use of the microtiter plate format, in conjunction with microplate scintillation counting, provides the ability to screen multiple samples simultaneously in a total assay time of less than 3 h. The assay format described in the current study provides for the quantitative determination of composite PST toxicity in seven shellfish extracts per 96-well microplate, each run in triplicate at three dilutions, covering toxicity ranges of approximately 35–6000 μg STX diHCl equiv./kg. In a high-throughput assay setting, multiple plates can be set up simultaneously, so that six assay plates can easily be accommodated each day by a single analyst, for a throughput of 42 samples/day. This compares favorably to an estimated throughput of 20–25 samples a day by the precolumn HPLC method (B. Niedzwiadek, Health Canada, personal communication) or 30–35 by mouse bioassay (B. Suarez, University of Chile, personal communication).

Collaborative Study

The focus of this study was to assess the performance of the RBA to determine PST toxicity in samples of commercially important shellfish at a range of concentrations below and above the regulatory limit. Twenty-one shellfish homogenates were included in the study, which represented 16 unique samples (Table 1). The homogenates included 12 naturally contaminated shellfish samples of different species collected from several geographic regions: blue mussel (*M. edulis*) from the U.S. east and west coasts, California mussel (*M. californianus*) from the U.S. west coast, chorito mussel (*M. chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method **959.08**), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**); however, final concentrations in µg/kg and µg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well CVs for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_o), and calculated values for these samples in the well (nM), in the extract (µg STX equiv./mL), and in the shellfish tissue (µg STX equiv./kg). Participants were also asked to report all raw count data so that all results could be analyzed by the coordinating laboratory using identical software (GraphPad Prism 4.0) to assess whether systematic differences in quantification arose from using different curve-fitting software. All data were reported via e-mail to the coordinating laboratory.

The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in dilutions and calculations and for use of the prescribed curve-fitting model. Obvious errors were corrected and the participant laboratory was consulted for concurrence. The reviewed results were then used for evaluation in the collaborative study.

Statistical Evaluation of the Collaborative Study

For each sample analyzed, outliers were first determined using the Grubbs test at a probability value of 1% (www.graphpad.com), with no more than one outlier removed, so that valid data remained from a minimum of eight laboratories. The mean, S_R, and RSD_R, and HorRat values were then calculated for each sample. For blind duplicates, the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0, was used to further evaluate for outliers and determine S_r and RSD_r. GraphPad Prism was used to determine correlation among the RBA, mouse bioassay, and HPLC results.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration.

All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, or International Isotopes Clearinghouse, Leawood, KS).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—See *Appendix*.

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation*.—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \cdot EC50\ Hill\ slope)}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (GraphPad Software, Inc.) is recommended. For the microplate counter users, receptor

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	–0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

$$(\text{nM STX equiv}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCL equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration). Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

	Microplate column											
Microplate row	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

Results and Discussion

Sample Characterization

All shellfish homogenates (MLV1–16) were analyzed by HPLC using the precolumn oxidation method (AOAC Method **2005.06**) to determine toxin congener profiles and quantify total PST as μg STX diHCl equiv./kg prior to initiation of the study (Table 2). It is noteworthy that the clear majority of samples, irrespective of shellfish species and location, were dominated largely by STX and GTX2,3 whereas the N1-hydroxylated congeners NEO and GTX1,4 were virtually absent, except in blue mussel from the U.S. west coast. The most unusual profile was observed in green mussel, which was dominated by the weakly toxic N-sulfo-carbamoyl congeners C1,2. The samples were analyzed by the AOAC mouse bioassay (AOAC Method **959.08**) by three laboratories that routinely perform the mouse bioassay for regulatory purposes (Table 3). The mouse bioassay detection limit is approximately 400 μg STX diHCl equiv./kg (one laboratory reported values as low as 290 μg STX equiv./kg). Because the study design included samples that bracketed the lower regulatory limit of 400 μg STX diHCl equiv./kg, several samples were reported as being below the mouse bioassay detection limit. For samples in which all values were above the detection threshold, the between-laboratory RSD_R of the mouse bioassay was 18.9%.

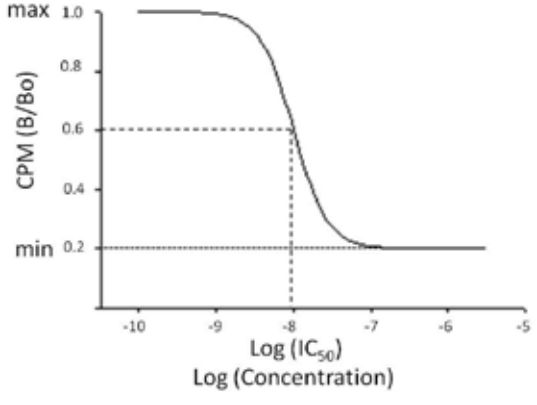


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

Data Reporting and Initial RBA Data Review

Nine of the 10 laboratories that received the study materials completed the study and reported results. All nine carried out the practice assay and reported results to the coordinating laboratory, which evaluated the results and provided feedback to the participating laboratories before initiating the full study. Following completion of the full study, the participating laboratories provided all raw and calculated data for each of

the three assays performed via e-mail to the coordinating laboratory. The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in sample dilutions and calculations, and for the use of the prescribed curve-fitting model. One laboratory used a sigmoidal curve-fitting model with the slope set to 1 (one-site binding curve in Prism), rather than the prescribed four-parameter logistic fit. In this case, the raw data were reanalyzed by the coordinating laboratory using the prescribed method. Obvious errors in calculation were corrected, such as accounting for the two-fold sample dilution resulting from the extraction process. In some cases, the participating laboratory carried out a fourth assay due to variability or inconsistency among dilutions for selected samples. In these cases, the value reported from the repeat (fourth) assay was used. One laboratory had consistent disagreement between the 1/50 and 1/200 dilutions when both fell within B/B_0 0.2–0.7. In all cases the 1/200 dilution overestimated almost two-fold relative to the 1/50 dilution, suggesting a systematic dilution error. In standard practice, these samples should be rerun. However, the instructions did not direct the participants to do so. Therefore, where there was corroborative evidence for the value reported by the 1/50 dilution, based on the 1/10 dilution, the 1/200 dilution was omitted. Where there was no basis on which to exclude the 1/200 value, an average value was calculated. This tended to result in an overestimate, and in two cases resulted in statistical outliers.

Overall Performance of the Method: Reproducibility

Table **2011.27A** summarizes the results obtained for 21 individual shellfish samples analyzed in three RBAs, determined by nine participating laboratories. Samples 1–7 were analyzed in the first assay, samples 8–14 in the second assay, and samples 15–21 in the third assay. Among these samples were five blind duplicates, treated here as individual unknown samples. One sample (marked by an footnote a in Table **2011.27A**) had a high variability in CPM between wells that was not attributable to any known cause, and was, therefore, omitted from analysis. Outliers identified by Grubbs test ($P < 0.01$) were excluded from the analysis (marked by footnote b in Table **2011.27A**). The overall RSD_R among all 21 independent samples was 33.2%, resulting in an average HorRat value of 2.0 (Table **2011.27A**). The HorRat values on individual samples ranged from 1.4 to 3.3, with a median value of 1.8. There was no apparent trend in reproducibility according to sample concentration or among shellfish species. If only the laboratories that are routine users of the RBA for PST (Laboratories 1–5) are included in the analysis, the average RSD_R is 23.1%, resulting in an average HorRat value of 1.4. Laboratory 9 tended to report the lowest values among the participating laboratories (14 of 21 samples), and although its individual sample values were not found to be statistical outliers, removing the results of this laboratory reduces all but one HorRat value (which remains unchanged), yielding an average HorRat value of 1.8 (range 1.0–2.8; Table **2011.27A**). Removal of any other single laboratory's results does not appreciably change the overall study performance. The reason for the systematically low values reported by Laboratory 9 is not clear, since the assay parameters fall well within those reported by the other laboratories. Given that assay parameters are within normal range, one possible source of systematic error could be incomplete extraction or pH adjustment of extracts, either of which would result in lower toxicity values.

A comparison of the RBA reproducibility with that of existing AOAC *Official Methods* is instructive. The AOAC collaborative study of the mouse bioassay (11), which entailed the analysis of seven samples representing three levels of STX-spiked shellfish by 11 participating laboratories, yielded a similar average RSD_R of 22%. More recent proficiency tests of the mouse bioassay performed in European regulatory laboratories report RSD_R of 2.3–38.3% on three samples run by eight laboratories (2) and RSD_R of 18.1–44.8% on two samples run by 20 laboratories (12). The mouse bioassay RSD_R values obtained in the current study ranged from 1.1 to 46.3% (average 19%) for three laboratories. The collaborative studies of the HPLC methods report reproducibility values for individual PST congeners, but do not report reproducibility of the composite toxic potency values. Collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an average RSD_R of 27.0% and HorRat value of 1.3 (range 0.8–2.1) for STX following C18 cleanup, but the reproducibility of other congeners varied considerably, with the maximum HorRat value (4.7), exceeding the highest HorRat value obtained by RBA (3.3).

Because composite toxic potency values were not reported in the studies of the HPLC methods, it is uncertain how this variability influences the composite toxic potency calculated from these methods. The average and ranges of HorRat values obtained for different congeners were: neoSTX–1.7 (range 1.2–2.5); dcSTX–1.1 (range 0.6–2.1); GTX1,4–1.9 (range 1.1–4.2), GTX2,3–1.4 (range 0.8–1.9); B1–1.1 (range 0.7–1.9); and C1,2–1.6 (range 0.9–4.5). Because of the variability obtained in neoSTX, GTX1,4, C3,4, and B2, AOAC Method **2005.06** calls for a second SPE-COOH cleanup of samples suspected of containing these congeners, after which reproducibility improved somewhat: neoSTX–1.8 (range 1.3–2.1); GTX1,4–1.3 (range 1.0–2.1); and C3,4–1.2 (range 0.8–1.8). The postcolumn oxidation HPLC method (AOAC Method **2011.02**) reported an average HorRat value of 0.6 for STX. In this method, neoSTX with an average HorRat of 1.9 (range 0.6–4.0) and GTX4 with an average HorRat of 1.6 (range 1.0–2.9) had reproducibility values that may affect the overall composite potency values. The maximum HorRat value (4.0) reported in this study also exceeded the maximum value reported in the RBA.

In summary, with the removal of Laboratory 9, the overall reproducibility of the RBA falls within the performance measures achieved by the established AOAC *Official Methods* for PST. The difference in reproducibility achieved by the laboratories that are routine users of the assay and participants who are not routine users of the method highlights the importance of training if this method were to be implemented in a regulatory setting.

Within-Laboratory Repeatability

Within-laboratory variability (RSD_r) was determined on five samples that were provided as blind duplicates. Participants were unaware that blind duplicates were included among the coded samples received. The duplicate samples were coded so that they were analyzed in separate assays, with different duplicate pairs falling into different assays (Table 1). One outlier was found among the results of the blind duplicates by Cochran's

test, $P < 0.025$ (Laboratory 7, sample MLV11) using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0. An overall RSD_r of 25.1% was observed, with an RSD_R of 32.9%, yielding a HorRat value of 2.0, similar to that of the overall study (Table **2011.27B**). When the performance of individual laboratories was evaluated separately, the average RSD_r was 22.2%, with individual laboratories varying from 11.8 to 34.4% (Table **2011.27C**). Routine users of the microplate format of the PST RBA (Laboratories 1–5) obtained an average RSD_r of 17.1%, which is similar to that obtained in the SLV study (10), and lower than that obtained by nonroutine users (Laboratories 6–9), which averaged 26.1% and ranged as high as 34.4%. The AOAC collaborative study of the mouse bioassay (11) did not report RSD_r; however, analysis of the data from that study using AOAC INTERNATIONAL's Interlaboratory Study Workbook for Blind Duplicates results in an average RSD_r of 16.5% for three STX-spiked samples. Proficiency testing of the mouse bioassay performed in eight French laboratories reported an average RSD_r of 8.3% on three samples (2). The analysis of blind duplicates in the collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an RSD_r of 15.2% for STX following SPE C18 cleanup and an average RSD_r of 16.4% across all congeners, which ranged from 6.0 to 31.7%. Following SPE–COOH cleanup, repeatability was similar, with RSD_r of 17.2% across all congeners. The intralaboratory repeatability values obtained in the postcolumn oxidation HPLC method (AOAC Method **2011.02**) averaged 6.4% for STX; most other congeners were similar, with neoSTX being the only congener that showed a somewhat higher RSD_r of 23.3%.

In summary, the within-laboratory repeatability of the RBA was found to be acceptable, with all but two laboratories achieving an RSD_r of 23.3% or less, and the routine users of the assay achieving an average RSD_r of 17.1%.

Spike Recovery

Three samples included in the study were homogenates of blue mussel spiked with STX diHCl at concentrations intended to bracket the regulatory limits of 800 μg STX equiv./kg used by most countries and 400 μg STX equiv./kg imposed in the Philippines. Nominal concentrations in the spiked samples were 200, 500, and 1200 μg STX equiv./kg. Also included in the study was the blue mussel homogenate to which the STX spikes had been added, which was determined to be negative for STX by the precolumn oxidation HPLC method. The negative control homogenate was reported as nondetectable by eight of nine laboratories. Recovery of spiked STX by the RBA was 84.4, 93.3, and 88.1%, respectively, for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, and yielded a slope of 0.87 and r^2 of 0.86 (Figure 2). In the current study, the mouse bioassay reported < detection limit, and 68.6 and 40.5% recovery for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels. The AOAC collaborative study of the mouse bioassay (11) reported recoveries of 62.3% at spike levels similar to those in the current study (equivalent to 1000 μg STX diHCl equiv./kg) but higher recoveries of 81.5 and 96.0% were achieved at higher spike levels equivalent to 4000 and 8000 μg STX diHCl equiv./kg.

The observed poor recovery in the mouse bioassay at concentrations near and below the regulatory limit has been observed in other studies (2), and has been attributed to a salt or protective effect of the shellfish matrix, which, for concentrations at or below the regulatory limit of 800 μg/kg, is injected undiluted into the mouse. The spike recovery observed in the precolumn HPLC method in this study is also somewhat low, with 54.0, 62, and 51.5% recovery at the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, respectively. The AOAC collaborative study of the precolumn HPLC method reported 74.4–76.8% at similar spike levels following SPE C18 cleanup and 63.7–68.2% following SPE-COOH cleanup (3, 4). In comparison, the postcolumn HPLC method reported 88–104% recovery of STX spiked at levels somewhat lower than the current study. The higher recovery of the RBA than the HPLC method in the current study may reflect the use of the 0.1 M HCl extraction method in the RBA as compared to the acetic acid extraction used in the HPLC methods.

We previously established in the SLV study that the RBA performs well with shellfish extracted using either method (10). In that study, the RBA reported slightly higher toxicity values for shellfish extracts made using the 0.1 M HCl method than the acetic acid extraction, yielding a correlation of 0.99 with a slope of 1.23 (10). The higher toxicity reported by the RBA in 0.1 M HCl extracts may reflect the hydrolysis of less toxic congeners to more toxic congeners.

Assay Parameters and Quality Metrics

Table **2011.27D** summarizes the assay parameters and quality metrics for all laboratories. Eight of nine laboratories used microplate scintillation counters. Laboratory 4 used the manual counting method in which the microplate well filters are punched out, using an eight-place punch system, into traditional 4 mL scintillation vials and counted. Its performance using the manual counting method (RSD_r 17.4%) was similar to or better than that of the laboratories using the microplate method, indicating that using the manual counting method does not affect the performance of the assay. Similarly, there was no apparent difference in assay parameters when the Packard Top Count (single detector) was used, compared to the Wallac Microbeta (coincidence detector), although the reference CPM values obtained on the Top Count generally were somewhat lower due to differences in counting efficiency inherent in the differences in detector geometry. Eight of nine laboratories used GraphPad Prism for curve-fitting, while only Laboratory 5 used Wallac MultiCalc software. Values reported by Laboratory 5 fell well within the range of values reported by laboratories using Prism.

All assays resulted in slopes between –0.8 and –1.2, as specified in the protocol. This specification reflects the fact that in a competitive binding assay for a ligand that interacts specifically at a single receptor site, the slope of the resulting standard curve should theoretically be 1.0. Although curve-fitting software packages often include a one-site binding curve that fixes the slope at 1.0, we specified in the protocol the use of the four-parameter logistic fit (also known as sigmoidal dose-response with variable slope), because it more readily identifies problems with the standard curve that may skew results. Laboratory 9 reported results using a one-site binding curve fit; in this case, the coordinating laboratory recalculated their raw data using the four-parameter logistic fit. The protocol also calls for RSD% < 30 on all standards. Most analysts did not experience variability problems in the standard wells. Infrequent high RSDs were most often associated with the well

Table 2. Congener profiles in shellfish homogenates included in the collaborative study[a]

Sample name	Species	STX	NEO	dcSTX	GTX1,4	GTX2,3	dcGTX2,3	B1	C1,2	C3,4	Total PSP	µg STX diHCl equiv./kg
MLV01	Surf clam	639.8		74.0		226.2	207.0				1146.9	894.3
MLV02	Almeja clam	298.3				1290.1		266.6			1855.0	802.1
MLV03	Chorito mussel	77.6				310.4					388.0	195.5
MLV04	Atlantic sea scallop	831.6				2785.6					3617.3	1890.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.9	1070.9
MLV10	Butter clam	1763.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	684.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHCl equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	890	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method **2006.02**, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10×SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0 <0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table **2011.27D**. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3×SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10×SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_R	RSD_R,%	Recovery, %
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

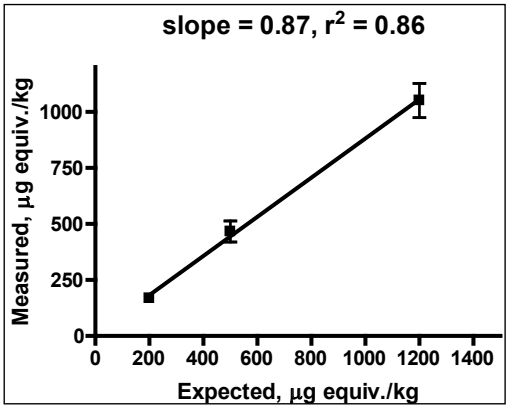


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table **2011.27E**). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8″ Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_o 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_o 0.2–0.7. When corrected for dilution, serial sample dilutions should yield similar quantification. The absence of linearity between sample dilutions indicates either error in dilution or sample matrix interference; however, at the minimum sample dilutions recommended in the proposed *Official Method*, matrix effects from shellfish homogenates have not been encountered (10). In the current study, the nonlinearity of dilutions experienced in several samples by Laboratory 8 was not observed by the other laboratories, suggesting a systematic sample dilution issue rather than a sample matrix problem. Although experienced in RBAs in general, Laboratory 8 had not previously run the microplate filtration format of the assay for PST.

Laboratory 9, which reported generally lower values than the other laboratories, although familiar with the assay, had not performed it in more than a year. The lower values reported do not appear to be associated with conduct of the assay, or scintillation conduct of the assay, or scintillation counting, since the assay metrics are well within the averages reported by the other laboratories. Insufficient boiling or pH adjustment of sample extracts are a possible explanation. These points identified by the study participants should be added to the critical steps identified in the SLV study (10) that can affect precision and accuracy of the assay results, including: (*1*) ensure that the water is strongly boiling during extraction; (*2*) carefully adjust pH of extracts; (*3*) ensure even distribution of the membrane preparation across the microplate by frequent vortex-mixing or pipetting before and during its addition to the plate; (*4*) the wells must clear within 2–5 s during filtration; (*5*) the wash buffer should be ice-cold to minimize the rate of toxin release from the receptor; and (*6*) following addition of scintillant to the wells, incubate a minimum of 30 min to ensure that the scintillant fully penetrates the filters before counting.

Recommendations

The collaborative study of the RBA for PST was completed by nine laboratories representing six countries. Collaborators quantified PST as a composite toxicity value reported in µg STX di HCl equiv./kg in a variety of shellfish species from different regions of the world, containing varied toxin congener profiles. The study included laboratories with extensive experience as well as others with little or no previous experience. The study also included both microplate and scintillation counters as end points, because either instrument type could potentially be used by test laboratories. The study demonstrates that the RBA yields adequate repeatability, reproducibility, and recovery for routine determination and monitoring of PST in shellfish. The greater precision attained by laboratories that received prior training on the RBA and routinely implement this assay suggests that

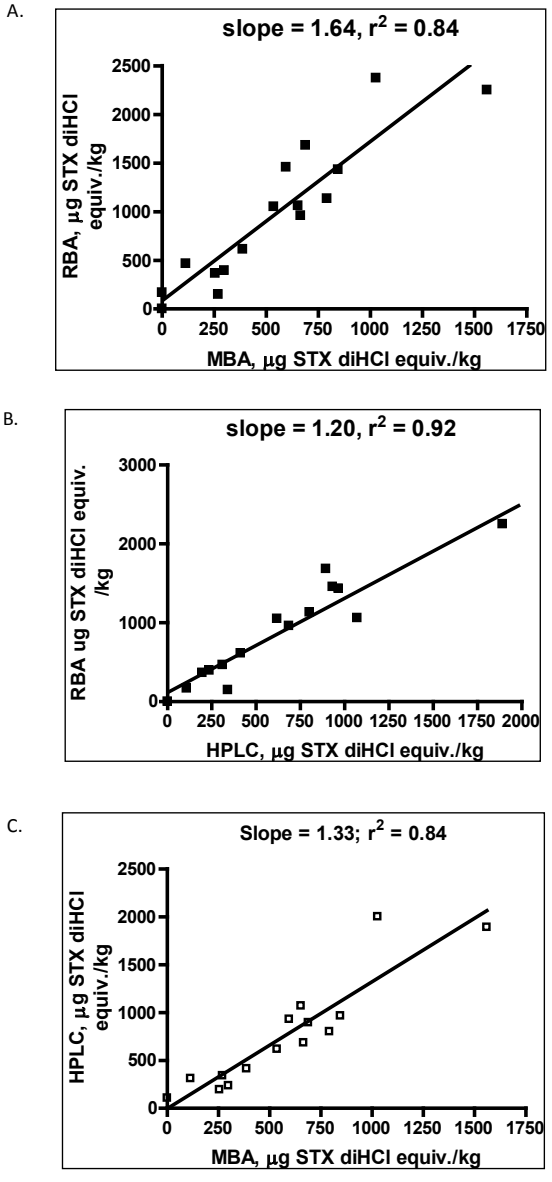


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

the overall interlaboratory reproducibility can be further improved. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the determination of PST in shellfish.

Acknowledgments

We would like to thank Laurie Bean (Maine Department of Marine Resources), Stacey DeGrasse (U.S. Food and Drug Administration), Patrick Holland (Cawthron Institute), Gregg Langlois (California Department of Health Services), Bob Lona (Washington State Department of Health), and Benjamin Suarez (University of Chile) for providing the shellfish material used in the study. We are grateful to Barbara Niedzwiadek and Dorothea Rawn (Health Canada) for HPLC analyses and to Laurie Bean and Darcie Couture (Maine Department of Marine Resources), Benjamin Saurez, and Supanoi Subsinserm (Thailand Department of Fisheries) for performing mouse bioassays. We are indebted to the following collaborators for their dedication and perseverance in carrying out the RBAs for this study:

Leanne Flewelling, Florida Wildlife and Marine Research Institute, St. Petersburg, FL

Stacey DeGrasse, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD

Patrick Holland and Paul McNabb, Cawthron Institute, Nelson, New Zealand

Gregg Langlois and Melisa Masuda, California Department of Public Health, Richmond, CA

Richard Lewis and Åsa Andersson, University of Queensland, Queensland, Australia

Christina Mikulski, NOAA Marine Biotoxins Program, Charleston, SC

Elvira Sombrito and Aileen DeLeon (Philippine Nuclear Research Institute, Manila, the Philippines

Kanitha Srisuksawad and Boonsom Porntepkasemsan, Thailand Nuclear Research Institute, Bangkok, Thailand

Aurelia Tubaro and Valeria Dell'Ovo, Dipartimento dei Materiali e Risorse Naturali, Trieste, Italy

This project was funded by the International Atomic Energy Agency Interregional Project INT7017 9001 and NOAA Marine Biotoxins Program Project 02E0017. FDA saxitoxin diHCl used for tritiation by IICH and STX diHCl reference standard (NIST RM 8642) were provided by Sherwood Hall, FDA Office of Seafoods.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) LeDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(3) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(5) *Official Methods of Analysis* (2011) AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.02**

(6) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425. http://dx.doi.org/10.1016/S0041-0101(02)00153-8

(7) Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(8) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667. http://dx.doi.org/10.1021/tx050277i

(9) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636. http://dx.doi.org/10.1016/S0041-0101(96)00189-4

(10) Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., & Rawn, D.F.K. (2009) *J. AOAC Int.* **92,** 1705–1713

(11) McFarran, E.F. (1959) *J. AOAC Int.* **42**, 263–271

(12) EU Reference Laboratory for Marine Biotoxins (2010) *Report*

on the EURLMB 2010 proficiency testing on saxitoxin group (PSP) toxins determination, Vigo, Spain

(13) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics,* http://www.eurachem.org/index.php/publications/guides/mv

(14) Kleivdahl, H., Kristiansen, S.-I., Nilson, M.A., Goksoyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(**a**) *Teflon/glass homogenizer*.—Motorized tapered Teflon pestle and glass tube, 15 mL.

(**b**) *Motorized tissue homogenizer*.—Polytron or small hand-held blender.

(**c**) *High-speed centrifuge and fixed angle rotor.*—Capable of 20 000 × *g* (rcf).

(**d**) *Centrifuge tubes*.—12–15 mL rated for >20 000 × *g* (rcf).

(**e**) *Plastic cryovials*.—2 mL.

(**f**) *Graduated beaker*.—300 or 500 mL.

(**g**) *Pipets*.—Disposable 5 and 10 mL.

(**h**) *Forceps*.

B. Reagents

(**a**) *20 Rat brains*.—Male, 6-week-old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA; http://hilltoplabs.com) or equivalent.

(**b**) *MOPS*.—pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G).

(**c**) *Choline chloride*.—100 mM (Sigma; Cat. No. C7527-500G).

(**d**) *Phenyl methylsulfonyl fluoride (PMSF)*.—Sigma; Cat. No. P7626.

(**e**) *Isopropanol*.

C. Procedure

(*1*) Prepare 1 L 100 mM MOPS buffer, pH 7.4, containing 100 mM choline chloride (detailed protocol in **E**, above) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol; dissolve 0.174 g PMSF in 10 mL isopropanol to make 100 mM stock. Aliquot and store at –20°C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh on the day of use.

(*2*) Remove medulla and cerebellum from each brain using forceps and discard. Place the cerebral cortex (*see* Figure 1) in a small amount of ice-cold buffer and place on ice.

(*3*) Place one cerebral cortex in 12.5 mL MOPS/choline Cl/PMSF, pH 7.4, in glass/teflon homogenizer (two brains in 25 mL buffer will fit into 30 mL homogenizer tube). Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes (more if necessary to homogenize brain; there should be no visible chunks remaining in the homogenate). Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

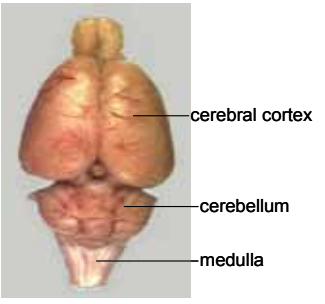


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20 000 × g for 15 min at 4°C.

(*5*) Aspirate the supernatant and resuspend the pellets in ice-cold MOPS/choline Cl/PMSF buffer, using an adequate amount (~5 mL) to fully resuspend the pellet (can use clean glass stir rod to break up pellet), not exceeding 10 mL per brain.

(*6*) Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume to 200 mL total (keep on ice).

(*7*) Keeping the beaker on ice, Polytron (or use a small hand-held blender at low speed) at 70% full speed for 20 s to obtain a consistent homogenate.

(*8*) Aliquot 2 mL/tube into cryovials. It is critical to keep the preparation well mixed while dispensing, e.g., prior to each aliquot to ensure equal allocation of protein/receptors to each vial. Keep cryotubes on ice.

(*9*) Freeze and store at –80°C. This preparation is stable for at least 6 months. Use a permanent marker to label the preparation date on the storage container.

D. Protein Assay

(**a**) Determine protein concentration of membrane preparation using Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) protein assay kit or equivalent protein assay (Thermo Fisher, Rockford, IL). The above protocol should yield 6–8 mg protein/mL of rat membrane preparation.

(**b**) Determine membrane dilution needed for the assay. The protein concentration in the daily working stock for the assay should be 1 mg/mL (this is diluted in the assay to yield 0.5 mg/mL in-assay concentration). Based on the protein concentration determined in the protein assay, determine the dilution needed to achieve 1 mg/mL. This is the dilution used in section **E**(**e**) above for all assays using this lot of membrane preparation. The protocol above typically yields a protein concentration that requires a dilution of 1/6–1/8. (Do not use less than 1/4 dilution or filtration wells may become clogged.) Protein concentration will need to be determined for each new batch of membrane preparation.

Proposal No. 17-106

Taylor & Francis
Taylor & Francis Group

Food Additives & Contaminants: Part A
2012, 1–10, iFirst

Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins

S.R. Ruberu*, G.W. Langlois, M. Masuda and S. Kusum Perera

Drinking Water and Radiation Laboratory Branch and Environmental Management Branch, California Department of Public Health, 850 Marina Bay Parkway, Richmond, CA 94804, USA

(*Received 13 April 2012; final version received 9 July 2012*)

The receptor-binding assay (RBA) method for determining saxatoxin (STX) and its numerous analogues, which cause paralytic shellfish poisoning (PSP) in humans, was evaluated in a single laboratory study. Each step of the assay preparation procedure including the performance of the multi-detector TopCount® instrument was evaluated for its contribution to method variability. The overall inherent RBA variability was determined to be 17%. Variability within the 12 detectors was observed; however, there was no reproducible pattern in detector performance. This observed variability among detectors could be attributed to other factors, such as pipetting errors. In an attempt to reduce the number of plates rejected due to excessive variability in the method's quality control parameters, a statistical approach was evaluated using either Grubbs' test or the Student's *t*-test for rejecting outliers in the measurement of triplicate wells. This approach improved the ratio of accepted versus rejected plates, saving cost and time for rerunning the assay. However, the potential reduction in accuracy and the lack of improvement in precision suggests caution when using this approach. The current study has recommended an alternate quality control procedure for accepting or rejecting plates in place of the criteria currently used in the published assay, or the alternative of outlier testing. The recommended procedure involves the development of control charts to monitor the critical parameters identified in the published method (QC sample, EC_{50}, slope of calibration curve), with the addition of a fourth critical parameter which is the top value (100% binding) of the calibration curve.

Keywords: receptor-binding assay; paralytic shellfish poisoning; saxitoxins; variability

Introduction

Coastal regions with a history of the occurrence of paralytic shellfish poisoning (PSP) toxins present unique challenges to the organisations responsible for protecting public health. The agencies responsible for monitoring these toxins in shellfish (e.g., mussels, oysters) and other seafood species have traditionally relied on the mouse bioassay (MBA) (American Public Health Association (APHA) 1970). Until recently this live animal assay has been the only method recognised by the National Shellfish Sanitation Program (NSSP) as administered by the US Food and Drug Administration (USFDA). The MBA has served these monitoring programmes well over the decades, but the continued use of live animals for toxin testing presents practical and ethical concerns. The MBA is also recognised as having relative poor accuracy and precision due to matrix effects at low dilutions and inherent differences in response among animals. As a result there has been a considerable amount of work and progress in the development of alternative methods including a receptor-binding assay (RBA) method (Doucette et al. 1997; Powell and Doucette 1999; Ruberu et al. 2003) and HPLC methods (Lawrence et al. 2005; van de Riet et al. 2009). The latter HPLC method has recently been accepted by the Interstate Shellfish Sanitation Conference (ISSC) and USFDA for use within the NSSP. The RBA method has recently been issued as an Official Method of Analysis (OMA) (number 2011-27) by the Association of Official Analytical Chemists (AOAC), but has yet to be presented to the ISSC for acceptance.

Most, if not all, monitoring programmes have similar requirements with respect to an acceptable replacement method for the MBA. Analytical turn-around time and sample throughput are critical factors for getting data into the hands of managers quickly, so decisions can be made regarding quarantines and notification of the public. Shellfish sample collection and shipment to an accredited laboratory can introduce significant time delays, often 24–48 h, placing the responsible agency at an immediate disadvantage in its efforts to protect consumers. Therefore, there is a need for a method that can provide data within hours of

*Corresponding author. Email: Shiyamalie.Ruberu@cdph.ca.gov

ISSN 1944–0049 print/ISSN 1944–0057 online

http://dx.doi.org/10.1080/19440049.2012.712063
http://www.tandfonline.com

sample arrival. The preferred method must also be capable of automation to accommodate a significant influx of samples when PSP levels begin increasing in a region. It is also highly preferable that the methodology be simple enough to be carried out by a trained technician, as opposed to the more technically sophisticated methods that require an experienced analyst with an advanced degree. Other desirable features include improved accuracy, precision and sensitivity relative to the current MBA. The lack of precision of the MBA creates ambiguity when results are close to the action level (80 μg of saxitoxin (STX) equivalents per 100 g of shellfish tissue, abbreviated as 80 μg/100 g). Replication would help alleviate this ambiguity but is usually impractical when large numbers of samples are being assayed and throughput time must be minimised.

The van de Riet HPLC method may be the alternative method of choice for some regulatory laboratories. One seemingly minor but very important practical consideration in this regard is the administrative location of the current MBA work. If this work is presently conducted in a laboratory section in which chemical instrumental analyses are also conducted (e.g., HPLC methods), then the adoption of the HPLC method for PSP toxins could be a relatively simple transition both technically and organisationally. However, if the MBA work is currently conducted in a microbiology setting, a number of obstacles may preclude adoption of a chemical instrumental method in favour of an assay format more familiar to the microbiologist, such as an immunoassay or receptor assay. The potential obstacles in these times of reduced resources include retraining or hiring new staff, purchasing of expensive equipment or transferring resources from one department to another. Although HPLC technology includes automation via autosamplers, other factors such as time for careful filtration makes the analytical time spent per sample long enough that results for many of the samples in the queue are not available until the following workday. Furthermore, at present there are standards commercially available through the National Research Council of Canada for 12 of the more than 30 analogues of STX. The cost of these standards, and the lack of a domestic supply, may be of concern for a regulatory laboratory that processes thousands of samples per year. The detailed, compound-specific information provided by the current HPLC methods will provide valuable insight into the toxin profile(s) present along a coastal region, but may not be essential for routine monitoring purposes. A quick and reliable estimate of total toxicity is what is typically needed by the public health manager.

An alternative method that may satisfy the criteria listed above is the RBA. This competitive binding assay (Doucette et al. 1997; Ruberu et al. 2003) uses the same AOAC sample extraction procedure used for the MBA. The 96-well plate format of the RBA allows testing of up to seven samples in triplicate, with three dilutions per sample to ensure the proper concentration range is represented. Multiple plates can be queued on the plate reader, with results from several successive plates available on the same day. In fairness, the MBA will likely provide results faster for the first several samples assayed, but will fail to meet the high throughput requirements during a major event due to the lack of automation. The RBA procedures are straightforward and can easily be performed by a trained technician. The reporting limit established in our laboratory for the RBA is significantly lower (4 μg/100 g tissue) than the detection limit of the MBA (35 μg/100 g tissue in the CDPH laboratory), illustrating the high sensitivity of the RBA method. Another advantage of the RBA is that it does not require careful filtration of samples prior to analysis as is the case with the HPLC method, reducing the time required for sample preparation. The majority of reagents are commonly available and relatively inexpensive, the exception being the tritiated STX needed for competitive binding. This reagent is not readily available through government services such as the National Institute of Standards and Technology (NIST), but is currently available commercially within the United States. Reliance on proprietary materials is always a point of concern for regulatory laboratories if there are no alternative sources available. A possible source of error in the RBA is the rat membrane synaptasome preparation. Not only is it a very inconvenient preparation procedure to carry out, but also due to its heterogeneity this membrane can be associated with high assay variability. One way to overcome this would be to have it available commercially as a standardised reagent.

Our previous experience with the RBA (Ruberu et al. 2003) was encouraging relative to the criteria mentioned above, and the precision of the method in our laboratory was found to be 10%. However, more recent work in our laboratory has suggested that method precision was no better than the MBA. Therefore, it was determined that a more detailed investigation into the various components of this assay was warranted in the hopes that method precision could be improved, facilitating the decision-making process for public health managers.

Materials and methods

Chemicals and reagents

- ^{3}H-STX diacetate in methanol (Lot #040616, 0.1 m Ci ml^{-1}, specific activity = 18.0 Ci mmol^{-1}) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA).

- FDA reference standard, STX dihydrochloride (Lot #088 100 μg ml^{-1} in 20% ethanol–water at pH 3.5) (USFDA, Office of Seafood, Laurel, MD, USA).
- Rat membrane synaptosome: the rat membrane preparation containing sodium channel receptors was composed of 20 brains from 6-week-old male Hotsman rats (Harlan Bioproducts, Indianapolis, IN, USA) and prepared according to the methodology of Doucette (Doucette et al. 1997). This preparation was divided into 2 ml aliquots and frozen at −70°C. A single aliquot was thawed for each RBA plate preparation.
- All reagents, standards and dilutions were prepared in 100 mM MOPS/100 mM choline Cl buffer at pH 7.4. To prepare this buffer, 20.9 g of MOPS (3-morpholinopropanesulfonic acid) and 13.96 g of choline chloride were dissolved in 900 ml of water, the pH adjusted to 7.4 and the final volume brought to 1 L with water.

Instrumentation

Scintillation counting was performed on a PerkinElmer Life and Analytical Sciences instruments TopCount® Model B. MicroScint-20 cocktail (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) was used as the scintillant for all RBA work.

Receptor binding assay (RBA) protocol

The RBA procedure involved the addition of 35 μl of MOPS/choline Cl buffer, 35 μl of unknown sample (or STX standard), 35 μl of ^{3}H-STX, and 105 μl of a 1:6 diluted synaptosome preparation, in this order, to a 96-well microtitre filtration plate. A typical plate outline is given in Figure 1. All calibration standards, QC samples, reference samples and shellfish sample extracts are run in triplicate on each plate. The first three columns of each plate were used to generate a calibration curve. Saxitoxin dihydrochloride standard was used for the calibration curve in the following final in-assay molar concentrations: 1×10^{-6}, 1×10^{-7}, 3×10^{-8}, 1×10^{-8}, 3×10^{-9}, 1×10^{-9}, 1×10^{-10} and 1×10^{-11}. Three wells per plate served as a reference blank, containing the material and reagents described above but omitting a source of non-radiolabelled STX. The reference blank establishes the maximum binding (B_{max}) for each plate. A quality control (QC) sample yielding an in-assay concentration of 3.0×10^{-9} M STX standard, independently made, was used as a daily QC check. All pipetting was carried out using a certified, calibrated eight-channel pipette. To achieve equilibrium binding, the plate was incubated for 1 h at 4°C, then filtered using a MultiScreen vacuum manifold system and rinsed with 200 μl of ice-cold (4°C) MOPS/choline Cl buffer to remove unbound toxin. To each well 50 μl of the scintillant (MicroScint®) were added, and the top of the plate sealed with tape. The prepared plate was placed inside the TopCount scintillation counter for 30 min. This allowed the scintillant to dark adapt and the contents to mix, prior to counting the receptor-bound ^{3}H-STX.

Criteria that must be met for assay acceptance are as follows: (1) the slope of the standard curve must be between 0.8 and 1.2, (2) the relative standard deviation (RSD) of counts per minute (CPM) for each standard must be <30%, and (3) the QC check must be ±30% of the in-assay concentration of 3.0×10^{-9} M STX. Criteria for sample acceptance and quantification are: (1) $B/B_0 = 0.3$–0.7 and (2) RSD of the sample CPM must be <30%.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1X10^{-6}	1X10^{-6}	1X10^{-6}	Ref Blank	Ref Blank	Ref Blank	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200
B	1X10^{-7}	1X10^{-7}	1X10^{-7}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
C	3X10^{-8}	3X10^{-8}	3X10^{-8}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
D	1X10^{-8}	1X10^{-8}	1X10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
E	3X10^{-9}	3X10^{-9}	3X10^{-9}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
F	1X10^{-9}	1X10^{-9}	1X10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
G	1X10^{-10}	1X10^{-10}	1X10^{-10}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
H	1X10^{-11}	1X10^{-11}	1X10^{-11}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50	QC	QC	QC

Figure 1. Layout of a typical 96-well plate used in RBA. The first three columns are used to generate the calibration curve. Six wells are used for quality control samples (QC) spiked at 3.0×10^{-9} M in assay concentration, three wells are used to determine maximum binding (ref blank) (B_{max}), and the rest of the wells are used for unknown samples (U).

Statistical analysis

MedCalc statistical software (MedCalc Software, Mariakerke, Belgium; Windows Version 10.4.8.0; http://www.medcalc.org) was used for all statistical analyses. Analysis of variance (ANOVA) was used to evaluate the significance of variability of mean counts among sequential plate readings and among detectors for a given plate reading. Plates found to have a significant difference among either sequential readings or detectors were subjected to post-hoc significance testing with the Student–Newman–Keuls (SNK) test for all pairwise comparisons. Prism (Graph Pad Software, Inc., La Jolla, California, USA) was used to generate the STX binding curves.

Results and discussion

Method variability study of RBA

Our laboratory has been following the RBA protocol as developed by Doucette et al. (1997) and later modified by Ruberu et al. (2003) for the detection of STXs in shellfish matrices. This methodology involves the competitive binding between STX analogues (in sample) and tritiated saxitoxin (^{3}H-STX) reagent. As we gained more experience with the assay it became clear that there were sources of variability that were not well understood. This involved unacceptable levels of variance among triplicate samples at a given dilution, variability in reference samples, and in QC standards placed at the beginning and end of each plate. The RBA requires pipetting of small volumes of reagents (35–100 µl) and is comprised of several independent steps, each of which is a potential source of variability. Our previous work (Ruberu et al. 2003) determined the RSD for assays of environmental samples to be 10%. In order to study the overall variability of the RBA with the goal of improving the method's precision, it was necessary to deconstruct the assay to its simplest components, then "rebuild" step by step, evaluating each step for its contribution to overall assay variability. Identified in this study are inherent differences among wells of the plate for replicate samples, heterogeneity of the rat membrane (binding sites) in each well, and the competitive binding process in each well. Given that each well acts as an independent experiment within a single plate, some amount of variability was expected for the measured CPMs among wells.

Another factor that can contribute to assay variability is the instrument's inherent variability among its 12 detectors, each of which reads a total of eight wells per plate. Detector normalisation is performed as part of routine maintenance of the instrument to minimise the variability that may exist among detectors. To understand detector variability it is important to know how the detectors are set up and which order the detectors read the wells. The TopCount® instrument has two rows of six detectors each. The plate is read starting from the top row A to bottom row H (Figure 1). When a plate is read, the first set of six detectors measure wells A1, A3, A5, A7, A9 and A11, then move down to read wells B1, B3, B5, B7, B9 and B11. Subsequently, wells C1, C3, C5, C7, C9, C11 and A2, A4, A6, A8, A10 and A12 are read simultaneously by both sets of detectors. This continues until the set of wells G2, G4, G6, G8, G10 and G12 and the last set of wells H2, H4, H6, H8, H10 and H12 have been read by the second set of detectors. Not all wells are read simultaneously. As such, with a 5-min count time per well, the time difference between the measurement of the first and last wells is about 50 min. This can be a substantial period with respect to dissolution between sample and cocktail. To evaluate this potential source of variability to the assay, the count data for the series of plates studied were grouped by detector and statistically analysed by ANOVA to determine if there was a significant difference among the 12 detectors and, if so, which detectors were responsible for this variability.

Instrument background plate

To determine the inherent background variability in counts among the wells of a single plate, all 96 wells were filled with 50 µl of MicroScint® cocktail and counted three times in succession with a 30-min dark adapt delay period prior to each measurement. Background counts ranged from 8 to 36 CPM, from 7 to 27 CPM, and from 8 to 27 CPM for the three consecutive readings with average counts of 17.9, 17.8 and 16.5 CPM respectively. The standard deviation (SD) for the three count cycles ranged between 4 and 5 CPM. Figure 2 shows the CPM variability of the instrument background plate with respect to each

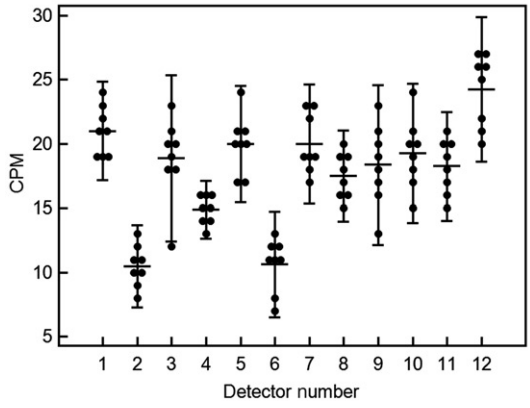


Figure 2. Plot of the instrument background plate second count cycle depicting the randomness of CPMs in the 96 wells. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

detector. Since there is no mixing of reagents involved in this plate, the variation seen here is attributed solely to counting statistics and to differences between the 12 detectors themselves. There was no significant pattern of variability observed for any single detector or to the time at which a well was counted. Although detectors 2 and 6 exhibited lower cpm values than the other ten detectors (Figure 2), the pattern of detector performance varied among the three sequential plate readings.

Blank plate

The next step was to determine the variability in counts among wells when a source of tritium was present. For this study all 96 wells were filled with 35 µl of ^{3}H-STX followed by 50 µl of MicroScint® cocktail. This blank plate was counted five times in succession with a 30-min dark adapt delay period prior to each counting cycle. Potential contributors to variability such as rat membrane preparation, competing non-labelled toxin, the competitive binding process itself and the washing/filtering step were absent.

Results showed a gradual increase in average CPM for the five sequential readings (Figure 3) with the greatest increase between the first ($CPM_{average} = 700$; RSD = 19%) and second ($CPM_{average} = 869$; RSD = 17%) measurements. The CPM stabilised with the next three readings ($CPM_{average} = 915$, 939, 954; RSD = 17%, 16%, 16%). A one-way ANOVA determined that there was a significant difference among the mean CPMs ($p < 0.001$) for the five counting cycles. SNK post-hoc significance testing for all pairwise comparisons determined that the first two plate readings were significantly different from one another ($p < 0.05$) and both were significantly different from plate readings three through five. It also showed that the last three plate readings were not significantly different from one another ($p > 0.05$). From these observations it is clear that the increase in CPM is due to mixing of the aqueous phase with the cocktail. An additional time of 3 h is needed to reach equilibrium in mixing. This is not a practical concern for the present assay because subsequent steps involve rinsing and filtering of each well prior to adding the cocktail, resulting in a single phase in each of the wells at the time of counting. However, shaking the RBA plate prior to the incubation step would make certain that all the reagents in the wells are properly mixed. These results give insight into the two-phase mixing process in a plate format. Unlike conventional liquid scintillation counting methods where 20 ml vials are vigorously shaken to obtain homogenous mixing prior to counting, in the plate format mixing can be an issue in obtaining reproducible results when assays with two phases are involved. This is further supported by looking at the first set of wells measured at the start of the count and the last set of wells measured (about 50 min later) within a single plate. The former (row A) had a $CPM_{average}$ of 447 while the latter (row H) had a $CPM_{average}$ of 723.

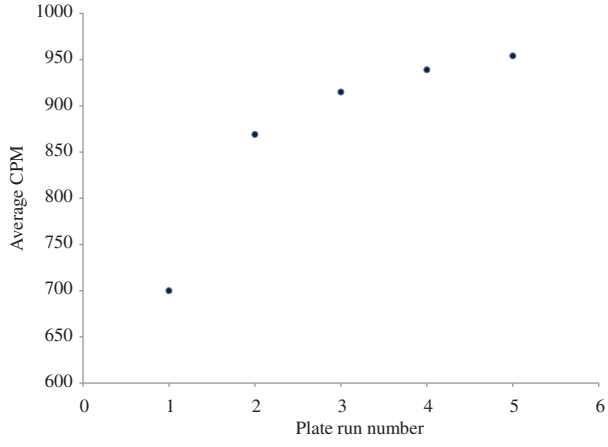


Figure 3. Graph of the blank plate five sequential readings showing a gradual increase in average CPM.

Reference plate (non-competitive binding)

The next step in reconstructing the assay involved introduction of binding sites for the ^{3}H-STX, i.e. non-competitive binding. In practice such a reference sample is run in triplicate on each RBA plate and the average CPM is used to determine maximum binding, B_{max}. This B_{max} value is used as the baseline and is compared with the sample CPM to generate the actual binding of samples. The reference plate was prepared by adding reagents in the following order: 35 µl of MOPS buffer, 35 µl of ^{3}H-STX and 105 µl of rat membrane preparation, then processed following the standard RBA protocol described above. This plate was measured three successive times.

The reference plate had a higher average CPM (1196 CPM) compared with the blank plate, with an RSD of 19%. Theoretically, the reference plate CPM values are expected to be lower than the blank plate, because the membrane binding sites would not retain all of the available ^{3}H-STX, with the excess being removed during the filtration step. The lower CPM of the blank plate is attributed to incomplete mixing of the ^{3}H-STX with the scintillation cocktail rather than the amount of tritiated toxin present. Since there is no aqueous phase in the reference plate, mixing does not become an issue. When the CPM values of the wells counted first (row A) are compared with those counted last (row H) there was no significant difference, which supports that phase mixing is absent. The comparable RSDs for the blank plate and the reference plate suggest that the addition of the rat membrane preparation, and the subsequent rinsing and filtering steps, do not contribute a significant amount of variability to

the assay. A one-way ANOVA determined that there was no significant difference among mean CPMs ($p > 0.3$) for the three sequential plate readings.

The reference plate, however, exhibited a significant difference among detectors (two-way ANOVA, $p < 0.001$). The same pattern in detector performance was observed for all three plate readings and the SNK pairwise comparisons determined that detector #12 was significantly different from all other detectors ($p < 0.001$) (Figure 4). The data from detector #12 were omitted and the statistical analysis repeated. The removal of this detector's data did not change the ANOVA outcome for sequential plate readings or detector variability.

QC plate (competitive binding)

To evaluate the added variance component associated with competitive binding, a non-labelled STX standard was added to compete with the ^{3}H-STX, creating a competition for binding sites. For the non-labelled STX, a solution at 1.8×10^{-8} M, with a final concentration of 3.0×10^{-9} M in assay, was used. The standard RBA plate configuration contains triplicates of this solution and their average CPM is used as the plate's QC sample. The reagents added per well for the QC plate were identical to the reference plate described above, with the addition of 35 µl of QC sample prior to the addition of 35 µl of the ^{3}H-STX. This plate was measured three successive times.

As expected, due to the introduction of competitive binding, the mean CPM of the QC plate was considerably lower than that of the reference plate (825 and 1196 CPM, respectively). Fewer binding sites for the radiolabelled toxin resulted in lower activity in the well after the rinsing and filtering steps. Triplicate counting of this plate gave an RSD of 17%. There appeared to be a slight decline in counts over the three successive plate readings (Figure 5). There was a slightly significant difference among successive plate readings ($p = 0.04$), which was due to a significant difference between the first and third plate readings ($p < 0.05$).

Consistent with the results of the previous plate, there was a significant difference among detectors (two-way ANOVA, $p < 0.001$). The SNK pairwise comparisons of detectors did not identify a single detector to be different from all others, however detector #11 differed significantly from seven other detectors ($p < 0.05$) and detector #12 differed significantly from five other detectors ($p < 0.05$).

Overall assay variability

Introduction of the heterogeneous rat membrane preparation increased RSD only slightly for triplicate readings from 16% (blank plate) to 19% (reference plate). This demonstrates that the number of receptor sites in each aliquot of the membrane preparation is fairly uniform and does not affect assay precision significantly. With the introduction of competitive binding (QC plate) the RSD remained in the same range: 17%. Overall, an inherent variability of approximately 17% is associated with this assay, which is independent of the addition of the membrane preparation or the non-radiolabelled STX and subsequent competitive binding process. When assay variability was evaluated with respect to individual detectors, there was no reproducible pattern in detector performance, although there were frequent occurrences of one or more detectors having significantly different CPM than the rest of the detectors for a given plate. The detectors with the lowest and highest levels of variability were different from plate to plate. Removal of data for a detector that was found to be significantly different from a majority of the remaining detectors did not change the outcome of the ANOVA for any of the series of plates. This detector variability observed

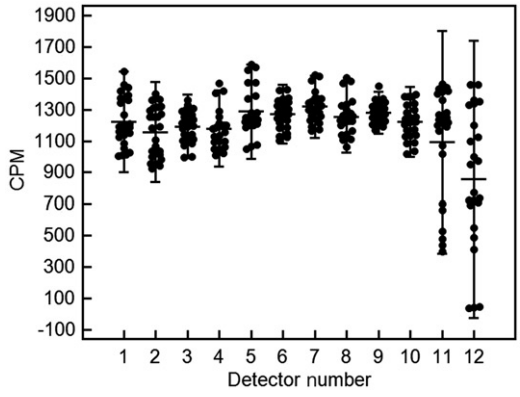


Figure 4. Scatter plot of the reference plate, first reading showing detector variability. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

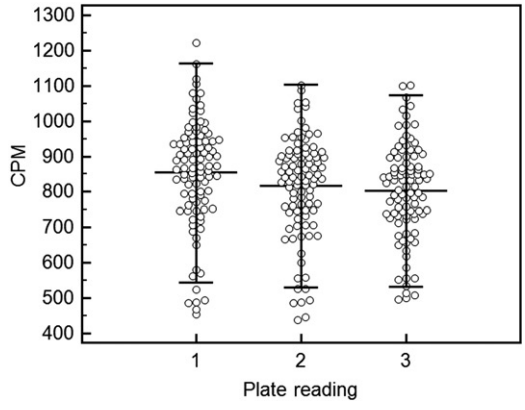


Figure 5. Results of the QC plate showing the variability of CPM in the three successive readings. For each run the mean and error bars at 2 SDs per plate are shown.

could be attributed to pipetting error along single rows. Since an eight-channel micropipette is used to add reagents to single rows, an error in one such addition will show up as a single detector inconsistency. This would erroneously label one or more detectors as being different to other detectors. Although our initial work (Ruberu et al. 2003) on the RBA showed an RSD of 10%; a more recent study (van Dolah et al. 2009) demonstrated an RSD of 17.7% comparable with the current finding of 17%. It should be noted that our initial work was conducted using a six-detector instrument and with more experienced analysts, which could be the reason for the lower RSD for that study.

RBA acceptance criteria

In a typical RBA plate, each sample (calibration standard sample, QC sample, unknown sample, reference sample) is run in triplicate and the average CPM value is used for further calculations. Triplicate samples, rather than duplicates, are run to improve the accuracy of this assay. According to the acceptance criteria of the RBA assay (van Dolah et al. 2012), a given set of triplicate sample data are rejected if the RSD exceeds 30%, requiring reanalysis of the rejected sample. On average about 10% of the samples analysed in our laboratory are rejected due to the high variance among the triplicate values. In addition, if the QC sample CPM has $>30\%$ RSD, then the entire plate must be rejected. This loss of data results in the need to prepare and run a new plate, increasing both the turnaround time for results and the cost of the assay. One possible way of preventing samples from being rejected is to identify and remove outliers within a set of replicates. By eliminating outliers, the variability of replicates may be reduced to an acceptable level ($<30\%$ RSD), preventing invalidation of the entire plate or of individual samples. Therefore, we investigated a statistical approach to eliminate outliers methodically.

Grubbs' test and Student's t-test

A comparison of statistical outlier tests concluded that the Grubbs' test (Grubbs 1969) and the Student's *t*-test (Sokal and Rolf 1981) were best suited for determining an outlier within a triplicate dataset. The Grubbs' test compares the suspected outlier to the mean of all replicates, including the suspected value. The Student's *t*-test compares the potential outlier to the mean of the remaining values. The Grubbs' test is therefore more conservative in approach and it would be expected that this test would identify fewer outliers than the Student's *t*-test. The Grubbs' test for triplicates determines that a value is an outlier if the calculated value (G) is greater than the critical value (Z) of 1.153 at a 95% confidence interval ($\alpha = 0.05$). The Student's *t*-test determines that a value is an outlier in a triplicate dataset if the calculated *t*-value is greater than the critical *t*-value of 12.706 ($\alpha = 0.05$). By running the triplicate sample data values through these statistical tests, an outlier can be determined in an unbiased fashion, possibly avoiding the rejection of the entire sample.

The two outlier tests were evaluated by analysing data of each plate reading for the experiments presented above. For example, statistical analysis of the second plate reading of the QC plate, which had an RSD of 17.5% with an average CPM value of 817, resulted in both tests identifying a total of four outliers (Table 1). The results calculated after the four outliers were removed gave an average of 823 CPM with a 17% RSD. Removing the outliers did not improve the RSD significantly but showed a slight increase in CPM. Other plates tested for outliers had similar results, with an insignificant lowering of the sample RSD and slight increases or decreases in CPM. Since the CPM values directly relate to STX concentration of a sample, it is possible that the removal of outliers could have an impact on the accuracy of the assay results.

In order to evaluate how outlier testing would impact results of actual shellfish samples, 17 samples were spiked with STX concentrations that ranged from 5 to 1000 µg/100 g shellfish tissue and were evaluated for outliers using both statistical tests. Each triplicate set of data were assessed in two ways: raw data (no outliers removed) and data with outliers removed. Each test identified the same outliers and, as expected, the *t*-test identified additional outliers that were not detected by the Grubbs' test. Removal of the outliers kept these samples from being rejected ($<30\%$ RSD in

Table 1. Results from two outlier tests for three successive readings of the QC plate.

	Raw data			Grubbs test outliers removed			Student's *t*-test outliers removed		RSD % change		
Plate reading	Mean CPM	SD	RSD	Mean CPM	SD	RSD	Mean CPM	SD	RSD	Grubbs	*t*-test
1	854	155	18	859	152	18	859	152	18	0.03	0.03
2	817	143	18	823	140	17	823	140	17	0.03	0.03
3	803	135	17	810	135	17	809	136	17	0.01	0.00

triplicate wells), however there was no significant improvement in the precision of the assay. The average recovery of STX was 118% and 113% for raw data and data with outliers removed, respectively. Although the assay accuracy was improved on average, some plates showed a decrease in accuracy after outliers were removed, indicating that the suspected outlier was closer to the actual value than the remaining data points. In practice, outlier testing would likely reduce the number of samples and plates rejected, thereby reducing the time required to report results and lowering the per sample cost of the assay. The potential negative effect on method accuracy suggests caution with this approach in the absence of tangible evidence of analytical error during plate preparation.

Control charts for RBA

A better way of identifying erroneous data is through the use of control charts, which are based on a laboratory's acceptable and attainable performance criteria for precision and accuracy for a given method. A control chart enables the laboratory to monitor its performance visually by updating the chart with data from each subsequent analytical run. In this way a control chart for each critical parameter of a method tracks the detection of data outside of the acceptable performance limits. Control charts are prepared by plotting the date or run number as the abscissa and the value of interest, e.g. STX concentration of the QC check sample estimated on each plate, as the ordinate. Performance limits are established by averaging at least 20 measurements that have acceptable individual statistics, setting control limits and identifying the range of variability for that parameter. Rather than setting an arbitrary acceptance limit of ±30% for recovery of the QC sample, each laboratory can establish control limits based on their performance to determine whether or not an RBA plate is acceptable. Typical control limits are based on the number of SDs from the estimated mean. Once the mean and SD have been determined, the parameters from each subsequent assay are added to the appropriate control chart to maintain a continuous record of performance. In addition to the detection of erroneous values that would indicate an unacceptable plate, control charts allow tracking of systematic changes in method performance (e.g. due to degrading stock solutions, changes in materials like plate manufacturers, etc.) as well.

Figure 6 shows control charts for three RBA parameters acquired from our laboratory over a period of 1 year: (1) QC check standard, (2) slope of the binding curve and (3) EC_{50}. For each of the three control charts, control limits were based on the mean ±2 SD of the first set of 20 acceptable data. For example, the estimated mean for the QC check standard (3.0 nM theoretical concentration) from the first 20 plates was 2.94 nM and the calculated SD was 0.962 nM, resulting in control limits of between 1 and 4.5 nM (Figure 6(a)). Two data points on this plot had QC check standard values that were found to be outside of the control limits, requiring those two plates to be rejected and the assay run again for those samples. In contrast, the current RBA protocol of ±30% calculates an acceptance limit of between 2.1 and 3.9 nM for the QC check standard, which would result in 12 data points out of control, hence the rejection of 12 RBA plates. The latter criterion is arbitrary since the accuracy of the QC sample is method, instrument and analyst specific and must be established per individual laboratory.

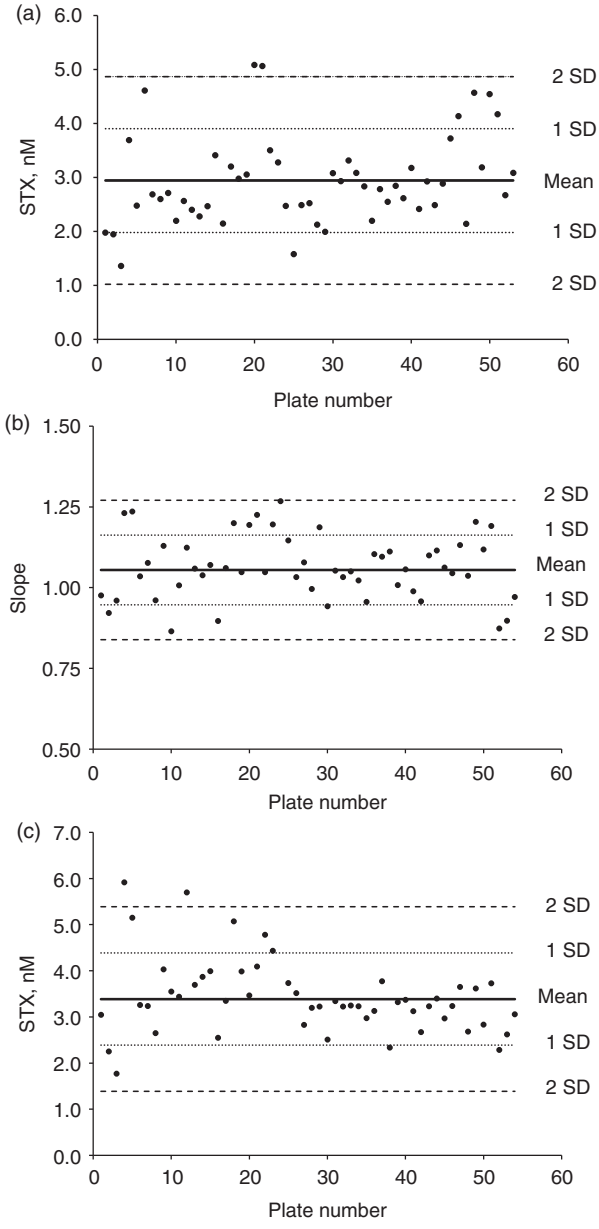


Figure 6. Control charts for (a) averaged daily QC samples on a plate, (b) slope of the binding curve and (c) EC_{50} per plate.

When the established control limits are exceeded and a trend is observed, results are investigated for method bias and potential mistakes, allowing corrective actions to be taken to address the root cause to prevent recurrence of the error. Figure 6(b) shows the control chart for the slope of the standard curve. The slope was demonstrated to be a very stable parameter, with tight control limits ranging from 0.8 to 1.3 with no data points being rejected. The EC_{50} parameter is shown in Figure 6(c). In this case a high variability is seen at the beginning of the chart and as the analyst gains more experience with the assay the EC_{50} value becomes more consistent with less variation.

An additional parameter that has been inconsistent and highly variable in the RBA is the maximum binding, or top value of the binding curve. Ideally, the standard binding curve should plateau at 100% binding. However, often times we observe the plateau significantly below (80%) or above (120%) this value. Such a large shift in the curve significantly affects the final results of STX concentration in a sample. It is sometimes caused by one or more of the lowest three standards being out of control, thus dragging the curve in one direction. The top plateau was monitored using a control chart (Figure 7). The 1 SD control limit established for this data was between 0.9 and 1.1, which is ±10% binding. We have observed that this parameter can have a significant effect on the outcome of the results and therefore recommend developing a control chart to monitor its performance. Currently the importance of the top plateau is overlooked and not considered as an assay performance acceptance criterion.

Conclusions

Previous studies from our laboratory demonstrated the RBA method for the detection of STXs to be very reliable and to have the potential of being an alternate regulatory test method for PSP. Our current work focused on identifying sources of variability associated with the RBA and evaluating alternate QC approaches for validating test plates. The assay variability work included evaluation of each step of the assay by deconstructing its procedural steps, and also assessing the instrument's detector variability. The overall variability of the assay was determined to be 17%. Results discussed above show that the variability within a plate arises from several factors, such as counting statistics, analyst variability, mixing of well contents with cocktail, and the inherent measurement technique of the TopCount®. It is not known if the same variability would be observed in other instruments with different numbers of detectors or with detectors placed in a different array. A pipetting error along a row by the eight-channel micropipette would point to single detector variability as well and would be hard to identify. It is recommended periodically to evaluate individual detector performance with either a reference plate or a QC plate format similar to that used in the current study.

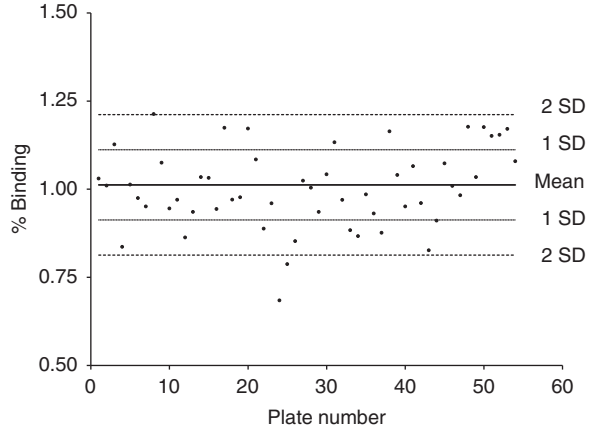


Figure 7. Control chart for the top plateau, maximum binding (B_{max}), of the calibration curve.

We have explored the use of two different outlier tests, Grubbs' test and Student's *t*-test, alone and in combination with the allowable procedure recommended in the NOAA protocol. Overall, removal of outliers lowers the RSD between replicate wells of a sample to $<30\%$, thus preventing that sample from being rejected. As a result it is expected that routine outlier testing would reduce the number of samples and plates rejected under the current QC criteria, which would help minimise the turnaround time between sample receipt and the reporting of results. The reduction in the number of rejected samples would also lower the cost per sample of the assay. Although some improvement in precision will be gained when an outlier is excluded, it is possible that accuracy will be diminished if the excluded value is closer to the actual target concentration. The potential negative effect on method accuracy suggests that the removal of suspected outliers should only be considered if it is suspected that there is an error associated with the sample(s) involved (e.g., a pipetting error).

Another avenue explored was the use of control charts for monitoring the three critical parameters of the RBA method, i.e. QC check standard, slope and EC_{50}. Establishing acceptable limits within each laboratory for respective parameters will ensure consistent performance over time, identify plates that must be rejected because one or more parameters are outside of the set control limits, and allow identifying and correcting process changes that would affect every assay. Establishing control limits for the maximum binding (B_{max}) as a fourth critical parameter for RBA performance is recommended. Such a development of associated control charts can be a part of the

laboratory's routine QC programme and is recommended as the primary quality control process for the RBA.

Acknowledgements

This study was supported under NOAA grant NA04NOS4780239 from the Monitoring and Event Response for Harmful Algal Bloom (MERHAB) programme. This is MERHAB Publication Number 152. The authors would like to thank Roger Ho for helping with the Graphpad Prism data analysis; Clive Kittredge and Vanessa Zubkousky for the preparation and analysis of plates; and American Radiolabeled Chemicals Inc. for providing ^{3}H-STX reagents. Special thanks to Sherwood Hall of the US Food and Drug Administration's Office of Regulatory Science for FDA STX reference standards and helpful discussions on this study. The authors would also like to thank Fran van Dolah and Greg Doucette for their guidance in the RBA method.

References

American Public Health Association (APHA). 1970. Recommended procedures for the examination of sea water and shellfish. 4th ed. New York (NY): American Public Health Association. p. 57–61.

Doucette GJ, Logan MM, Ramsdell JS, van Dolah FM. 1997. Development and preliminary validation of a microtiter plate-based receptor-binding assay for paralytic shellfish poisoning toxins. Toxicon. 35(5):625–636.

Grubbs FE. 1969. Procedures for detecting outlying observations in samples. Technometrics. 11(1):1–21.

Lawrence JF, Niedzwiadek B, Menard C. 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J AOAC Int. 88:1714–1732.

Powell CL, Doucette GJ. 1999. A receptor-binding assay for paralytic shellfish poisoning toxins: recent advances and applications. Natural Toxin. 7:393–400.

Ruberu SR, Liu YG, Wong CT, Perera KS, Langlois GW, Doucette GJ, Powell CL. 2003. Receptor-binding assay for paralytic shellfish poisoning toxins: optimization and inter-laboratory comparison. J AOAC Int. 86(4):737–745.

Sokal RR, Rolf FJ. 1981. Biometry. 2nd ed. New York (NY): W. H. Freeman. p. 229–231.

van de Riet JM, Gibbs RS, Chou FW, Muggah PM, Rourke WA, Burns G. 2009. Liquid chromatographic post-column oxidation method for analysis of paralytic shellfish toxins in mussels, clams, scallops, and oysters: single-laboratory validation. J AOAC Int. 92(6): 1690–1704.

van Dolah FM, Fire SA, Leighfield TA, Mikulski CM, Doucette GJ. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor-binding assay: a collaborative study. J AOAC Int. 95(3):795–812.

van Dolah FM, Leighfield TA, Doucette GJ, Bean L, Niedzwiadek B, Rawn DFK. 2009. Single-laboratory validation of the microplate receptor-binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. 92(6):1705–1713.

Proposal No. 17-107

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Leanne J. Flewelling
Affiliation	Florida Fish and Wildlife Conservation Commission
Address Line 1	100 8th Avenue SE
Address Line 2	
City, State, Zip	St. Petersburg, Florida, 33701
Phone	727-502-4891
Fax	727-550-4222
Email	leanne.flewelling@myfwc.com
Proposal Subject	ISSC Method Application and Single Lab Validation of an Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in hard clams, sunray venus clams, and oysters.
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	This submission proposes that the MARBIONC brevetoxin ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method. Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay (i.e., Growing Area closing or re-opening, controlled relay, and end product testing of controlled harvest as permitted within a State Authority's marine biotoxin contingency program). Samples failing by ELISA would either require additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. ELISA could also be used as a screening method to initiate precautionary closures. Requested changes: Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests 4. Approved Limited Use Methods for Marine Biotoxin Testing Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP) Add columns for Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP) and for Application: Controlled Harvest end product testing Add MARBIONC brevetoxin ELISA to table for all applications except Dockside Testing with the following footnote: MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters: a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.

	b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. See attached proposed revisions to Table 4. Approved Limited Use Methods for Marine Biotoxin Testing
Public Health Significance	Brevetoxins produced by K. brevis are toxic to humans. Filter-feeding bivalves accumulate brevetoxins during blooms, and ingestion of contaminated shellfish can cause NSP in humans. Symptoms of NSP typically begin three to six hours after ingestion and may include nausea, diarrhea, tingling of lips or tongue, muscle ache, lack of coordination, temperature reversal, and vertigo. In severe cases, a feeling of constriction in the throat may occur. Individuals with NSP may require hospitalization but usually recover within days. To prevent NSP, shellfish harvesting areas are closed when K. brevis concentrations exceed 5,000 cells/L and are re-opened once K. brevis levels decrease and testing demonstrates that shellfish are no longer toxic. However, the APHA mouse bioassay - the only approved method for NSP testing - has many drawbacks, and the delays caused by the time required to analyze samples (two days) and low sample throughput compound economic losses. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed.
Cost Information	Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Dockside Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest end product testing Sample Type: Shellfish
Abraxis Shipboard ELISA[3]		X			X		
JRT[2]		X		X	X	X	
HPLC[1]	X			X		X	
Reveal 2.0 ASP[4]	X			X	X	X	
RBA[5]		X		X	X	X	
MARBIONC Brevetoxin ELISA[6]			X	X		X	X

Footnotes:

[1]M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Biosciences, Technical Report #64, National Research Council Canada #33001. This method may also be used direct without cleanup.

[2]Jellett Rapid Test for PSP, Jellett Rapid Testing Ltd.

a. Method can be used to determine when to perform a mouse bioassay in a previously closed area.
b. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.
c. A positive result shall be used for a precautionary closure.

[3]Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of Actions, Proposal 05-111 (page 15) and 09-107 (page 140).

[4]Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 2013 Summary of Actions Proposal 13-112.

[5]Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114

[6]MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:

a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish
Name of the Method Developer	The ELISA Kit was developed by UNCW and is sold through MARBIONC. The method was optimized and submitted for use with molluscan shellfish by Leanne Flewelling, Florida Fish and Wildlife Conservation Commission.
Developer Contact Information	Florida Fish and Wildlife Conservation Commission 100 8th Avenue SE St. Petersburg, FL 33701 (727) 502-4891 leanne.flewelling@myfwc.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Blooms of the dinoflagellate *Karenia brevis* threaten the productive Gulf of Mexico shellfish industry. Brevetoxins produced by *K. brevis* are toxic to humans and can result in Neurotoxic Shellfish Poisoning (NSP) if contaminated shellfish are eaten. To prevent NSP, shellfish harvesting areas (SHAs) are closed when *K. brevis* concentrations exceed 5,000 cells/L and are re-opened once *K. brevis* levels decrease and testing demonstrates that shellfish are no longer toxic. This biotoxin plan successfully prevents occurrences of NSP from lawfully harvested shellfish, but NSP closures come at a steep economic cost to the shellfish industry. The APHA mouse bioassay - the only NSSP approved method for regulatory NSP testing - has many drawbacks. The delays caused by the time required to analyze samples (two full days) and very low sample throughput delay re-openings and add to economic losses. The assay is nonspecific, imprecise, and not calibrated against known levels of brevetoxins. It is costly in terms of labor and supplies, and the use of live animals is both undesirable and increasingly unacceptable. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed. Among the many chemical and biological methods developed for brevetoxin detection, enzyme-linked immunosorbent assays (ELISAs) have performed well. The method proposed here was the first commercially-available brevetoxin ELISA to be offered. The assay uses goat anti-brevetoxin antibodies developed by Trainer and Baden (1991) and is based on the indirect competitive assay developed in 2002 by Naar et al. (2002). The kit is marketed by MARBIONC Development Group (MDG), which is based at the University of North

		Carolina at Wilmington. This assay is widely and routinely used to monitor brevetoxins in Florida's marine systems and to diagnose human, marine mammal, and other animal exposure to brevetoxins. This method is much faster than the mouse bioassay, more user-friendly, more sensitive, more specific to brevetoxins, less expensive, and does not involve the use of live animals.
2. What is the intended purpose of the method?		The proposed use for the MARBIONC ELISA is as a Limited Use Method for determination of NSP toxin levels in hard clams, sunray venus clams, and oysters. Applications include Growing Area Survey & Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing as permitted within a State Authority's marine biotoxin contingency program. We propose that the ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters, at or below the estimated equivalent to one-half the 20 MU/100 g guidance level) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method (currently, the NSP mouse bioassay). Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay including: Growing Area Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing. Samples failing by ELISA would either require additional testing by NSP mouse bioassay or could support the same management actions as samples failing by NSP mouse bioassay. ELISA could also be used as a screening method to initiate precautionary closures.
3. Is there an acknowledged need for this method in the NSSP?		Yes, the ISSC Laboratory Committee has specified the need for qualitative or semi-quantitative (screening) and quantitative/confirmatory methods of analysis for all toxins and for each commercially-harvested bivalve species.
4. What type of method? i.e. chemical, molecular, culture, etc.		ELISA is a biological method that uses biological components (antibodies) to detect toxins. Detection relies on structural recognition of a region of the toxin molecule shared by PbTx-2-type brevetoxins (the most abundant forms) and provides an overall estimate of toxin content.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish.
Method Scope		This ELISA is a high-throughput, sensitive, accurate, quantitative assay for NSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Limited Use Method for the purposes of screening for NSP toxins in hard clams, sunray venus clams, and oysters.
References		Original method reference: Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG. 2002. A competitive ELISA to detect brevetoxins from

		Karenia brevis (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110(2):179-185. Antibody development reference: Trainer VL, Baden DG. 1991. An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29(11):1387-1394. Epitope identification reference: Melinek R, Rein KS, Schultz DR, Baden DG. 1994. Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32(8):883-90. Other relevant publications: Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK. 2004. Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. p. 300-302. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44:677-685. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (Crassostrea virginica). Toxicon 52(1):32-8. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH. 2015. Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (Mercenaria sp.) exposed to *Karenia brevis* blooms. Toxicon 96:82-88.
Principle		In this indirect competitive ELISA based on Naar et al. (2002), a 96-well ELISA plate is coated with protein-linked brevetoxin, and any remaining binding sites in the wells are blocked. Goat anti-brevetoxin antibodies are then incubated with samples or standards in the plate wells. The antibodies will react with the brevetoxins in the samples or standards or will be immobilized on the plate. Antibodies that are not attached to the plate after incubation are washed out during subsequent rinses. Antibodies immobilized on the plate are detected through steps linking the antibodies to horse radish peroxidase (HRP)-linked secondary antibodies, and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine), which yields a blue color that changes to yellow (Amax = 450nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For quick screening, more samples can be run on one plate

		(up to 40).
Any Proprietary Aspects		Methods of production of key kit reagents (brevetoxin-BSA conjugate and anti-brevetoxin antibodies) are proprietary (MDG).
Equipment Required		Equipment required: Balance capable of measuring to 0.1g Number 10 sieve Laboratory blender Vortex mixer Centrifuge capable of 3,000x*g,* with rotor for 15 mL Microplate reader with filter for measurement at 450 nm Multichannel pipettor (50-200 µL) Individual pipettors (10-1000 µL) Orbital microplate shaker Refrigerator/freezer Consumables required: Disposable glass test tubes Disposable plastic dilution tubes (96-well cluster format) 15-ml and 50-ml polypropylene centrifuge tubes Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (- substitution NOT recommended) Microplate sealing film Assorted pipet tips Solution basins Aluminum foil
Reagents Required		Included in MARBIONC ELISA Kit: • Reagent A: BSA-linked PbTx-3 • Reagent C: Goat anti-brevetoxin Ab • Reagent D: HRP-linked anti-goat secondary Ab • Brevetoxin standard (PbTx-3) Reagents required but not included: • Methanol • Reagent B: Superblock Blocking Buffer • Phosphate Buffered Saline, pH 7.4 • Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 • Gelatin • 3,3'5,5'-Tetramethylbenzidine (TMB) • Sulfuric acid stop solution (H_2SO_4, 0.5M) • Nanopure water (or equivalent quality water)
Sample Collection, Preservation and Storage Requirements		At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis. The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are sucked from shell being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for brevetoxins (see protocol in Appendix A). Samples must be processed within 24 hours of shucking.
Safety Requirements		General chemical safety requirements (e.g., personal protective equipment including gloves, safety glasses,

		and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure		See protocol detailed in Appendix A.
Quality Control Steps Specific for this Method		Acceptance of assay results is dependent on meeting the following criteria: Absorbance of reference wells (Amax) must be ≥ 0.6. %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%. Acceptance of sample results is dependent on meeting the following criteria: %CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (within the linear range of the assay; 20-70% inhibition) must be <20%. %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%.
C. Validation Criteria		
1. Accuracy / Trueness		Accuracy /trueness was determined by calculating the closeness of agreement between the test results and targeted value. Calculated % accuracy/trueness: Oysters: 96.27% Hard Clams: 98.39% Sunray Venus Clams: 95.12% Data and details in Appendix B
2. Measurement Uncertainty		Two-sided, 95% confidence intervals for the difference in concentrations between the reference and the spiked samples: Oysters: -0.0057 – 0.1137 Hard Clams: 0.0603 – 0.1898 Sunray Venus Clams: 0.0783 – 0.2487 Data and details in Appendix B
3. Precision Characteristics (repeatability and reproducibility)		Repeatability was assessed using duplicate determinations of 10 samples spiked with PbTx-3 to three levels (0.4, 1, and 4 ppm). %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams. Data and details in Appendix C
4. Recovery		The recovery of the method was consistent over the range of concentrations examined to determine Precision. The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams. Data and details in Appendix C
5. Specificity		Potentially interfering substances examined in this study included three types of microalgae (two types commonly used as food for hatchery raised bivalves and a non-brevetoxin producing *Karenia* species) as well as okadaic acid (a potentially co-occurring polyether dinoflagellate toxin). Two-sided t-tests indicated no significant difference in brevetoxin measurements in the presence or absence of these substances. Data and details in Appendix D
6. Working and Linear Ranges		The overall or dynamic linear range of this method results from a combination of the linear range of the assay standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate. The linear range of the ELISA standard curve varied slightly among two lots of kit reagents examined. One lot yielded a range of 0.21-1.04 ng PbTx-3/mL and a second lot yielded a range of 0.30-1.38 ng PbTx-3/mL. The overall or dynamic linear range of the method as

		described for this proposal (in PbTx-3 equivalents) is from 0.12 ppm to 26.62 ppm for the June 2014 kit lot and up to 35.33 ppm for the June 2016 kit lot. Data and details in Appendix E
7. Limit of Detection		The calculated assay LOD is 0.1 ng/mL. At the lowest sample dilution of 1:400, the LOD for brevetoxin in shellfish is 0.04 ppm. Data and details in Appendix E
8. Limit of Quantitation / Sensitivity		The calculated assay LOQ is 0.3 ng/mL. At the lowest sample dilution of 1:400, the LOQ for brevetoxin in shellfish is 0.12 ppm. Data and details in Appendix E
9. Ruggedness		Results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min). Significant differences were observed only with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities of 1.0 ± 30%. Data and details in Appendix F
10. Matrix Effects		Brevetoxin-free samples (10 samples per species) for this study were obtained from shellfish harvest areas along Florida's Gulf coast that infrequently experience *K.. brevis* blooms during periods when *K. brevis* was verified to be absent. Farmed hard clams and sunray venus clams were sourced from Cedar Key, FL and were provided by a Shellfish Aquaculture Extension Agent and as well as local clam farmers. Hard clams were collected from 10 different locations over four days. Sunray venus clams were collected from two locations over six days. Wild oysters were collected by Florida Department of Agriculture and Consumer Services staff from five sites in Apalachicola Bay over nine days. At the lowest dilution (1:400), all samples tested <LOD and no matrix effects were observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Comparative data for 501 samples (173 oyster, 277 hard clam, and 51 sunray venus clam) are presented in Appendix G. For several reasons discussed in Appendix G, comparing NSP mouse bioassay and ELISA data is not straightforward, and analytical NSP methods of any type are unlikely to ever completely agree with mouse bioassay results. There was a very wide range of concentrations measured by ELISA in samples testing <20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay. In samples testing < 20MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. Where quantitative results were obtained by both mouse bioassay and ELISA (i.e., in samples testing ≥ 20 MU/100 g), significant positive correlations were observed. Using linear regression, the 20 MU/100 g equivalent by ELISA was predicted to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams (in PbTx-3 equivalents). Across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.
D. Other Information		
1. Cost of the Method		Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).
2. Special Technical Skills Required to Perform the Method		General laboratory skills are required: reagent preparation, pipetting, basic equipment operation, data analysis using curve-fitting software, basic calculations.
3. Special Equipment Required and Associated Cost		Microplate reader with filter for measurement at 450 nm. Costs range, but basic readers start at approximately $5,000, and a used plate reader can be purchased for less than $1,000.
4. Abbreviations and Acronyms Defined		Ab Antibody BSA Bovine Serum Albumin ELISA Enzyme-linked Immunosorbent Assay HRP Horse radish peroxidase MDG MARBIONC Development Group NSP Neurotoxic Shellfish Poisoning PBS Phosphate Buffered Saline PBS-Tween Phosphate Buffered Saline with Tween 20 (0.05%) PbTx Brevetoxin PGT Phosphate Buffered Saline with gelatin (5%) Tween 20 (0.05%) TMB 3,3'5,5'-Tetramethylbenzidine
5. Details of Turn Around Times (time involved to complete the method)		The ELISA takes approximately 6 hours to complete, and one practiced analyst can comfortably process up to 4 plates per day.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research

		Institute's HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. The Laboratory Manager, Laboratory Safety Officer, Laboratory Secondary Staff and field staff are responsible for implementing QA/QC procedures outlined in the manual. Key practices include the use of Standard Operating Procedures, standard methods, training, quality control, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines. **From the manufacturer (MARBIONC):** Each time new kit reagents are made from stocks, QC ELISAs are run and compared to previous assays. A standard ELISA set is retained to compare all new kits back to. New reagent stocks are given lot numbers. When new reagents are made (e.g. purified antibodies or PbTx-BSA conjugate), the ELISAs are designed with the new reagents to maintain continuity with previous kit lots. Kits are manufactured in a controlled environment to maintain cleanliness and avoid any cross contamination. Kits and kit components are validated. Kit and kit components are serialized to maintain traceability. Higher-level Good Manufacturing Processes are in process and as new reagents are produced, they will conform to requirements to allow for overall implementation of quality systems. Supply: MARBIONC Development Group, LLC has a future vision and is currently working to maintain an adequate supply of reagents. Sufficient supplies are on hand to cover current and projected increased demand for the foreseeable future (approximately 10-15 yrs). MARBIONC is committed to providing the kits for research and commercial use and has also committed to provide resources for the resupply of kit components in advance of the time when such components may be required.
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:

A total of 526 samples were tested using both ELISA and the NSP mouse bioassay (Table G1). Results of individual samples are contained in Table G2. Although additional data exists (both published and unpublished) comparing this ELISA with NSP mouse bioassay results, extraction methods have been modified over time. The data presented here includes only samples that were extracted for ELISA using 80% methanol with no additional clean-up. Almost all of the samples (495 of 526, 94%) were extracted and assayed in duplicate, and the mean is reported in the table. The mean %CV of duplicate analyses was 6.2%.

Table G1. Summary of comparative data using both NSP mouse bioassay and ELISA.

Shellfish Matrix	Total Samples	Mouse Bioassay < 20 MU/100g	Mouse Bioassay ≥ 20 MU/100g
Oysters	197	135 (69%)	62 (31%)
Hard Clams	277	238 (86%)	39 (14%)
Sunray Venus Clams	52	22 (42%)	30 (58%)

Table G2. Sample information and results of NSP mouse bioassay and ELISA

Sample ID	Shellfish Matrix	Harvest Area	Sample Date	MU/100g	ELISA (ppm)
HABB070327-017	oyster	Pine Island Sound	3/26/2007	<20	6.60
HABB070403-002	oyster	Pine Island Sound	4/2/2007	<20	5.26
HABB071115-001	oyster	St. Johns	11/14/2007	33.75	7.26
HABB071115-002	oyster	St. Johns	11/14/2007	38.63	16.31
HABB071128-004	oyster	St. Johns	11/27/2007	27.37	6.53
HABB071212-003	oyster	St. Johns	12/11/2007	<20	3.40
HABB080214-001	oyster	Alabama	2/8/2008	<20	0.52
HABB091117-001	oyster	Pine Island Sound	11/16/2009	<20	0.66
HABB091202-001	oyster	Pine Island Sound	12/1/2009	<20	0.42
HABB091202-002	oyster	Pine Island Sound	12/1/2009	<20	0.29
HABB100105-001	oyster	Pine Island Sound	1/4/2010	36.38	9.44
HABB100112-003	oyster	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-004	oyster	Pine Island Sound	1/11/2010	26.04	6.07
HABB100113-001	oyster	Gasparilla Sound	1/12/2010	<20	1.21
HABB100113-002	oyster	Gasparilla Sound	1/12/2010	<20	1.66
HABB100120-001	oyster	Pine Island Sound	1/19/2010	<20	<LOD
HABB100120-002	oyster	Pine Island Sound	1/19/2010	<20	2.34
HABB100224-001	oyster	Pine Island Sound	2/23/2010	<20	1.83
HABB100224-002	oyster	Pine Island Sound	2/23/2010	<20	1.01
HABB111026-003	oyster	Pine Island Sound	10/25/2011	<20	<LOD
HABB111026-004	oyster	Pine Island Sound	10/25/2011	<20	1.99
HABB111103-001	oyster	Gasparilla Sound	11/2/2011	33.31	9.57
HABB111103-002	oyster	Gasparilla Sound	11/2/2011	28.19	6.50
HABB111109-001	oyster	Pine Island Sound	11/8/2011	<20	0.53
HABB111109-002	oyster	Pine Island Sound	11/8/2011	32.93	10.09
HABB111115-001	oyster	Gasparilla Sound	11/14/2011	<20	4.80
HABB111115-002	oyster	Gasparilla Sound	11/14/2011	<20	2.98
HABB111122-002	oyster	Lemon Bay	11/21/2011	<20	7.76
HABB111213-001	oyster	Pine Island Sound	12/12/2011	<20	2.04
HABB111213-002	oyster	Pine Island Sound	12/12/2011	<20	1.71
HABB111220-001	oyster	Pine Island Sound	12/19/2011	<20	10.83
HABB111220-002	oyster	Pine Island Sound	12/19/2011	<20	3.85
HABB120124-003	oyster	Pine Island Sound	1/23/2012	<20	3.94
HABB120124-004	oyster	Pine Island Sound	1/23/2012	<20	1.31
HABB120131-001	oyster	Ten Thousand Islands	1/30/2012	37.70	14.01
HABB120214-001	oyster	Ten Thousand Islands	2/13/2012	22.80	6.19
HABB120214-002	oyster	Pine Island Sound	2/13/2012	<20	8.25
HABB120214-003	oyster	Pine Island Sound	2/13/2012	<20	1.79
HABB120221-001	oyster	Ten Thousand Islands	2/20/2012	27.43	6.72
HABB120228-001	oyster	Ten Thousand Islands	2/27/2012	<20	4.42
HABB121113-002	oyster	Lower Tampa Bay	11/6/2012	34.08	4.32
HABB130212-004	oyster	Lower Tampa Bay	11/14/2012	34.99	22.43
HABB130205-003	oyster	Lower Tampa Bay	2/4/2013	<20	3.28
HABB130409-001	oyster	Gasparilla Sound	4/8/2013	31.56	8.17
HABB130409-002	oyster	Gasparilla Sound	4/8/2013	29.65	15.40
HABB130501-001	oyster	Gasparilla Sound	4/30/2013	32.21	5.07
HABB130501-002	oyster	Gasparilla Sound	4/30/2013	24.07	3.26

HABB130501-003	oyster	Ten Thousand Islands	4/30/2013	<20	0.77
HABB130508-002	oyster	Gasparilla Sound	5/7/2013	<20	4.91
HABB130508-003	oyster	Gasparilla Sound	5/7/2013	<20	3.00
HABB130508-005	oyster	Lemon Bay	5/7/2013	<20	3.92
HABB130515-001	oyster	Pine Island Sound	5/14/2013	<20	3.17
HABB130515-002	oyster	Pine Island Sound	5/14/2013	<20	3.24
HABB130604-002	oyster	Sarasota Bay	6/3/2013	<20	2.43
HABB131210-001	oyster	Gasparilla Sound	12/9/2013	<20	4.52
HABB131210-002	oyster	Gasparilla Sound	12/9/2013	<20	0.79
HABB131210-003	oyster	Pine Island Sound	12/9/2013	<20	1.99
HABB131217-001	oyster	Pine Island Sound	12/16/2013	<20	2.03
HABB131217-002	oyster	Pine Island Sound	12/16/2013	<20	1.51
HABB131217-003	oyster	Matlacha	12/16/2013	<20	0.18
HABB131218-009	oyster	Lemon Bay	12/17/2013	<20	1.63
HABB141021-001	oyster	Suwannee Sound	10/20/2014	<20	4.62
HABB141021-002	oyster	Suwannee Sound	10/20/2014	<20	5.02
HABB141021-003	oyster	Suwannee Sound	10/20/2014	<20	3.34
HABB141022-002	oyster	Horseshoe Beach	10/21/2014	27.89	5.02
HABB141022-003	oyster	Horseshoe Beach	10/21/2014	<20	<LOD
HABB141028-001	oyster	Horseshoe Beach	10/27/2014	<20	4.44
HABB141028-002	oyster	Horseshoe Beach	10/27/2014	<20	5.20
HABB141028-003	oyster	Horseshoe Beach	10/27/2014	22.56	5.73
HABB141104-001	oyster	Horseshoe Beach	11/3/2014	<20	3.53
HABB141118-001	oyster	Gasparilla Sound	11/17/2014	<20	1.07
HABB141118-002	oyster	Gasparilla Sound	11/17/2014	<20	0.45
HABB141124-004	oyster	Pine Island Sound	11/23/2014	<20	2.57
HABB141209-001	oyster	Pine Island Sound	12/8/2014	<20	0.91
HABB141209-002	oyster	Pine Island Sound	12/8/2014	<20	2.49
HABB141216-001	oyster	Ten Thousand Islands	12/15/2014	<20	1.13
HABB151014-002	oyster	Indian Lagoon	10/13/2015	<20	0.84
HABB151119-001	oyster	East Bay	10/29/2015	94.60	25.50
HABB151103-001	oyster	Indian Lagoon	11/2/2015	<20	1.99
HABB151103-002	oyster	Pine Island Sound	11/2/2015	<20	0.98
HABB151103-003	oyster	Pine Island Sound	11/2/2015	<20	<LOD
HABB151110-001	oyster	Gasparilla Sound	11/9/2015	<20	1.34
HABB151110-002	oyster	Gasparilla Sound	11/9/2015	<20	3.87
HABB151117-001	oyster	East Bay	11/16/2015	34.05	7.08
HABB151117-002	oyster	North Bay	11/16/2015	<20	1.59
HABB151124-001	oyster	East Bay	11/23/2015	25.03	5.77
HABB151202-001	oyster	East Bay	12/1/2015	34.84	7.44
HABB151208-001	oyster	West Bay	12/7/2015	33.07	3.57
HABB151208-002	oyster	East Bay	12/7/2015	28.14	5.09
HABB151208-003	oyster	East Bay	12/7/2015	35.47	13.95
HABB151216-001	oyster	East Bay	12/15/2015	33.37	5.04
HABB151216-002	oyster	West Bay	12/15/2015	30.10	5.55
HABB151217-001	oyster	Gasparilla Sound	12/16/2015	<20	2.27
HABB151217-002	oyster	Gasparilla Sound	12/16/2015	26.79	4.73
HABB151217-003	oyster	Pine Island Sound	12/16/2015	31.47	3.96
HABB151217-004	oyster	Pine Island Sound	12/16/2015	20.21	3.56
HABB151222-001	oyster	Gasparilla Sound	12/21/2015	<20	4.31
HABB151222-002	oyster	Gasparilla Sound	12/21/2015	<20	1.77
HABB160105-001	oyster	Pine Island Sound	1/4/2016	<20	2.28

HABB160105-002	oyster	Pine Island Sound	1/4/2016	<20	2.17
HABB160105-003	oyster	Apalachicola Bay	1/4/2016	<20	3.27
HABB160105-004	oyster	Apalachicola Bay	1/4/2016	<20	2.52
HABB160106-001	oyster	East Bay	1/5/2016	30.63	2.45
HABB160106-002	oyster	North Bay	1/5/2016	17.07	7.91
HABB160112-001	oyster	West Bay	1/11/2016	22.35	3.28
HABB160112-002	oyster	North Bay	1/11/2016	23.94	7.28
HABB160112-003	oyster	West Bay	1/11/2016	35.43	12.59
HABB160113-001	oyster	Pensacola Bay	1/12/2016	<20	2.13
HABB160114-001	oyster	Apalachicola Bay	1/12/2016	<20	1.88
HABB160114-002	oyster	Indian Lagoon	1/12/2016	21.84	10.53
HABB160120-001	oyster	East Bay	1/19/2016	<20	2.02
HABB160120-002	oyster	North Bay	1/19/2016	<20	6.41
HABB160120-003	oyster	Mississippi	1/19/2016	<20	0.16
HABB160120-004	oyster	Mississippi	1/19/2016	<20	0.33
HABB160120-005	oyster	Mississippi	1/19/2016	<20	0.23
HABB160120-006	oyster	Mississippi	1/19/2016	<20	0.41
HABB160120-007	oyster	Mississippi	1/19/2016	<20	1.22
HABB160120-008	oyster	Mississippi	1/19/2016	<20	0.88
HABB160121-001	oyster	Indian Lagoon	1/20/2016	22.20	9.84
HABB160126-001	oyster	West Bay	1/25/2016	30.18	9.37
HABB160126-002	oyster	West Bay	1/25/2016	16.69	2.82
HABB160127-001	oyster	Alabama	1/25/2016	<20	3.17
HABB160127-002	oyster	Alabama	1/25/2016	<20	2.23
HABB160127-003	oyster	Alabama	1/25/2016	<20	3.11
HABB160127-004	oyster	Alabama	1/25/2016	<20	0.36
HABB160127-005	oyster	Alabama	1/25/2016	<20	0.42
HABB160128-001	oyster	East Bay	1/27/2016	<20	3.00
HABB160202-001	oyster	West Bay	2/1/2016	29.32	5.96
HABB160203-001	oyster	St. Joseph Bay	2/2/2016	28.40	14.20
HABB160203-002	oyster	Louisiana	2/2/2016	<20	0.29
HABB160203-003	oyster	Louisiana	2/2/2016	<20	0.77
HABB160203-004	oyster	Louisiana	2/2/2016	<20	0.84
HABB160203-005	oyster	Louisiana	2/2/2016	<20	1.08
HABB160203-006	oyster	Louisiana	2/2/2016	<20	0.33
HABB160203-007	oyster	Louisiana	2/2/2016	<20	0.29
HABB160204-001	oyster	Indian Lagoon	2/2/2016	<20	4.22
HABB160211-001	oyster	West Bay	2/10/2016	<20	5.56
HABB160223-001	oyster	Pine Island Sound	2/22/2016	31.66	6.77
HABB160223-005	oyster	St. Joseph Bay	2/22/2016	<20	12.37
HABB160224-001	oyster	Pine Island Sound	2/23/2016	<20	0.94
HABB160301-001	oyster	Alabama	2/29/2016	<20	1.72
HABB160302-001	oyster	Pine Island Sound	3/1/2016	<20	4.02
HABB160303-002	oyster	Gasparilla Sound	3/2/2016	19.81	5.07
HABB160308-001	oyster	Lower Tampa Bay	3/7/2016	23.53	10.51
HABB160309-001	oyster	Choctawhatchee Bay	3/8/2016	<20	0.60
HABB160317-001	oyster	Pine Island Sound	3/16/2016	25.90	3.87
HABB160317-002	oyster	Pine Island Sound	3/16/2016	<20	3.03
HABB160322-001	oyster	Lower Tampa Bay	3/22/2016	<20	4.33
HABB160328-002	oyster	Lower Tampa Bay	3/28/2016	<20	4.87
HABB160330-001	oyster	Pine Island Sound	3/29/2016	26.26	4.88
HABB160330-002	oyster	Pine Island Sound	3/29/2016	<20	2.19

HABB160407-002	oyster	Lower Tampa Bay	4/6/2016	<20	3.99
HABB160407-004	oyster	Pine Island Sound	4/7/2016	<20	3.00
HABB160411-013	oyster	Lower Tampa Bay	4/11/2016	<20	3.83
HABB160418-002	oyster	Lower Tampa Bay	4/18/2016	<20	2.76
HABB160421-002	oyster	Pine Island Sound	4/20/2016	23.66	3.01
HABB160421-003	oyster	Pine Island Sound	4/20/2016	<20	1.71
HABB160427-001	oyster	Pine Island Sound	4/26/2016	<20	3.37
HABB160427-002	oyster	Pine Island Sound	4/26/2016	<20	1.71
HABB160502-001	oyster	Boca Ceiga Bay	5/2/2016	21.65	4.59
HABB160505-001	oyster	Gasparilla Sound	5/4/2016	<20	2.70
HABB160505-002	oyster	Gasparilla Sound	5/4/2016	<20	1.67
HABB160510-001	oyster	Boca Ceiga Bay	5/10/2016	16.23	4.11
HABB161011-002	oyster	Lower Tampa Bay	10/10/2016	<20	0.74
HABB161018-002	oyster	Lower Tampa Bay	10/17/2016	<20	1.57
HABB161114-002	oyster	Lower Tampa Bay	11/14/2016	156.08	47.60
HABB170104-003	oyster	Pine Island Sound	1/3/2017	30.23	9.64
HABB170105-001	oyster	Lower Tampa Bay	1/4/2017	<20	2.31
HABB170110-001	oyster	Lower Tampa Bay	1/9/2017	<20	0.84
HABB170110-004	oyster	Gasparilla Sound	1/9/2017	28.32	8.43
HABB170111-001	oyster	Ten Thousand Islands	1/10/2017	19.63	3.14
HABB170111-002	oyster	Matlacha Pass	1/10/2017	<20	1.58
HABB170111-003	oyster	Pine Island Sound	1/10/2017	30.71	7.37
HABB170118-002	oyster	Gasparilla Sound	1/17/2017	29.46	6.65
HABB170119-003	oyster	Pine Island Sound	1/18/2017	33.87	5.64
HABB170119-004	oyster	Myakka River	1/18/2017	31.00	4.56
HABB170125-001	oyster	Gasparilla Sound	1/24/2017	<20	4.06
HABB170125-003	oyster	Pine Island Sound	1/24/2017	<20	4.31
HABB170131-002	oyster	Gasparilla Sound	1/30/2017	36.73	9.68
HABB170201-002	oyster	Myakka River	1/31/2017	22.45	3.56
HABB170207-002	oyster	Gasparilla Sound	2/6/2017	31.32	8.12
HABB170213-002	oyster	Lower Tampa Bay	2/13/2017	<20	1.47
HABB170214-004	oyster	Pine Island Sound	2/13/2017	<20	2.01
HABB170221-001	oyster	Myakka River	2/20/2017	<20	2.08
HABB170222-001	oyster	Gasparilla Sound	2/21/2017	42.30	10.51
HABB170307-002	oyster	Gasparilla Sound	3/6/2017	29.03	5.11
HABB170314-002	oyster	Gasparilla Sound	3/13/2017	<20	2.55
HABB170315-002	oyster	Lower Tampa Bay	3/14/2017	<20	2.21
HABB170322-002	oyster	Gasparilla Sound	3/21/2017	<20	2.49
HABB170405-001	oyster	Boca Ceiga Bay	4/4/2017	31.35	6.80
HABB170410-005	oyster	Gasparilla Sound	4/10/2017	<20	1.23
HABB170412-001	oyster	Pine Island Sound	4/11/2017	25.73	3.56
HABB170418-001	oyster	Pine Island Sound	4/17/2017	19.01	2.35
HABB170419-001	oyster	Lower Tampa Bay	4/18/2017	<20	5.89
HABB170419-002	oyster	Lower Tampa Bay	4/18/2017	<20	3.72
HABB170425-001	oyster	Gasparilla Sound	4/24/2017	25.81	4.13
HABB170425-002	oyster	Gasparilla Sound	4/24/2017	34.91	8.27
HABB080108-001	hard clam	Volusia County	1/7/2008	<20	0.97
HABB080108-002	hard clam	Volusia County	1/7/2008	<20	0.77
HABB080108-003	hard clam	Mosquito Lagoon	1/7/2008	52.8	4.2
HABB080109-003	hard clam	North Indian River	1/8/2008	<20	2.69
HABB080109-004	hard clam	Indian River Body F	1/8/2008	<20	0.14
HABB080115-001	hard clam	Mosquito Lagoon	1/14/2008	46.26	4

HABB080115-002	hard clam	Indian River Body A	1/14/2008	<20	1.18
HABB080115-003	hard clam	Indian River Body A	1/14/2008	38.66	4.44
HABB080123-022	hard clam	St. Lucie County	1/22/2008	<20	0.93
HABB080123-023	hard clam	Mosquito Lagoon	1/22/2008	<20	3.05
HABB080123-024	hard clam	Indian River Body A	1/22/2008	<20	2.35
HABB080123-025	hard clam	Indian River Body B	1/22/2008	<20	1.16
HABB090519-001	hard clam	Indian River Body F	5/18/2009	<20	<LOD
HABB091109-001	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-002	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-003	hard clam	Pine Island Sound	11/9/2009	<20	<LOD
HABB091109-004	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB100105-002	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-003	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-004	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-005	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100112-001	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-002	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100118-001	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-002	hard clam	Pine Island Sound	1/18/2010	<20	0.06
HABB100118-003	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-004	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB111011-001	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-002	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-003	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-004	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111018-001	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-002	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-003	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-004	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111024-001	hard clam	Pine Island Sound	10/23/2011	<20	<LOD
HABB111122-001	hard clam	Gasparilla Sound	11/21/2011	<20	4.13
HABB111206-001	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111206-002	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111213-003	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB111213-004	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB120104-001	hard clam	Pine Island Sound	1/4/2012	<20	0.63
HABB120104-002	hard clam	Pine Island Sound	1/4/2012	<20	0.66
HABB120109-001	hard clam	Pine Island Sound	1/9/2012	<20	0.63
HABB120109-002	hard clam	Pine Island Sound	1/9/2012	<20	0.48
HABB120117-001	hard clam	Pine Island Sound	1/16/2012	<20	0.24
HABB120117-002	hard clam	Pine Island Sound	1/16/2012	<20	0.23
HABB120124-001	hard clam	Pine Island Sound	1/23/2012	<20	0.14
HABB120124-002	hard clam	Pine Island Sound	1/23/2012	<20	0.13
HABB120131-003	hard clam	Ten Thousand Islands	1/25/2012	<20	1.39
HABB120131-004	hard clam	Ten Thousand Islands	1/25/2012	<20	1.49
HABB121002-001	hard clam	Gasparilla Sound	10/1/2012	37.63	12.68
HABB121002-002	hard clam	Gasparilla Sound	10/1/2012	<20	0.25
HABB121003-001	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121003-002	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121009-001	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-002	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-003	hard clam	Pine Island Sound	10/8/2012	<20	<LOD

HABB121009-004	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-005	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-006	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121016-001	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-002	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-003	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-004	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-005	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-006	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121017-001	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121017-002	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121023-005	hard clam	Pine Island Sound	10/22/2012	<20	0.28
HABB121023-006	hard clam	Pine Island Sound	10/22/2012	<20	0.26
HABB121023-007	hard clam	Pine Island Sound	10/22/2012	<20	0.18
HABB121023-008	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-009	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-010	hard clam	Pine Island Sound	10/22/2012	<20	0.22
HABB121024-001	hard clam	Lower Tampa Bay	10/23/2012	<20	0.92
HABB121024-002	hard clam	Lower Tampa Bay	10/23/2012	<20	1.05
HABB121024-003	hard clam	Lower Tampa Bay	10/23/2012	<20	0.7
HABB121024-004	hard clam	Lower Tampa Bay	10/23/2012	<20	0.66
Habb121024-005	hard clam	Pine Island Sound	10/23/2012	<20	0.18
HABB121024-006	hard clam	Pine Island Sound	10/23/2012	<20	0.23
HABB121030-001	hard clam	Lower Tampa Bay	10/29/2012	<20	0.5
HABB121030-002	hard clam	Lower Tampa Bay	10/29/2012	<20	0.34
HABB121030-003	hard clam	Pine Island Sound	10/29/2012	<20	1.2
HABB121030-004	hard clam	Pine Island Sound	10/29/2012	<20	0.88
HABB121113-001	hard clam	Lower Tampa Bay	11/6/2012	<20	1.78
HABB130212-003	hard clam	Lower Tampa Bay	11/14/2012	<20	<LOD
HABB121120-001	hard clam	Pine Island Sound	11/19/2012	<20	2.16
HABB121127-001	hard clam	Sarasota Bay	11/26/2012	<20	0.7
HABB121127-002	hard clam	Pine Island Sound	11/26/2012	<20	0.88
HABB121127-003	hard clam	Pine Island Sound	11/26/2012	<20	2.01
HABB121127-004	hard clam	Pine Island Sound	11/26/2012	<20	1.82
HABB121211-001	hard clam	Pine Island Sound	12/10/2012	<20	0.63
HABB121211-002	hard clam	Pine Island Sound	12/10/2012	<20	0.52
HABB121211-003	hard clam	Pine Island Sound	12/10/2012	<20	1.01
HABB121211-004	hard clam	Pine Island Sound	12/10/2012	<20	1.31
HABB121218-001	hard clam	Pine Island Sound	12/17/2012	<20	1.19
HABB121218-002	hard clam	Pine Island Sound	12/17/2012	<20	5.6
HABB121218-003	hard clam	Pine Island Sound	12/17/2012	<20	0.86
HABB121218-004	hard clam	Pine Island Sound	12/17/2012	<20	0.99
HABB121218-005	hard clam	Pine Island Sound	12/17/2012	<20	0.58
HABB121218-006	hard clam	Pine Island Sound	12/17/2012	<20	0.5
HABB121218-007	hard clam	Lower Tampa Bay	12/18/2012	<20	2.01
HABB121218-008	hard clam	Lower Tampa Bay	12/18/2012	<20	2.34
HABB121227-026	hard clam	Lower Tampa Bay	12/26/2012	23.59	3
HABB121227-027	hard clam	Lower Tampa Bay	12/26/2012	22.19	2.34
HABB121227-028	hard clam	Pine Island Sound	12/26/2012	<20	0.45
HABB121227-029	hard clam	Pine Island Sound	12/26/2012	<20	0.44
HABB130103-001	hard clam	Pine Island Sound	1/2/2013	<20	0.74
HABB130103-002	hard clam	Pine Island Sound	1/2/2013	<20	0.82

HABB130103-003	hard clam	Pine Island Sound	1/2/2013	22.09	2.18
HABB130103-004	hard clam	Pine Island Sound	1/2/2013	21.64	2.45
HABB130103-005	hard clam	Pine Island Sound	1/2/2013	<20	0.66
HABB130103-006	hard clam	Pine Island Sound	1/2/2013	<20	0.87
HABB130108-001	hard clam	Pine Island Sound	1/7/2013	<20	0.72
HABB130108-002	hard clam	Pine Island Sound	1/7/2013	<20	0.85
HABB130108-003	hard clam	Pine Island Sound	1/7/2013	<20	1.09
HABB130108-004	hard clam	Pine Island Sound	1/7/2013	<20	0.83
HABB130109-001	hard clam	Lower Tampa Bay	1/8/2013	20.2	4.38
HABB130109-002	hard clam	Lower Tampa Bay	1/8/2013	<20	1.96
HABB130109-003	hard clam	Lower Tampa Bay	1/8/2013	<20	1.51
HABB130115-003	hard clam	Pine Island Sound	1/14/2013	<20	1.07
HABB130115-004	hard clam	Pine Island Sound	1/14/2013	<20	1.74
HABB130122-001	hard clam	Lower Tampa Bay	1/22/2013	<20	1.57
HABB130122-002	hard clam	Lower Tampa Bay	1/22/2013	<20	1.54
HABB130130-001	hard clam	Lower Tampa Bay	1/28/2013	<20	1.8
HABB130130-002	hard clam	Lower Tampa Bay	1/28/2013	<20	1.82
HABB130205-001	hard clam	Lower Tampa Bay	2/4/2013	<20	1.41
HABB130205-002	hard clam	Lower Tampa Bay	2/4/2013	<20	1.44
HABB130212-001	hard clam	Pine Island Sound	2/11/2013	21.01	4.16
HABB130212-005	hard clam	Pine Island Sound	2/11/2013	29.23	5.68
HABB130226-002	hard clam	Pine Island Sound	2/24/2013	49.23	8.44
HABB130226-003	hard clam	Pine Island Sound	2/24/2013	44.71	8.37
HABB130226-004	hard clam	Pine Island Sound	2/24/2013	84.59	16.18
HABB130226-005	hard clam	Pine Island Sound	2/24/2013	39.34	9.89
HABB130226-006	hard clam	Pine Island Sound	2/24/2013	38.23	4.83
HABB130226-007	hard clam	Pine Island Sound	2/24/2013	27.18	4.82
HABB130226-008	hard clam	Pine Island Sound	2/24/2013	68.19	7.04
HABB130226-009	hard clam	Pine Island Sound	2/24/2013	<20	2.55
HABB130226-010	hard clam	Pine Island Sound	2/24/2013	44.16	6.33
HABB151007-002	hard clam	Pine Island Sound	2/25/2013	92.65	9.84
HABB130306-005	hard clam	Pine Island Sound	3/4/2013	<20	4.57
HABB130319-006	hard clam	Pine Island Sound	3/8/2013	<20	2.81
HABB130312-004	hard clam	Pine Island Sound	3/11/2013	205.34	37.33
HABB130312-005	hard clam	Pine Island Sound	3/11/2013	24.95	3.87
HABB130312-006	hard clam	Pine Island Sound	3/11/2013	<20	2.51
HABB130312-007	hard clam	Pine Island Sound	3/11/2013	<20	2.39
HABB130313-007	hard clam	Pine Island Sound	3/11/2013	36.89	3.26
HABB130313-008	hard clam	Pine Island Sound	3/11/2013	<20	1.73
HABB130313-001	hard clam	Pine Island Sound	3/12/2013	<20	1.93
HABB130313-002	hard clam	Pine Island Sound	3/12/2013	<20	2.46
HABB130313-003	hard clam	Pine Island Sound	3/12/2013	<20	2.47
HABB130313-004	hard clam	Pine Island Sound	3/12/2013	<20	2.35
HABB130319-007	hard clam	Pine Island Sound	3/13/2013	<20	2.24
HABB130319-004	hard clam	Pine Island Sound	3/18/2013	<20	2.14
HABB130319-005	hard clam	Pine Island Sound	3/18/2013	<20	4.2
HABB130319-012	hard clam	Pine Island Sound	3/18/2013	22.55	2.79
HABB140725-001	hard clam	Ten Thousand Islands	3/20/2013	<20	3.89
HABB130326-003	hard clam	Pine Island Sound	3/25/2013	<20	1.58
HABB130326-004	hard clam	Pine Island Sound	3/25/2013	<20	1.39
HABB130326-005	hard clam	Pine Island Sound	3/25/2013	<20	1.71
HABB130326-006	hard clam	Pine Island Sound	3/25/2013	<20	1.65

HABB130326-009	hard clam	Pine Island Sound	3/25/2013	<20	1.57
HABB130326-010	hard clam	Pine Island Sound	3/25/2013	<20	1.62
HABB130326-011	hard clam	Pine Island Sound	3/25/2013	<20	1.47
HABB130326-012	hard clam	Pine Island Sound	3/25/2013	<20	1.42
HABB130326-013	hard clam	Gasparilla Sound	3/25/2013	84.16	16.89
HABB130326-014	hard clam	Pine Island Sound	3/25/2013	75.9	16.4
HABB130403-002	hard clam	Pine Island Sound	3/29/2013	<20	2.23
HABB130402-001	hard clam	Pine Island Sound	4/1/2013	<20	2.05
HABB130402-002	hard clam	Pine Island Sound	4/1/2013	<20	1.98
HABB130402-003	hard clam	Pine Island Sound	4/1/2013	25.2	3.5
HABB130402-004	hard clam	Pine Island Sound	4/1/2013	24.3	2.92
HABB130402-005	hard clam	Pine Island Sound	4/1/2013	<20	1.4
HABB130402-006	hard clam	Pine Island Sound	4/1/2013	<20	1.27
HABB130402-007	hard clam	Pine Island Sound	4/1/2013	<20	1.55
HABB130402-008	hard clam	Pine Island Sound	4/1/2013	<20	3.51
HABB130402-009	hard clam	Pine Island Sound	4/1/2013	<20	3.27
HABB130409-003	hard clam	Pine Island Sound	4/8/2013	<20	0.97
HABB130409-004	hard clam	Pine Island Sound	4/8/2013	<20	1.17
HABB130409-008	hard clam	Pine Island Sound	4/8/2013	<20	1.81
HABB130409-009	hard clam	Pine Island Sound	4/8/2013	<20	1.09
HABB130409-010	hard clam	Pine Island Sound	4/8/2013	<20	0.85
HABB130409-011	hard clam	Pine Island Sound	4/8/2013	<20	3.82
HABB130409-012	hard clam	Pine Island Sound	4/8/2013	<20	4.12
HABB130409-013	hard clam	Pine Island Sound	4/8/2013	<20	3.81
HABB130409-014	hard clam	Pine Island Sound	4/8/2013	35.6	4.29
HABB130409-015	hard clam	Pine Island Sound	4/8/2013	<20	1.69
HABB130409-016	hard clam	Pine Island Sound	4/8/2013	<20	1.52
HABB130410-001	hard clam	Pine Island Sound	4/9/2013	<20	1.82
HABB130410-002	hard clam	Pine Island Sound	4/9/2013	<20	1.91
HABB130410-003	hard clam	Pine Island Sound	4/9/2013	<20	1.69
HABB130416-006	hard clam	Pine Island Sound	4/15/2013	<20	0.83
HABB130416-007	hard clam	Pine Island Sound	4/15/2013	<20	0.81
HABB130417-001	hard clam	Pine Island Sound	4/16/2013	<20	1.09
HABB130417-002	hard clam	Pine Island Sound	4/16/2013	<20	1.24
HABB130417-004	hard clam	Pine Island Sound	4/16/2013	<20	1.37
HABB130417-005	hard clam	Pine Island Sound	4/16/2013	<20	1.28
HABB130423-001	hard clam	Pine Island Sound	4/22/2013	<20	1.02
HABB130423-002	hard clam	Pine Island Sound	4/22/2013	<20	1.06
HABB130423-003	hard clam	Pine Island Sound	4/22/2013	<20	0.98
HABB130424-001	hard clam	Pine Island Sound	4/24/2013	<20	0.93
HABB130424-002	hard clam	Pine Island Sound	4/24/2013	<20	1
HABB130424-003	hard clam	Pine Island Sound	4/24/2013	<20	0.86
HABB130508-004	hard clam	Lemon Bay	5/7/2013	<20	17.33
HABB131113-001	hard clam	Pine Island Sound	11/12/2013	<20	0.5
HABB131113-002	hard clam	Pine Island Sound	11/12/2013	<20	0.32
HABB131113-003	hard clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-006	hard clam	Pine Island Sound	11/12/2013	<20	0.41
HABB131113-007	hard clam	Pine Island Sound	11/12/2013	<20	0.38
HABB131113-008	hard clam	Pine Island Sound	11/12/2013	<20	0.44
HABB131119-001	hard clam	Pine Island Sound	11/18/2013	<20	1.96
HABB131119-002	hard clam	Pine Island Sound	11/18/2013	<20	1.71
HABB131119-003	hard clam	Pine Island Sound	11/18/2013	<20	1.78

HABB131126-001	hard clam	Pine Island Sound	11/25/2013	<20	0.3
HABB131126-002	hard clam	Pine Island Sound	11/25/2013	<20	0.29
HABB131126-003	hard clam	Pine Island Sound	11/25/2013	<20	0.28
HABB131126-004	hard clam	Pine Island Sound	11/25/2013	<20	0.59
HABB131126-005	hard clam	Pine Island Sound	11/25/2013	<20	0.69
HABB131126-006	hard clam	Pine Island Sound	11/25/2013	<20	0.68
HABB131203-001	hard clam	Pine Island Sound	12/2/2013	<20	0.23
HABB131203-002	hard clam	Pine Island Sound	12/2/2013	<20	0.24
HABB131203-003	hard clam	Pine Island Sound	12/2/2013	<20	0.21
HABB131203-004	hard clam	Pine Island Sound	12/2/2013	<20	0.31
HABB131203-005	hard clam	Pine Island Sound	12/2/2013	<20	0.33
HABB131203-006	hard clam	Pine Island Sound	12/2/2013	<20	0.38
HABB131210-004	hard clam	Pine Island Sound	12/9/2013	<20	0.35
HABB131210-005	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131210-006	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131211-012	hard clam	Gasparilla Sound	12/10/2013	<20	0.84
HABB131218-010	hard clam	Gasparilla Sound	12/17/2013	36.91	8.96
HABB141014-001	hard clam	Cedar Key	10/13/2014	<20	0.33
HABB141014-002	hard clam	Cedar Key	10/13/2014	<20	0.31
HABB141014-003	hard clam	Cedar Key	10/13/2014	<20	0.42
HABB141113-002	hard clam	Pine Island Sound	11/12/2014	<20	0.34
HABB141113-003	hard clam	Pine Island Sound	11/12/2014	<20	0.44
HABB141113-004	hard clam	Pine Island Sound	11/12/2014	<20	0.69
HABB141113-005	hard clam	Pine Island Sound	11/12/2014	<20	0.7
HABB141113-006	hard clam	Pine Island Sound	11/12/2014	<20	0.66
HABB141113-007	hard clam	Pine Island Sound	11/12/2014	<20	0.62
HABB141119-001	hard clam	Pine Island Sound	11/18/2014	<20	0.15
HABB141119-002	hard clam	Pine Island Sound	11/18/2014	<20	0.13
HABB141119-003	hard clam	Pine Island Sound	11/18/2014	<20	0.2
HABB141119-004	hard clam	Pine Island Sound	11/18/2014	<20	0.18
HABB141119-005	hard clam	Pine Island Sound	11/18/2014	<20	0.23
HABB141119-006	hard clam	Pine Island Sound	11/18/2014	<20	0.25
HABB141124-001	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-002	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-003	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB160202-002	hard clam	Pine Island Sound	2/1/2016	<20	0.92
HABB160209-017	hard clam	Gasparilla Sound	2/8/2016	76.77	10.82
HABB160209-018	hard clam	Gasparilla Sound	2/8/2016	42.61	9.68
HABB160209-019	hard clam	Gasparilla Sound	2/8/2016	85.99	10
HABB160223-003	hard clam	Pine Island Sound	2/22/2016	<20	0.44
HABB160301-002	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-003	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-004	hard clam	Pine Island Sound	2/29/2016	<20	0.33
HABB160301-005	hard clam	Pine Island Sound	2/29/2016	<20	0.37
HABB160302-002	hard clam	Pine Island Sound	3/1/2016	<20	0.6
HABB160302-003	hard clam	Pine Island Sound	3/1/2016	<20	0.65
HABB160308-002	hard clam	Lower Tampa Bay	3/7/2016	40.05	6.21
HABB160322-002	hard clam	Lower Tampa Bay	3/22/2016	25	5.12
HABB160328-001	hard clam	Lower Tampa Bay	3/28/2016	35.83	4.9
HABB160407-001	hard clam	Lower Tampa Bay	4/6/2016	29.59	4.36
HABB160407-003	hard clam	Pine Island Sound	4/7/2016	<20	0.5
HABB160411-012	hard clam	Lower Tampa Bay	4/11/2016	<20	1.36

HABB160418-001	hard clam	Lower Tampa Bay	4/18/2016	<20	1.76
HABB160601-001	hard clam	Lemon Bay	5/31/2016	<20	0.43
HABB161011-001	hard clam	Lower Tampa Bay	10/10/2016	<20	1.16
HABB161013-001	hard clam	Gasparilla Sound	10/12/2016	<20	0.54
HABB161018-001	hard clam	Lower Tampa Bay	10/17/2016	<20	2.07
HABB170104-001	Hard clam	Pine Island Sound	1/3/2017	<20	1.66
HABB170104-002	Hard clam	Pine Island Sound	1/3/2017	<20	1
HABB170105-002	Hard clam	Lower Tampa Bay	1/4/2017	35.96	2.22
HABB170110-002	hard clam	Lower Tampa Bay	1/9/2017	<20	1.58
HABB170110-003	hard clam	Gasparilla Sound	1/9/2017	20.26	2.35
HABB131125-020	hard clam	Composite		<20	3.9
HABB130115-001	sunray venus clam	Pine Island Sound	1/14/2013	<20	1.85
HABB130212-002	sunray venus clam	Pine Island Sound	2/11/2013	34.13	12.04
HABB130212-005	sunray venus clam	Pine Island Sound	2/11/2013	39.09	19.74
HABB130226-001	sunray venus clam	Pine Island Sound	2/24/2013	42.41	15.41
HABB130226-011	sunray venus clam	Pine Island Sound	2/24/2013	<20	5.58
HABB130228-001	sunray venus clam	Pine Island Sound	2/25/2013	32.17	9.93
HABB130227-001	sunray venus clam	Pine Island Sound	2/26/2013	42.9	13.01
HABB130227-002	sunray venus clam	Pine Island Sound	2/26/2013	34.97	19.09
HABB130228-003	sunray venus clam	Pine Island Sound	2/27/2013	27.54	17.94
HABB130319-009	sunray venus clam	Pine Island Sound	3/8/2013	<20	3.13
HABB130312-001	sunray venus clam	Pine Island Sound	3/11/2013	27.65	6.59
HABB130312-002	sunray venus clam	Pine Island Sound	3/11/2013	26.33	7.39
HABB130312-003	sunray venus clam	Pine Island Sound	3/11/2013	28.7	5.16
HABB130312-009	sunray venus clam	Pine Island Sound	3/11/2013	<20	5.38
HABB150921-001	sunray venus clam	Pine Island Sound	3/11/2013	31.33	5.3
HABB130319-010	sunray venus clam	Pine Island Sound	3/13/2013	<20	3.1
HABB130319-001	sunray venus clam	Pine Island Sound	3/18/2013	22.05	4.48
HABB130319-002	sunray venus clam	Pine Island Sound	3/18/2013	20.67	4.28
HABB130319-003	sunray venus clam	Pine Island Sound	3/18/2013	27.85	7.69
HABB130319-011	sunray venus clam	Pine Island Sound	3/18/2013	25.87	5.43
HABB130326-001	sunray venus clam	Pine Island Sound	3/25/2013	23.16	3.48
HABB130326-002	sunray venus clam	Pine Island Sound	3/25/2013	22.36	3.4
HABB130326-007	sunray venus clam	Pine Island Sound	3/25/2013	24.4	4.44
HABB130326-008	sunray venus clam	Pine Island Sound	3/25/2013	22.5	3.35
HABB130409-006	sunray venus clam	Pine Island Sound	4/8/2013	22.84	2.53
HABB130409-020	sunray venus clam	Pine Island Sound	4/8/2013	<20	2.16
HABB130409-021	sunray venus clam	Pine Island Sound	4/8/2013	23.91	2.69
HABB130410-004	sunray venus clam	Pine Island Sound	4/9/2013	<20	2.18
HABB130410-005	sunray venus clam	Pine Island Sound	4/9/2013	<20	1.84
HABB130416-002	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.47
HABB130416-003	sunray venus clam	Pine Island Sound	4/15/2013	<20	0.99
HABB130416-004	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.48
HABB130417-006	sunray venus clam	Pine Island Sound	4/16/2013	<20	1.62
HABB130604-003	sunray venus clam	Pine Island Sound	6/3/2013	<20	0.56
HABB131113-004	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-005	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.24
HABB131125-019	sunray venus clam	Alligator Harbor	11/22/2013	25.88	4.11
HABB151120-001	sunray venus clam	Sarasota Bay	11/18/2015	33.21	11.05
HABB151120-002	sunray venus clam	Sarasota Bay	11/18/2015	33.58	12.11
HABB151207-001	sunray venus clam	Sarasota Bay	12/7/2015	53.21	14.47
HABB160111-002	Sunray venus clam	Lower Tampa Bay	12/15/2015	33.34	6.37

HABB160111-001	sunray venus clam	Sarasota Bay	1/6/2016	<20	2.77
HABB160202-003	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160202-004	sunray venus clam	Pine Island Sound	2/1/2016	<20	2.74
HABB160202-005	sunray venus clam	Pine Island Sound	2/1/2016	19.77	2.14
HABB160202-006	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160223-004	sunray venus clam	Pine Island Sound	2/22/2016	27.66	2.16
HABB160330-004	sunray venus clam	Lower Tampa Bay	3/16/2016	36.48	3.38
HABB160330-005	sunray venus clam	Lower Tampa Bay	3/16/2016	33.04	3.41
HABB161213-019	sunray venus clam	MML lab exposure	9/6/2016	<20	2.63
HABB161213-021	sunray venus clam	MML lab exposure	12/7/2016	20.66	4.04
HABB161213-022	sunray venus clam	MML exp control	12/7/2016	<20	<LOD

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method

Variance of the data generated from the officially recognized analytical method

Variance of the data generated from the proposed alternative analytical method

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____

Is there a significant difference between the analytical methods Y/N

Comparative data for NSP mouse bioassays and ELISAs cannot be evaluated as described above. Please see below for additional discussion and comparisons permitted by the data presented above.

Brevetoxins in bivalves

At least nine brevetoxin congeners have been isolated from *K. brevis*[1]. PbTx-1 and PbTx-2 are presumed to be the parent toxins from which all other brevetoxins are derived via substitutions on the terminal ring. Consequently, brevetoxins are grouped into two types according to their backbone structure. Brevetoxin A-type (PbTx-1-type) toxins possess a 10-ring backbone, and brevetoxin B-type (PbTx-2-type) toxins possess an 11-ring backbone (Fig. G1). Although brevetoxin A-type toxins are more potent, the brevetoxin B-type toxins are much more abundant[2]. Polar derivatives identified in both culture and bloom materials have further increased the number of known brevetoxin structures[3,4].

In bivalves, the more reactive forms of brevetoxin are rapidly transformed into brevetoxin metabolites[3,5] that are generally the products of reduction, oxidation, and conjugation to other molecules including taurine, cysteine, cysteine sulfoxide, amino acids and fatty acids[5-7]. Literally dozens of metabolites have been identified in shellfish. Most modifications to brevetoxins occur at the side chain on the terminal ether ring that differentiates the brevetoxin congeners, resulting in an assortment of conjugates with either an A-type or B-type of backbone. Brevetoxin metabolites are known to contribute to NSP toxicity [3,6-8], but their individual potency varies. Toxicity information is available for only a small subset of the dozens of characterized metabolites. Some common shellfish metabolites are less potent than parent brevetoxins, while a few have demonstrated higher toxicities [7,9,10]. Different rates of tissue uptake and elimination of brevetoxin metabolites have also been described and may factor into their variable potencies[11].

The complexity of brevetoxins and their metabolic products is the primary reasons that so little progress has been made on moving away from the NSP mouse bioassay. Of the many chemical and biological methods evaluated for measuring brevetoxins in bivalves, those that recognize molecular structure (i.e., ELISAs and liquid chromatography-mass spectroscopy [LC-MS]) have outperformed activity-based assays (i.e., receptor-binding and cytotoxicity assay), demonstrating less variability and better agreement with mouse bioassays[7,12,13].

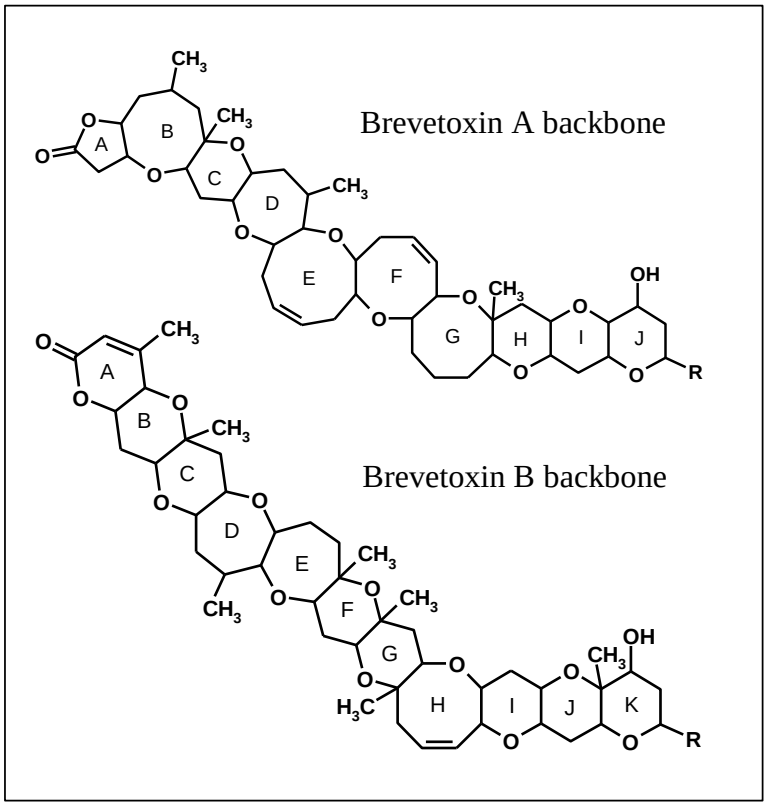


Figure G1. Brevetoxin backbone structures.

An LC-MS method has been developed by the FDA Gulf Coast Seafood Lab and will be submitted to the ISSC for consideration as an alternative to the mouse bioassay. LC-MS can provide confirmation of toxins detected by other assays, and sample throughput is higher compared to the mouse bioassay. However, the large number of brevetoxin metabolites in bivalves will necessitate a targeted approach. For routine analysis as a part of monitoring and management, it is not practical to attempt to identify and quantify them all. Nor is this even possible, given the lack of available standards for almost all metabolites. In the Gulf of Mexico, the most important commercial species are eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*). In oysters, the brevetoxin profile is dominated by the cysteine metabolites S-desoxy-BTX-B2 and BTX-B2[3,5,12]. These were also the major metabolites identified in hard clams, along

with BTX-B1, a taurine conjugate[14,15]. Sunray venus clams (*Macrocallista nimbosa*), a relatively new aquaculture product gaining popularity in Florida, have been less well-studied, but analyses thus far indicate that this species metabolizes brevetoxins similarly to hard clams (Fig. G2), with the cysteine and taurine conjugates representing the major metabolites (Fig. G3).

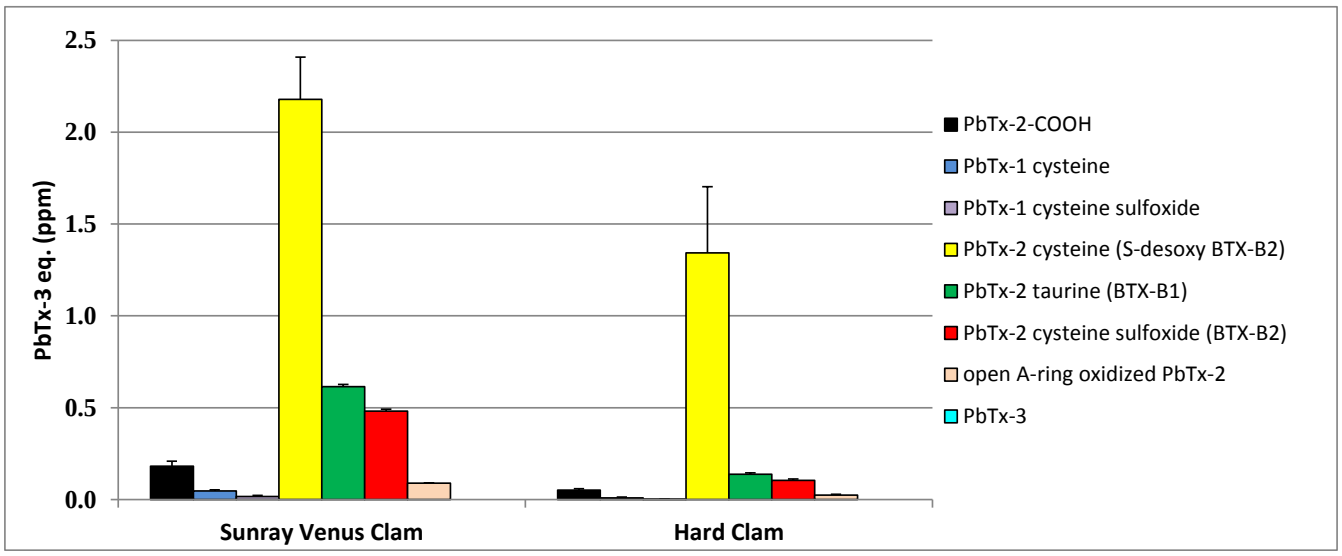


Figure G2. Brevetoxin metabolites identified by LC-MS in laboratory-exposed sunray venus and hard clams. (Error bars=standard deviation, n=3. Unpublished data provided by Dr. R. Pierce, Mote Marine Laboratory.)

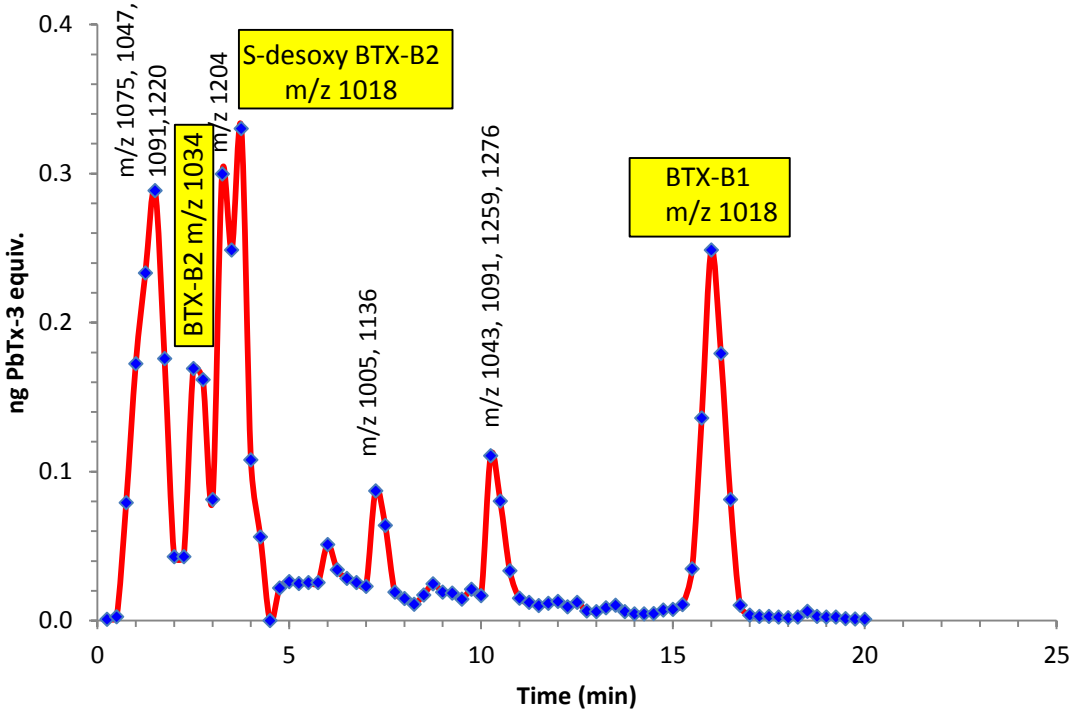


Figure G3. Chromatograms of brevetoxin metabolites in sunray venus clams based on ELISA of LC-fractionated shellfish extracts. (Unpublished data provided by Dr. A. Abraham, USFDA.)

Oral toxicity to mammals has not been assessed for any of the brevetoxin conjugates. Nevertheless, the cysteine and taurine metabolites were found to be excellent biomarkers of composite B-type brevetoxins as determined by ELISA for these species[12,14]. Based on these studies, the FDA's LC-MS protocol targets these three metabolites as biomarkers for NSP toxicity in oysters and clams.

LC-MS analyses require expensive instrumentation and highly technical expertise and are further limited by the time required for each sample to run. Where high throughput is required, the speed and cost-effectiveness of ELISA makes it a more attractive screening method.

MARBIONC Brevetoxin Competitive ELISA

The MARBIONC ELISA kit used in this method validation is the same kit that was used in the method comparisons and bivalve studies cited above. The method is based on the activity of anti-brevetoxin goat polyclonal antibodies, which were produced using a PbTx-3-KLH (keyhole limpet hemocyanin) conjugate[16]. The recognition epitope is believed to include the last four rings (excluding the side chain) of the brevetoxin B type toxins[17,18] (Fig. G4). This specific region is maintained in all brevetoxin B type toxins including in the secondary metabolites identified thus far. However, cross-reactivity of these antibodies have only been assessed for a few metabolites.

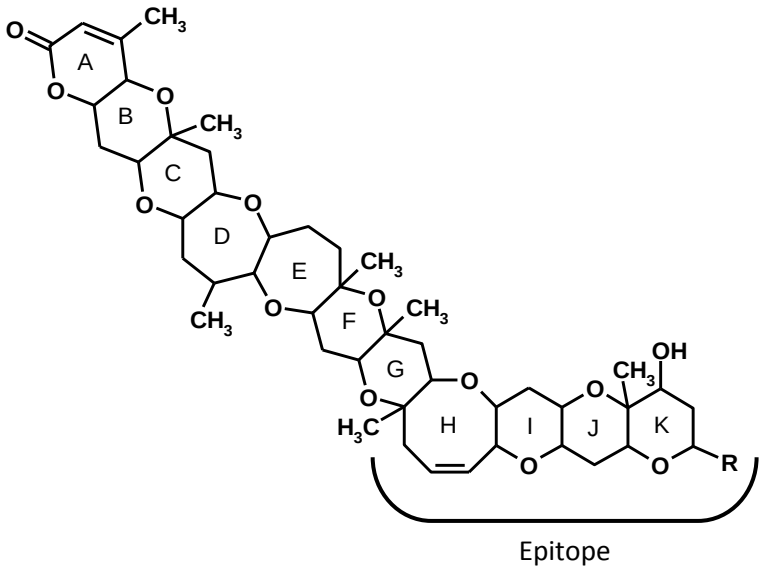


Figure G4. Brevetoxin B backbone with recognition epitope of anti-brevetoxin goat polyclonal antibodies

When this ELISA method was originally published, similar cross-reactivities were reported for PbTx-2, PbTx-3 and PbTx-9, which all share the B-type backbone[18] (Fig G5). MARBIONC reports cross-reactivities of 100% for PbTx-3, 97% for PbTx-2, 105% for oxidized-PbTx-2, and 7% for PbTx-1 at 10 ng/mL (Fig. G6).

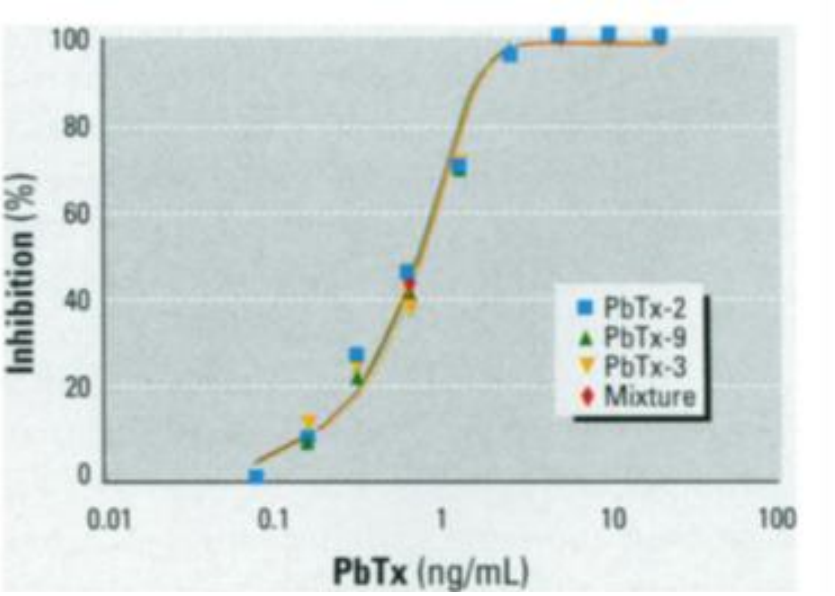


Figure G5. Figure taken from Naar et al.[18]. Anti-brevetoxin antibody cross-reaction with PbTx-2, PbTx-3, PbTx-9, and a mixture of the three toxins.

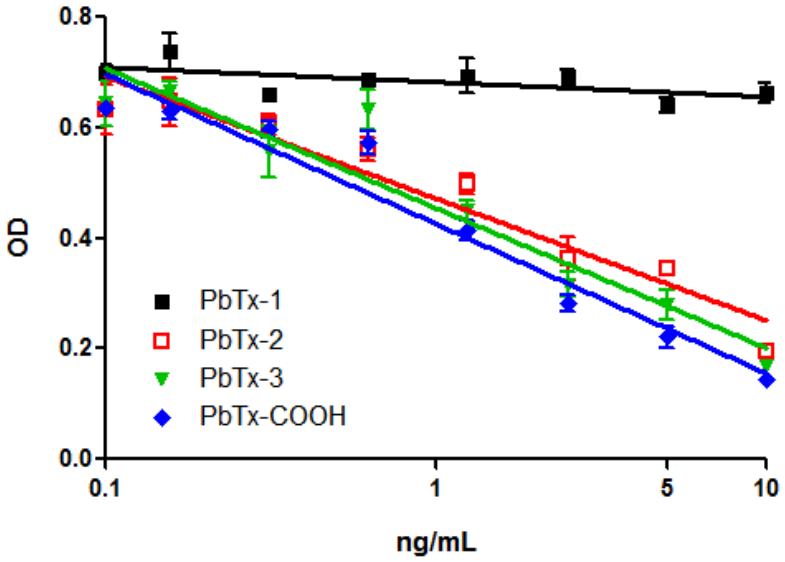


Figure G6. Figure provided by MARBIONC demonstrating degrees of anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2, PbTx-3, and oxidized-PbTx-2 (PbTx-COOH).

Competitive curves generated by L. Flewelling (FWC) are consistent with this, with calculated cross-reactivities (at 50% inhibition) of 97% for PbTx-2 and 2.4% for PbTx-1, relative to PbTx-3 (100%) (Fig. G7).

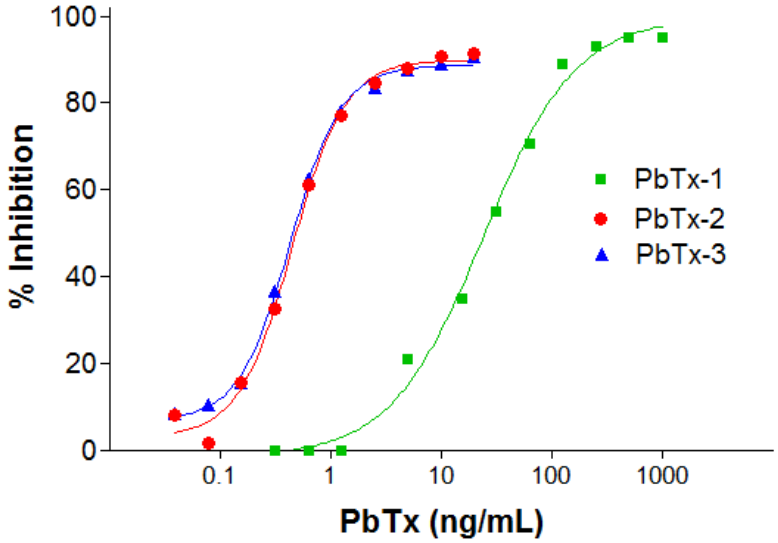


Figure G7. Anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2 and PbTx-3 (FWC data).

We also assessed the cross-reactivity of two shellfish metabolites (Fig. G8). The cross-reactivity of the cysteine conjugate S-desoxy BTX-B2 (provided by the FDA Gulf Coast Seafood Laboratory) was found to be 133% relative to PbTx-3. Cross reactivity of the brevetoxin lipid conjugate N-palmitoyl BTX-B2 (or BTX-B4, described in Bottein et al.[19] and provided by NOAA Center for Coastal Environmental Health and Biomolecular Research) was much lower (2.5%).

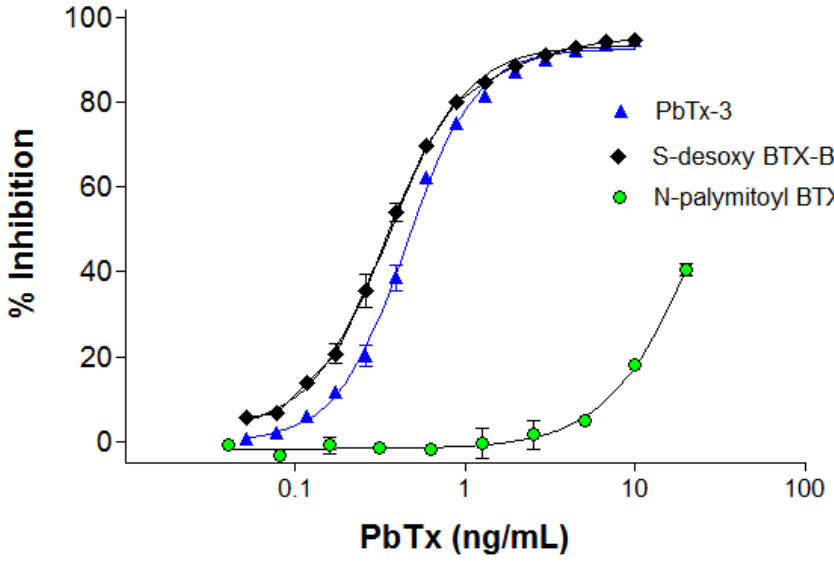


Figure G8. Anti-brevetoxin antibody cross-reaction with B-type brevetoxin metabolites S-desoxy BTX-B2 and N-palymitoyl BTX-B2 compared to PbTx-3 (FWC data).

The relatively low cross-reactivity of the antibodies with PbTx-1 (and presumably PbTx-1-derived conjugates) and with N-palmitoyl BTX-B2 indicates that ELISA results can underestimate of the total amount of brevetoxin and brevetoxin metabolites present in a sample. PbTx-1 is more potent than PbTx-2; however, the parent toxins PbTx-1 and -2 are not found in shellfish, and PbTx-2 type toxins consistently dominate the toxin profile in both *Karenia brevis* cells[20-22] and shellfish[22], typically accounting for 75% or more of the total toxins present. Additionally, although lipid conjugates of brevetoxin are thought to contribute substantially to NSP toxicity, these are derivatives of (and co-occur with) the more abundant amino acid metabolites that dominate the profile of toxic oysters and clams. The ELISA readily detects these forms, which have been identified as excellent biomarkers of NSP toxicity in oysters and clams. Therefore, the limited cross-reactivity of the ELISA with PbTx-1 and with N-palmitoyl BTX-B2 does not diminish the potential for the ELISA to perform successfully within a management program as proposed here.

In recent years, other brevetoxin ELISA kits have been introduced to the market, but prior to implementation into shellfish monitoring each kit would require individual evaluation of antibody cross-reactivity with dominant brevetoxin metabolites and comparisons with currently approved methods.

ELISA vs Mouse Bioassay

Currently, the only approved method for NSP testing is the APHA mouse bioassay[23]. The method is based on the bioassay developed by McFarren et al.[24] more than 50 years ago using toxic shellfish collected during an NSP outbreak in 1963. One mouse unit (MU) is the amount of crude lipid extract that will kill, on average, 50% of 20-g test mice in 15.5 hours. It is important to note that this method has never been validated, and the guidance limit used today (20 MU per 100g) is not based on any toxicological studies, but rather was described as the level of sensitivity of the test for 20g mice observed for 6 hours, which was deemed to be the longest reasonable observation time for the sake of accuracy and expediency. This guidance limit has proven to be effective, as no cases of NSP from legally harvested shellfish have been documented in Florida since the monitoring program began in the 1970's.

Comparing NSP mouse bioassay and ELISA data is not straightforward. The assays measure NSP toxins in very different ways. The mouse bioassay assesses toxicity by measuring the response of mice injected with a crude lipid extract of shellfish. This extract, prepared by repeated partitioning of acidified shellfish homogenate with diethyl ether, contains only a subset of the toxins present[7,25,26]. The method is semi-quantitative, yielding numerical results only at values ≥20 MU per 100g. Conversely, the ELISA is much more sensitive and yields continuous data to much lower concentrations, quantifying (relative to PbTx-3) a more comprehensive collection of brevetoxins and metabolites (regardless of potency) using antibodies that recognize a portion of the brevetoxin B-type backbone structure. Given that the NSP mouse bioassay measures only a subset of the toxins present, is semi-quantitative, has never been appropriately validated, and is not calibrated against known brevetoxin concentrations, a robust agreement of numerical results is unlikely to be achieved by any method.

At present, there is no validated brevetoxin equivalent of 'mouse units' in shellfish. Early work by Baden and Mende [27] established the toxicity of purified PbTx-2 and -3 dissolved in saline to mice intraperitoneally and calculated an LD50 (amount of toxin that kills half of the mice in 24 hours) of 0.2 mg/kg (similar for both toxins). This dose was used to derive a PbTx-2 "equivalent" of 4 µg per 20g-mouse and has since been extended to estimate the brevetoxin concentration in shellfish with a measured toxicity of 20 MU per 100g as 0.8 mg PbTx-2 equivalents per kg shellfish [13,16]. This number appears in several guidance documents; however**,** the extensive metabolism of brevetoxins in shellfish was unknown when the estimated equivalence was first proposed. We now know that shellfish exposed to *K. brevis* blooms contain a mixture of toxins with a multiplicity of potencies. In many cases the metabolites are less toxic, but in some cases they are more toxic. For these reasons, the use of this equivalent for brevetoxins in shellfish is inappropriate and has been acknowledged to be of little use for practical application[7].

Because a biomarker or indicator of toxicity approach is currently necessary for NSP, future NSP guidance limits may vary with the method used and may also vary across shellfish species. An appropriate non-mouse unit guidance limit for brevetoxins in shellfish will provide a level of protection for human health equal to that provided by the existing federal NSP guidance limit of 20 MU per 100 g shellfish. We know from existing data derived from naturally incurred eastern oysters and hard clams that such a limit *as measured using the MARBIONC ELISA with PbTx-3 as a standard* would exceed 0.8 mg per kg shellfish for these species.

Comparison of NSP Mouse Bioassay and ELISA results

Where quantitative results were obtained by both mouse bioassay and ELISA, Spearman rank correlation analysis was used to assess the correlation of brevetoxin concentrations measured by both methods for each shellfish matrix (Table G3). Significant correlations were observed in all cases.

Table G3. Spearman rank correlation coefficients (and p-values) for brevetoxin concentrations measured by NSP bioassay and ELISA

	Spearman rank correlation coefficient	p-value
oysters	0.5590	< 0.0001
hard clams	0.7866	< 0.0001
sunray venus clams	0.6859	< 0.0001

(From this portion on, changes to address early LMC comments are underway, and an updated Appendix G will be submitted.)

Given the differences between the assays and what they measure, strong agreement between numerical results was not expected. Nevertheless, the data were analyzed using linear regression analysis to estimate predicted concentrations by ELISA for samples testing at 20 MU per 100g (Fig. G9). Removal of the outlying (high) mouse bioassay results for oysters (>50MU) and clams (>100MU) that influenced the regression lines lowered the R-squared values, but slopes did not change appreciably. The 20 MU/100 g equivalent by ELISA was estimated to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams.

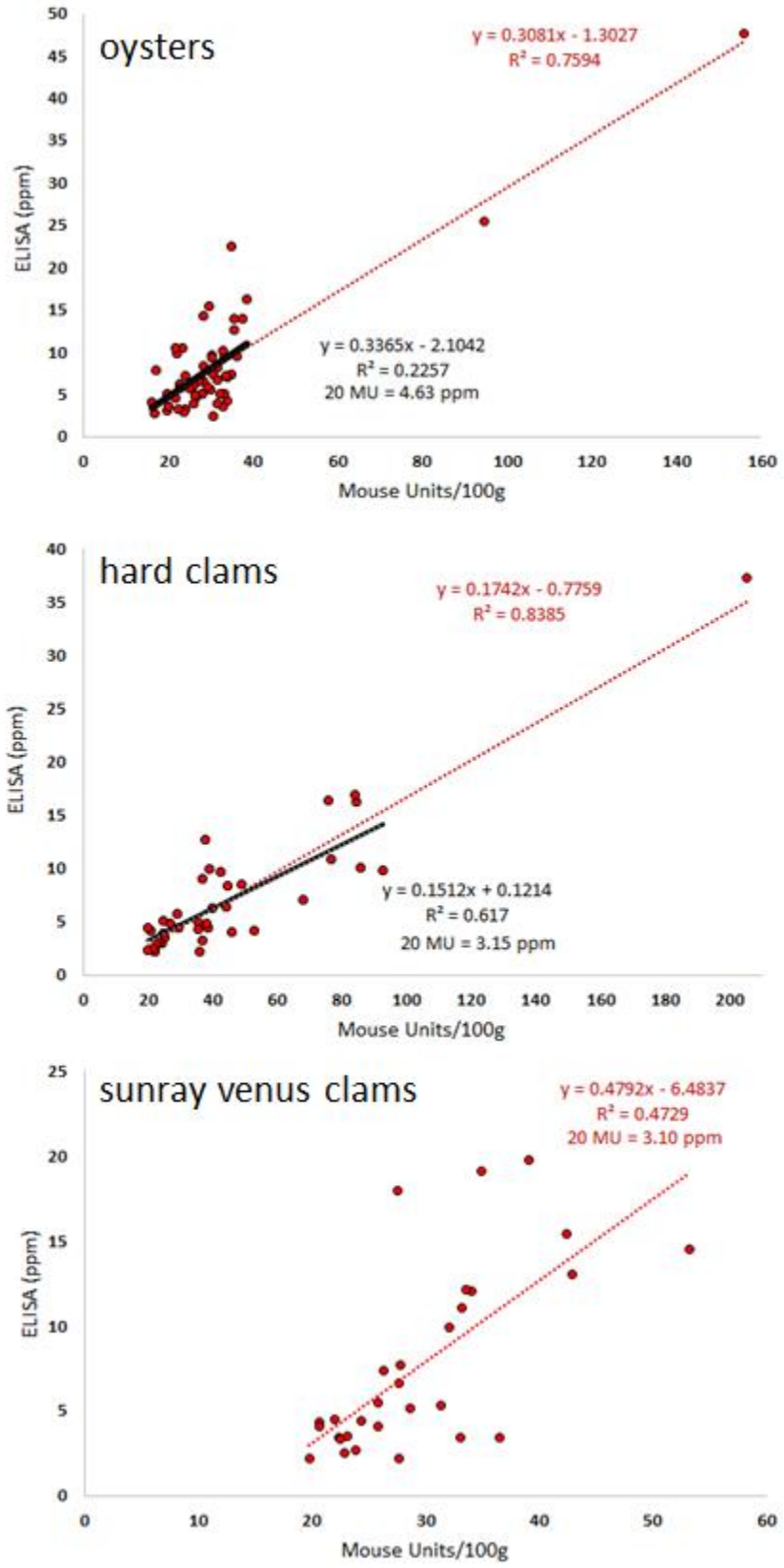


Figure G9. NSP mouse bioassay vs. ELISA results in oyster, hard clams, and sunray venus clams.

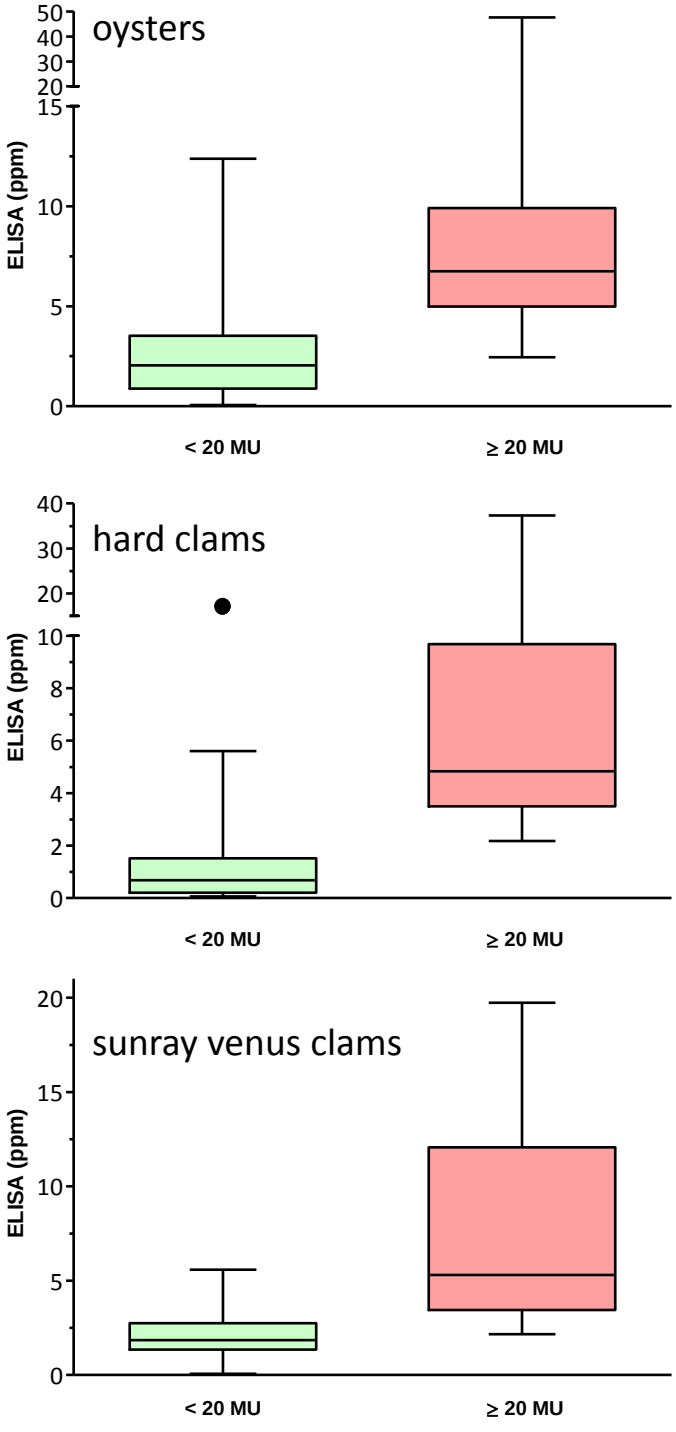


Figure G10. Boxplots (whiskers min to max) of ELISA results for samples testing < 20 MU/100g and ≥ 20 MU/100g in oysters, hard clams, and sunray venus clams. A value of 0.06ppm (half the limit of detection[LOD]) was substituted for ELISA results that were <LOD.

Boxplots were created to visualize the distribution of the data for samples testing < 20 MU/100g and ≥ 20 MU/100g (Fig. G10). There was a very wide range of concentrations measured by ELISA in samples testing < 20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay.

Brevetoxin metabolites are persistent in shellfish, and some level is frequently measured in bivalves from *K. brevis* endemic areas that have tested safe by mouse bioassay. In samples testing <20 MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. The highest concentrations were measured in wild oysters and hard clams, presumably due to repeated exposure to *K. brevis* and retention of the more persisitent metabolites across multiple bloom seasons. Farmed clams are brevetoxin-free when they are placed on lease sites, and their residence time in natural waters is short. These bivalves do not experience multiple successive bloom seasons. The maximum concentration measured in farmed clams that were < 20 MU was 4.6 ppm and in sunray venus clams was 5.6 ppm.

Importantly, across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.

As the only Approved Method, the NSP mouse bioassay is currently the only yardstick to which new methods can be compared. The mouse bioassay is semi-quantitative, not calibrated, and detects only that subset of compounds in shellfish that are ether-extractable. Analytical and screening NSP methods are unlikely to ever completely agree with mouse bioassay results, and expectations for

comparisons of proposed alternate methods with the mouse bioassay should be guaged accordingly, with a goal of achieving an equal measure of safety rather than perfect alignment of results and management actions on a sample by sample basis.

The results of our Single Lab Validation demonstrate that this assay generates specific, precise, and repeatable results. Additionally, ELISA results of naturally incurred shellfish compare very well with LC-MS analyses targeting the dominant metabolites found in eastern oysters and hard clams from the Gulf of Mexico (S-desoxy-BTX-B2, BTX-B2, and BTX-B1; Fig. G11).

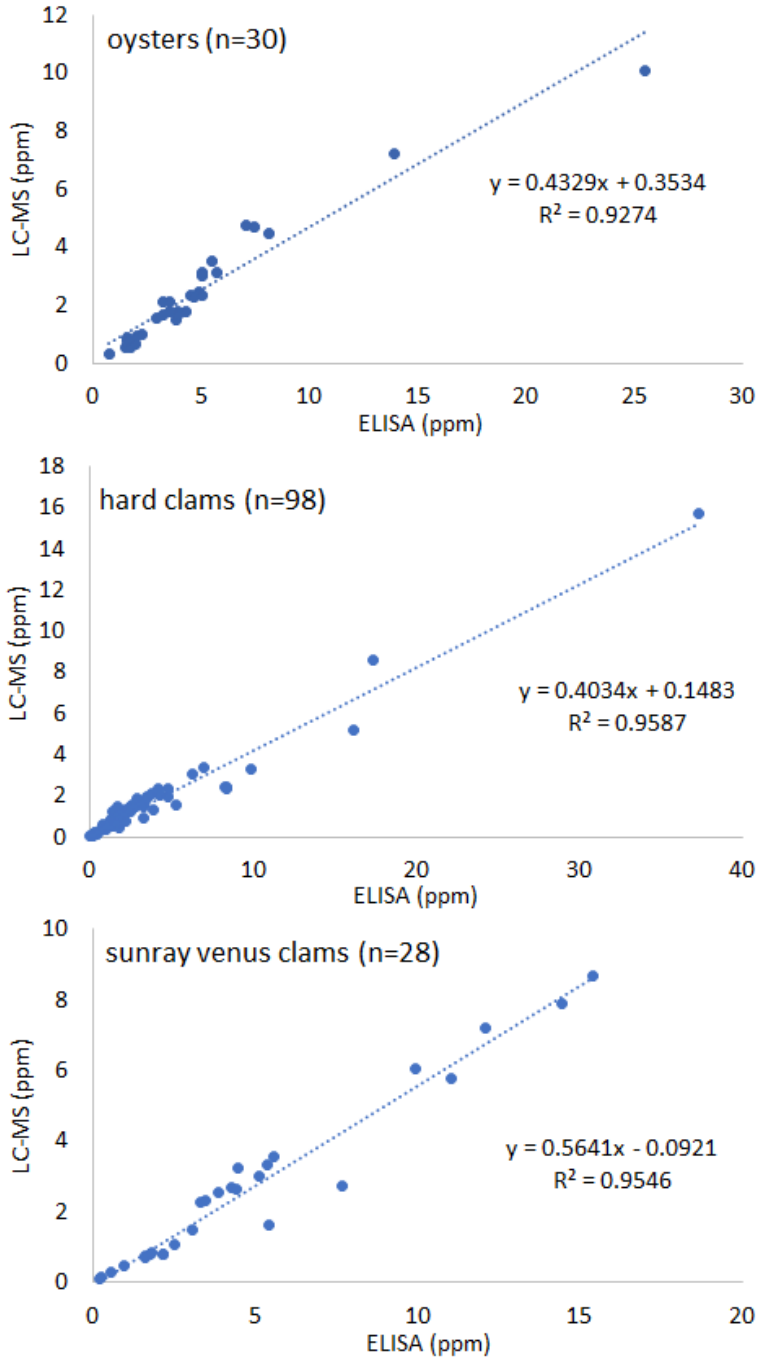


Figure G11. Comparison of NSP ELISA and LC-MS analysis of naturally incurred shellfish. LC-MS data generated and provided by A. Abraham, USFDA Gulf Coast Seafood Laboratory, using samples from this study.

Given the shortcomings and limitations of the mouse bioassay and the hardship this method imposes on both resource managers and industry, the move towards alternate methods must begin.

We propose that ELISA be approved for use as Limited Use Method such that samples would "pass" NSP rapid screening by ELISA when ELISA results are at or below a threshold representing no more than half of the level predicted in samples testing close to 20 MU/100g and below the lowest level measured in samples that have tested greater than or equal to 20 MU/100g (i.e., yielding no false negatives when applied to the existing dataset).

Thresholds of 1.8 ppm in oysters and 1.6 ppm in hard clams and sunray venus clams are proposed. The approach to derive the threshold was to approximate the ELISA equivalent of half of 20 MU/100 g and to ensure that the threshold would yield no false negatives. To protect against false negatives, the proposed thresholds are no more than 75% of the lowest concentration in the dataset that yielded a positive mouse bioassay. In hard clams and sunray venus clams, 1.6 ppm approximates half of the predicted 20 MU/100 g equivalent and is 75% of the lowest level measured in clams that failed mouse bioassay (2.18 ppm). For oysters, 1.80 is less than half of the estimated 20 MU/100 g equivalent and is 73% of the lowest level measured in oysters that failed mouse bioassay (2.45 ppm). These thresholds are not proposed as new guidance or actions limits for NSP, but rather as screening thresholds **specific to the MARBIONC ELISA (using PbTx-3 as a standard)** below which we have confidence that oysters and clams would yield <20 MU/100g and above which testing by mouse bioassay (or other future Approved Method) would be required.

Applying these thresholds to the comparative data set presented here would produce no false negatives (no samples testing greater than or equal to 20 MU/100 exceeded these levels by ELISA). Among the subset of samples testing < 20 MU/100g, ELISA results exceeded the thresholds (and would necessitate additional testing by NSP mouse bioassay) for 56% of oyster samples, 22% of hard clam samples, and 68% of sunray venus clam samples. The high proportion of <20 MU sunray venus clams above the threshold is an artifact of our sample set. Because sunray venus clams are relatively new to Florida aquaculture, our sample size is smaller, and collections during and following *K. brevis* blooms have been targeted in recent years to generate quantitative mouse bioassay data for comparisons.

As a first step away from total reliance on the NSP mouse bioassay, the proposed thresholds are conservative, and they may need to be revised in the future when more data and/or other approved methods are available, but they would have eliminated the need for 246 of the 501 bioassays (49%) conducted and represented in this data set. Having this method available as an approved option for NSP testing would greatly benefit all Gulf States. In 2015, a *K. brevis* affected the entire northern Gulf of Mexico, resulting in simultaneous closures of shellfish harvest areas in Florida, Alabama, Mississippi, and Louisiana. Because Alabama, Mississippi, and Louisiana experience these blooms infrequently, they lack the capacity to conduct NSP mouse bioassays. Therefore, sample testing to reopen harvest areas in these states after the bloom had dissipated was coordinated by our lab in Florida with the assistance of Resource Access International in Maine. While this cooperative effort was successful, it was a heavy burden on Florida, taking five weeks following bloom termination to complete and unnecessarily extending closures in these states. In every case, samples submitted by the other states passed by mouse bioassay (contained < 20 MU/100g), and if screening by ELISA had been an approved option, bioassays would not have been necessary in Mississippi or Louisiana, where NSP levels of oyster samples tested by ELISA ranged from 0.16 to 1.22 ppm.

References:

1. Baden DG, Bourdelais AJ, Jacocks H, Michelliza S, Naar J (2005) Natural and derivative brevetoxins: historical background, multiplicity, and effects. Environ Health Perspect 113: 621-625.
2. Baden DG (1989) Brevetoxins: unique polyether dinoflagellate toxins. FASEB J 3: 1807-1817.
3. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW (2004) Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44: 677-685.
4. Abraham A, Plakas SM, Wang Z, Jester ELE, El Said KR, Granade HR, Henry MS, Blum PC, Pierce RH, Dickey RW (2006) Characterization of polar brevetoxin derivatives isolated from Karenia brevis cultures and natural blooms. Toxicon 48: 104-115.
5. Wang Z, Plakas SM, Said KRE, Jester ELE, Granade HR, Dickey RW (2004) LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). Toxicon 43: 455-465.
6. Ishida H, Nozawa A, Nukaya H, Rhodes L, McNabb P, Holland PT, Tsuji K (2006) Brevetoxin metabolism in shellfish associated with neurotoxic shellfish poisoning. In: Njapeu H, Trujillo S, van Egmond HP, Park DL, editors. Mycotoxins and Phycotoxins: Advances in Determination, Toxicology, and Exposure Management. The Netherlands Wageningen Academic Publishers. pp. 297-307.
7. Plakas SM, Dickey RW (2010) Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. Toxicon 56: 137-149.
8. Poli MA, Musser SM, Dickey RW, Eilers PP, Hall S (2000) Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. Toxicon 38: 981-993.
9. Morohashi A, Satake M, Naoki H, Kaspar HF, Oshima Y, Yasumoto T (1999) Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat Toxins 7: 45-48.
10. Ishida H, Nozawa A, Totoribe K, Muramatsu N, Nukaya H, Tsuji K, Yamaguchi K, Yasumoto T, Kaspar H, Berkett N, Kosuge T (1995) Brevetoxn B_1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi*. Tetrahedron Lett 36: 725-728.
11. Leighfield TA, Muha N, Ramsdell JS (2014) Tissue distribution of amino acid- and lipid-brevetoxins after intravenous administration to C57BL/6 mice. Chem Res Toxicol 27: 1166-1175.
12. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R (2008) Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). Toxicon 52: 32-38.
13. Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK (2004) Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 300-302.
14. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH (2015) Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (*Mercenaria* sp.) exposed to *Karenia brevis* blooms. Toxicon 96: 82-88.
15. Abraham A, Wang Y, El Said KR, Plakas SM (2012) Characterization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. Toxicon 60: 1030-1040.
16. Trainer VL, Baden DG (1991) An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29: 1387-1394.
17. Melinek R, Rein KS, Schultz DR, Baden DG (1994) Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32: 883-890.

18. Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG (2002) A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110: 179-185.
19. Bottein MY, Fuquay JM, Munday R, Selwood AI, van Ginkel R, Miles CO, Loader JI, Wilkins AL, Ramsdell JS (2010) Bioassay methods for detection of N-palmitoylbrevetoxin-B2 (BTX-B4). Toxicon 55: 497-506.
20. Baden DG, Tomas CR (1988) Variations in major toxin composition for six clones of *Ptychodiscus brevis*. Toxicon 26: 961-963.
21. Corcoran AA, Richardson B, Flewelling LJ (2014) Effects of nutrient-limiting supply ratios on toxin content of *Karenia brevis* grown in continuous culture. Harmful Algae 39: 334-341.
22. Pierce RH, Henry MS (2008) Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. Ecotoxicology. pp. 623-631.
23. APHA (1970) Subcommittee on Laboratory Methods for the Examination of Shellfish. Method for the bioassay of *Gymnodinium breve* toxin(s) in shellfish. Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition. Washington, D.C.: American Public Health Association. pp. 61-66.
24. McFarren EF, Tanabe H, Silva FJ, Wilson WB, Campbell JE, Lewis KH (1965) The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3: 111-123.
25. Naar J, Kubanek J, Weidner AL, Flewelling LJ, Bourdelais A, Steidinger K, Baden DG (2004) Brevetoxin depuration in shellfish via production of non-toxic metabolites: consequences for seafood safety and the environmental fate of brevetoxins. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 488-490.
26. Dickey R, Jester E, Granade R, Mowdy D, Moncreiff C, Rebarchik D, Robl M, Musser S, Poli M (1999) Monitoring brevetoxins during a *Gymnodinium breve* red tide: Comparison of sodium channel specific cytoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. Nat Toxins 7: 157-165.
27. Baden DG, Mende TJ (1982) Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. Toxicon 20: 457-461.

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

Data for demonstrating the ruggedness of the new or modified method:
For this study, results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min).

1) Different lots of ELISA kit reagents:

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	Jun-14 lot	Jun-16 lot
oyster	OY01	0.4	0.38	0.36
oyster	OY02	0.4	0.38	0.36
oyster	OY03	1	0.92	0.90
oyster	OY04	1	0.88	0.88
oyster	OY05	2	1.87	1.88
oyster	OY06	2	1.84	2.04
oyster	OY07	4	3.78	3.66
oyster	OY08	4	3.88	4.54
oyster	OY09	8	7.67	7.86
oyster	OY10	8	8.05	7.73
hard clam	HC01	0.4	0.38	0.39
hard clam	HC02	0.4	0.39	0.38
hard clam	HC03	1	1.08	0.92
hard clam	HC04	1	0.97	0.97
hard clam	HC05	2	1.97	1.92

hard clam	HC06	2	2.16	1.85
hard clam	HC07	4	3.78	4.05
hard clam	HC08	4	3.90	3.83
hard clam	HC09	8	7.86	7.69
hard clam	HC10	8	7.79	8.16
sunray venus clam	SV01	0.4	0.35	0.33
sunray venus clam	SV02	0.4	0.39	0.38
sunray venus clam	SV03	1	1.03	0.89
sunray venus clam	SV04	1	1.05	1.00
sunray venus clam	SV05	2	2.05	1.89
sunray venus clam	SV06	2	1.97	1.95
sunray venus clam	SV07	4	3.62	4.23
sunray venus clam	SV08	4	3.82	4.22
sunray venus clam	SV09	8	7.57	7.38
sunray venus clam	SV10	8	8.34	7.85

2) Incubation of ELISA plates throughout the procedure at ambient laboratory temperature (21-22°C) vs. in a heated plate shaker (25°C):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	21-22°C	25°C
oyster	OY01	0.4	0.36	0.37
oyster	OY02	0.4	0.36	0.40
oyster	OY03	1	0.90	0.88
oyster	OY04	1	0.88	0.84
oyster	OY05	2	1.88	1.96
oyster	OY06	2	2.04	1.94
oyster	OY07	4	3.66	3.72
oyster	OY08	4	4.54	4.56
oyster	OY09	8	7.86	8.08
oyster	OY10	8	7.73	8.31
hard clam	HC01	0.4	0.39	0.39
hard clam	HC02	0.4	0.38	0.37
hard clam	HC03	1	0.92	0.91
hard clam	HC04	1	0.97	0.86
hard clam	HC05	2	1.92	2.07
hard clam	HC06	2	1.85	1.87
hard clam	HC07	4	4.05	4.06
hard clam	HC08	4	3.83	4.17
hard clam	HC09	8	7.69	7.96
hard clam	HC10	8	8.16	8.26
sunray venus clam	SV01	0.4	0.33	0.35
sunray venus clam	SV02	0.4	0.38	0.40
sunray venus clam	SV03	1	0.89	0.92
sunray venus clam	SV04	1	1.00	0.94
sunray venus clam	SV05	2	1.89	2.24
sunray venus clam	SV06	2	1.95	1.86

sunray venus clam	SV07	4	4.23	4.08
sunray venus clam	SV08	4	4.22	4.19
sunray venus clam	SV09	8	7.38	7.03
sunray venus clam	SV10	8	7.85	7.49

3) Duration of sample and primary antibody (reagent C) incubation (60 min vs. 90 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	60 min C	90 min C
oyster	OY01	0.4	0.37	0.38
oyster	OY02	0.4	0.39	0.38
oyster	OY03	1	0.92	0.92
oyster	OY04	1	0.92	0.88
oyster	OY05	2	1.61	1.87
oyster	OY06	2	1.62	1.84
oyster	OY07	4	3.28	3.78
oyster	OY08	4	3.51	3.88
oyster	OY09	8	7.94	7.67
oyster	OY10	8	7.99	8.05
hard clam	HC01	0.4	0.40	0.38
hard clam	HC02	0.4	0.39	0.39
hard clam	HC03	1	1.02	1.08
hard clam	HC04	1	1.07	0.97
hard clam	HC05	2	1.84	1.97
hard clam	HC06	2	1.97	2.16
hard clam	HC07	4	3.65	3.78
hard clam	HC08	4	3.40	3.90
hard clam	HC09	8	7.44	7.86
hard clam	HC10	8	7.89	7.79
sunray venus clam	SV01	0.4	0.37	0.35
sunray venus clam	SV02	0.4	0.40	0.39
sunray venus clam	SV03	1	0.94	1.03
sunray venus clam	SV04	1	0.95	1.05
sunray venus clam	SV05	2	2.11	2.05
sunray venus clam	SV06	2	2.07	1.97
sunray venus clam	SV07	4	3.89	3.62
sunray venus clam	SV08	4	3.73	3.82
sunray venus clam	SV09	8	7.84	7.57
sunray venus clam	SV10	8	7.89	8.34

4) Duration of TMB color development step (7 min vs 13 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	TMB 7 min	TMB 13 min
oyster	OY01	0.4	0.35	0.48
oyster	OY02	0.4	0.36	0.36
oyster	OY03	1	0.91	1.13
oyster	OY04	1	0.91	0.89
oyster	OY05	2	1.69	2.05
oyster	OY06	2	1.86	1.90
oyster	OY07	4	3.84	4.05
oyster	OY08	4	3.88	4.61
oyster	OY09	8	7.89	8.35
oyster	OY10	8	7.90	7.63
hard clam	HC01	0.4	0.34	0.42
hard clam	HC02	0.4	0.37	0.37
hard clam	HC03	1	0.95	1.13
hard clam	HC04	1	0.93	0.93
hard clam	HC05	2	1.78	2.22
hard clam	HC06	2	1.64	1.80
hard clam	HC07	4	3.74	4.45
hard clam	HC08	4	3.62	4.37
hard clam	HC09	8	7.52	7.48
hard clam	HC10	8	7.94	7.55
sunray venus clam	SV01	0.4	0.35	0.39
sunray venus clam	SV02	0.4	0.38	0.44
sunray venus clam	SV03	1	0.94	0.97
sunray venus clam	SV04	1	0.93	1.17
sunray venus clam	SV05	2	1.84	2.13
sunray venus clam	SV06	2	1.76	1.81
sunray venus clam	SV07	4	3.66	3.90
sunray venus clam	SV08	4	3.76	4.04
sunray venus clam	SV09	8	7.88	7.50
sunray venus clam	SV10	8	7.95	8.14

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
   - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

See tables on next page

Significant differences were observed with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities of 1.0 ± 30%.

Value for the test of symmetry of the distribution of batch/lot 1 data _____

Value for the test of symmetry of the distribution of batch/lot 2 data _____

Variance of batch/lot 1 data _____

Variance of batch/lot 2 data _____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples? Y/N

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	Jun14 lot	Jun16 lot	Jun14 lot	Jun16 lot			
oyster	-0.32402 (0.750)	-0.07704 (0.906)	0.2281	0.2350	1.030	0.302	no
hard clam	-0.10448 (0.916)	-0.26257 (0.856)	0.2366	0.2483	1.049	0.708	no
sunray venus clam	-0.27735 (0.804)	-0.17249 (0.852)	0.2327	0.2471	1.062	0.465	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	21-22°C	25°C	21-22°C	25°C			
oyster	-0.07704 (0.974)	-0.20833 (0.822)	0.2350	0.2465	1.049	0.741	no
hard clam	-0.26257 (0.740)	-0.18657 (0.874)	0.2483	0.2483	1.000	0.287	no
sunray venus clam	-0.17249 (0.820)	-0.37325 (0.764)	0.2471	0.2333	1.059	0.754	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	60 min C	90 min C	60 min C	90 min C			
oyster	-0.13316 (0.866)	-0.32402 (0.780)	0.2160	0.2281	1.056	0.219	no
hard clam	0.25186 (0.772)	-0.10448 (0.912)	0.2301	0.2366	1.028	0.099	no
sunray venus clam	-0.42338 (0.680)	-0.27735 (0.734)	0.2326	0.2327	1.000	0.982	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	TMB 7 min	TMB 13 min	TMB 7 min	TMB 13 min			
oyster	0.07922 (0.892)	-0.13022 (0.922)	0.2388	0.2297	1.040	0.014	yes
hard clam	-0.00274 (0.958)	-0.04028 (0.982)	0.2460	0.2302	1.069	0.030	yes
sunray venus clam	-0.03460 (0.990)	-0.06355 (0.940)	0.2387	0.2187	1.092	0.011	yes

*m-out-of-n bootstrap symmetry test by Miao, Gel, and Gastwirth (2006)

Miao, W., Y. R. Gel, and J. L. Gastwirth. "A New Test of Symmetry about an Unknown Median. Random Walk." *Sequential Analysis and Related Topics-A Festschrift in Honor of Yuan-Shih Chow. Eds.: Agnes Hsiung, Cun-Hui Zhang, and Zhiliang Ying, World Scientific Publisher, Singapore* (2006).

VALIDATION CRITERIA

Linear Range is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

Limit of Detection is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

Limit of Quantitation/Sensitivity is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. 10^a, 10^b…10^n) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed (10^a, 10^b…10^n).

Data:
Sample type
Working range/Range of interest: 0.4-8 ppm
Range in spiking levels used: 0.4 ppm, 1 ppm, 4 ppm, 8 ppm, 12 ppm
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

Response is the signal data (absorbance, fluorescence, Ct value), colonies, plaques, etc resulting from the analysis.

For shellfish samples repeat for each tissue type of interest.

DATA HANDLING

Linear Range
To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.

8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary: See below for explanation

Linear range of the method as implemented 0.12 ppm to 35.33 ppm
The limit of detection of the method as implemented 0.040 ppm
The limit of quantitation/sensitivity of the method as implemented 0.12 ppm

Data was generated as directed above (ten samples spiked to five levels, analyzed in triplicate plus one blank aliquot) for each matrix type examined, but this data could not be analyzed as described in the data handling portion of this SOP. (Although most of the data was not used to determine linearity and LOQ/LOD, it is provided at the end of this Appendix.)

This ELISA kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the maximum absorbance of the reference wells (Amax). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared and analyzed. Assay response is converted to concentration by comparison to a standard curve, and the final sample concentration is the product of the concentration measured in the assay and the dilution factor. As a result, similar responses (signal data) can be measured for very different sample concentrations.

The overall or dynamic linear range of this method results from a combination of: 1) the linear range of the assay standard curve, 2) the assay limit of quantitation, and 3) the range of sample dilutions on the plate.

1) Linear Range of Assay

To evaluate the linear range of the assay, 7-point PbTx-3 standard curves (ranging from 0.08 to 5.0 ng/mL) from 60 ELISA plates run in this validation were generated using the sigmoidal dose-response (variable slope), or four-parameter logistic, curve fitting equation in Prism 5 (GraphPad Software). The upper and lower plateaus of the curves were then applied to formulae derived by Sebaugh and McCray[1] to define the "bend points" of the standard curves, the beginning and end of the linear concentration--response region, expressed both in terms of % inhibition (1-A/Amax x 100) and concentration (Table E.1). The assays included data generated using two different kit lots: June 2014 (n=34) and June 2016 (n=26). We found that the position of the standard curves and the linear range defined by the bend points differed between the two kit lots (Fig. E1). Such shifts can be achieved with the same kit lot by altering dilutions of key reagents (A and C). Therefore, we believe that the differences we observed in kit lots were due to minor concentration variations in the supplied reagents A and/or C. However, comparative analyses of spiked samples were not significantly different between the two kit lots (see Appendix F: Ruggedness).

Table E1. Average bend points (± standard deviation), expressed as % inhibition and concentration, defining the linear range of standard curves generated using two lots of ELISA kit reagents.

	% inhibition		ng PbTx-3/mL	
	Jun-14 Lot	Jun-16 Lot	Jun-14 Lot	Jun-16 Lot
lower bend point	17.34 ± 2.47	16.76 ± 2.73	0.21 ± 0.04	0.30 ± 0.06
upper bend point	76.91 ± 2.07	74.19 ± 1.68	1.04 ± 0.14	1.38 ± 0.16

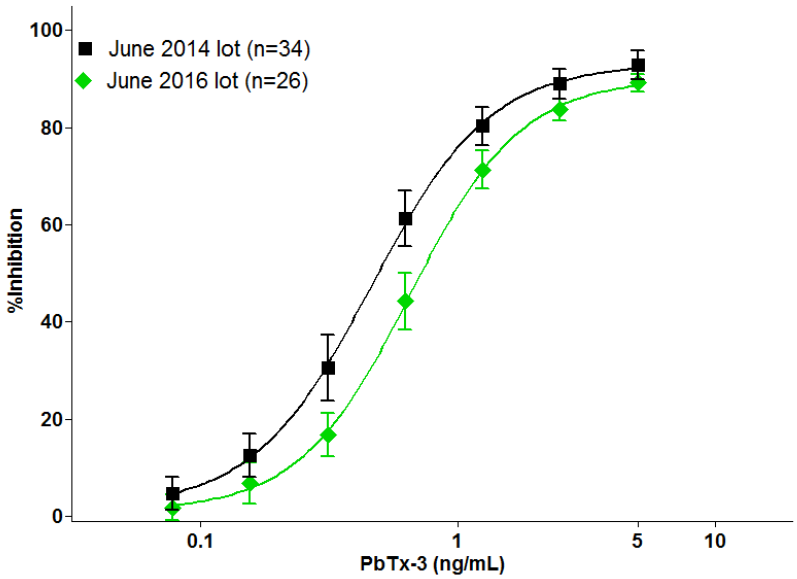


Figure E1. Average of multiple semi-log standard curves generated using two lots of ELISA kit reagents. Error bars represent standard deviation of independent curves prepared and assayed on different plates or days.

Using the June 2014 lot, two additional 14-point standard curves were assayed on different days to generate curves with more points that fell along the linear portion of the curve (Fig. E2). The bend points from these 14-point curves (16%-76%) were similar to those derived from the routine standard curves (Table E1).

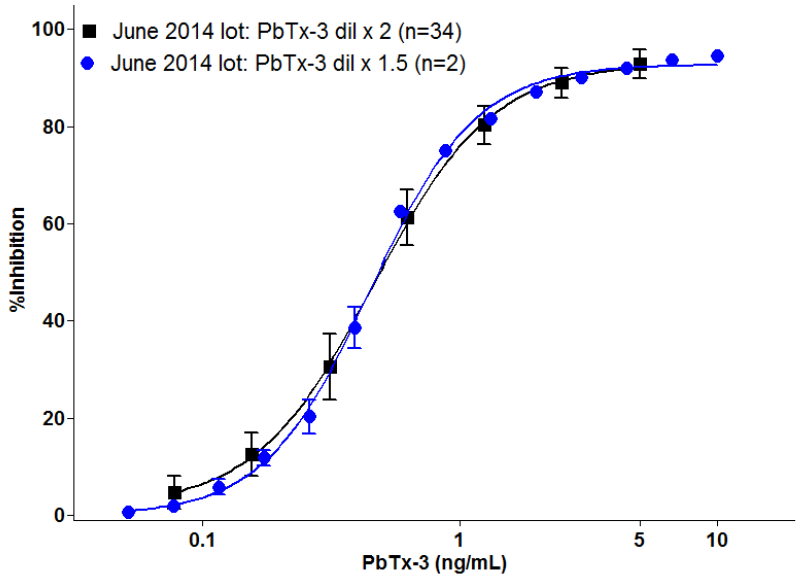


Figure E2. Comparison of 7-point and 14-point PbTx-3 standard curves. Error bars represent standard deviation of independent curves prepared and assays on different plates/days.

To verify linearity within the range defined by the bend points, multiple dilutions of shellfish samples spiked to 0.4 ppm with PbTx-3 were quantified. Ten samples were used for each matrix type, and three replicates per sample were extracted and analyzed. These assays were performed using the June 2014 kit lot. As written in the method protocol, the shellfish extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume). The extract is then diluted another 40 times in ELISA buffer for the initial dilution, and six additional dilutions are prepared by serial dilution by two, yielding a total of seven dilutions (from 1:400 to 1:25,600) for each sample.
At the 0.4 ppm spike level, the expected value of the first three dilutions are 1.0, 0.5, and 0.25 ng/mL, which are all within the linear range of the June 2014 kit lot as defined by the bend points. The expected and mean measured values of the three dilutions are listed in Table E2. Linear regression yielded r^2 values of 0.94-0.97 (Fig. E3).

Table E2. Expected concentrations and mean of concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Shellfish were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed.

	dilution	expected	mean measured	SD	%CV
oyster	400	1.00	0.935	0.079	8.4%
	800	0.50	0.471	0.038	8.1%
	1600	0.25	0.229	0.034	14.8%
hard clam	400	1.00	0.893	0.081	9.1%
	800	0.50	0.456	0.055	12.1%
	1600	0.25	0.233	0.033	14.2%
sunray venus clam	400	1.00	0.911	0.098	10.8%
	800	0.50	0.455	0.059	13.0%
	1600	0.25	0.234	0.030	12.8%

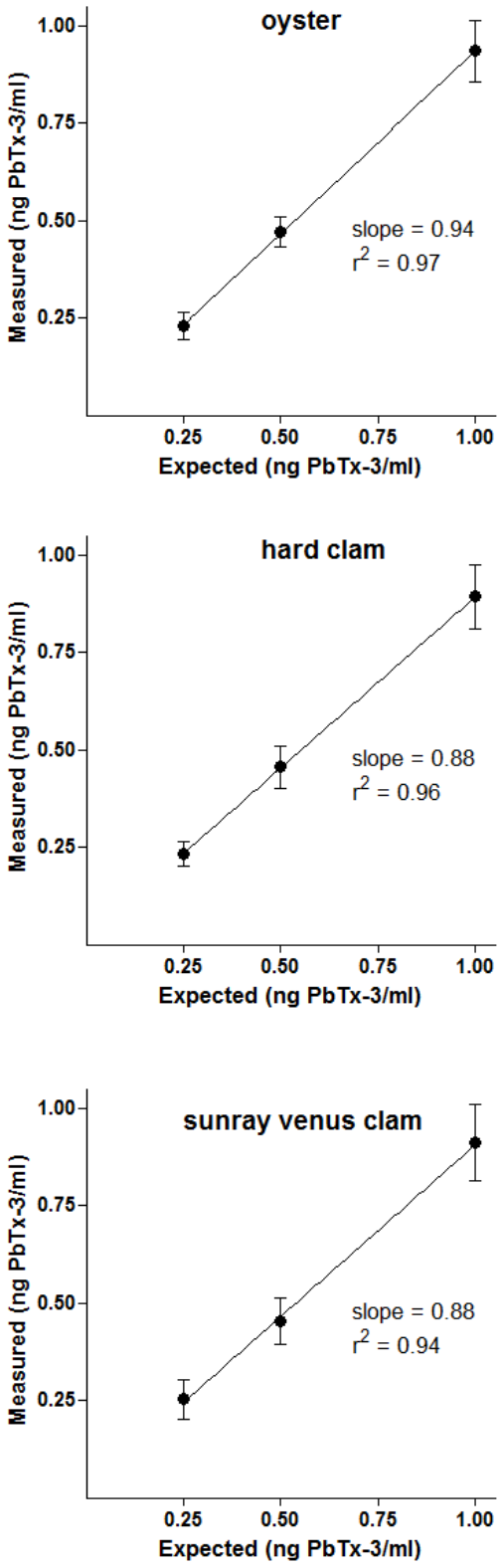


Figure E3. Expected concentrations vs. mean concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Oyster, hard clams, and sunray venus clams were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed. Error bars represent standard deviation.

Limit of Detection and Limit of Quantitation

Blank samples consistently yielded assay responses that were not quantifiable. Therefore, the standard deviation of results from the 1:1600 dilution of shellfish reported in Table E2 above were used to derive the limit of detection (LOD) and limit of quantitation (LOQ) expressed as 3x and 10x the standard deviation, respectively. This dilution consistently yielded a signal (% inhibition) closest to the lower bend point (17%) and was the lowest quantifiable dilution.

For all three matrix types, the standard deviation at the 1:1600 dilution was approximately 0.03. Calculated assay LOD and LOQ are 0.1 and 0.3 ng/mL, respectively. At the lowest sample dilution of 1:400, the LOD and LOQ for brevetoxin in shellfish are 40 and 120 ng/g or 0.04 and 0.12 ppm.

Dynamic linear range

The overall or dynamic linear range of this method is a combination of the linear range of the standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate (from 400 to 25,600). Using the LOQ calculated above, which falls with the linear portion of the standard curve identified for both lots of kits used in this study, and the upper bend points identified for each kit lot, the overall or dynamic linear range of the method is from 120 ng PbTx-3 eq./g or 0.12 ppm up to 26,624 or 26.62ppm for the June 2014 kit lot and up to 35,328 ng PbTx-3 eq. per g or 35.33 ppm for the June 2016 kit lot.

References:

1. Sebaugh JL, McCray PD (2003) Defining the linear portion of a sigmoid-shaped curve: bend points. Pharmaceutical Statistics 2: 167-174.

Results of spiking experiments: ten samples were spiked to five levels and analyzed in triplicate (plus one blank aliquot) for each matrix type examined.

Data Summary:

spike conc (ppm)	Average concentration (ppm) Average recovery (%) Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%
12	10.63	11.03	12.74
	89%	92%	106%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY01	12	10.60	10.97	10.73
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY02	12	10.77	10.26	10.54
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY03	12	10.83	11.13	11.11
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY04	12	10.90	10.08	9.94
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36

oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY05	12	10.17	10.38	9.68
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY06	12	11.62	10.71	11.36
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY07	12	10.68	11.42	10.75
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY08	12	10.68	11.00	10.46
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04
oyster	OY09	12	11.09	10.44	10.78
oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
oyster	OY10	12	9.58	9.75	10.34
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC01	12	9.89	9.80	10.75
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC02	12	11.14	11.33	11.63
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC03	12	10.26	11.20	10.25
hard clam	HC04	0	<LD		

hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC04	12	11.50	11.92	11.74
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC05	12	10.78	9.82	11.27
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC06	12	11.18	11.40	12.08
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC07	12	10.94	10.50	10.70
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC08	12	10.53	10.76	10.98
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40
hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC09	12	12.38	10.77	10.84
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
hard clam	HC10	12	10.99	11.31	12.19
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV01	12	11.61	12.13	11.52
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV02	12	12.57	11.57	12.71

sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV03	12	12.95	12.02	13.17
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV04	12	12.51	11.70	13.43
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV05	12	13.32	12.34	13.47
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV06	12	14.52	12.76	13.90
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV07	12	11.91	13.74	11.53
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70
sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV08	12	11.73	14.16	12.19
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV09	12	13.18	14.53	12.87
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94
sunray venus clam	SV10	12	12.62	12.50	12.98

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank , the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:

Potentially interfering substances examined in this study included two types of microalgae – the flagellate *Isochrysis* aff. *galbana* and the cryptophyte *Rhodomonas lens* – that are commonly fed to aquaculture-reared bivalves at the age/size at which they are ready to be relocated from the hatchery to the aquaculture zones. With the capacity to ingest as many as 10^9 cells per day, it is reasonable to predict there may be some bioaccumulation of cell constituents over time, and that they may still be present when the bivalves are harvested. Cells were added to a concentration of 100 million cells per g of shellfish.

Also examined was okadaic acid, a dinoflagellate toxin produced by some species of *Dinophysis* and *Prorocentrum*. These organisms are present in waters where *Karenia brevis* occurs, and potentially both toxins could be present. Both brevetoxin and okadaic acid are polyether toxins, so cross-reactivity with okadaic acid was investigated. Okadaic acid was added to a concentration of 1.5 µg per g of shellfish (or 1.5 ppm), which is roughly ten times above the current US guidance limit of 0.16 ppm.

The final substance to be examined was *Karenia mikimotoi*, a dinoflagellate that is closely related to *Karenia brevis*. *K. mikimotoi* produces bioactive compounds, but brevetoxin production has not been documented in this species. *Karenia* blooms are often mixed species blooms with two or more *Karenia* species present, although *K. brevis* is typically dominant. *K. mikimotoi* cells were added to a concentration of 500,000 cells per g.

Interfering organism/compound/toxin:

A *Isochrysis* aff. *galbana* (100 million cells per g shellfish)
B *Rhodomonas lens* (100 million cells per g shellfish)
C Okadaic acid (1.5 µg per g shellfish)
D *Karenia mikimotoi* (500,000 cells per g shellfish)

PbTx-3 spike concentration: 0.4 ppm

oyster

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.39	0.41	0.41	0.38	0.39	<LD
0.38	0.38	0.41	0.38	0.40	
0.42	0.39	0.39	0.37	0.43	
0.34	0.38	0.42	0.37	0.37	
0.39	0.44	0.40	0.35	0.42	

hard clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.36	0.40	0.40	0.38	0.40	<LD
0.38	0.38	0.39	0.40	0.32	
0.39	0.40	0.37	0.37	0.38	
0.35	0.36	0.38	0.37	0.33	
0.38	0.40	0.39	0.38	0.37	

sunray venus clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.33	0.41	0.42	0.41	0.35	<LD
0.35	0.39	0.38	0.40	0.41	
0.38	0.38	0.36	0.35	0.35	
0.35	0.37	0.34	0.39	0.40	
0.38	0.43	0.40	0.39	0.41	

Data handling for demonstrating specificity of the new or modified method

The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SI_{avg}) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Oyster

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.96	no	0.352
B	*Rhodomonas lens*	0.94	no	0.254
C	Okadaic acid	1.03	no	0.490
D	*Karenia mikimotoi*	0.95	no	0.061

Hard clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.97	no	0.164
B	*Rhodomonas lens*	0.97	no	0.230
C	Okadaic acid	0.98	no	0.374
D	*Karenia mikimotoi*	1.04	no	0.364

Sunray venus clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.91	no	0.055
B	*Rhodomonas lens*	0.95	no	0.311
C	Okadaic acid	0.93	no	0.205
D	*Karenia mikimotoi*	0.94	no	0.230

VALIDATION CRITERIA

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Recovery is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^3 and 10^5).

Data:
Working Range: 0.4 - 4 ppm
Sample Type: Oyster, Hard Clam, Sunray Venus Clam
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

matrix type	sample	spike level	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)
hard clam	HC01	blank	0	<LD	
hard clam	HC01	L	0.4	0.33	0.32
hard clam	HC01	M	1	0.98	0.93
hard clam	HC01	H	4	3.85	3.79
hard clam	HC02	blank	0	<LD	
hard clam	HC02	L	0.4	0.35	0.33
hard clam	HC02	M	1	0.92	0.89
hard clam	HC02	H	4	3.82	3.36
hard clam	HC03	blank	0	<LD	
hard clam	HC03	L	0.4	0.35	0.33
hard clam	HC03	M	1	0.91	0.91
hard clam	HC03	H	4	3.55	3.36
hard clam	HC04	blank	0	<LD	
hard clam	HC04	L	0.4	0.33	0.3
hard clam	HC04	M	1	0.91	0.91
hard clam	HC04	H	4	4.66	3.99
hard clam	HC05	blank	0	<LD	
hard clam	HC05	L	0.4	0.32	0.33
hard clam	HC05	M	1	0.92	0.89

hard clam	HC05	H	4	3.49	4.03
hard clam	HC06	blank	0	<LD	
hard clam	HC06	L	0.4	0.44	0.44
hard clam	HC06	M	1	0.84	0.92
hard clam	HC06	H	4	4.15	4.25
hard clam	HC07	blank	0	<LD	
hard clam	HC07	L	0.4	0.42	0.43
hard clam	HC07	M	1	1	1.01
hard clam	HC07	H	4	4.05	4.12
hard clam	HC08	blank	0	<LD	
hard clam	HC08	L	0.4	0.35	0.37
hard clam	HC08	M	1	0.92	1
hard clam	HC08	H	4	4.23	3.55
hard clam	HC09	blank	0	<LD	
hard clam	HC09	L	0.4	0.4	0.39
hard clam	HC09	M	1	0.93	0.91
hard clam	HC09	H	4	3.98	4.26
hard clam	HC10	blank	0	<LD	
hard clam	HC10	L	0.4	0.36	0.39
hard clam	HC10	M	1	0.97	0.98
hard clam	HC10	H	4	4.54	3.98
oyster	OY01	blank	0	<LD	
oyster	OY01	L	0.4	0.38	0.38
oyster	OY01	M	1	0.99	0.95
oyster	OY01	H	4	4.07	4.12
oyster	OY02	blank	0	<LD	
oyster	OY02	L	0.4	0.39	0.39
oyster	OY02	M	1	0.94	0.95
oyster	OY02	H	4	3.87	3.85
oyster	OY03	blank	0	<LD	
oyster	OY03	L	0.4	0.44	0.42
oyster	OY03	M	1	0.8	0.77
oyster	OY03	H	4	3.57	3.92
oyster	OY04	blank	0	<LD	
oyster	OY04	L	0.4	0.37	0.35
oyster	OY04	M	1	1	0.85
oyster	OY04	H	4	4.17	4.14
oyster	OY05	blank	0	<LD	
oyster	OY05	L	0.4	0.36	0.38
oyster	OY05	M	1	0.77	0.89
oyster	OY05	H	4	4.22	4.06
oyster	OY06	blank	0	<LD	
oyster	OY06	L	0.4	0.31	0.33
oyster	OY06	M	1	0.91	0.92
oyster	OY06	H	4	3.36	3.48
oyster	OY07	blank	0	<LD	

oyster	OY07	L	0.4	0.4	0.4
oyster	OY07	M	1	0.88	1.05
oyster	OY07	H	4	3.9	4.21
oyster	OY08	blank	0	<LD	
oyster	OY08	L	0.4	0.46	0.44
oyster	OY08	M	1	1.05	1.03
oyster	OY08	H	4	3.86	4.03
oyster	OY09	blank	0	<LD	
oyster	OY09	L	0.4	0.43	0.37
oyster	OY09	M	1	1.06	0.92
oyster	OY09	H	4	3.74	3.94
oyster	OY10	blank	0	<LD	
oyster	OY10	L	0.4	0.36	0.38
oyster	OY10	M	1	0.94	0.99
oyster	OY10	H	4	4.24	4.28
sunray venus clam	SV01	blank	0	<LD	
sunray venus clam	SV01	L	0.4	0.36	0.37
sunray venus clam	SV01	M	1	0.94	0.98
sunray venus clam	SV01	H	4	3.89	3.95
sunray venus clam	SV02	blank	0	<LD	
sunray venus clam	SV02	L	0.4	0.32	0.34
sunray venus clam	SV02	M	1	1	0.97
sunray venus clam	SV02	H	4	4.09	3.6
sunray venus clam	SV03	blank	0	<LD	
sunray venus clam	SV03	L	0.4	0.38	0.36
sunray venus clam	SV03	M	1	1	0.98
sunray venus clam	SV03	H	4	4.15	3.71
sunray venus clam	SV04	blank	0	<LD	
sunray venus clam	SV04	L	0.4	0.32	0.32
sunray venus clam	SV04	M	1	1.11	1.01
sunray venus clam	SV04	H	4	4.28	4.45
sunray venus clam	SV05	blank	0	<LD	
sunray venus clam	SV05	L	0.4	0.29	0.3
sunray venus clam	SV05	M	1	1.13	1.08
sunray venus clam	SV05	H	4	4.19	3.98
sunray venus clam	SV06	blank	0	<LD	
sunray venus clam	SV06	L	0.4	0.36	0.33
sunray venus clam	SV06	M	1	0.84	0.87
sunray venus clam	SV06	H	4	4.03	3.67
sunray venus clam	SV07	blank	0	<LD	
sunray venus clam	SV07	L	0.4	0.41	0.41
sunray venus clam	SV07	M	1	0.93	0.91
sunray venus clam	SV07	H	4	4.1	3.62
sunray venus clam	SV08	blank	0	<LD	
sunray venus clam	SV08	L	0.4	0.43	0.42
sunray venus clam	SV08	M	1	0.95	0.92

sunray venus clam	SV08	H	4	4.03	3.82
sunray venus clam	SV09	blank	0	<LD	
sunray venus clam	SV09	L	0.4	0.44	0.35
sunray venus clam	SV09	M	1	0.86	1.03
sunray venus clam	SV09	H	4	4.36	3.87
sunray venus clam	SV10	blank	0	<LD	
sunray venus clam	SV10	L	0.4	0.4	0.38
sunray venus clam	SV10	M	1	1.15	1
sunray venus clam	SV10	H	4	4.22	3.95

DATA HANDLING

Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		
Total	29		

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary: **Details Below**

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **N**
- At what concentrations is the one way analysis of variance significant? **NA**
- What is the overall percent recovery of the MPN based method under study? **Oysters 97.6%, Hard Clams 97.2%, Sunray Venus Clams 99.0 %**

Working Range of the assay

The overall working range of this ELISA assay is a combination of the linear range of the standard curve and the range of sample dilutions on the plate. This kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the Amax (see linearity). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared. The extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume), and the extract is diluted another 40X for the initial ELISA dilution (yielding a starting dilution factor of 400).

With data showing samples that tested at 20 MU were on the order of 3-4 ppm by ELISA, and anticipating a critical threshold value of approximately half of that, the “low” “medium” and “high” levels selected for this portion were 0.4, 1, and 4 ppm. Samples spiked to these levels were quantitated at dilution factors ranging from 1,600-12,800.

Data Handling Results

Precision

Nested ANOVA: Following this data handling procedure and using log transformed data, the variance ratio (F) at the 95% confidence interval for the variance components: samples/concentrations in samples is not significant. The specified variance ratio for the components: concentrations in samples /determinations within concentrations is significant for all matrices. However, even using the best possible *mock* data, the specified variance ratio is significant. Therefore this approach may not be appropriate for evaluating this data set.

Oysters	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.030086	9	0.003343	0.006402	1	0
concentrations in samples	10.443037	20	0.522152	995.3691	5.93E-37	99.80
determinations within concentrations	0.015737	30	0.000525			0.20
total	10.488860	59				100
Hard Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.036326	9	0.004036	0.007381	1	0
concentrations in samples	10.936302	20	0.546815	1038.552	3.14E-37	99.81
determinations within concentrations	0.015796	30	0.000527			0.19
total	10.988423	59				100
Sunray Venus Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.015388	9	0.001710	0.0031	1	0
concentrations in samples	11.028937	20	0.551447	799.5186	1.57E-35	99.75
determinations within concentrations	0.020692	30	0.000690			0.25
total	11.065016	59				100

Similarly, calculating the %CV for the whole data set as specified above may not be appropriate. Theoretically, if the recoveries were all perfectly 100%, the %CV of the full data set per species would be 89%. Within each spike concentration, %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams.

Oysters	n	%CV
all spike levels/reps	**60**	**90.40**
low (0.4ppm)	20	9.74
med (1ppm)	20	9.45
high (4ppm)	20	6.53
Hard Clams	n	%CV
all spike levels/reps	**60**	**91.53**
low (0.4ppm)	20	11.97
med (1ppm)	20	4.69
high (4ppm)	20	9.16
Sunray Venus Clams	n	%CV
all spike levels/reps	**60**	**90.21**
low (0.4ppm)	20	12.06
med (1ppm)	20	8.81
high (4ppm)	20	6.02

Recovery

Performing a one-way ANOVA as specified above yielded F test results for each matrix type that were not significant at the 95% confidence interval, suggesting that the recovery of the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method.

oysters	sum of squares	d.f	mean square	Fs	P
concentration	0.015062	2	0.007531	0.33193	0.72042
error	0.612573	27	0.022688		
total	0.627634	29			
hard clams	sum of squares	d.f	mean square	Fs	P
concentration	0.004995	2	0.002498	0.08340	0.92022
error	0.808525	27	0.029945		
total	0.813520	29			
sunray venus clams	sum of squares	d.f	mean square	Fs	P
concentration	0.005632	2	0.002816	0.24244	0.78640
error	0.313593	27	0.011615		
total	0.319224	29			

The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams.

Matrix	Avg Spike Conc (ppm)	Avg Rep Conc (ppm)	% Recovery
Oysters	1.8	1.76	97.62
Hard Clams	1.8	1.75	97.17
Sunray Venus Clams	1.8	1.78	98.99

VALIDATION CRITERIA

Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

Data:
Working Range: 0.4-8ppm
Sample Type: Hard Clam, Oyster, Sunray Venus Clam
Agar used to determine spike concentration _____
Organism used for spiking: PbTx-3

Data used for Accuracy are from 10 samples per matrix type, each spiked to 4 concentrations, extracted and analyzed in triplicate with blanks (for each matrix type, n = 120 plus blanks). The stock solution used for spiking was considered the reference and was used for the ELISA positive control/standard curve.

Data summary:

Average concentration (ppm)
Average recovery (%)

spike conc (ppm)	Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36
oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04

oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC04	0	<LD		
hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40

hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70

sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94

DATA HANDLING

Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.
Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

To determine the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is worked-up in the following manner.

1. Convert plate counts to logs.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average reference concentration of the analyte/measurand used to spike the samples; or, for microbiological methods calculate the average plate count of the data in logs. The average plate count represents the average reference concentration for the microbiological method.
4. Calculate the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
5. Divide the average concentration calculated from the spiked samples by the average reference concentration.
6. Multiply the quotient by 100. This provides an estimate in percent of the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations of importance to the intended application of the method.

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary:

Calculated % accuracy/trueness: Oysters: 96.27%
Hard Clams: 98.39%
Sunray Venus Clams: 95.12%

Calculated measurement uncertainty: Oysters: -0.0057 – 0.1137
Hard Clams: 0.0603 – 0.1898
Sunray Venus Clams: 0.0783 – 0.2487

MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish

Principle of Analysis

In this indirect competitive ELISA based on Naar et al. (2002), a 96-well ELISA plate is coated with protein-linked brevetoxin, and any remaining binding sites in the wells are blocked. Polyclonal goat anti-brevetoxin antibodies are then incubated with samples or standards in the plate wells. The antibodies will react with the brevetoxins in the samples or standards or will be immobilized on the plate. Antibodies that are not attached to the plate after incubation are washed out during subsequent rinses. Antibodies immobilized on the plate are detected through steps linking the antibodies to horse radish peroxidase (HRP)-linked secondary antibodies and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine [TMB]), which yields a blue color (Amax = 370 nm and 652 nm) that changes to yellow (Amax = 450 nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin that was present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For qualitative (+/-) screening, more samples can be run on one plate (up to 40).

Included in MARBIONC ELISA Kit (store in freezer):

- Reagent A BSA-linked PbTx-3
- Reagent C Goat anti-brevetoxin Ab
- Reagent D HRP-linked anti-goat secondary Ab
- Brevetoxin standard (PbTx-3, 10 μg)

Reagents required but not included (Brands and product numbers are for convenience. Unless otherwise noted, equivalents are acceptable)**:**

- Methanol (ACS grade or better)
- Reagent B: Superblock Blocking Buffer (Thermo Scientific 37545)
- Phosphate Buffered Saline, pH 7.4 (PBS, Sigma P-3813)
- Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 (PBS-Tween, Sigma P-3563)
- Gelatin (Sigma G-6144)
- 3,3'5,5'-Tetramethylbenzidine (TMB, Sigma T0440)
- Sulfuric acid stop solution (H_2SO_4, 0.5M)
- Nanopure water (or equivalent quality water)

Consumables needed:

- Disposable glass test tubes
- Disposable plastic dilution tubes (96-well cluster format)
- 15-ml and 50-ml graduated polypropylene centrifuge tubes
- Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (**substitution NOT recommended**)
- Microplate sealing film
- Assorted pipet tips
- Solution basins
- Aluminum foil

Equipment needed:

Balance capable of measuring to 0.1g
Number 10 sieve
Laboratory blender
Vortex mixer
Centrifuge capable of 3,000*xg*, with rotor for 15 mL centrifuge tubes
Microplate reader with filter for measurement at 450 nm
Multichannel pipettor (100-300 µl), individual pipettors (10-1000 µl)
Orbital microplate shaker
Refrigerator (4°C)/freezer (-20°C)

Pre-Assay Preparation

In advance: PbTx-3 for positive control. Each set of kit reagents (15-plate supply) comes with 10 µg of PbTx-3 for use as a positive control.
Stock solution (1 µg/ml): Dissolve in 10 ml of 100% methanol. Store at -20°C. (May be stored for up to 1 year.)
Working solution (100 ng/ml): From this stock, dilute 1 ml to 10 ml with 100% methanol. Store at -20°C. (May be used for several months.)

80% aqueous methanol. Add 800 ml of methanol to a 1L graduated cylinder and bring to 1L with Nanopure water (or equivalent quality water). Good for up to 1 year.

5% gelatin stock solution. Dissolve 5 g gelatin in 100 ml Nanopure water - stir on heated stir plate until clear. Portion into 15-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

SuperBlock - Dissolve 1 pouch in 200 ml Nanopure water. Portion 50-ml aliquots into 50-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

PBS, pH 7.4 1 L - Dissolve 1 pouch of PBS powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

PBS-Tween (0.05% Tween), pH 7.4 1L - Dissolve 1 pouch of PBS-Tween powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

Make fresh daily:
PGT (PBS, 0.05% Tween, 0.5% gelatin) - Immerse a tube of stock gelatin in warm water for a few minutes to liquefy. Pour 5 ml gelatin into a 50-ml centrifuge tube and fill to 50 ml with PBS-Tween. Make one tube per plate.

Shellfish Sample Preparation (follows requirements for the NSP mouse bioassay)

At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis.

The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are sucked from shell being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for brevetoxins. Samples must be processed within 24 hours of shucking.

Rapid Extraction of Shellfish for Brevetoxins

1. Weigh 1.0 g of homogenized shellfish into a 15-mL polypropylene centrifuge tube.
2. Add 9mL of 80% aqueous methanol, and cap tightly.
3. Vortex for 2 minutes at highest speed.
4. Centrifuge at a minimum of 3000xg for 10 minutes.
5. Pour off supernatant into clean, labeled graduated 15-mL centrifuge tube.
6. Bring the volume of the supernatant to 10mL with 80% methanol.
7. Vortex for 15 seconds to mix.
8. Transfer to a clean labeled glass vial and store at -20°C until assayed.

ELISA Protocol

****IMPORTANT NOTE**** Kit Reagents A, C, and D are diluted in a glycerol solution to prevent freezing. To avoid pipetting error due to viscosity, only place the very tip of the pipet into the vial to withdraw the desired amount. DO NOT PRE-RINSE THE TIP. Submerge the tip into the buffer when dispensing, and rinse the tip several times with buffer to ensure complete transfer.

Step 1 - Reagent A
Shake vial of Reagent A gently by hand. Dilute Reagent A. 1:300 (or as specified in kit instructions) in **PBS**. (For 1 plate, add 40 µl of A to 12 ml **PBS**; for 2 plates, add 80 µl A to 24 ml **PBS**).

Fill each well of a 96-well Maxisorp Immunoplates with 100 µl of diluted Reagent A. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature. After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl **PBS**. (**No Tween for this step**.)

Step 2 - Reagent B
Fill each well with 250 µl of Reagent B-Blocking Buffer. Cover with microplate sealing film, and incubate on plate shaker for 30 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 3 - Sample and positive control dilutions *(This step can be done while Step 1 and 2 are incubating.)*

Note: Sample extracts and PbTx-3 working solution should be brought to room temperature before diluting.

Arrange dilution tubes in a rack according to plate layout - see below. Eight (8) tubes are needed for each sample or positive control.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Pos Ctrl (PbTx-3)
A	tube A	tube A	tube A	tube A	tube A	tube A
B	tube B	tube B	tube B	tube B	tube B	tube B
C	tube C	tube C	tube C	tube C	tube C	tube C
D	tube D	tube D	tube D	tube D	tube D	tube D
E	tube E	tube E	tube E	tube E	tube E	tube E
F	tube F	tube F	tube F	tube F	tube F	tube F
G	tube G	tube G	tube G	tube G	tube G	tube G
H	tube H	tube H	tube H	tube H	tube H	tube H

Leave dilution tubes in row **A** empty. To all other tubes in rows **B-H** (for both samples and Pos Ctrl) add 250 µl of PGT. For each sample, add 975µl of PGT to a small glass test tube. Add 25 µl of sample extract to the tube, and vortex briefly to mix. Transfer 250 µl of this diluted extract into dilution tube **A**. Withdraw another 250 µl from the glass tube, place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.** Do this for each sample.

Positive Control (PbTx-3)
To make the positive control, add 950µl of PGT to a small glass test tube. Add 50 µl of brevetoxin working solution (at 100 ng PbTx-3/ml) to the tube (50 µl PbTx-3 + 950 ul PGT= 5 ng PbTx-3/ml). (This is sufficient for up to two plates.) For each plate, transfer 250 µl of diluted PbTx-3 into dilution tube **A**. Withdraw another 250 µl from the glass tube and place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.**
(Tube H are PGT only and will serve as Reference Wells for maximum absorbance in the absence of brevetoxin.)

Step 4 - Transfer Samples On to Plate

After the plate has been blocked and washed (after Step 2 is complete), use a multichannel pipette to transfer the diluted samples and standards to the plate.

Fill wells of the microplate with 100 µl of each tube **in duplicate** (side by side wells), according to the figure below.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Pos. Ctrl.	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	PbTx-3	5 ng/ml
B	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	PbTx-3	2.5 ng/nl
C	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	PbTx-3	1.25 ng/ml
D	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	PbTx-3	0.625 ng/ml
E	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	PbTx-3	0.31 mg/ml
F	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	PbTx-3	0.156 ng/ml
G	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	PbTx-3	0.078 ng/ml
H	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT

Step 5 - Reagent C

Dilute Reagent **C** 1:300 (or as specified in kit instructions)

(For 1 plate, add 40 µl of A to 12 ml PGT; for 2 plates, add 80 µl A to 24 ml PGT)

To each well add 100 µl of diluted Reagent C. Cover with microplate sealing film, and shake the plate on the plate shaker for 90 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 6 - Reagent D

Dilute Reagent **D** 1:800 (or as specified in kit instructions)

(For 1 plate, add 15 µl of D to 12 ml PGT; for 2 plates, add 30 µl D to 24 ml PGT.)

Fill each well with 100 µl of diluted Reagent D. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature.

(When you get to this step – aliquot 12 ml of TMB per plate into a 15 or 50-ml centrifuge tube and warm to room temperature. Keep the tube in the dark (do not expose to light).

After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl PBS-Tween. **Then rinse each well one time with 300 µl PBS to ensure no Tween remains on the plate.**

Step 7 - TMB

Fill each well with 100 µl of TMB. Cover the plate with a piece of aluminum foil and incubate for 5-7 minutes. Stop the reaction by adding 100 µl of 0.5M H_2SO_4 to each well. The blue color in the wells should turn yellow. Read the plate at 450 nm.

Note: The stop time may vary with kit reagent lots and bottles of TMB. The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities (at 450 nm) of 1.0 ± 30%.

Calculations

Presence of brevetoxin in the sample will prevent color development in the well. Toxin can be quantified by converting absorbance values to % color inhibition and comparing to the positive control.

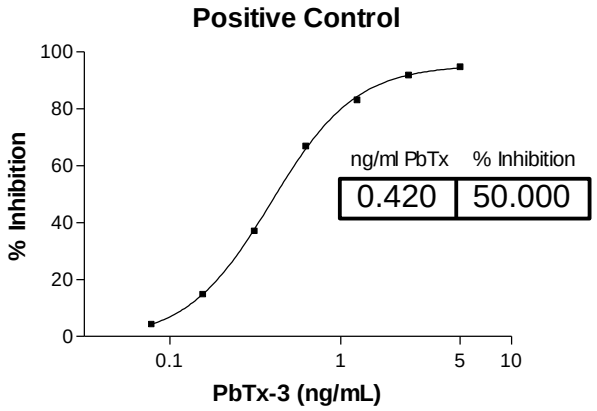
1. Average the values of the duplicate wells for each dilution, and determine the % color inhibition using the following equation:

   % inhibition = [1 - (Avg of dups/Amax)] x 100%

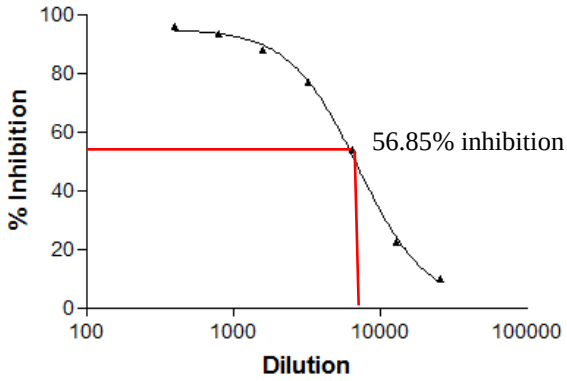
   where Amax is the average absorbance of the reference wells (PGT only) oriented below the sample or standard dilutions.

2. Using the 4-parameter logistic (4PL) curve in a curve-fitting program like Prism or SigmaPlot, fit a curve to the positive control with ng toxin/ml on the x-axis (log scale), and % inhibition on the y-axis (linear scale).
3. Determine the concentration for sample dilutions falling within the linear portion of the standard curve.
4. Multiply the concentration by the sample dilution and divide by 1000 to obtain PbTx-3 eq. results in ppm.

Example Standard Curve (50% inhibition = 0.42 ng PbTx-3/ml)
The control curve should be steep. On the linear part of the curve, the space between the dilutions (on the y-axis) is large. There should be clear plateaus at the top and bottom of the curve.



Example Sample Serial Dilution
Sample curves plotted with dilution on the x-axis (log scale), and % inhibition on the y-axis (linear scale) should have the same features. There should be a clear plateau either at the top or the bottom (or both). Shallow curves with no plateaus or linear curves with little space between points indicate interference in the assay, and results should be discarded.



For a sample with % inhibition of 56.85% at dilution of 1:6,400, the interpolated concentration = 0.495 ng/mL

[PbTx-3 eq] = 0.495 ng/ml x 6400 = 3168 ng/ml or 3.17 ppm

Quality Control Criteria

Acceptance of **assay results** is dependent on meeting the following criteria:

- Absorbance of reference wells must be (Amax) ≥ 0.6. (Optimal absorbance is 1.0 ± 30%.)
- %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%.

If either criteria are not met, re-run the ELISA plate.

Acceptance of **sample results** is dependent on meeting the following criteria:

- %CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (within the linear range of the assay; 20-70% inhibition) must be <20%.
- %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%. (A 20% or greater disparity between the calculated concentrations of two different dilutions of the same sample indicates assay interference or dilution error.)

If either criteria are not met, re-run the sample.

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Dockside Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest end product testing Sample Type: Shellfish
Abraxis Shipboard ELISA[3]		X			X		
JRT[2]		X		X	X	X	
HPLC[1]	X			X		X	
Reveal 2.0 ASP[4]	X			X	X	X	
RBA[5]		X		X	X	X	
MARBIONC Brevetoxin ELISA[6]			X	X		X	X

Footnotes:

[1]M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Biosciences, Technical Report #64, National Research Council Canada #33001. This method may also be used direct without cleanup.

[2]Jellett Rapid Test for PSP, Jellett Rapid Testing Ltd.

a. Method can be used to determine when to perform a mouse bioassay in a previously closed area.
b. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.
c. A positive result shall be used for a precautionary closure.

[3]Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of Actions, Proposal 05-111 (page 15) and 09-107 (page 140).

[4]Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 2013 Summary of Actions Proposal 13-112.

[5]Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114

[6]MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:

a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish
Name of the Method Developer	The ELISA Kit was developed by UNCW and is sold through MARBIONC. The method was optimized and submitted for use with molluscan shellfish by Leanne Flewelling, Florida Fish and Wildlife Conservation Commission.
Developer Contact Information	Florida Fish and Wildlife Conservation Commission 100 8th Avenue SE St. Petersburg, FL 33701 (727) 502-4891 leanne.flewelling@myfwc.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Blooms of the dinoflagellate *Karenia brevis* threaten the productive Gulf of Mexico shellfish industry. Brevetoxins produced by *K. brevis* are toxic to humans and can result in Neurotoxic Shellfish Poisoning (NSP) if contaminated shellfish are eaten. To prevent NSP, shellfish harvesting areas (SHAs) are closed when *K. brevis* concentrations exceed 5,000 cells/L and are re-opened once *K. brevis* levels decrease and testing demonstrates that shellfish are no longer toxic. This biotoxin plan successfully prevents occurrences of NSP from lawfully harvested shellfish, but NSP closures come at a steep economic cost to the shellfish industry. The APHA mouse bioassay - the only NSSP approved method for regulatory NSP testing - has many drawbacks. The delays caused by the time required to analyze samples (two full days) and very low sample throughput delay re-openings and add to economic losses. The assay is nonspecific, imprecise, and not calibrated against known levels of brevetoxins. It is costly in terms of labor and supplies, and the use of live animals is both undesirable and increasingly unacceptable. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed. Among the many chemical and biological methods developed for brevetoxin detection, enzyme-linked immunosorbent assays (ELISAs) have performed well. The method proposed here was the first commercially-available brevetoxin ELISA to be offered. The assay uses goat anti-brevetoxin antibodies developed by Trainer and Baden (1991) and is based on the indirect competitive assay developed in 2002 by Naar et al. (2002). The kit is marketed by MARBIONC Development Group (MDG), which is based at the University of North

		Carolina at Wilmington. This assay is widely and routinely used to monitor brevetoxins in Florida's marine systems and to diagnose human, marine mammal, and other animal exposure to brevetoxins. This method is much faster than the mouse bioassay, more user-friendly, more sensitive, more specific to brevetoxins, less expensive, and does not involve the use of live animals.
2. What is the intended purpose of the method?		The proposed use for the MARBIONC ELISA is as a Limited Use Method for determination of NSP toxin levels in hard clams, sunray venus clams, and oysters. Applications include Growing Area Survey & Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing as permitted within a State Authority's marine biotoxin contingency program. We propose that the ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters, at or below the estimated equivalent to one-half the 20 MU/100 g guidance level) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method (currently, the NSP mouse bioassay). Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay including: Growing Area Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing. Samples failing by ELISA would either require additional testing by NSP mouse bioassay or could support the same management actions as samples failing by NSP mouse bioassay. ELISA could also be used as a screening method to initiate precautionary closures.
3. Is there an acknowledged need for this method in the NSSP?		Yes, the ISSC Laboratory Committee has specified the need for qualitative or semi-quantitative (screening) and quantitative/confirmatory methods of analysis for all toxins and for each commercially-harvested bivalve species.
4. What type of method? i.e. chemical, molecular, culture, etc.		ELISA is a biological method that uses biological components (antibodies) to detect toxins. Detection relies on structural recognition of a region of the toxin molecule shared by PbTx-2-type brevetoxins (the most abundant forms) and provides an overall estimate of toxin content.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish.
Method Scope		This ELISA is a high-throughput, sensitive, accurate, quantitative assay for NSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Limited Use Method for the purposes of screening for NSP toxins in hard clams, sunray venus clams, and oysters.
References		Original method reference: Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG. 2002. A competitive ELISA to detect brevetoxins from

		Karenia brevis (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110(2):179-185. Antibody development reference: Trainer VL, Baden DG. 1991. An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29(11):1387-1394. Epitope identification reference: Melinek R, Rein KS, Schultz DR, Baden DG. 1994. Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32(8):883-90. Other relevant publications: Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK. 2004. Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. p. 300-302. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44:677-685. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (Crassostrea virginica). Toxicon 52(1):32-8. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH. 2015. Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (Mercenaria sp.) exposed to *Karenia brevis* blooms. Toxicon 96:82-88.
Principle		In this indirect competitive ELISA based on Naar et al. (2002), a 96-well ELISA plate is coated with protein-linked brevetoxin, and any remaining binding sites in the wells are blocked. Goat anti-brevetoxin antibodies are then incubated with samples or standards in the plate wells. The antibodies will react with the brevetoxins in the samples or standards or will be immobilized on the plate. Antibodies that are not attached to the plate after incubation are washed out during subsequent rinses. Antibodies immobilized on the plate are detected through steps linking the antibodies to horse radish peroxidase (HRP)-linked secondary antibodies, and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine), which yields a blue color that changes to yellow (Amax = 450nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For quick screening, more samples can be run on one plate

		(up to 40).
Any Proprietary Aspects		Methods of production of key kit reagents (brevetoxin-BSA conjugate and anti-brevetoxin antibodies) are proprietary (MDG).
Equipment Required		Equipment required: Balance capable of measuring to 0.1g Number 10 sieve Laboratory blender Vortex mixer Centrifuge capable of 3,000x*g,* with rotor for 15 mL Microplate reader with filter for measurement at 450 nm Multichannel pipettor (50-200 µL) Individual pipettors (10-1000 µL) Orbital microplate shaker Refrigerator/freezer Consumables required: Disposable glass test tubes Disposable plastic dilution tubes (96-well cluster format) 15-ml and 50-ml polypropylene centrifuge tubes Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (- substitution NOT recommended) Microplate sealing film Assorted pipet tips Solution basins Aluminum foil
Reagents Required		Included in MARBIONC ELISA Kit: • Reagent A: BSA-linked PbTx-3 • Reagent C: Goat anti-brevetoxin Ab • Reagent D: HRP-linked anti-goat secondary Ab • Brevetoxin standard (PbTx-3) Reagents required but not included: • Methanol • Reagent B: Superblock Blocking Buffer • Phosphate Buffered Saline, pH 7.4 • Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 • Gelatin • 3,3'5,5'-Tetramethylbenzidine (TMB) • Sulfuric acid stop solution (H_2SO_4, 0.5M) • Nanopure water (or equivalent quality water)
Sample Collection, Preservation and Storage Requirements		At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis. The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are sucked from shell being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for brevetoxins (see protocol in Appendix A). Samples must be processed within 24 hours of shucking.
Safety Requirements		General chemical safety requirements (e.g., personal protective equipment including gloves, safety glasses,

		and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure		See protocol detailed in Appendix A.
Quality Control Steps Specific for this Method		Acceptance of assay results is dependent on meeting the following criteria: Absorbance of reference wells (Amax) must be ≥ 0.6. %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%. Acceptance of sample results is dependent on meeting the following criteria: %CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (within the linear range of the assay; 20-70% inhibition) must be <20%. %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%.
C. Validation Criteria		
1. Accuracy / Trueness		Accuracy /trueness was determined by calculating the closeness of agreement between the test results and targeted value. Calculated % accuracy/trueness: Oysters: 96.27% Hard Clams: 98.39% Sunray Venus Clams: 95.12% Data and details in Appendix B
2. Measurement Uncertainty		Two-sided, 95% confidence intervals for the difference in concentrations between the reference and the spiked samples: Oysters: -0.0057 – 0.1137 Hard Clams: 0.0603 – 0.1898 Sunray Venus Clams: 0.0783 – 0.2487 Data and details in Appendix B
3. Precision Characteristics (repeatability and reproducibility)		Repeatability was assessed using duplicate determinations of 10 samples spiked with PbTx-3 to three levels (0.4, 1, and 4 ppm). %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams. Data and details in Appendix C
4. Recovery		The recovery of the method was consistent over the range of concentrations examined to determine Precision. The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams. Data and details in Appendix C
5. Specificity		Potentially interfering substances examined in this study included three types of microalgae (two types commonly used as food for hatchery raised bivalves and a non-brevetoxin producing *Karenia* species) as well as okadaic acid (a potentially co-occurring polyether dinoflagellate toxin). Two-sided t-tests indicated no significant difference in brevetoxin measurements in the presence or absence of these substances. Data and details in Appendix D
6. Working and Linear Ranges		The overall or dynamic linear range of this method results from a combination of the linear range of the assay standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate. The linear range of the ELISA standard curve varied slightly among two lots of kit reagents examined. One lot yielded a range of 0.21-1.04 ng PbTx-3/mL and a second lot yielded a range of 0.30-1.38 ng PbTx-3/mL. The overall or dynamic linear range of the method as

		described for this proposal (in PbTx-3 equivalents) is from 0.12 ppm to 26.62 ppm for the June 2014 kit lot and up to 35.33 ppm for the June 2016 kit lot. Data and details in Appendix E
7. Limit of Detection		The calculated assay LOD is 0.1 ng/mL. At the lowest sample dilution of 1:400, the LOD for brevetoxin in shellfish is 0.04 ppm. Data and details in Appendix E
8. Limit of Quantitation / Sensitivity		The calculated assay LOQ is 0.3 ng/mL. At the lowest sample dilution of 1:400, the LOQ for brevetoxin in shellfish is 0.12 ppm. Data and details in Appendix E
9. Ruggedness		Results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min). Significant differences were observed only with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities of 1.0 ± 30%. Data and details in Appendix F
10. Matrix Effects		Brevetoxin-free samples (10 samples per species) for this study were obtained from shellfish harvest areas along Florida's Gulf coast that infrequently experience *K.. brevis* blooms during periods when *K. brevis* was verified to be absent. Farmed hard clams and sunray venus clams were sourced from Cedar Key, FL and were provided by a Shellfish Aquaculture Extension Agent and as well as local clam farmers. Hard clams were collected from 10 different locations over four days. Sunray venus clams were collected from two locations over six days. Wild oysters were collected by Florida Department of Agriculture and Consumer Services staff from five sites in Apalachicola Bay over nine days. At the lowest dilution (1:400), all samples tested <LOD and no matrix effects were observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Comparative data for 501 samples (173 oyster, 277 hard clam, and 51 sunray venus clam) are presented in Appendix G. For several reasons discussed in Appendix G, comparing NSP mouse bioassay and ELISA data is not straightforward, and analytical NSP methods of any type are unlikely to ever completely agree with mouse bioassay results. There was a very wide range of concentrations measured by ELISA in samples testing <20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay. In samples testing < 20MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. Where quantitative results were obtained by both mouse bioassay and ELISA (i.e., in samples testing ≥ 20 MU/100 g), significant positive correlations were observed. Using linear regression, the 20 MU/100 g equivalent by ELISA was predicted to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams (in PbTx-3 equivalents). Across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.
D. Other Information		
1. Cost of the Method		Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).
2. Special Technical Skills Required to Perform the Method		General laboratory skills are required: reagent preparation, pipetting, basic equipment operation, data analysis using curve-fitting software, basic calculations.
3. Special Equipment Required and Associated Cost		Microplate reader with filter for measurement at 450 nm. Costs range, but basic readers start at approximately $5,000, and a used plate reader can be purchased for less than $1,000.
4. Abbreviations and Acronyms Defined		Ab Antibody BSA Bovine Serum Albumin ELISA Enzyme-linked Immunosorbent Assay HRP Horse radish peroxidase MDG MARBIONC Development Group NSP Neurotoxic Shellfish Poisoning PBS Phosphate Buffered Saline PBS-Tween Phosphate Buffered Saline with Tween 20 (0.05%) PbTx Brevetoxin PGT Phosphate Buffered Saline with gelatin (5%) Tween 20 (0.05%) TMB 3,3'5,5'-Tetramethylbenzidine
5. Details of Turn Around Times (time involved to complete the method)		The ELISA takes approximately 6 hours to complete, and one practiced analyst can comfortably process up to 4 plates per day.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research

		Institute's HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. The Laboratory Manager, Laboratory Safety Officer, Laboratory Secondary Staff and field staff are responsible for implementing QA/QC procedures outlined in the manual. Key practices include the use of Standard Operating Procedures, standard methods, training, quality control, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines. **From the manufacturer (MARBIONC):** Each time new kit reagents are made from stocks, QC ELISAs are run and compared to previous assays. A standard ELISA set is retained to compare all new kits back to. New reagent stocks are given lot numbers. When new reagents are made (e.g. purified antibodies or PbTx-BSA conjugate), the ELISAs are designed with the new reagents to maintain continuity with previous kit lots. Kits are manufactured in a controlled environment to maintain cleanliness and avoid any cross contamination. Kits and kit components are validated. Kit and kit components are serialized to maintain traceability. Higher-level Good Manufacturing Processes are in process and as new reagents are produced, they will conform to requirements to allow for overall implementation of quality systems. Supply: MARBIONC Development Group, LLC has a future vision and is currently working to maintain an adequate supply of reagents. Sufficient supplies are on hand to cover current and projected increased demand for the foreseeable future (approximately 10-15 yrs). MARBIONC is committed to providing the kits for research and commercial use and has also committed to provide resources for the resupply of kit components in advance of the time when such components may be required.
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish

Principle of Analysis

In this indirect competitive ELISA based on Naar et al. (2002), a 96-well ELISA plate is coated with protein-linked brevetoxin, and any remaining binding sites in the wells are blocked. Polyclonal goat anti-brevetoxin antibodies are then incubated with samples or standards in the plate wells. The antibodies will react with the brevetoxins in the samples or standards or will be immobilized on the plate. Antibodies that are not attached to the plate after incubation are washed out during subsequent rinses. Antibodies immobilized on the plate are detected through steps linking the antibodies to horse radish peroxidase (HRP)-linked secondary antibodies and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine [TMB]), which yields a blue color (Amax = 370 nm and 652 nm) that changes to yellow (Amax = 450 nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin that was present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For qualitative (+/-) screening, more samples can be run on one plate (up to 40).

Included in MARBIONC ELISA Kit (store in freezer):

- Reagent A BSA-linked PbTx-3
- Reagent C Goat anti-brevetoxin Ab
- Reagent D HRP-linked anti-goat secondary Ab
- Brevetoxin standard (PbTx-3, 10 μg)

Reagents required but not included (Brands and product numbers are for convenience. Unless otherwise noted, equivalents are acceptable)**:**

- Methanol (ACS grade or better)
- Reagent B: Superblock Blocking Buffer (Thermo Scientific 37545)
- Phosphate Buffered Saline, pH 7.4 (PBS, Sigma P-3813)
- Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 (PBS-Tween, Sigma P-3563)
- Gelatin (Sigma G-6144)
- 3,3'5,5'-Tetramethylbenzidine (TMB, Sigma T0440)
- Sulfuric acid stop solution (H_2SO_4, 0.5M)
- Nanopure water (or equivalent quality water)

Consumables needed:

- Disposable glass test tubes
- Disposable plastic dilution tubes (96-well cluster format)
- 15-ml and 50-ml graduated polypropylene centrifuge tubes
- Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (**substitution NOT recommended**)
- Microplate sealing film
- Assorted pipet tips
- Solution basins
- Aluminum foil

Equipment needed:

Balance capable of measuring to 0.1g
Number 10 sieve
Laboratory blender
Vortex mixer
Centrifuge capable of 3,000*xg*, with rotor for 15 mL centrifuge tubes
Microplate reader with filter for measurement at 450 nm
Multichannel pipettor (100-300 µl), individual pipettors (10-1000 µl)
Orbital microplate shaker
Refrigerator (4°C)/freezer (-20°C)

Pre-Assay Preparation

In advance: PbTx-3 for positive control. Each set of kit reagents (15-plate supply) comes with 10 µg of PbTx-3 for use as a positive control.
Stock solution (1 µg/ml): Dissolve in 10 ml of 100% methanol. Store at -20°C. (May be stored for up to 1 year.)
Working solution (100 ng/ml): From this stock, dilute 1 ml to 10 ml with 100% methanol. Store at -20°C. (May be used for several months.)

80% aqueous methanol. Add 800 ml of methanol to a 1L graduated cylinder and bring to 1L with Nanopure water (or equivalent quality water). Good for up to 1 year.

5% gelatin stock solution. Dissolve 5 g gelatin in 100 ml Nanopure water - stir on heated stir plate until clear. Portion into 15-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

SuperBlock - Dissolve 1 pouch in 200 ml Nanopure water. Portion 50-ml aliquots into 50-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

PBS, pH 7.4 1 L - Dissolve 1 pouch of PBS powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

PBS-Tween (0.05% Tween), pH 7.4 1L - Dissolve 1 pouch of PBS-Tween powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

Make fresh daily:
PGT (PBS, 0.05% Tween, 0.5% gelatin) - Immerse a tube of stock gelatin in warm water for a few minutes to liquefy. Pour 5 ml gelatin into a 50-ml centrifuge tube and fill to 50 ml with PBS-Tween. Make one tube per plate.

Shellfish Sample Preparation (follows requirements for the NSP mouse bioassay)

At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis.

The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are sucked from shell being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for brevetoxins. Samples must be processed within 24 hours of shucking.

Rapid Extraction of Shellfish for Brevetoxins

1. Weigh 1.0 g of homogenized shellfish into a 15-mL polypropylene centrifuge tube.
2. Add 9mL of 80% aqueous methanol, and cap tightly.
3. Vortex for 2 minutes at highest speed.
4. Centrifuge at a minimum of 3000xg for 10 minutes.
5. Pour off supernatant into clean, labeled graduated 15-mL centrifuge tube.
6. Bring the volume of the supernatant to 10mL with 80% methanol.
7. Vortex for 15 seconds to mix.
8. Transfer to a clean labeled glass vial and store at -20°C until assayed.

ELISA Protocol

****IMPORTANT NOTE**** Kit Reagents A, C, and D are diluted in a glycerol solution to prevent freezing. To avoid pipetting error due to viscosity, only place the very tip of the pipet into the vial to withdraw the desired amount. DO NOT PRE-RINSE THE TIP. Submerge the tip into the buffer when dispensing, and rinse the tip several times with buffer to ensure complete transfer.

Step 1 - Reagent A
Shake vial of Reagent A gently by hand. Dilute Reagent A. 1:300 (or as specified in kit instructions) in **PBS**. (For 1 plate, add 40 µl of A to 12 ml **PBS**; for 2 plates, add 80 µl A to 24 ml **PBS**).

Fill each well of a 96-well Maxisorp Immunoplates with 100 µl of diluted Reagent A. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature. After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl **PBS**. (**No Tween for this step**.)

Step 2 - Reagent B
Fill each well with 250 µl of Reagent B-Blocking Buffer. Cover with microplate sealing film, and incubate on plate shaker for 30 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 3 - Sample and positive control dilutions *(This step can be done while Step 1 and 2 are incubating.)*

Note: Sample extracts and PbTx-3 working solution should be brought to room temperature before diluting.

Arrange dilution tubes in a rack according to plate layout - see below. Eight (8) tubes are needed for each sample or positive control.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Pos Ctrl (PbTx-3)
A	tube A	tube A	tube A	tube A	tube A	tube A
B	tube B	tube B	tube B	tube B	tube B	tube B
C	tube C	tube C	tube C	tube C	tube C	tube C
D	tube D	tube D	tube D	tube D	tube D	tube D
E	tube E	tube E	tube E	tube E	tube E	tube E
F	tube F	tube F	tube F	tube F	tube F	tube F
G	tube G	tube G	tube G	tube G	tube G	tube G
H	tube H	tube H	tube H	tube H	tube H	tube H

Leave dilution tubes in row **A** empty. To all other tubes in rows **B-H** (for both samples and Pos Ctrl) add 250 µl of PGT. For each sample, add 975µl of PGT to a small glass test tube. Add 25 µl of sample extract to the tube, and vortex briefly to mix. Transfer 250 µl of this diluted extract into dilution tube **A**. Withdraw another 250 µl from the glass tube, place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.** Do this for each sample.

Positive Control (PbTx-3)
To make the positive control, add 950µl of PGT to a small glass test tube. Add 50 µl of brevetoxin working solution (at 100 ng PbTx-3/ml) to the tube (50 µl PbTx-3 + 950 ul PGT= 5 ng PbTx-3/ml). (This is sufficient for up to two plates.) For each plate, transfer 250 µl of diluted PbTx-3 into dilution tube **A**. Withdraw another 250 µl from the glass tube and place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.**
(Tube H are PGT only and will serve as Reference Wells for maximum absorbance in the absence of brevetoxin.)

Step 4 - Transfer Samples On to Plate

After the plate has been blocked and washed (after Step 2 is complete), use a multichannel pipette to transfer the diluted samples and standards to the plate.

Fill wells of the microplate with 100 µl of each tube **in duplicate** (side by side wells), according to the figure below.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Pos. Ctrl.	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	PbTx-3	5 ng/ml
B	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	PbTx-3	2.5 ng/nl
C	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	PbTx-3	1.25 ng/ml
D	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	PbTx-3	0.625 ng/ml
E	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	PbTx-3	0.31 mg/ml
F	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	PbTx-3	0.156 ng/ml
G	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	PbTx-3	0.078 ng/ml
H	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT

Step 5 - Reagent C

Dilute Reagent **C** 1:300 (or as specified in kit instructions)

(For 1 plate, add 40 µl of A to 12 ml PGT; for 2 plates, add 80 µl A to 24 ml PGT)

To each well add 100 µl of diluted Reagent C. Cover with microplate sealing film, and shake the plate on the plate shaker for 90 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 6 - Reagent D

Dilute Reagent **D** 1:800 (or as specified in kit instructions)

(For 1 plate, add 15 µl of D to 12 ml PGT; for 2 plates, add 30 µl D to 24 ml PGT.)

Fill each well with 100 µl of diluted Reagent D. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature.

(When you get to this step – aliquot 12 ml of TMB per plate into a 15 or 50-ml centrifuge tube and warm to room temperature. Keep the tube in the dark (do not expose to light).

After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl PBS-Tween. **Then rinse each well one time with 300 µl PBS to ensure no Tween remains on the plate.**

Step 7 - TMB

Fill each well with 100 µl of TMB. Cover the plate with a piece of aluminum foil and incubate for 5-7 minutes. Stop the reaction by adding 100 µl of 0.5M H_2SO_4 to each well. The blue color in the wells should turn yellow. Read the plate at 450 nm.

Note: The stop time may vary with kit reagent lots and bottles of TMB. The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities (at 450 nm) of 1.0 ± 30%.

Calculations

Presence of brevetoxin in the sample will prevent color development in the well. Toxin can be quantified by converting absorbance values to % color inhibition and comparing to the positive control.

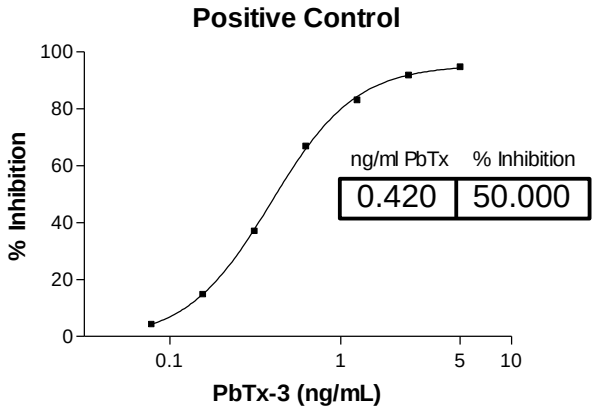
1. Average the values of the duplicate wells for each dilution, and determine the % color inhibition using the following equation:

   % inhibition = [1 - (Avg of dups/Amax)] x 100%

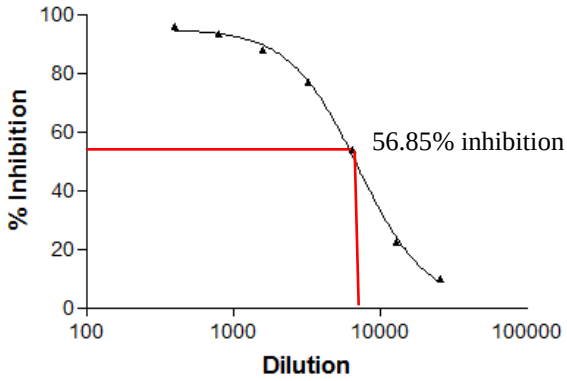
   where Amax is the average absorbance of the reference wells (PGT only) oriented below the sample or standard dilutions.

2. Using the 4-parameter logistic (4PL) curve in a curve-fitting program like Prism or SigmaPlot, fit a curve to the positive control with ng toxin/ml on the x-axis (log scale), and % inhibition on the y-axis (linear scale).
3. Determine the concentration for sample dilutions falling within the linear portion of the standard curve.
4. Multiply the concentration by the sample dilution and divide by 1000 to obtain PbTx-3 eq. results in ppm.

Example Standard Curve (50% inhibition = 0.42 ng PbTx-3/ml)
The control curve should be steep. On the linear part of the curve, the space between the dilutions (on the y-axis) is large. There should be clear plateaus at the top and bottom of the curve.



Example Sample Serial Dilution
Sample curves plotted with dilution on the x-axis (log scale), and % inhibition on the y-axis (linear scale) should have the same features. There should be a clear plateau either at the top or the bottom (or both). Shallow curves with no plateaus or linear curves with little space between points indicate interference in the assay, and results should be discarded.



For a sample with % inhibition of 56.85% at dilution of 1:6,400, the interpolated concentration = 0.495 ng/mL

[PbTx-3 eq] = 0.495 ng/ml x 6400 = 3168 ng/ml or 3.17 ppm

Quality Control Criteria

Acceptance of **assay results** is dependent on meeting the following criteria:

- Absorbance of reference wells must be (Amax) ≥ 0.6. (Optimal absorbance is 1.0 ± 30%.)
- %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%.

If either criteria are not met, re-run the ELISA plate.

Acceptance of **sample results** is dependent on meeting the following criteria:

- %CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (within the linear range of the assay; 20-70% inhibition) must be <20%.
- %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%. (A 20% or greater disparity between the calculated concentrations of two different dilutions of the same sample indicates assay interference or dilution error.)

If either criteria are not met, re-run the sample.

VALIDATION CRITERIA

Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

Data:
Working Range: 0.4-8ppm
Sample Type: Hard Clam, Oyster, Sunray Venus Clam
Agar used to determine spike concentration _____
Organism used for spiking: PbTx-3

Data used for Accuracy are from 10 samples per matrix type, each spiked to 4 concentrations, extracted and analyzed in triplicate with blanks (for each matrix type, n = 120 plus blanks). The stock solution used for spiking was considered the reference and was used for the ELISA positive control/standard curve.

Data summary:

Average concentration (ppm)
Average recovery (%)

spike conc (ppm)	Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36
oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04

oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC04	0	<LD		
hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40

hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70

sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94

DATA HANDLING

Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.
Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

To determine the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is worked-up in the following manner.

1. Convert plate counts to logs.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average reference concentration of the analyte/measurand used to spike the samples; or, for microbiological methods calculate the average plate count of the data in logs. The average plate count represents the average reference concentration for the microbiological method.
4. Calculate the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
5. Divide the average concentration calculated from the spiked samples by the average reference concentration.
6. Multiply the quotient by 100. This provides an estimate in percent of the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations of importance to the intended application of the method.

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary:

Calculated % accuracy/trueness: Oysters: 96.27%
Hard Clams: 98.39%
Sunray Venus Clams: 95.12%

Calculated measurement uncertainty: Oysters: -0.0057 – 0.1137
Hard Clams: 0.0603 – 0.1898
Sunray Venus Clams: 0.0783 – 0.2487

VALIDATION CRITERIA

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Recovery is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^3 and 10^5).

Data:
Working Range: 0.4 - 4 ppm
Sample Type: Oyster, Hard Clam, Sunray Venus Clam
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

matrix type	sample	spike level	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)
hard clam	HC01	blank	0	<LD	
hard clam	HC01	L	0.4	0.33	0.32
hard clam	HC01	M	1	0.98	0.93
hard clam	HC01	H	4	3.85	3.79
hard clam	HC02	blank	0	<LD	
hard clam	HC02	L	0.4	0.35	0.33
hard clam	HC02	M	1	0.92	0.89
hard clam	HC02	H	4	3.82	3.36
hard clam	HC03	blank	0	<LD	
hard clam	HC03	L	0.4	0.35	0.33
hard clam	HC03	M	1	0.91	0.91
hard clam	HC03	H	4	3.55	3.36
hard clam	HC04	blank	0	<LD	
hard clam	HC04	L	0.4	0.33	0.3
hard clam	HC04	M	1	0.91	0.91
hard clam	HC04	H	4	4.66	3.99
hard clam	HC05	blank	0	<LD	
hard clam	HC05	L	0.4	0.32	0.33
hard clam	HC05	M	1	0.92	0.89

hard clam	HC05	H	4	3.49	4.03
hard clam	HC06	blank	0	<LD	
hard clam	HC06	L	0.4	0.44	0.44
hard clam	HC06	M	1	0.84	0.92
hard clam	HC06	H	4	4.15	4.25
hard clam	HC07	blank	0	<LD	
hard clam	HC07	L	0.4	0.42	0.43
hard clam	HC07	M	1	1	1.01
hard clam	HC07	H	4	4.05	4.12
hard clam	HC08	blank	0	<LD	
hard clam	HC08	L	0.4	0.35	0.37
hard clam	HC08	M	1	0.92	1
hard clam	HC08	H	4	4.23	3.55
hard clam	HC09	blank	0	<LD	
hard clam	HC09	L	0.4	0.4	0.39
hard clam	HC09	M	1	0.93	0.91
hard clam	HC09	H	4	3.98	4.26
hard clam	HC10	blank	0	<LD	
hard clam	HC10	L	0.4	0.36	0.39
hard clam	HC10	M	1	0.97	0.98
hard clam	HC10	H	4	4.54	3.98
oyster	OY01	blank	0	<LD	
oyster	OY01	L	0.4	0.38	0.38
oyster	OY01	M	1	0.99	0.95
oyster	OY01	H	4	4.07	4.12
oyster	OY02	blank	0	<LD	
oyster	OY02	L	0.4	0.39	0.39
oyster	OY02	M	1	0.94	0.95
oyster	OY02	H	4	3.87	3.85
oyster	OY03	blank	0	<LD	
oyster	OY03	L	0.4	0.44	0.42
oyster	OY03	M	1	0.8	0.77
oyster	OY03	H	4	3.57	3.92
oyster	OY04	blank	0	<LD	
oyster	OY04	L	0.4	0.37	0.35
oyster	OY04	M	1	1	0.85
oyster	OY04	H	4	4.17	4.14
oyster	OY05	blank	0	<LD	
oyster	OY05	L	0.4	0.36	0.38
oyster	OY05	M	1	0.77	0.89
oyster	OY05	H	4	4.22	4.06
oyster	OY06	blank	0	<LD	
oyster	OY06	L	0.4	0.31	0.33
oyster	OY06	M	1	0.91	0.92
oyster	OY06	H	4	3.36	3.48
oyster	OY07	blank	0	<LD	

oyster	OY07	L	0.4	0.4	0.4
oyster	OY07	M	1	0.88	1.05
oyster	OY07	H	4	3.9	4.21
oyster	OY08	blank	0	<LD	
oyster	OY08	L	0.4	0.46	0.44
oyster	OY08	M	1	1.05	1.03
oyster	OY08	H	4	3.86	4.03
oyster	OY09	blank	0	<LD	
oyster	OY09	L	0.4	0.43	0.37
oyster	OY09	M	1	1.06	0.92
oyster	OY09	H	4	3.74	3.94
oyster	OY10	blank	0	<LD	
oyster	OY10	L	0.4	0.36	0.38
oyster	OY10	M	1	0.94	0.99
oyster	OY10	H	4	4.24	4.28
sunray venus clam	SV01	blank	0	<LD	
sunray venus clam	SV01	L	0.4	0.36	0.37
sunray venus clam	SV01	M	1	0.94	0.98
sunray venus clam	SV01	H	4	3.89	3.95
sunray venus clam	SV02	blank	0	<LD	
sunray venus clam	SV02	L	0.4	0.32	0.34
sunray venus clam	SV02	M	1	1	0.97
sunray venus clam	SV02	H	4	4.09	3.6
sunray venus clam	SV03	blank	0	<LD	
sunray venus clam	SV03	L	0.4	0.38	0.36
sunray venus clam	SV03	M	1	1	0.98
sunray venus clam	SV03	H	4	4.15	3.71
sunray venus clam	SV04	blank	0	<LD	
sunray venus clam	SV04	L	0.4	0.32	0.32
sunray venus clam	SV04	M	1	1.11	1.01
sunray venus clam	SV04	H	4	4.28	4.45
sunray venus clam	SV05	blank	0	<LD	
sunray venus clam	SV05	L	0.4	0.29	0.3
sunray venus clam	SV05	M	1	1.13	1.08
sunray venus clam	SV05	H	4	4.19	3.98
sunray venus clam	SV06	blank	0	<LD	
sunray venus clam	SV06	L	0.4	0.36	0.33
sunray venus clam	SV06	M	1	0.84	0.87
sunray venus clam	SV06	H	4	4.03	3.67
sunray venus clam	SV07	blank	0	<LD	
sunray venus clam	SV07	L	0.4	0.41	0.41
sunray venus clam	SV07	M	1	0.93	0.91
sunray venus clam	SV07	H	4	4.1	3.62
sunray venus clam	SV08	blank	0	<LD	
sunray venus clam	SV08	L	0.4	0.43	0.42
sunray venus clam	SV08	M	1	0.95	0.92

sunray venus clam	SV08	H	4	4.03	3.82
sunray venus clam	SV09	blank	0	<LD	
sunray venus clam	SV09	L	0.4	0.44	0.35
sunray venus clam	SV09	M	1	0.86	1.03
sunray venus clam	SV09	H	4	4.36	3.87
sunray venus clam	SV10	blank	0	<LD	
sunray venus clam	SV10	L	0.4	0.4	0.38
sunray venus clam	SV10	M	1	1.15	1
sunray venus clam	SV10	H	4	4.22	3.95

DATA HANDLING

Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		
Total	29		

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary: **Details Below**

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **N**
- At what concentrations is the one way analysis of variance significant? **NA**
- What is the overall percent recovery of the MPN based method under study? **Oysters 97.6%, Hard Clams 97.2%, Sunray Venus Clams 99.0 %**

Working Range of the assay

The overall working range of this ELISA assay is a combination of the linear range of the standard curve and the range of sample dilutions on the plate. This kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the Amax (see linearity). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared. The extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume), and the extract is diluted another 40X for the initial ELISA dilution (yielding a starting dilution factor of 400).

With data showing samples that tested at 20 MU were on the order of 3-4 ppm by ELISA, and anticipating a critical threshold value of approximately half of that, the “low” “medium” and “high” levels selected for this portion were 0.4, 1, and 4 ppm. Samples spiked to these levels were quantitated at dilution factors ranging from 1,600-12,800.

Data Handling Results

Precision

Nested ANOVA: Following this data handling procedure and using log transformed data, the variance ratio (F) at the 95% confidence interval for the variance components: samples/concentrations in samples is not significant. The specified variance ratio for the components: concentrations in samples /determinations within concentrations is significant for all matrices. However, even using the best possible *mock* data, the specified variance ratio is significant. Therefore this approach may not be appropriate for evaluating this data set.

Oysters	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.030086	9	0.003343	0.006402	1	0
concentrations in samples	10.443037	20	0.522152	995.3691	5.93E-37	99.80
determinations within concentrations	0.015737	30	0.000525			0.20
total	10.488860	59				100
Hard Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.036326	9	0.004036	0.007381	1	0
concentrations in samples	10.936302	20	0.546815	1038.552	3.14E-37	99.81
determinations within concentrations	0.015796	30	0.000527			0.19
total	10.988423	59				100
Sunray Venus Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.015388	9	0.001710	0.0031	1	0
concentrations in samples	11.028937	20	0.551447	799.5186	1.57E-35	99.75
determinations within concentrations	0.020692	30	0.000690			0.25
total	11.065016	59				100

Similarly, calculating the %CV for the whole data set as specified above may not be appropriate. Theoretically, if the recoveries were all perfectly 100%, the %CV of the full data set per species would be 89%. Within each spike concentration, %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams.

Oysters	n	%CV
all spike levels/reps	**60**	**90.40**
low (0.4ppm)	20	9.74
med (1ppm)	20	9.45
high (4ppm)	20	6.53
Hard Clams	n	%CV
all spike levels/reps	**60**	**91.53**
low (0.4ppm)	20	11.97
med (1ppm)	20	4.69
high (4ppm)	20	9.16
Sunray Venus Clams	n	%CV
all spike levels/reps	**60**	**90.21**
low (0.4ppm)	20	12.06
med (1ppm)	20	8.81
high (4ppm)	20	6.02

Recovery

Performing a one-way ANOVA as specified above yielded F test results for each matrix type that were not significant at the 95% confidence interval, suggesting that the recovery of the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method.

oysters	sum of squares	d.f	mean square	Fs	P
concentration	0.015062	2	0.007531	0.33193	0.72042
error	0.612573	27	0.022688		
total	0.627634	29			
hard clams	sum of squares	d.f	mean square	Fs	P
concentration	0.004995	2	0.002498	0.08340	0.92022
error	0.808525	27	0.029945		
total	0.813520	29			
sunray venus clams	sum of squares	d.f	mean square	Fs	P
concentration	0.005632	2	0.002816	0.24244	0.78640
error	0.313593	27	0.011615		
total	0.319224	29			

The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams.

Matrix	Avg Spike Conc (ppm)	Avg Rep Conc (ppm)	% Recovery
Oysters	1.8	1.76	97.62
Hard Clams	1.8	1.75	97.17
Sunray Venus Clams	1.8	1.78	98.99

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank , the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:

Potentially interfering substances examined in this study included two types of microalgae – the flagellate *Isochrysis* aff. *galbana* and the cryptophyte *Rhodomonas lens* – that are commonly fed to aquaculture-reared bivalves at the age/size at which they are ready to be relocated from the hatchery to the aquaculture zones. With the capacity to ingest as many as 10^9 cells per day, it is reasonable to predict there may be some bioaccumulation of cell constituents over time, and that they may still be present when the bivalves are harvested. Cells were added to a concentration of 100 million cells per g of shellfish.

Also examined was okadaic acid, a dinoflagellate toxin produced by some species of *Dinophysis* and *Prorocentrum*. These organisms are present in waters where *Karenia brevis* occurs, and potentially both toxins could be present. Both brevetoxin and okadaic acid are polyether toxins, so cross-reactivity with okadaic acid was investigated. Okadaic acid was added to a concentration of 1.5 µg per g of shellfish (or 1.5 ppm), which is roughly ten times above the current US guidance limit of 0.16 ppm.

The final substance to be examined was *Karenia mikimotoi*, a dinoflagellate that is closely related to *Karenia brevis*. *K. mikimotoi* produces bioactive compounds, but brevetoxin production has not been documented in this species. *Karenia* blooms are often mixed species blooms with two or more *Karenia* species present, although *K. brevis* is typically dominant. *K. mikimotoi* cells were added to a concentration of 500,000 cells per g.

Interfering organism/compound/toxin:

A *Isochrysis* aff. *galbana* (100 million cells per g shellfish)
B *Rhodomonas lens* (100 million cells per g shellfish)
C Okadaic acid (1.5 µg per g shellfish)
D *Karenia mikimotoi* (500,000 cells per g shellfish)

PbTx-3 spike concentration: 0.4 ppm

oyster

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.39	0.41	0.41	0.38	0.39	<LD
0.38	0.38	0.41	0.38	0.40	
0.42	0.39	0.39	0.37	0.43	
0.34	0.38	0.42	0.37	0.37	
0.39	0.44	0.40	0.35	0.42	

hard clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.36	0.40	0.40	0.38	0.40	<LD
0.38	0.38	0.39	0.40	0.32	
0.39	0.40	0.37	0.37	0.38	
0.35	0.36	0.38	0.37	0.33	
0.38	0.40	0.39	0.38	0.37	

sunray venus clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.33	0.41	0.42	0.41	0.35	<LD
0.35	0.39	0.38	0.40	0.41	
0.38	0.38	0.36	0.35	0.35	
0.35	0.37	0.34	0.39	0.40	
0.38	0.43	0.40	0.39	0.41	

Data handling for demonstrating specificity of the new or modified method

The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SI_{avg}) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Oyster

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.96	no	0.352
B	*Rhodomonas lens*	0.94	no	0.254
C	Okadaic acid	1.03	no	0.490
D	*Karenia mikimotoi*	0.95	no	0.061

Hard clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.97	no	0.164
B	*Rhodomonas lens*	0.97	no	0.230
C	Okadaic acid	0.98	no	0.374
D	*Karenia mikimotoi*	1.04	no	0.364

Sunray venus clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.91	no	0.055
B	*Rhodomonas lens*	0.95	no	0.311
C	Okadaic acid	0.93	no	0.205
D	*Karenia mikimotoi*	0.94	no	0.230

VALIDATION CRITERIA

Linear Range is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

Limit of Detection is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

Limit of Quantitation/Sensitivity is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. 10^a, 10^b…10^n) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed (10^a, 10^b…10^n).

Data:
Sample type
Working range/Range of interest: 0.4-8 ppm
Range in spiking levels used: 0.4 ppm, 1 ppm, 4 ppm, 8 ppm, 12 ppm
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

Response is the signal data (absorbance, fluorescence, Ct value), colonies, plaques, etc resulting from the analysis.

For shellfish samples repeat for each tissue type of interest.

DATA HANDLING

Linear Range
To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.

8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary: See below for explanation

Linear range of the method as implemented 0.12 ppm to 35.33 ppm
The limit of detection of the method as implemented 0.040 ppm
The limit of quantitation/sensitivity of the method as implemented 0.12 ppm

Data was generated as directed above (ten samples spiked to five levels, analyzed in triplicate plus one blank aliquot) for each matrix type examined, but this data could not be analyzed as described in the data handling portion of this SOP. (Although most of the data was not used to determine linearity and LOQ/LOD, it is provided at the end of this Appendix.)

This ELISA kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the maximum absorbance of the reference wells (Amax). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared and analyzed. Assay response is converted to concentration by comparison to a standard curve, and the final sample concentration is the product of the concentration measured in the assay and the dilution factor. As a result, similar responses (signal data) can be measured for very different sample concentrations.

The overall or dynamic linear range of this method results from a combination of: 1) the linear range of the assay standard curve, 2) the assay limit of quantitation, and 3) the range of sample dilutions on the plate.

1) Linear Range of Assay

To evaluate the linear range of the assay, 7-point PbTx-3 standard curves (ranging from 0.08 to 5.0 ng/mL) from 60 ELISA plates run in this validation were generated using the sigmoidal dose-response (variable slope), or four-parameter logistic, curve fitting equation in Prism 5 (GraphPad Software). The upper and lower plateaus of the curves were then applied to formulae derived by Sebaugh and McCray[1] to define the "bend points" of the standard curves, the beginning and end of the linear concentration--response region, expressed both in terms of % inhibition (1-A/Amax x 100) and concentration (Table E.1). The assays included data generated using two different kit lots: June 2014 (n=34) and June 2016 (n=26). We found that the position of the standard curves and the linear range defined by the bend points differed between the two kit lots (Fig. E1). Such shifts can be achieved with the same kit lot by altering dilutions of key reagents (A and C). Therefore, we believe that the differences we observed in kit lots were due to minor concentration variations in the supplied reagents A and/or C. However, comparative analyses of spiked samples were not significantly different between the two kit lots (see Appendix F: Ruggedness).

Table E1. Average bend points (± standard deviation), expressed as % inhibition and concentration, defining the linear range of standard curves generated using two lots of ELISA kit reagents.

	% inhibition		ng PbTx-3/mL	
	Jun-14 Lot	Jun-16 Lot	Jun-14 Lot	Jun-16 Lot
lower bend point	17.34 ± 2.47	16.76 ± 2.73	0.21 ± 0.04	0.30 ± 0.06
upper bend point	76.91 ± 2.07	74.19 ± 1.68	1.04 ± 0.14	1.38 ± 0.16

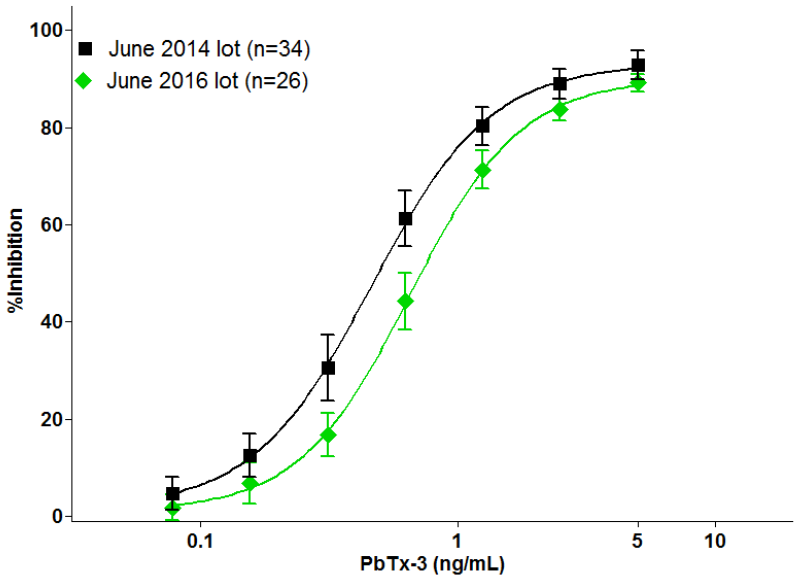


Figure E1. Average of multiple semi-log standard curves generated using two lots of ELISA kit reagents. Error bars represent standard deviation of independent curves prepared and assayed on different plates or days.

Using the June 2014 lot, two additional 14-point standard curves were assayed on different days to generate curves with more points that fell along the linear portion of the curve (Fig. E2). The bend points from these 14-point curves (16%-76%) were similar to those derived from the routine standard curves (Table E1).

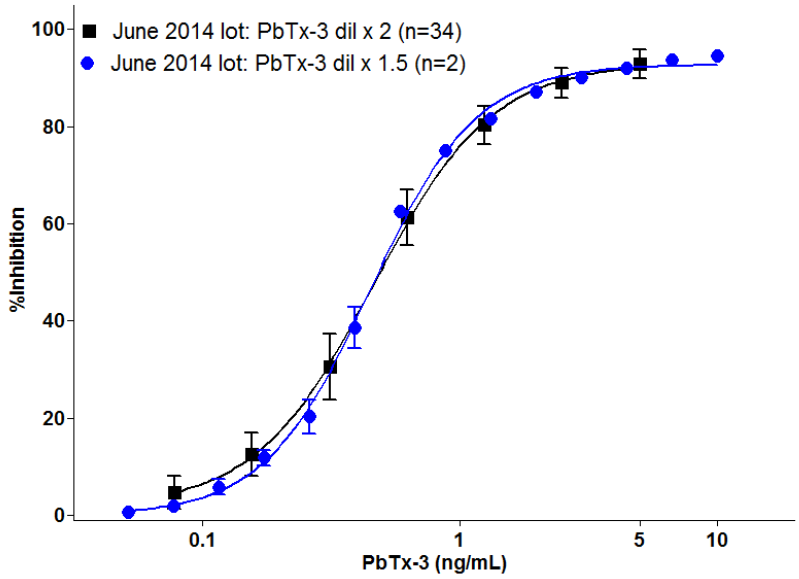


Figure E2. Comparison of 7-point and 14-point PbTx-3 standard curves. Error bars represent standard deviation of independent curves prepared and assays on different plates/days.

To verify linearity within the range defined by the bend points, multiple dilutions of shellfish samples spiked to 0.4 ppm with PbTx-3 were quantified. Ten samples were used for each matrix type, and three replicates per sample were extracted and analyzed. These assays were performed using the June 2014 kit lot. As written in the method protocol, the shellfish extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume). The extract is then diluted another 40 times in ELISA buffer for the initial dilution, and six additional dilutions are prepared by serial dilution by two, yielding a total of seven dilutions (from 1:400 to 1:25,600) for each sample.
At the 0.4 ppm spike level, the expected value of the first three dilutions are 1.0, 0.5, and 0.25 ng/mL, which are all within the linear range of the June 2014 kit lot as defined by the bend points. The expected and mean measured values of the three dilutions are listed in Table E2. Linear regression yielded r^2 values of 0.94-0.97 (Fig. E3).

Table E2. Expected concentrations and mean of concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Shellfish were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed.

	dilution	expected	mean measured	SD	%CV
oyster	400	1.00	0.935	0.079	8.4%
	800	0.50	0.471	0.038	8.1%
	1600	0.25	0.229	0.034	14.8%
hard clam	400	1.00	0.893	0.081	9.1%
	800	0.50	0.456	0.055	12.1%
	1600	0.25	0.233	0.033	14.2%
sunray venus clam	400	1.00	0.911	0.098	10.8%
	800	0.50	0.455	0.059	13.0%
	1600	0.25	0.234	0.030	12.8%

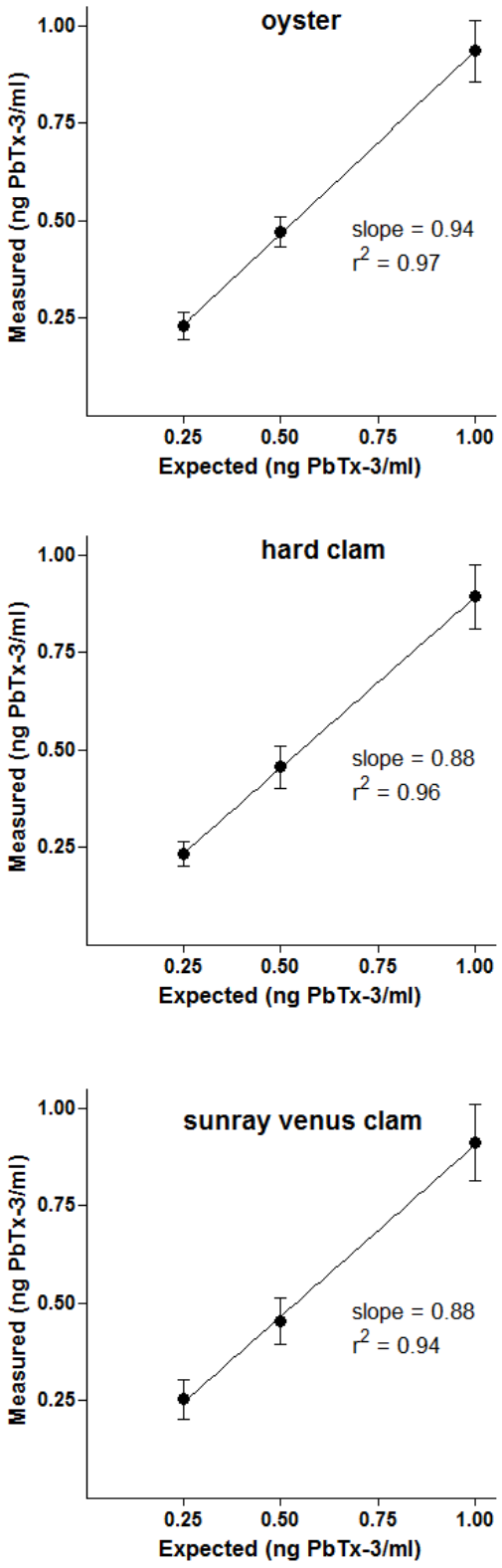


Figure E3. Expected concentrations vs. mean concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Oyster, hard clams, and sunray venus clams were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed. Error bars represent standard deviation.

Limit of Detection and Limit of Quantitation

Blank samples consistently yielded assay responses that were not quantifiable. Therefore, the standard deviation of results from the 1:1600 dilution of shellfish reported in Table E2 above were used to derive the limit of detection (LOD) and limit of quantitation (LOQ) expressed as 3x and 10x the standard deviation, respectively. This dilution consistently yielded a signal (% inhibition) closest to the lower bend point (17%) and was the lowest quantifiable dilution.

For all three matrix types, the standard deviation at the 1:1600 dilution was approximately 0.03. Calculated assay LOD and LOQ are 0.1 and 0.3 ng/mL, respectively. At the lowest sample dilution of 1:400, the LOD and LOQ for brevetoxin in shellfish are 40 and 120 ng/g or 0.04 and 0.12 ppm.

Dynamic linear range

The overall or dynamic linear range of this method is a combination of the linear range of the standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate (from 400 to 25,600). Using the LOQ calculated above, which falls with the linear portion of the standard curve identified for both lots of kits used in this study, and the upper bend points identified for each kit lot, the overall or dynamic linear range of the method is from 120 ng PbTx-3 eq./g or 0.12 ppm up to 26,624 or 26.62ppm for the June 2014 kit lot and up to 35,328 ng PbTx-3 eq. per g or 35.33 ppm for the June 2016 kit lot.

References:

1. Sebaugh JL, McCray PD (2003) Defining the linear portion of a sigmoid-shaped curve: bend points. Pharmaceutical Statistics 2: 167-174.

Results of spiking experiments: ten samples were spiked to five levels and analyzed in triplicate (plus one blank aliquot) for each matrix type examined.

Data Summary:

Average concentration (ppm)
Average recovery (%)

spike conc (ppm)	Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%
12	10.63	11.03	12.74
	89%	92%	106%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY01	12	10.60	10.97	10.73
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY02	12	10.77	10.26	10.54
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY03	12	10.83	11.13	11.11
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY04	12	10.90	10.08	9.94
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36

oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY05	12	10.17	10.38	9.68
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY06	12	11.62	10.71	11.36
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY07	12	10.68	11.42	10.75
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY08	12	10.68	11.00	10.46
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04
oyster	OY09	12	11.09	10.44	10.78
oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
oyster	OY10	12	9.58	9.75	10.34
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC01	12	9.89	9.80	10.75
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC02	12	11.14	11.33	11.63
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC03	12	10.26	11.20	10.25
hard clam	HC04	0	<LD		

hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC04	12	11.50	11.92	11.74
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC05	12	10.78	9.82	11.27
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC06	12	11.18	11.40	12.08
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC07	12	10.94	10.50	10.70
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC08	12	10.53	10.76	10.98
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40
hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC09	12	12.38	10.77	10.84
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
hard clam	HC10	12	10.99	11.31	12.19
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV01	12	11.61	12.13	11.52
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV02	12	12.57	11.57	12.71

sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV03	12	12.95	12.02	13.17
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV04	12	12.51	11.70	13.43
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV05	12	13.32	12.34	13.47
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV06	12	14.52	12.76	13.90
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV07	12	11.91	13.74	11.53
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70
sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV08	12	11.73	14.16	12.19
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV09	12	13.18	14.53	12.87
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94
sunray venus clam	SV10	12	12.62	12.50	12.98

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

Data for demonstrating the ruggedness of the new or modified method:
For this study, results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min).

1) Different lots of ELISA kit reagents:

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	Jun-14 lot	Jun-16 lot
oyster	OY01	0.4	0.38	0.36
oyster	OY02	0.4	0.38	0.36
oyster	OY03	1	0.92	0.90
oyster	OY04	1	0.88	0.88
oyster	OY05	2	1.87	1.88
oyster	OY06	2	1.84	2.04
oyster	OY07	4	3.78	3.66
oyster	OY08	4	3.88	4.54
oyster	OY09	8	7.67	7.86
oyster	OY10	8	8.05	7.73
hard clam	HC01	0.4	0.38	0.39
hard clam	HC02	0.4	0.39	0.38
hard clam	HC03	1	1.08	0.92
hard clam	HC04	1	0.97	0.97
hard clam	HC05	2	1.97	1.92

hard clam	HC06	2	2.16	1.85
hard clam	HC07	4	3.78	4.05
hard clam	HC08	4	3.90	3.83
hard clam	HC09	8	7.86	7.69
hard clam	HC10	8	7.79	8.16
sunray venus clam	SV01	0.4	0.35	0.33
sunray venus clam	SV02	0.4	0.39	0.38
sunray venus clam	SV03	1	1.03	0.89
sunray venus clam	SV04	1	1.05	1.00
sunray venus clam	SV05	2	2.05	1.89
sunray venus clam	SV06	2	1.97	1.95
sunray venus clam	SV07	4	3.62	4.23
sunray venus clam	SV08	4	3.82	4.22
sunray venus clam	SV09	8	7.57	7.38
sunray venus clam	SV10	8	8.34	7.85

2) Incubation of ELISA plates throughout the procedure at ambient laboratory temperature (21-22°C) vs. in a heated plate shaker (25°C):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	21-22°C	25°C
oyster	OY01	0.4	0.36	0.37
oyster	OY02	0.4	0.36	0.40
oyster	OY03	1	0.90	0.88
oyster	OY04	1	0.88	0.84
oyster	OY05	2	1.88	1.96
oyster	OY06	2	2.04	1.94
oyster	OY07	4	3.66	3.72
oyster	OY08	4	4.54	4.56
oyster	OY09	8	7.86	8.08
oyster	OY10	8	7.73	8.31
hard clam	HC01	0.4	0.39	0.39
hard clam	HC02	0.4	0.38	0.37
hard clam	HC03	1	0.92	0.91
hard clam	HC04	1	0.97	0.86
hard clam	HC05	2	1.92	2.07
hard clam	HC06	2	1.85	1.87
hard clam	HC07	4	4.05	4.06
hard clam	HC08	4	3.83	4.17
hard clam	HC09	8	7.69	7.96
hard clam	HC10	8	8.16	8.26
sunray venus clam	SV01	0.4	0.33	0.35
sunray venus clam	SV02	0.4	0.38	0.40
sunray venus clam	SV03	1	0.89	0.92
sunray venus clam	SV04	1	1.00	0.94
sunray venus clam	SV05	2	1.89	2.24
sunray venus clam	SV06	2	1.95	1.86

sunray venus clam	SV07	4	4.23	4.08
sunray venus clam	SV08	4	4.22	4.19
sunray venus clam	SV09	8	7.38	7.03
sunray venus clam	SV10	8	7.85	7.49

3) Duration of sample and primary antibody (reagent C) incubation (60 min vs. 90 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	60 min C	90 min C
oyster	OY01	0.4	0.37	0.38
oyster	OY02	0.4	0.39	0.38
oyster	OY03	1	0.92	0.92
oyster	OY04	1	0.92	0.88
oyster	OY05	2	1.61	1.87
oyster	OY06	2	1.62	1.84
oyster	OY07	4	3.28	3.78
oyster	OY08	4	3.51	3.88
oyster	OY09	8	7.94	7.67
oyster	OY10	8	7.99	8.05
hard clam	HC01	0.4	0.40	0.38
hard clam	HC02	0.4	0.39	0.39
hard clam	HC03	1	1.02	1.08
hard clam	HC04	1	1.07	0.97
hard clam	HC05	2	1.84	1.97
hard clam	HC06	2	1.97	2.16
hard clam	HC07	4	3.65	3.78
hard clam	HC08	4	3.40	3.90
hard clam	HC09	8	7.44	7.86
hard clam	HC10	8	7.89	7.79
sunray venus clam	SV01	0.4	0.37	0.35
sunray venus clam	SV02	0.4	0.40	0.39
sunray venus clam	SV03	1	0.94	1.03
sunray venus clam	SV04	1	0.95	1.05
sunray venus clam	SV05	2	2.11	2.05
sunray venus clam	SV06	2	2.07	1.97
sunray venus clam	SV07	4	3.89	3.62
sunray venus clam	SV08	4	3.73	3.82
sunray venus clam	SV09	8	7.84	7.57
sunray venus clam	SV10	8	7.89	8.34

4) Duration of TMB color development step (7 min vs 13 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	TMB 7 min	TMB 13 min
oyster	OY01	0.4	0.35	0.48
oyster	OY02	0.4	0.36	0.36
oyster	OY03	1	0.91	1.13
oyster	OY04	1	0.91	0.89
oyster	OY05	2	1.69	2.05
oyster	OY06	2	1.86	1.90
oyster	OY07	4	3.84	4.05
oyster	OY08	4	3.88	4.61
oyster	OY09	8	7.89	8.35
oyster	OY10	8	7.90	7.63
hard clam	HC01	0.4	0.34	0.42
hard clam	HC02	0.4	0.37	0.37
hard clam	HC03	1	0.95	1.13
hard clam	HC04	1	0.93	0.93
hard clam	HC05	2	1.78	2.22
hard clam	HC06	2	1.64	1.80
hard clam	HC07	4	3.74	4.45
hard clam	HC08	4	3.62	4.37
hard clam	HC09	8	7.52	7.48
hard clam	HC10	8	7.94	7.55
sunray venus clam	SV01	0.4	0.35	0.39
sunray venus clam	SV02	0.4	0.38	0.44
sunray venus clam	SV03	1	0.94	0.97
sunray venus clam	SV04	1	0.93	1.17
sunray venus clam	SV05	2	1.84	2.13
sunray venus clam	SV06	2	1.76	1.81
sunray venus clam	SV07	4	3.66	3.90
sunray venus clam	SV08	4	3.76	4.04
sunray venus clam	SV09	8	7.88	7.50
sunray venus clam	SV10	8	7.95	8.14

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
   - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

See tables on next page

Significant differences were observed with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities of 1.0 ± 30%.

Value for the test of symmetry of the distribution of batch/lot 1 data _____

Value for the test of symmetry of the distribution of batch/lot 2 data _____

Variance of batch/lot 1 data _____

Variance of batch/lot 2 data _____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples? Y/N

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	Jun14 lot	Jun16 lot	Jun14 lot	Jun16 lot			
oyster	-0.32402 (0.750)	-0.07704 (0.906)	0.2281	0.2350	1.030	0.302	no
hard clam	-0.10448 (0.916)	-0.26257 (0.856)	0.2366	0.2483	1.049	0.708	no
sunray venus clam	-0.27735 (0.804)	-0.17249 (0.852)	0.2327	0.2471	1.062	0.465	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	21-22°C	25°C	21-22°C	25°C			
oyster	-0.07704 (0.974)	-0.20833 (0.822)	0.2350	0.2465	1.049	0.741	no
hard clam	-0.26257 (0.740)	-0.18657 (0.874)	0.2483	0.2483	1.000	0.287	no
sunray venus clam	-0.17249 (0.820)	-0.37325 (0.764)	0.2471	0.2333	1.059	0.754	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	60 min C	90 min C	60 min C	90 min C			
oyster	-0.13316 (0.866)	-0.32402 (0.780)	0.2160	0.2281	1.056	0.219	no
hard clam	0.25186 (0.772)	-0.10448 (0.912)	0.2301	0.2366	1.028	0.099	no
sunray venus clam	-0.42338 (0.680)	-0.27735 (0.734)	0.2326	0.2327	1.000	0.982	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	TMB 7 min	TMB 13 min	TMB 7 min	TMB 13 min			
oyster	0.07922 (0.892)	-0.13022 (0.922)	0.2388	0.2297	1.040	0.014	yes
hard clam	-0.00274 (0.958)	-0.04028 (0.982)	0.2460	0.2302	1.069	0.030	yes
sunray venus clam	-0.03460 (0.990)	-0.06355 (0.940)	0.2387	0.2187	1.092	0.011	yes

*m-out-of-n bootstrap symmetry test by Miao, Gel, and Gastwirth (2006)

Miao, W., Y. R. Gel, and J. L. Gastwirth. "A New Test of Symmetry about an Unknown Median. Random Walk." *Sequential Analysis and Related Topics-A Festschrift in Honor of Yuan-Shih Chow. Eds.: Agnes Hsiung, Cun-Hui Zhang, and Zhiliang Ying, World Scientific Publisher, Singapore* (2006).

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:

A total of 526 samples were tested using both ELISA and the NSP mouse bioassay (Table G1). Results of individual samples are contained in Table G2. Although additional data exists (both published and unpublished) comparing this ELISA with NSP mouse bioassay results, extraction methods have been modified over time. The data presented here includes only samples that were extracted for ELISA using 80% methanol with no additional clean-up. Almost all of the samples (495 of 526, 94%) were extracted and assayed in duplicate, and the mean is reported in the table. The mean %CV of duplicate analyses was 6.2%.

Table G1. Summary of comparative data using both NSP mouse bioassay and ELISA.

Shellfish Matrix	Total Samples	Mouse Bioassay < 20 MU/100g	Mouse Bioassay ≥ 20 MU/100g
Oysters	197	135 (69%)	62 (31%)
Hard Clams	277	238 (86%)	39 (14%)
Sunray Venus Clams	52	22 (42%)	30 (58%)

Table G2. Sample information and results of NSP mouse bioassay and ELISA

Sample ID	Shellfish Matrix	Harvest Area	Sample Date	MU/100g	ELISA (ppm)
HABB070327-017	oyster	Pine Island Sound	3/26/2007	<20	6.60
HABB070403-002	oyster	Pine Island Sound	4/2/2007	<20	5.26
HABB071115-001	oyster	St. Johns	11/14/2007	33.75	7.26
HABB071115-002	oyster	St. Johns	11/14/2007	38.63	16.31
HABB071128-004	oyster	St. Johns	11/27/2007	27.37	6.53
HABB071212-003	oyster	St. Johns	12/11/2007	<20	3.40
HABB080214-001	oyster	Alabama	2/8/2008	<20	0.52
HABB091117-001	oyster	Pine Island Sound	11/16/2009	<20	0.66
HABB091202-001	oyster	Pine Island Sound	12/1/2009	<20	0.42
HABB091202-002	oyster	Pine Island Sound	12/1/2009	<20	0.29
HABB100105-001	oyster	Pine Island Sound	1/4/2010	36.38	9.44
HABB100112-003	oyster	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-004	oyster	Pine Island Sound	1/11/2010	26.04	6.07
HABB100113-001	oyster	Gasparilla Sound	1/12/2010	<20	1.21
HABB100113-002	oyster	Gasparilla Sound	1/12/2010	<20	1.66
HABB100120-001	oyster	Pine Island Sound	1/19/2010	<20	<LOD
HABB100120-002	oyster	Pine Island Sound	1/19/2010	<20	2.34
HABB100224-001	oyster	Pine Island Sound	2/23/2010	<20	1.83
HABB100224-002	oyster	Pine Island Sound	2/23/2010	<20	1.01
HABB111026-003	oyster	Pine Island Sound	10/25/2011	<20	<LOD
HABB111026-004	oyster	Pine Island Sound	10/25/2011	<20	1.99
HABB111103-001	oyster	Gasparilla Sound	11/2/2011	33.31	9.57
HABB111103-002	oyster	Gasparilla Sound	11/2/2011	28.19	6.50
HABB111109-001	oyster	Pine Island Sound	11/8/2011	<20	0.53
HABB111109-002	oyster	Pine Island Sound	11/8/2011	32.93	10.09
HABB111115-001	oyster	Gasparilla Sound	11/14/2011	<20	4.80
HABB111115-002	oyster	Gasparilla Sound	11/14/2011	<20	2.98
HABB111122-002	oyster	Lemon Bay	11/21/2011	<20	7.76
HABB111213-001	oyster	Pine Island Sound	12/12/2011	<20	2.04
HABB111213-002	oyster	Pine Island Sound	12/12/2011	<20	1.71
HABB111220-001	oyster	Pine Island Sound	12/19/2011	<20	10.83
HABB111220-002	oyster	Pine Island Sound	12/19/2011	<20	3.85
HABB120124-003	oyster	Pine Island Sound	1/23/2012	<20	3.94
HABB120124-004	oyster	Pine Island Sound	1/23/2012	<20	1.31
HABB120131-001	oyster	Ten Thousand Islands	1/30/2012	37.70	14.01
HABB120214-001	oyster	Ten Thousand Islands	2/13/2012	22.80	6.19
HABB120214-002	oyster	Pine Island Sound	2/13/2012	<20	8.25
HABB120214-003	oyster	Pine Island Sound	2/13/2012	<20	1.79
HABB120221-001	oyster	Ten Thousand Islands	2/20/2012	27.43	6.72
HABB120228-001	oyster	Ten Thousand Islands	2/27/2012	<20	4.42
HABB121113-002	oyster	Lower Tampa Bay	11/6/2012	34.08	4.32
HABB130212-004	oyster	Lower Tampa Bay	11/14/2012	34.99	22.43
HABB130205-003	oyster	Lower Tampa Bay	2/4/2013	<20	3.28
HABB130409-001	oyster	Gasparilla Sound	4/8/2013	31.56	8.17
HABB130409-002	oyster	Gasparilla Sound	4/8/2013	29.65	15.40
HABB130501-001	oyster	Gasparilla Sound	4/30/2013	32.21	5.07
HABB130501-002	oyster	Gasparilla Sound	4/30/2013	24.07	3.26

HABB130501-003	oyster	Ten Thousand Islands	4/30/2013	<20	0.77
HABB130508-002	oyster	Gasparilla Sound	5/7/2013	<20	4.91
HABB130508-003	oyster	Gasparilla Sound	5/7/2013	<20	3.00
HABB130508-005	oyster	Lemon Bay	5/7/2013	<20	3.92
HABB130515-001	oyster	Pine Island Sound	5/14/2013	<20	3.17
HABB130515-002	oyster	Pine Island Sound	5/14/2013	<20	3.24
HABB130604-002	oyster	Sarasota Bay	6/3/2013	<20	2.43
HABB131210-001	oyster	Gasparilla Sound	12/9/2013	<20	4.52
HABB131210-002	oyster	Gasparilla Sound	12/9/2013	<20	0.79
HABB131210-003	oyster	Pine Island Sound	12/9/2013	<20	1.99
HABB131217-001	oyster	Pine Island Sound	12/16/2013	<20	2.03
HABB131217-002	oyster	Pine Island Sound	12/16/2013	<20	1.51
HABB131217-003	oyster	Matlacha	12/16/2013	<20	0.18
HABB131218-009	oyster	Lemon Bay	12/17/2013	<20	1.63
HABB141021-001	oyster	Suwannee Sound	10/20/2014	<20	4.62
HABB141021-002	oyster	Suwannee Sound	10/20/2014	<20	5.02
HABB141021-003	oyster	Suwannee Sound	10/20/2014	<20	3.34
HABB141022-002	oyster	Horseshoe Beach	10/21/2014	27.89	5.02
HABB141022-003	oyster	Horseshoe Beach	10/21/2014	<20	<LOD
HABB141028-001	oyster	Horseshoe Beach	10/27/2014	<20	4.44
HABB141028-002	oyster	Horseshoe Beach	10/27/2014	<20	5.20
HABB141028-003	oyster	Horseshoe Beach	10/27/2014	22.56	5.73
HABB141104-001	oyster	Horseshoe Beach	11/3/2014	<20	3.53
HABB141118-001	oyster	Gasparilla Sound	11/17/2014	<20	1.07
HABB141118-002	oyster	Gasparilla Sound	11/17/2014	<20	0.45
HABB141124-004	oyster	Pine Island Sound	11/23/2014	<20	2.57
HABB141209-001	oyster	Pine Island Sound	12/8/2014	<20	0.91
HABB141209-002	oyster	Pine Island Sound	12/8/2014	<20	2.49
HABB141216-001	oyster	Ten Thousand Islands	12/15/2014	<20	1.13
HABB151014-002	oyster	Indian Lagoon	10/13/2015	<20	0.84
HABB151119-001	oyster	East Bay	10/29/2015	94.60	25.50
HABB151103-001	oyster	Indian Lagoon	11/2/2015	<20	1.99
HABB151103-002	oyster	Pine Island Sound	11/2/2015	<20	0.98
HABB151103-003	oyster	Pine Island Sound	11/2/2015	<20	<LOD
HABB151110-001	oyster	Gasparilla Sound	11/9/2015	<20	1.34
HABB151110-002	oyster	Gasparilla Sound	11/9/2015	<20	3.87
HABB151117-001	oyster	East Bay	11/16/2015	34.05	7.08
HABB151117-002	oyster	North Bay	11/16/2015	<20	1.59
HABB151124-001	oyster	East Bay	11/23/2015	25.03	5.77
HABB151202-001	oyster	East Bay	12/1/2015	34.84	7.44
HABB151208-001	oyster	West Bay	12/7/2015	33.07	3.57
HABB151208-002	oyster	East Bay	12/7/2015	28.14	5.09
HABB151208-003	oyster	East Bay	12/7/2015	35.47	13.95
HABB151216-001	oyster	East Bay	12/15/2015	33.37	5.04
HABB151216-002	oyster	West Bay	12/15/2015	30.10	5.55
HABB151217-001	oyster	Gasparilla Sound	12/16/2015	<20	2.27
HABB151217-002	oyster	Gasparilla Sound	12/16/2015	26.79	4.73
HABB151217-003	oyster	Pine Island Sound	12/16/2015	31.47	3.96
HABB151217-004	oyster	Pine Island Sound	12/16/2015	20.21	3.56
HABB151222-001	oyster	Gasparilla Sound	12/21/2015	<20	4.31
HABB151222-002	oyster	Gasparilla Sound	12/21/2015	<20	1.77
HABB160105-001	oyster	Pine Island Sound	1/4/2016	<20	2.28

HABB160105-002	oyster	Pine Island Sound	1/4/2016	<20	2.17
HABB160105-003	oyster	Apalachicola Bay	1/4/2016	<20	3.27
HABB160105-004	oyster	Apalachicola Bay	1/4/2016	<20	2.52
HABB160106-001	oyster	East Bay	1/5/2016	30.63	2.45
HABB160106-002	oyster	North Bay	1/5/2016	17.07	7.91
HABB160112-001	oyster	West Bay	1/11/2016	22.35	3.28
HABB160112-002	oyster	North Bay	1/11/2016	23.94	7.28
HABB160112-003	oyster	West Bay	1/11/2016	35.43	12.59
HABB160113-001	oyster	Pensacola Bay	1/12/2016	<20	2.13
HABB160114-001	oyster	Apalachicola Bay	1/12/2016	<20	1.88
HABB160114-002	oyster	Indian Lagoon	1/12/2016	21.84	10.53
HABB160120-001	oyster	East Bay	1/19/2016	<20	2.02
HABB160120-002	oyster	North Bay	1/19/2016	<20	6.41
HABB160120-003	oyster	Mississippi	1/19/2016	<20	0.16
HABB160120-004	oyster	Mississippi	1/19/2016	<20	0.33
HABB160120-005	oyster	Mississippi	1/19/2016	<20	0.23
HABB160120-006	oyster	Mississippi	1/19/2016	<20	0.41
HABB160120-007	oyster	Mississippi	1/19/2016	<20	1.22
HABB160120-008	oyster	Mississippi	1/19/2016	<20	0.88
HABB160121-001	oyster	Indian Lagoon	1/20/2016	22.20	9.84
HABB160126-001	oyster	West Bay	1/25/2016	30.18	9.37
HABB160126-002	oyster	West Bay	1/25/2016	16.69	2.82
HABB160127-001	oyster	Alabama	1/25/2016	<20	3.17
HABB160127-002	oyster	Alabama	1/25/2016	<20	2.23
HABB160127-003	oyster	Alabama	1/25/2016	<20	3.11
HABB160127-004	oyster	Alabama	1/25/2016	<20	0.36
HABB160127-005	oyster	Alabama	1/25/2016	<20	0.42
HABB160128-001	oyster	East Bay	1/27/2016	<20	3.00
HABB160202-001	oyster	West Bay	2/1/2016	29.32	5.96
HABB160203-001	oyster	St. Joseph Bay	2/2/2016	28.40	14.20
HABB160203-002	oyster	Louisiana	2/2/2016	<20	0.29
HABB160203-003	oyster	Louisiana	2/2/2016	<20	0.77
HABB160203-004	oyster	Louisiana	2/2/2016	<20	0.84
HABB160203-005	oyster	Louisiana	2/2/2016	<20	1.08
HABB160203-006	oyster	Louisiana	2/2/2016	<20	0.33
HABB160203-007	oyster	Louisiana	2/2/2016	<20	0.29
HABB160204-001	oyster	Indian Lagoon	2/2/2016	<20	4.22
HABB160211-001	oyster	West Bay	2/10/2016	<20	5.56
HABB160223-001	oyster	Pine Island Sound	2/22/2016	31.66	6.77
HABB160223-005	oyster	St. Joseph Bay	2/22/2016	<20	12.37
HABB160224-001	oyster	Pine Island Sound	2/23/2016	<20	0.94
HABB160301-001	oyster	Alabama	2/29/2016	<20	1.72
HABB160302-001	oyster	Pine Island Sound	3/1/2016	<20	4.02
HABB160303-002	oyster	Gasparilla Sound	3/2/2016	19.81	5.07
HABB160308-001	oyster	Lower Tampa Bay	3/7/2016	23.53	10.51
HABB160309-001	oyster	Choctawhatchee Bay	3/8/2016	<20	0.60
HABB160317-001	oyster	Pine Island Sound	3/16/2016	25.90	3.87
HABB160317-002	oyster	Pine Island Sound	3/16/2016	<20	3.03
HABB160322-001	oyster	Lower Tampa Bay	3/22/2016	<20	4.33
HABB160328-002	oyster	Lower Tampa Bay	3/28/2016	<20	4.87
HABB160330-001	oyster	Pine Island Sound	3/29/2016	26.26	4.88
HABB160330-002	oyster	Pine Island Sound	3/29/2016	<20	2.19

HABB160407-002	oyster	Lower Tampa Bay	4/6/2016	<20	3.99
HABB160407-004	oyster	Pine Island Sound	4/7/2016	<20	3.00
HABB160411-013	oyster	Lower Tampa Bay	4/11/2016	<20	3.83
HABB160418-002	oyster	Lower Tampa Bay	4/18/2016	<20	2.76
HABB160421-002	oyster	Pine Island Sound	4/20/2016	23.66	3.01
HABB160421-003	oyster	Pine Island Sound	4/20/2016	<20	1.71
HABB160427-001	oyster	Pine Island Sound	4/26/2016	<20	3.37
HABB160427-002	oyster	Pine Island Sound	4/26/2016	<20	1.71
HABB160502-001	oyster	Boca Ceiga Bay	5/2/2016	21.65	4.59
HABB160505-001	oyster	Gasparilla Sound	5/4/2016	<20	2.70
HABB160505-002	oyster	Gasparilla Sound	5/4/2016	<20	1.67
HABB160510-001	oyster	Boca Ceiga Bay	5/10/2016	16.23	4.11
HABB161011-002	oyster	Lower Tampa Bay	10/10/2016	<20	0.74
HABB161018-002	oyster	Lower Tampa Bay	10/17/2016	<20	1.57
HABB161114-002	oyster	Lower Tampa Bay	11/14/2016	156.08	47.60
HABB170104-003	oyster	Pine Island Sound	1/3/2017	30.23	9.64
HABB170105-001	oyster	Lower Tampa Bay	1/4/2017	<20	2.31
HABB170110-001	oyster	Lower Tampa Bay	1/9/2017	<20	0.84
HABB170110-004	oyster	Gasparilla Sound	1/9/2017	28.32	8.43
HABB170111-001	oyster	Ten Thousand Islands	1/10/2017	19.63	3.14
HABB170111-002	oyster	Matlacha Pass	1/10/2017	<20	1.58
HABB170111-003	oyster	Pine Island Sound	1/10/2017	30.71	7.37
HABB170118-002	oyster	Gasparilla Sound	1/17/2017	29.46	6.65
HABB170119-003	oyster	Pine Island Sound	1/18/2017	33.87	5.64
HABB170119-004	oyster	Myakka River	1/18/2017	31.00	4.56
HABB170125-001	oyster	Gasparilla Sound	1/24/2017	<20	4.06
HABB170125-003	oyster	Pine Island Sound	1/24/2017	<20	4.31
HABB170131-002	oyster	Gasparilla Sound	1/30/2017	36.73	9.68
HABB170201-002	oyster	Myakka River	1/31/2017	22.45	3.56
HABB170207-002	oyster	Gasparilla Sound	2/6/2017	31.32	8.12
HABB170213-002	oyster	Lower Tampa Bay	2/13/2017	<20	1.47
HABB170214-004	oyster	Pine Island Sound	2/13/2017	<20	2.01
HABB170221-001	oyster	Myakka River	2/20/2017	<20	2.08
HABB170222-001	oyster	Gasparilla Sound	2/21/2017	42.30	10.51
HABB170307-002	oyster	Gasparilla Sound	3/6/2017	29.03	5.11
HABB170314-002	oyster	Gasparilla Sound	3/13/2017	<20	2.55
HABB170315-002	oyster	Lower Tampa Bay	3/14/2017	<20	2.21
HABB170322-002	oyster	Gasparilla Sound	3/21/2017	<20	2.49
HABB170405-001	oyster	Boca Ceiga Bay	4/4/2017	31.35	6.80
HABB170410-005	oyster	Gasparilla Sound	4/10/2017	<20	1.23
HABB170412-001	oyster	Pine Island Sound	4/11/2017	25.73	3.56
HABB170418-001	oyster	Pine Island Sound	4/17/2017	19.01	2.35
HABB170419-001	oyster	Lower Tampa Bay	4/18/2017	<20	5.89
HABB170419-002	oyster	Lower Tampa Bay	4/18/2017	<20	3.72
HABB170425-001	oyster	Gasparilla Sound	4/24/2017	25.81	4.13
HABB170425-002	oyster	Gasparilla Sound	4/24/2017	34.91	8.27
HABB080108-001	hard clam	Volusia County	1/7/2008	<20	0.97
HABB080108-002	hard clam	Volusia County	1/7/2008	<20	0.77
HABB080108-003	hard clam	Mosquito Lagoon	1/7/2008	52.8	4.2
HABB080109-003	hard clam	North Indian River	1/8/2008	<20	2.69
HABB080109-004	hard clam	Indian River Body F	1/8/2008	<20	0.14
HABB080115-001	hard clam	Mosquito Lagoon	1/14/2008	46.26	4

HABB080115-002	hard clam	Indian River Body A	1/14/2008	<20	1.18
HABB080115-003	hard clam	Indian River Body A	1/14/2008	38.66	4.44
HABB080123-022	hard clam	St. Lucie County	1/22/2008	<20	0.93
HABB080123-023	hard clam	Mosquito Lagoon	1/22/2008	<20	3.05
HABB080123-024	hard clam	Indian River Body A	1/22/2008	<20	2.35
HABB080123-025	hard clam	Indian River Body B	1/22/2008	<20	1.16
HABB090519-001	hard clam	Indian River Body F	5/18/2009	<20	<LOD
HABB091109-001	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-002	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-003	hard clam	Pine Island Sound	11/9/2009	<20	<LOD
HABB091109-004	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB100105-002	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-003	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-004	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-005	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100112-001	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-002	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100118-001	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-002	hard clam	Pine Island Sound	1/18/2010	<20	0.06
HABB100118-003	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-004	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB111011-001	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-002	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-003	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-004	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111018-001	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-002	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-003	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-004	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111024-001	hard clam	Pine Island Sound	10/23/2011	<20	<LOD
HABB111122-001	hard clam	Gasparilla Sound	11/21/2011	<20	4.13
HABB111206-001	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111206-002	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111213-003	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB111213-004	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB120104-001	hard clam	Pine Island Sound	1/4/2012	<20	0.63
HABB120104-002	hard clam	Pine Island Sound	1/4/2012	<20	0.66
HABB120109-001	hard clam	Pine Island Sound	1/9/2012	<20	0.63
HABB120109-002	hard clam	Pine Island Sound	1/9/2012	<20	0.48
HABB120117-001	hard clam	Pine Island Sound	1/16/2012	<20	0.24
HABB120117-002	hard clam	Pine Island Sound	1/16/2012	<20	0.23
HABB120124-001	hard clam	Pine Island Sound	1/23/2012	<20	0.14
HABB120124-002	hard clam	Pine Island Sound	1/23/2012	<20	0.13
HABB120131-003	hard clam	Ten Thousand Islands	1/25/2012	<20	1.39
HABB120131-004	hard clam	Ten Thousand Islands	1/25/2012	<20	1.49
HABB121002-001	hard clam	Gasparilla Sound	10/1/2012	37.63	12.68
HABB121002-002	hard clam	Gasparilla Sound	10/1/2012	<20	0.25
HABB121003-001	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121003-002	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121009-001	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-002	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-003	hard clam	Pine Island Sound	10/8/2012	<20	<LOD

HABB121009-004	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-005	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-006	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121016-001	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-002	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-003	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-004	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-005	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-006	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121017-001	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121017-002	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121023-005	hard clam	Pine Island Sound	10/22/2012	<20	0.28
HABB121023-006	hard clam	Pine Island Sound	10/22/2012	<20	0.26
HABB121023-007	hard clam	Pine Island Sound	10/22/2012	<20	0.18
HABB121023-008	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-009	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-010	hard clam	Pine Island Sound	10/22/2012	<20	0.22
HABB121024-001	hard clam	Lower Tampa Bay	10/23/2012	<20	0.92
HABB121024-002	hard clam	Lower Tampa Bay	10/23/2012	<20	1.05
HABB121024-003	hard clam	Lower Tampa Bay	10/23/2012	<20	0.7
HABB121024-004	hard clam	Lower Tampa Bay	10/23/2012	<20	0.66
Habb121024-005	hard clam	Pine Island Sound	10/23/2012	<20	0.18
HABB121024-006	hard clam	Pine Island Sound	10/23/2012	<20	0.23
HABB121030-001	hard clam	Lower Tampa Bay	10/29/2012	<20	0.5
HABB121030-002	hard clam	Lower Tampa Bay	10/29/2012	<20	0.34
HABB121030-003	hard clam	Pine Island Sound	10/29/2012	<20	1.2
HABB121030-004	hard clam	Pine Island Sound	10/29/2012	<20	0.88
HABB121113-001	hard clam	Lower Tampa Bay	11/6/2012	<20	1.78
HABB130212-003	hard clam	Lower Tampa Bay	11/14/2012	<20	<LOD
HABB121120-001	hard clam	Pine Island Sound	11/19/2012	<20	2.16
HABB121127-001	hard clam	Sarasota Bay	11/26/2012	<20	0.7
HABB121127-002	hard clam	Pine Island Sound	11/26/2012	<20	0.88
HABB121127-003	hard clam	Pine Island Sound	11/26/2012	<20	2.01
HABB121127-004	hard clam	Pine Island Sound	11/26/2012	<20	1.82
HABB121211-001	hard clam	Pine Island Sound	12/10/2012	<20	0.63
HABB121211-002	hard clam	Pine Island Sound	12/10/2012	<20	0.52
HABB121211-003	hard clam	Pine Island Sound	12/10/2012	<20	1.01
HABB121211-004	hard clam	Pine Island Sound	12/10/2012	<20	1.31
HABB121218-001	hard clam	Pine Island Sound	12/17/2012	<20	1.19
HABB121218-002	hard clam	Pine Island Sound	12/17/2012	<20	5.6
HABB121218-003	hard clam	Pine Island Sound	12/17/2012	<20	0.86
HABB121218-004	hard clam	Pine Island Sound	12/17/2012	<20	0.99
HABB121218-005	hard clam	Pine Island Sound	12/17/2012	<20	0.58
HABB121218-006	hard clam	Pine Island Sound	12/17/2012	<20	0.5
HABB121218-007	hard clam	Lower Tampa Bay	12/18/2012	<20	2.01
HABB121218-008	hard clam	Lower Tampa Bay	12/18/2012	<20	2.34
HABB121227-026	hard clam	Lower Tampa Bay	12/26/2012	23.59	3
HABB121227-027	hard clam	Lower Tampa Bay	12/26/2012	22.19	2.34
HABB121227-028	hard clam	Pine Island Sound	12/26/2012	<20	0.45
HABB121227-029	hard clam	Pine Island Sound	12/26/2012	<20	0.44
HABB130103-001	hard clam	Pine Island Sound	1/2/2013	<20	0.74
HABB130103-002	hard clam	Pine Island Sound	1/2/2013	<20	0.82

HABB130103-003	hard clam	Pine Island Sound	1/2/2013	22.09	2.18
HABB130103-004	hard clam	Pine Island Sound	1/2/2013	21.64	2.45
HABB130103-005	hard clam	Pine Island Sound	1/2/2013	<20	0.66
HABB130103-006	hard clam	Pine Island Sound	1/2/2013	<20	0.87
HABB130108-001	hard clam	Pine Island Sound	1/7/2013	<20	0.72
HABB130108-002	hard clam	Pine Island Sound	1/7/2013	<20	0.85
HABB130108-003	hard clam	Pine Island Sound	1/7/2013	<20	1.09
HABB130108-004	hard clam	Pine Island Sound	1/7/2013	<20	0.83
HABB130109-001	hard clam	Lower Tampa Bay	1/8/2013	20.2	4.38
HABB130109-002	hard clam	Lower Tampa Bay	1/8/2013	<20	1.96
HABB130109-003	hard clam	Lower Tampa Bay	1/8/2013	<20	1.51
HABB130115-003	hard clam	Pine Island Sound	1/14/2013	<20	1.07
HABB130115-004	hard clam	Pine Island Sound	1/14/2013	<20	1.74
HABB130122-001	hard clam	Lower Tampa Bay	1/22/2013	<20	1.57
HABB130122-002	hard clam	Lower Tampa Bay	1/22/2013	<20	1.54
HABB130130-001	hard clam	Lower Tampa Bay	1/28/2013	<20	1.8
HABB130130-002	hard clam	Lower Tampa Bay	1/28/2013	<20	1.82
HABB130205-001	hard clam	Lower Tampa Bay	2/4/2013	<20	1.41
HABB130205-002	hard clam	Lower Tampa Bay	2/4/2013	<20	1.44
HABB130212-001	hard clam	Pine Island Sound	2/11/2013	21.01	4.16
HABB130212-005	hard clam	Pine Island Sound	2/11/2013	29.23	5.68
HABB130226-002	hard clam	Pine Island Sound	2/24/2013	49.23	8.44
HABB130226-003	hard clam	Pine Island Sound	2/24/2013	44.71	8.37
HABB130226-004	hard clam	Pine Island Sound	2/24/2013	84.59	16.18
HABB130226-005	hard clam	Pine Island Sound	2/24/2013	39.34	9.89
HABB130226-006	hard clam	Pine Island Sound	2/24/2013	38.23	4.83
HABB130226-007	hard clam	Pine Island Sound	2/24/2013	27.18	4.82
HABB130226-008	hard clam	Pine Island Sound	2/24/2013	68.19	7.04
HABB130226-009	hard clam	Pine Island Sound	2/24/2013	<20	2.55
HABB130226-010	hard clam	Pine Island Sound	2/24/2013	44.16	6.33
HABB151007-002	hard clam	Pine Island Sound	2/25/2013	92.65	9.84
HABB130306-005	hard clam	Pine Island Sound	3/4/2013	<20	4.57
HABB130319-006	hard clam	Pine Island Sound	3/8/2013	<20	2.81
HABB130312-004	hard clam	Pine Island Sound	3/11/2013	205.34	37.33
HABB130312-005	hard clam	Pine Island Sound	3/11/2013	24.95	3.87
HABB130312-006	hard clam	Pine Island Sound	3/11/2013	<20	2.51
HABB130312-007	hard clam	Pine Island Sound	3/11/2013	<20	2.39
HABB130313-007	hard clam	Pine Island Sound	3/11/2013	36.89	3.26
HABB130313-008	hard clam	Pine Island Sound	3/11/2013	<20	1.73
HABB130313-001	hard clam	Pine Island Sound	3/12/2013	<20	1.93
HABB130313-002	hard clam	Pine Island Sound	3/12/2013	<20	2.46
HABB130313-003	hard clam	Pine Island Sound	3/12/2013	<20	2.47
HABB130313-004	hard clam	Pine Island Sound	3/12/2013	<20	2.35
HABB130319-007	hard clam	Pine Island Sound	3/13/2013	<20	2.24
HABB130319-004	hard clam	Pine Island Sound	3/18/2013	<20	2.14
HABB130319-005	hard clam	Pine Island Sound	3/18/2013	<20	4.2
HABB130319-012	hard clam	Pine Island Sound	3/18/2013	22.55	2.79
HABB140725-001	hard clam	Ten Thousand Islands	3/20/2013	<20	3.89
HABB130326-003	hard clam	Pine Island Sound	3/25/2013	<20	1.58
HABB130326-004	hard clam	Pine Island Sound	3/25/2013	<20	1.39
HABB130326-005	hard clam	Pine Island Sound	3/25/2013	<20	1.71
HABB130326-006	hard clam	Pine Island Sound	3/25/2013	<20	1.65

HABB130326-009	hard clam	Pine Island Sound	3/25/2013	<20	1.57
HABB130326-010	hard clam	Pine Island Sound	3/25/2013	<20	1.62
HABB130326-011	hard clam	Pine Island Sound	3/25/2013	<20	1.47
HABB130326-012	hard clam	Pine Island Sound	3/25/2013	<20	1.42
HABB130326-013	hard clam	Gasparilla Sound	3/25/2013	84.16	16.89
HABB130326-014	hard clam	Pine Island Sound	3/25/2013	75.9	16.4
HABB130403-002	hard clam	Pine Island Sound	3/29/2013	<20	2.23
HABB130402-001	hard clam	Pine Island Sound	4/1/2013	<20	2.05
HABB130402-002	hard clam	Pine Island Sound	4/1/2013	<20	1.98
HABB130402-003	hard clam	Pine Island Sound	4/1/2013	25.2	3.5
HABB130402-004	hard clam	Pine Island Sound	4/1/2013	24.3	2.92
HABB130402-005	hard clam	Pine Island Sound	4/1/2013	<20	1.4
HABB130402-006	hard clam	Pine Island Sound	4/1/2013	<20	1.27
HABB130402-007	hard clam	Pine Island Sound	4/1/2013	<20	1.55
HABB130402-008	hard clam	Pine Island Sound	4/1/2013	<20	3.51
HABB130402-009	hard clam	Pine Island Sound	4/1/2013	<20	3.27
HABB130409-003	hard clam	Pine Island Sound	4/8/2013	<20	0.97
HABB130409-004	hard clam	Pine Island Sound	4/8/2013	<20	1.17
HABB130409-008	hard clam	Pine Island Sound	4/8/2013	<20	1.81
HABB130409-009	hard clam	Pine Island Sound	4/8/2013	<20	1.09
HABB130409-010	hard clam	Pine Island Sound	4/8/2013	<20	0.85
HABB130409-011	hard clam	Pine Island Sound	4/8/2013	<20	3.82
HABB130409-012	hard clam	Pine Island Sound	4/8/2013	<20	4.12
HABB130409-013	hard clam	Pine Island Sound	4/8/2013	<20	3.81
HABB130409-014	hard clam	Pine Island Sound	4/8/2013	35.6	4.29
HABB130409-015	hard clam	Pine Island Sound	4/8/2013	<20	1.69
HABB130409-016	hard clam	Pine Island Sound	4/8/2013	<20	1.52
HABB130410-001	hard clam	Pine Island Sound	4/9/2013	<20	1.82
HABB130410-002	hard clam	Pine Island Sound	4/9/2013	<20	1.91
HABB130410-003	hard clam	Pine Island Sound	4/9/2013	<20	1.69
HABB130416-006	hard clam	Pine Island Sound	4/15/2013	<20	0.83
HABB130416-007	hard clam	Pine Island Sound	4/15/2013	<20	0.81
HABB130417-001	hard clam	Pine Island Sound	4/16/2013	<20	1.09
HABB130417-002	hard clam	Pine Island Sound	4/16/2013	<20	1.24
HABB130417-004	hard clam	Pine Island Sound	4/16/2013	<20	1.37
HABB130417-005	hard clam	Pine Island Sound	4/16/2013	<20	1.28
HABB130423-001	hard clam	Pine Island Sound	4/22/2013	<20	1.02
HABB130423-002	hard clam	Pine Island Sound	4/22/2013	<20	1.06
HABB130423-003	hard clam	Pine Island Sound	4/22/2013	<20	0.98
HABB130424-001	hard clam	Pine Island Sound	4/24/2013	<20	0.93
HABB130424-002	hard clam	Pine Island Sound	4/24/2013	<20	1
HABB130424-003	hard clam	Pine Island Sound	4/24/2013	<20	0.86
HABB130508-004	hard clam	Lemon Bay	5/7/2013	<20	17.33
HABB131113-001	hard clam	Pine Island Sound	11/12/2013	<20	0.5
HABB131113-002	hard clam	Pine Island Sound	11/12/2013	<20	0.32
HABB131113-003	hard clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-006	hard clam	Pine Island Sound	11/12/2013	<20	0.41
HABB131113-007	hard clam	Pine Island Sound	11/12/2013	<20	0.38
HABB131113-008	hard clam	Pine Island Sound	11/12/2013	<20	0.44
HABB131119-001	hard clam	Pine Island Sound	11/18/2013	<20	1.96
HABB131119-002	hard clam	Pine Island Sound	11/18/2013	<20	1.71
HABB131119-003	hard clam	Pine Island Sound	11/18/2013	<20	1.78

HABB131126-001	hard clam	Pine Island Sound	11/25/2013	<20	0.3
HABB131126-002	hard clam	Pine Island Sound	11/25/2013	<20	0.29
HABB131126-003	hard clam	Pine Island Sound	11/25/2013	<20	0.28
HABB131126-004	hard clam	Pine Island Sound	11/25/2013	<20	0.59
HABB131126-005	hard clam	Pine Island Sound	11/25/2013	<20	0.69
HABB131126-006	hard clam	Pine Island Sound	11/25/2013	<20	0.68
HABB131203-001	hard clam	Pine Island Sound	12/2/2013	<20	0.23
HABB131203-002	hard clam	Pine Island Sound	12/2/2013	<20	0.24
HABB131203-003	hard clam	Pine Island Sound	12/2/2013	<20	0.21
HABB131203-004	hard clam	Pine Island Sound	12/2/2013	<20	0.31
HABB131203-005	hard clam	Pine Island Sound	12/2/2013	<20	0.33
HABB131203-006	hard clam	Pine Island Sound	12/2/2013	<20	0.38
HABB131210-004	hard clam	Pine Island Sound	12/9/2013	<20	0.35
HABB131210-005	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131210-006	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131211-012	hard clam	Gasparilla Sound	12/10/2013	<20	0.84
HABB131218-010	hard clam	Gasparilla Sound	12/17/2013	36.91	8.96
HABB141014-001	hard clam	Cedar Key	10/13/2014	<20	0.33
HABB141014-002	hard clam	Cedar Key	10/13/2014	<20	0.31
HABB141014-003	hard clam	Cedar Key	10/13/2014	<20	0.42
HABB141113-002	hard clam	Pine Island Sound	11/12/2014	<20	0.34
HABB141113-003	hard clam	Pine Island Sound	11/12/2014	<20	0.44
HABB141113-004	hard clam	Pine Island Sound	11/12/2014	<20	0.69
HABB141113-005	hard clam	Pine Island Sound	11/12/2014	<20	0.7
HABB141113-006	hard clam	Pine Island Sound	11/12/2014	<20	0.66
HABB141113-007	hard clam	Pine Island Sound	11/12/2014	<20	0.62
HABB141119-001	hard clam	Pine Island Sound	11/18/2014	<20	0.15
HABB141119-002	hard clam	Pine Island Sound	11/18/2014	<20	0.13
HABB141119-003	hard clam	Pine Island Sound	11/18/2014	<20	0.2
HABB141119-004	hard clam	Pine Island Sound	11/18/2014	<20	0.18
HABB141119-005	hard clam	Pine Island Sound	11/18/2014	<20	0.23
HABB141119-006	hard clam	Pine Island Sound	11/18/2014	<20	0.25
HABB141124-001	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-002	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-003	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB160202-002	hard clam	Pine Island Sound	2/1/2016	<20	0.92
HABB160209-017	hard clam	Gasparilla Sound	2/8/2016	76.77	10.82
HABB160209-018	hard clam	Gasparilla Sound	2/8/2016	42.61	9.68
HABB160209-019	hard clam	Gasparilla Sound	2/8/2016	85.99	10
HABB160223-003	hard clam	Pine Island Sound	2/22/2016	<20	0.44
HABB160301-002	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-003	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-004	hard clam	Pine Island Sound	2/29/2016	<20	0.33
HABB160301-005	hard clam	Pine Island Sound	2/29/2016	<20	0.37
HABB160302-002	hard clam	Pine Island Sound	3/1/2016	<20	0.6
HABB160302-003	hard clam	Pine Island Sound	3/1/2016	<20	0.65
HABB160308-002	hard clam	Lower Tampa Bay	3/7/2016	40.05	6.21
HABB160322-002	hard clam	Lower Tampa Bay	3/22/2016	25	5.12
HABB160328-001	hard clam	Lower Tampa Bay	3/28/2016	35.83	4.9
HABB160407-001	hard clam	Lower Tampa Bay	4/6/2016	29.59	4.36
HABB160407-003	hard clam	Pine Island Sound	4/7/2016	<20	0.5
HABB160411-012	hard clam	Lower Tampa Bay	4/11/2016	<20	1.36

HABB160418-001	hard clam	Lower Tampa Bay	4/18/2016	<20	1.76
HABB160601-001	hard clam	Lemon Bay	5/31/2016	<20	0.43
HABB161011-001	hard clam	Lower Tampa Bay	10/10/2016	<20	1.16
HABB161013-001	hard clam	Gasparilla Sound	10/12/2016	<20	0.54
HABB161018-001	hard clam	Lower Tampa Bay	10/17/2016	<20	2.07
HABB170104-001	Hard clam	Pine Island Sound	1/3/2017	<20	1.66
HABB170104-002	Hard clam	Pine Island Sound	1/3/2017	<20	1
HABB170105-002	Hard clam	Lower Tampa Bay	1/4/2017	35.96	2.22
HABB170110-002	hard clam	Lower Tampa Bay	1/9/2017	<20	1.58
HABB170110-003	hard clam	Gasparilla Sound	1/9/2017	20.26	2.35
HABB131125-020	hard clam	Composite		<20	3.9
HABB130115-001	sunray venus clam	Pine Island Sound	1/14/2013	<20	1.85
HABB130212-002	sunray venus clam	Pine Island Sound	2/11/2013	34.13	12.04
HABB130212-005	sunray venus clam	Pine Island Sound	2/11/2013	39.09	19.74
HABB130226-001	sunray venus clam	Pine Island Sound	2/24/2013	42.41	15.41
HABB130226-011	sunray venus clam	Pine Island Sound	2/24/2013	<20	5.58
HABB130228-001	sunray venus clam	Pine Island Sound	2/25/2013	32.17	9.93
HABB130227-001	sunray venus clam	Pine Island Sound	2/26/2013	42.9	13.01
HABB130227-002	sunray venus clam	Pine Island Sound	2/26/2013	34.97	19.09
HABB130228-003	sunray venus clam	Pine Island Sound	2/27/2013	27.54	17.94
HABB130319-009	sunray venus clam	Pine Island Sound	3/8/2013	<20	3.13
HABB130312-001	sunray venus clam	Pine Island Sound	3/11/2013	27.65	6.59
HABB130312-002	sunray venus clam	Pine Island Sound	3/11/2013	26.33	7.39
HABB130312-003	sunray venus clam	Pine Island Sound	3/11/2013	28.7	5.16
HABB130312-009	sunray venus clam	Pine Island Sound	3/11/2013	<20	5.38
HABB150921-001	sunray venus clam	Pine Island Sound	3/11/2013	31.33	5.3
HABB130319-010	sunray venus clam	Pine Island Sound	3/13/2013	<20	3.1
HABB130319-001	sunray venus clam	Pine Island Sound	3/18/2013	22.05	4.48
HABB130319-002	sunray venus clam	Pine Island Sound	3/18/2013	20.67	4.28
HABB130319-003	sunray venus clam	Pine Island Sound	3/18/2013	27.85	7.69
HABB130319-011	sunray venus clam	Pine Island Sound	3/18/2013	25.87	5.43
HABB130326-001	sunray venus clam	Pine Island Sound	3/25/2013	23.16	3.48
HABB130326-002	sunray venus clam	Pine Island Sound	3/25/2013	22.36	3.4
HABB130326-007	sunray venus clam	Pine Island Sound	3/25/2013	24.4	4.44
HABB130326-008	sunray venus clam	Pine Island Sound	3/25/2013	22.5	3.35
HABB130409-006	sunray venus clam	Pine Island Sound	4/8/2013	22.84	2.53
HABB130409-020	sunray venus clam	Pine Island Sound	4/8/2013	<20	2.16
HABB130409-021	sunray venus clam	Pine Island Sound	4/8/2013	23.91	2.69
HABB130410-004	sunray venus clam	Pine Island Sound	4/9/2013	<20	2.18
HABB130410-005	sunray venus clam	Pine Island Sound	4/9/2013	<20	1.84
HABB130416-002	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.47
HABB130416-003	sunray venus clam	Pine Island Sound	4/15/2013	<20	0.99
HABB130416-004	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.48
HABB130417-006	sunray venus clam	Pine Island Sound	4/16/2013	<20	1.62
HABB130604-003	sunray venus clam	Pine Island Sound	6/3/2013	<20	0.56
HABB131113-004	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-005	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.24
HABB131125-019	sunray venus clam	Alligator Harbor	11/22/2013	25.88	4.11
HABB151120-001	sunray venus clam	Sarasota Bay	11/18/2015	33.21	11.05
HABB151120-002	sunray venus clam	Sarasota Bay	11/18/2015	33.58	12.11
HABB151207-001	sunray venus clam	Sarasota Bay	12/7/2015	53.21	14.47
HABB160111-002	Sunray venus clam	Lower Tampa Bay	12/15/2015	33.34	6.37

HABB160111-001	sunray venus clam	Sarasota Bay	1/6/2016	<20	2.77
HABB160202-003	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160202-004	sunray venus clam	Pine Island Sound	2/1/2016	<20	2.74
HABB160202-005	sunray venus clam	Pine Island Sound	2/1/2016	19.77	2.14
HABB160202-006	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160223-004	sunray venus clam	Pine Island Sound	2/22/2016	27.66	2.16
HABB160330-004	sunray venus clam	Lower Tampa Bay	3/16/2016	36.48	3.38
HABB160330-005	sunray venus clam	Lower Tampa Bay	3/16/2016	33.04	3.41
HABB161213-019	sunray venus clam	MML lab exposure	9/6/2016	<20	2.63
HABB161213-021	sunray venus clam	MML lab exposure	12/7/2016	20.66	4.04
HABB161213-022	sunray venus clam	MML exp control	12/7/2016	<20	<LOD

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method

Variance of the data generated from the officially recognized analytical method

Variance of the data generated from the proposed alternative analytical method

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____

Is there a significant difference between the analytical methods Y/N

Comparative data for NSP mouse bioassays and ELISAs cannot be evaluated as described above. Please see below for additional discussion and comparisons permitted by the data presented above.

Brevetoxins in bivalves

At least nine brevetoxin congeners have been isolated from *K. brevis*[1]. PbTx-1 and PbTx-2 are presumed to be the parent toxins from which all other brevetoxins are derived via substitutions on the terminal ring. Consequently, brevetoxins are grouped into two types according to their backbone structure. Brevetoxin A-type (PbTx-1-type) toxins possess a 10-ring backbone, and brevetoxin B-type (PbTx-2-type) toxins possess an 11-ring backbone (Fig. G1). Although brevetoxin A-type toxins are more potent, the brevetoxin B-type toxins are much more abundant[2]. Polar derivatives identified in both culture and bloom materials have further increased the number of known brevetoxin structures[3,4].

In bivalves, the more reactive forms of brevetoxin are rapidly transformed into brevetoxin metabolites[3,5] that are generally the products of reduction, oxidation, and conjugation to other molecules including taurine, cysteine, cysteine sulfoxide, amino acids and fatty acids[5-7]. Literally dozens of metabolites have been identified in shellfish. Most modifications to brevetoxins occur at the side chain on the terminal ether ring that differentiates the brevetoxin congeners, resulting in an assortment of conjugates with either an A-type or B-type of backbone. Brevetoxin metabolites are known to contribute to NSP toxicity [3,6-8], but their individual potency varies. Toxicity information is available for only a small subset of the dozens of characterized metabolites. Some common shellfish metabolites are less potent than parent brevetoxins, while a few have demonstrated higher toxicities [7,9,10]. Different rates of tissue uptake and elimination of brevetoxin metabolites have also been described and may factor into their variable potencies[11].

The complexity of brevetoxins and their metabolic products is the primary reasons that so little progress has been made on moving away from the NSP mouse bioassay. Of the many chemical and biological methods evaluated for measuring brevetoxins in bivalves, those that recognize molecular structure (i.e., ELISAs and liquid chromatography-mass spectroscopy [LC-MS]) have outperformed activity-based assays (i.e., receptor-binding and cytotoxicity assay), demonstrating less variability and better agreement with mouse bioassays[7,12,13].

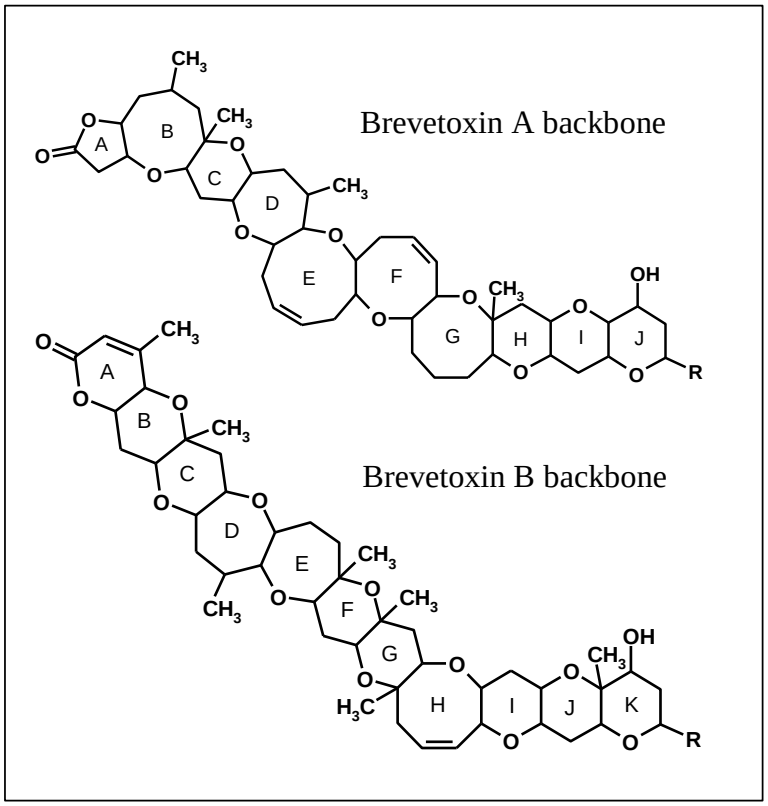


Figure G1. Brevetoxin backbone structures.

An LC-MS method has been developed by the FDA Gulf Coast Seafood Lab and will be submitted to the ISSC for consideration as an alternative to the mouse bioassay. LC-MS can provide confirmation of toxins detected by other assays, and sample throughput is higher compared to the mouse bioassay. However, the large number of brevetoxin metabolites in bivalves will necessitate a targeted approach. For routine analysis as a part of monitoring and management, it is not practical to attempt to identify and quantify them all. Nor is this even possible, given the lack of available standards for almost all metabolites. In the Gulf of Mexico, the most important commercial species are eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*). In oysters, the brevetoxin profile is dominated by the cysteine metabolites S-desoxy-BTX-B2 and BTX-B2[3,5,12]. These were also the major metabolites identified in hard clams, along

with BTX-B1, a taurine conjugate[14,15]. Sunray venus clams (*Macrocallista nimbosa*), a relatively new aquaculture product gaining popularity in Florida, have been less well-studied, but analyses thus far indicate that this species metabolizes brevetoxins similarly to hard clams (Fig. G2), with the cysteine and taurine conjugates representing the major metabolites (Fig. G3).

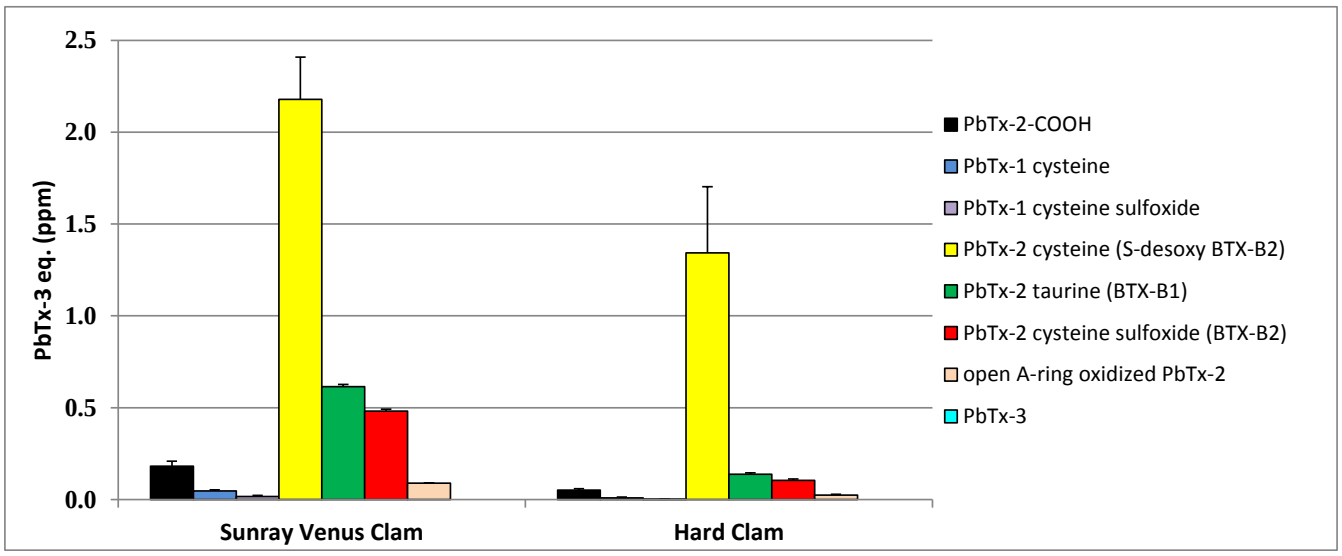


Figure G2. Brevetoxin metabolites identified by LC-MS in laboratory-exposed sunray venus and hard clams. (Error bars=standard deviation, n=3. Unpublished data provided by Dr. R. Pierce, Mote Marine Laboratory.)

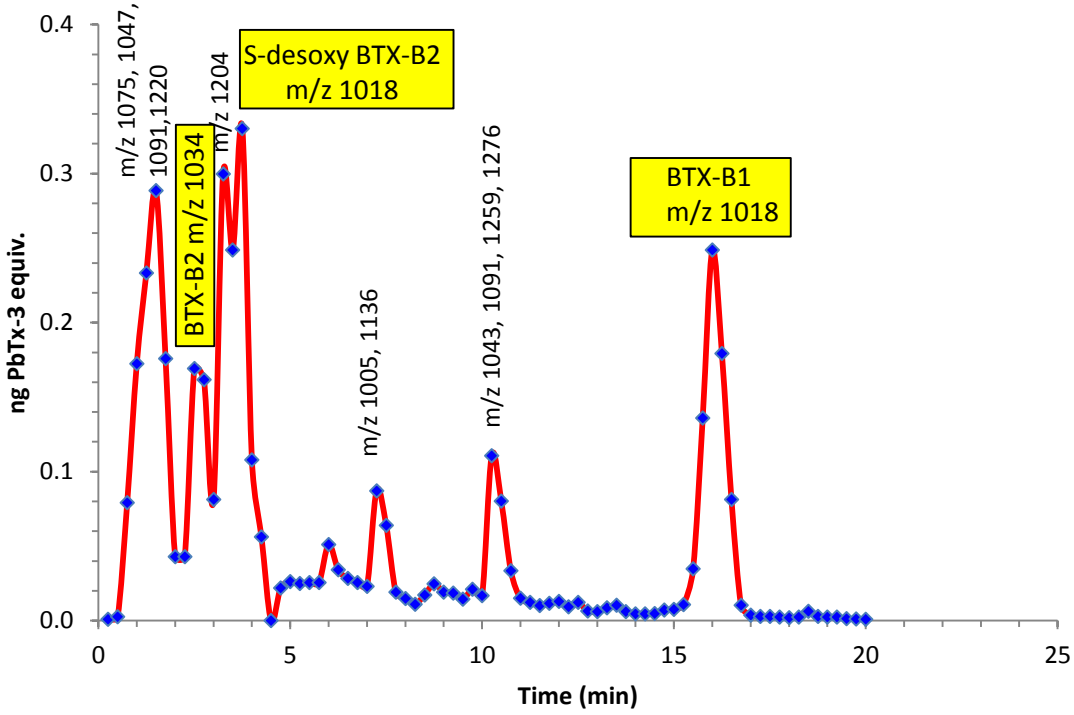


Figure G3. Chromatograms of brevetoxin metabolites in sunray venus clams based on ELISA of LC-fractionated shellfish extracts. (Unpublished data provided by Dr. A. Abraham, USFDA.)

Oral toxicity to mammals has not been assessed for any of the brevetoxin conjugates. Nevertheless, the cysteine and taurine metabolites were found to be excellent biomarkers of composite B-type brevetoxins as determined by ELISA for these species[12,14]. Based on these studies, the FDA's LC-MS protocol targets these three metabolites as biomarkers for NSP toxicity in oysters and clams.

LC-MS analyses require expensive instrumentation and highly technical expertise and are further limited by the time required for each sample to run. Where high throughput is required, the speed and cost-effectiveness of ELISA makes it a more attractive screening method.

MARBIONC Brevetoxin Competitive ELISA

The MARBIONC ELISA kit used in this method validation is the same kit that was used in the method comparisons and bivalve studies cited above. The method is based on the activity of anti-brevetoxin goat polyclonal antibodies, which were produced using a PbTx-3-KLH (keyhole limpet hemocyanin) conjugate[16]. The recognition epitope is believed to include the last four rings (excluding the side chain) of the brevetoxin B type toxins[17,18] (Fig. G4). This specific region is maintained in all brevetoxin B type toxins including in the secondary metabolites identified thus far. However, cross-reactivity of these antibodies have only been assessed for a few metabolites.

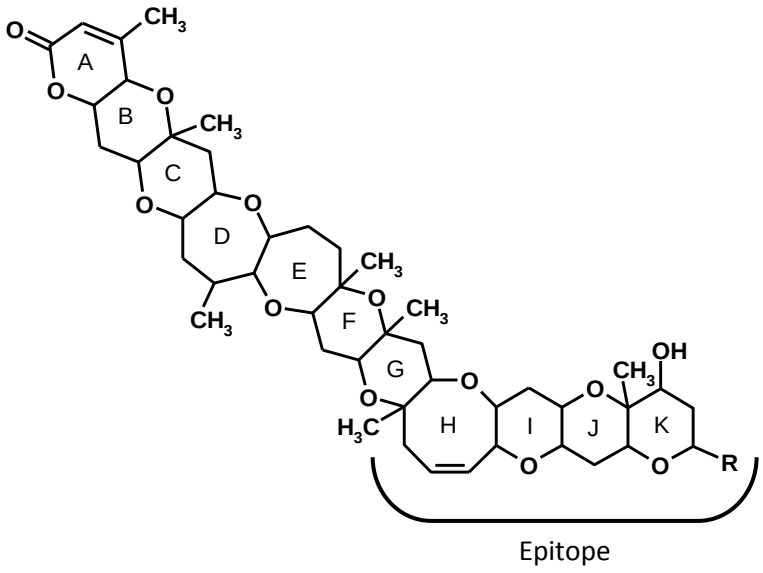


Figure G4. Brevetoxin B backbone with recognition epitope of anti-brevetoxin goat polyclonal antibodies

When this ELISA method was originally published, similar cross-reactivities were reported for PbTx-2, PbTx-3 and PbTx-9, which all share the B-type backbone[18] (Fig G5). MARBIONC reports cross-reactivities of 100% for PbTx-3, 97% for PbTx-2, 105% for oxidized-PbTx-2, and 7% for PbTx-1 at 10 ng/mL (Fig. G6).

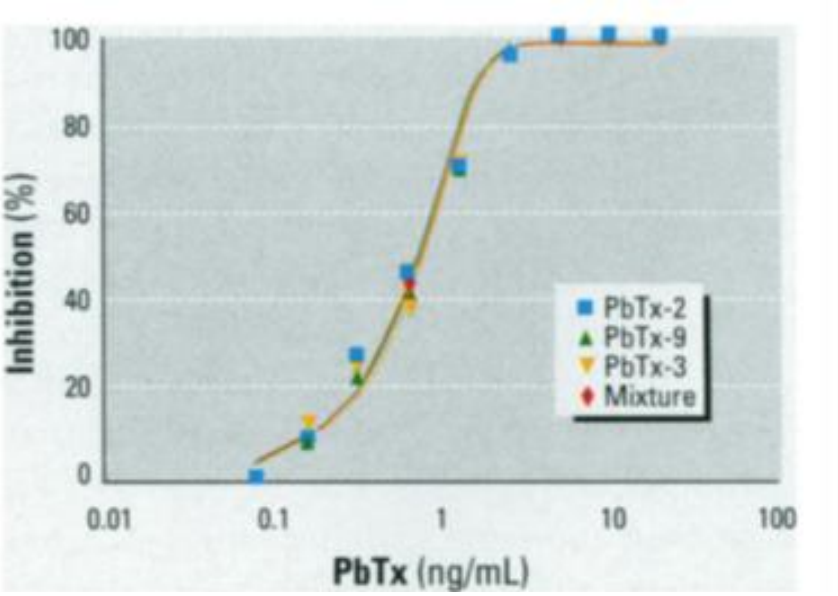


Figure G5. Figure taken from Naar et al.[18]. Anti-brevetoxin antibody cross-reaction with PbTx-2, PbTx-3, PbTx-9, and a mixture of the three toxins.

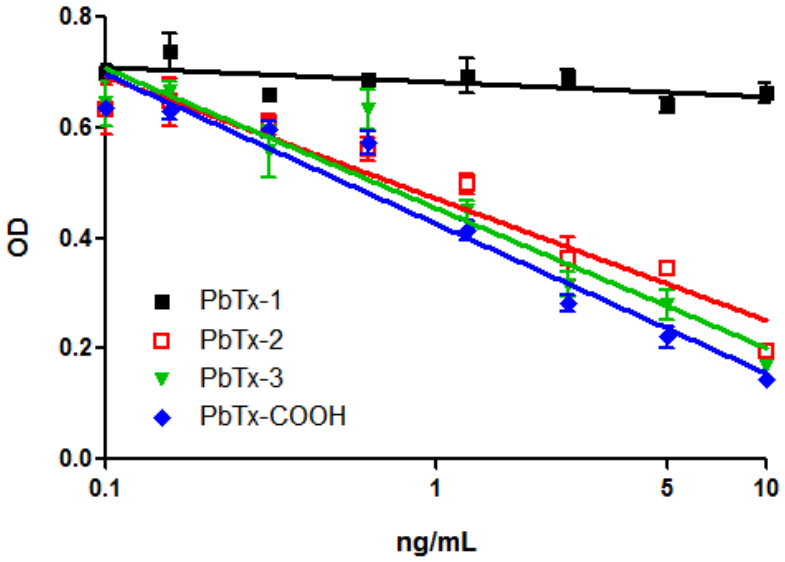


Figure G6. Figure provided by MARBIONC demonstrating degrees of anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2, PbTx-3, and oxidized-PbTx-2 (PbTx-COOH).

Competitive curves generated by L. Flewelling (FWC) are consistent with this, with calculated cross-reactivities (at 50% inhibition) of 97% for PbTx-2 and 2.4% for PbTx-1, relative to PbTx-3 (100%) (Fig. G7).

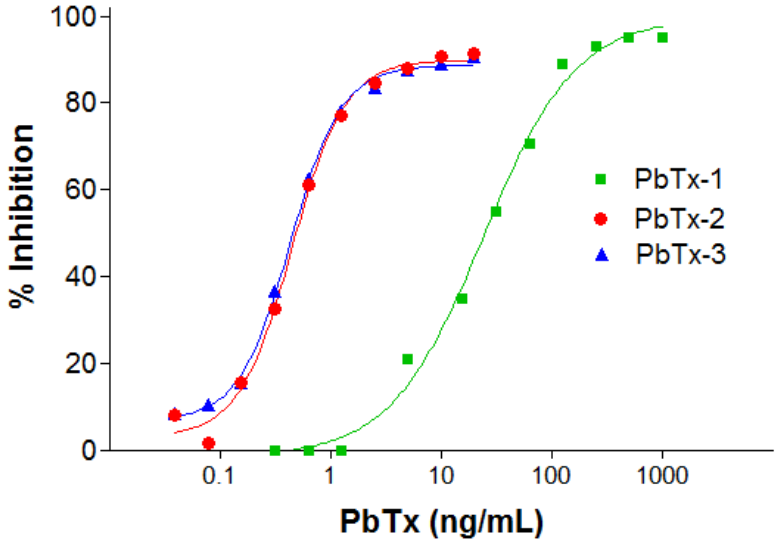


Figure G7. Anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2 and PbTx-3 (FWC data).

We also assessed the cross-reactivity of two shellfish metabolites (Fig. G8). The cross-reactivity of the cysteine conjugate S-desoxy BTX-B2 (provided by the FDA Gulf Coast Seafood Laboratory) was found to be 133% relative to PbTx-3. Cross reactivity of the brevetoxin lipid conjugate N-palmitoyl BTX-B2 (or BTX-B4, described in Bottein et al.[19] and provided by NOAA Center for Coastal Environmental Health and Biomolecular Research) was much lower (2.5%).

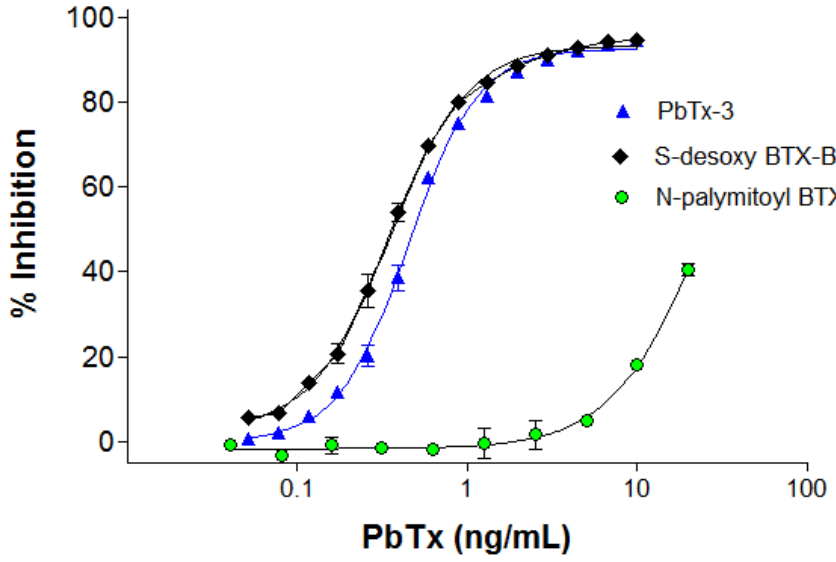


Figure G8. Anti-brevetoxin antibody cross-reaction with B-type brevetoxin metabolites S-desoxy BTX-B2 and N-palymitoyl BTX-B2 compared to PbTx-3 (FWC data).

The relatively low cross-reactivity of the antibodies with PbTx-1 (and presumably PbTx-1-derived conjugates) and with N-palmitoyl BTX-B2 indicates that ELISA results can underestimate of the total amount of brevetoxin and brevetoxin metabolites present in a sample. PbTx-1 is more potent than PbTx-2; however, the parent toxins PbTx-1 and -2 are not found in shellfish, and PbTx-2 type toxins consistently dominate the toxin profile in both *Karenia brevis* cells[20-22] and shellfish[22], typically accounting for 75% or more of the total toxins present. Additionally, although lipid conjugates of brevetoxin are thought to contribute substantially to NSP toxicity, these are derivatives of (and co-occur with) the more abundant amino acid metabolites that dominate the profile of toxic oysters and clams. The ELISA readily detects these forms, which have been identified as excellent biomarkers of NSP toxicity in oysters and clams. Therefore, the limited cross-reactivity of the ELISA with PbTx-1 and with N-palmitoyl BTX-B2 does not diminish the potential for the ELISA to perform successfully within a management program as proposed here.

In recent years, other brevetoxin ELISA kits have been introduced to the market, but prior to implementation into shellfish monitoring each kit would require individual evaluation of antibody cross-reactivity with dominant brevetoxin metabolites and comparisons with currently approved methods.

ELISA vs Mouse Bioassay

Currently, the only approved method for NSP testing is the APHA mouse bioassay[23]. The method is based on the bioassay developed by McFarren et al.[24] more than 50 years ago using toxic shellfish collected during an NSP outbreak in 1963. One mouse unit (MU) is the amount of crude lipid extract that will kill, on average, 50% of 20-g test mice in 15.5 hours. It is important to note that this method has never been validated, and the guidance limit used today (20 MU per 100g) is not based on any toxicological studies, but rather was described as the level of sensitivity of the test for 20g mice observed for 6 hours, which was deemed to be the longest reasonable observation time for the sake of accuracy and expediency. This guidance limit has proven to be effective, as no cases of NSP from legally harvested shellfish have been documented in Florida since the monitoring program began in the 1970's.

Comparing NSP mouse bioassay and ELISA data is not straightforward. The assays measure NSP toxins in very different ways. The mouse bioassay assesses toxicity by measuring the response of mice injected with a crude lipid extract of shellfish. This extract, prepared by repeated partitioning of acidified shellfish homogenate with diethyl ether, contains only a subset of the toxins present[7,25,26]. The method is semi-quantitative, yielding numerical results only at values ≥20 MU per 100g. Conversely, the ELISA is much more sensitive and yields continuous data to much lower concentrations, quantifying (relative to PbTx-3) a more comprehensive collection of brevetoxins and metabolites (regardless of potency) using antibodies that recognize a portion of the brevetoxin B-type backbone structure. Given that the NSP mouse bioassay measures only a subset of the toxins present, is semi-quantitative, has never been appropriately validated, and is not calibrated against known brevetoxin concentrations, a robust agreement of numerical results is unlikely to be achieved by any method.

At present, there is no validated brevetoxin equivalent of 'mouse units' in shellfish. Early work by Baden and Mende [27] established the toxicity of purified PbTx-2 and -3 dissolved in saline to mice intraperitoneally and calculated an LD50 (amount of toxin that kills half of the mice in 24 hours) of 0.2 mg/kg (similar for both toxins). This dose was used to derive a PbTx-2 "equivalent" of 4 µg per 20g-mouse and has since been extended to estimate the brevetoxin concentration in shellfish with a measured toxicity of 20 MU per 100g as 0.8 mg PbTx-2 equivalents per kg shellfish [13,16]. This number appears in several guidance documents; however**,** the extensive metabolism of brevetoxins in shellfish was unknown when the estimated equivalence was first proposed. We now know that shellfish exposed to *K. brevis* blooms contain a mixture of toxins with a multiplicity of potencies. In many cases the metabolites are less toxic, but in some cases they are more toxic. For these reasons, the use of this equivalent for brevetoxins in shellfish is inappropriate and has been acknowledged to be of little use for practical application[7].

Because a biomarker or indicator of toxicity approach is currently necessary for NSP, future NSP guidance limits may vary with the method used and may also vary across shellfish species. An appropriate non-mouse unit guidance limit for brevetoxins in shellfish will provide a level of protection for human health equal to that provided by the existing federal NSP guidance limit of 20 MU per 100 g shellfish. We know from existing data derived from naturally incurred eastern oysters and hard clams that such a limit *as measured using the MARBIONC ELISA with PbTx-3 as a standard* would exceed 0.8 mg per kg shellfish for these species.

Comparison of NSP Mouse Bioassay and ELISA results

Where quantitative results were obtained by both mouse bioassay and ELISA, Spearman rank correlation analysis was used to assess the correlation of brevetoxin concentrations measured by both methods for each shellfish matrix (Table G3). Significant correlations were observed in all cases.

Table G3. Spearman rank correlation coefficients (and p-values) for brevetoxin concentrations measured by NSP bioassay and ELISA

	Spearman rank correlation coefficient	p-value
oysters	0.5590	< 0.0001
hard clams	0.7866	< 0.0001
sunray venus clams	0.6859	< 0.0001

(From this portion on, changes to address early LMC comments are underway, and an updated Appendix G will be submitted.)

Given the differences between the assays and what they measure, strong agreement between numerical results was not expected. Nevertheless, the data were analyzed using linear regression analysis to estimate predicted concentrations by ELISA for samples testing at 20 MU per 100g (Fig. G9). Removal of the outlying (high) mouse bioassay results for oysters (>50MU) and clams (>100MU) that influenced the regression lines lowered the R-squared values, but slopes did not change appreciably. The 20 MU/100 g equivalent by ELISA was estimated to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams.

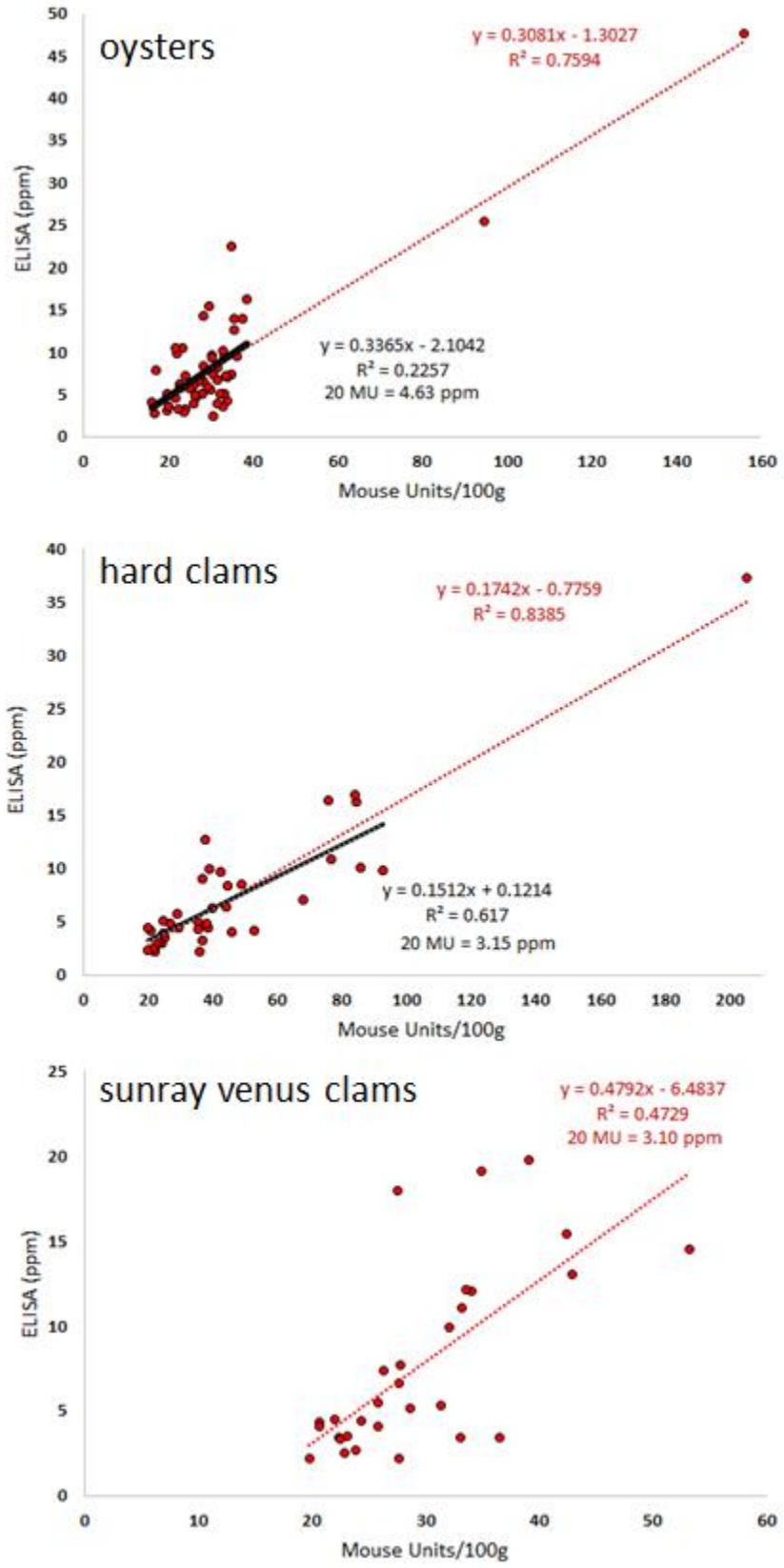


Figure G9. NSP mouse bioassay vs. ELISA results in oyster, hard clams, and sunray venus clams.

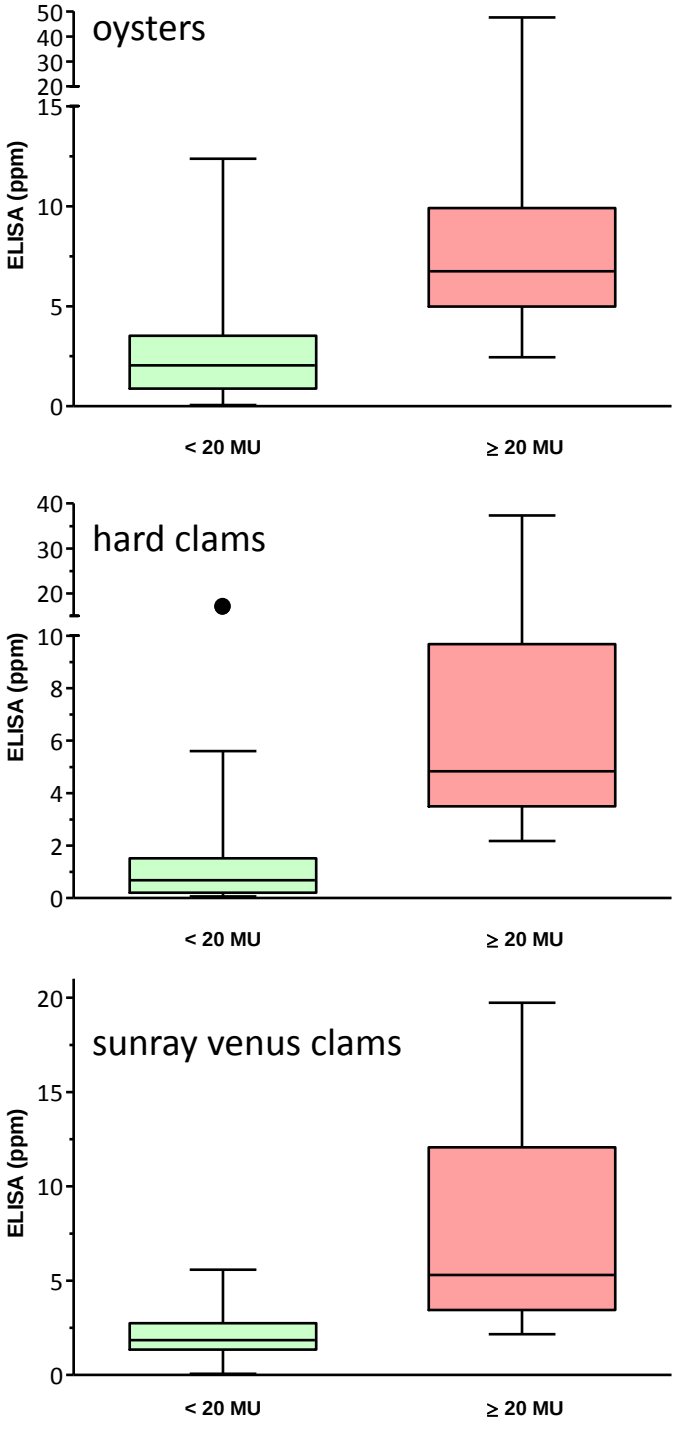


Figure G10. Boxplots (whiskers min to max) of ELISA results for samples testing < 20 MU/100g and ≥ 20 MU/100g in oysters, hard clams, and sunray venus clams. A value of 0.06ppm (half the limit of detection[LOD]) was substituted for ELISA results that were <LOD.

Boxplots were created to visualize the distribution of the data for samples testing < 20 MU/100g and ≥ 20 MU/100g (Fig. G10). There was a very wide range of concentrations measured by ELISA in samples testing < 20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay.

Brevetoxin metabolites are persistent in shellfish, and some level is frequently measured in bivalves from *K. brevis* endemic areas that have tested safe by mouse bioassay. In samples testing <20 MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. The highest concentrations were measured in wild oysters and hard clams, presumably due to repeated exposure to *K. brevis* and retention of the more persisitent metabolites across multiple bloom seasons. Farmed clams are brevetoxin-free when they are placed on lease sites, and their residence time in natural waters is short. These bivalves do not experience multiple successive bloom seasons. The maximum concentration measured in farmed clams that were < 20 MU was 4.6 ppm and in sunray venus clams was 5.6 ppm.

Importantly, across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.

As the only Approved Method, the NSP mouse bioassay is currently the only yardstick to which new methods can be compared. The mouse bioassay is semi-quantitative, not calibrated, and detects only that subset of compounds in shellfish that are ether-extractable. Analytical and screening NSP methods are unlikely to ever completely agree with mouse bioassay results, and expectations for

comparisons of proposed alternate methods with the mouse bioassay should be guaged accordingly, with a goal of achieving an equal measure of safety rather than perfect alignment of results and management actions on a sample by sample basis.

The results of our Single Lab Validation demonstrate that this assay generates specific, precise, and repeatable results. Additionally, ELISA results of naturally incurred shellfish compare very well with LC-MS analyses targeting the dominant metabolites found in eastern oysters and hard clams from the Gulf of Mexico (S-desoxy-BTX-B2, BTX-B2, and BTX-B1; Fig. G11).

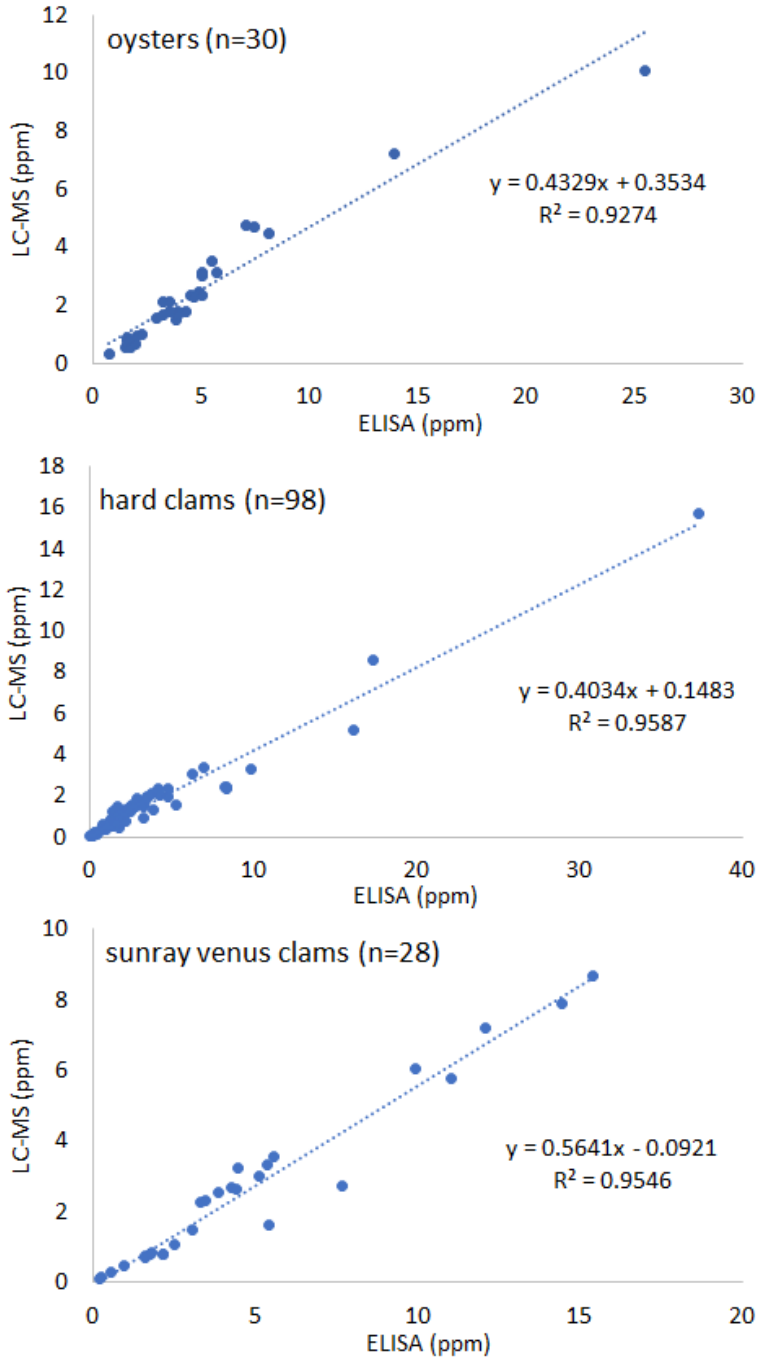


Figure G11. Comparison of NSP ELISA and LC-MS analysis of naturally incurred shellfish. LC-MS data generated and provided by A. Abraham, USFDA Gulf Coast Seafood Laboratory, using samples from this study.

Given the shortcomings and limitations of the mouse bioassay and the hardship this method imposes on both resource managers and industry, the move towards alternate methods must begin.

We propose that ELISA be approved for use as Limited Use Method such that samples would "pass" NSP rapid screening by ELISA when ELISA results are at or below a threshold representing no more than half of the level predicted in samples testing close to 20 MU/100g and below the lowest level measured in samples that have tested greater than or equal to 20 MU/100g (i.e., yielding no false negatives when applied to the existing dataset).

Thresholds of 1.8 ppm in oysters and 1.6 ppm in hard clams and sunray venus clams are proposed. The approach to derive the threshold was to approximate the ELISA equivalent of half of 20 MU/100 g and to ensure that the threshold would yield no false negatives. To protect against false negatives, the proposed thresholds are no more than 75% of the lowest concentration in the dataset that yielded a positive mouse bioassay. In hard clams and sunray venus clams, 1.6 ppm approximates half of the predicted 20 MU/100 g equivalent and is 75% of the lowest level measured in clams that failed mouse bioassay (2.18 ppm). For oysters, 1.80 is less than half of the estimated 20 MU/100 g equivalent and is 73% of the lowest level measured in oysters that failed mouse bioassay (2.45 ppm). These thresholds are not proposed as new guidance or actions limits for NSP, but rather as screening thresholds **specific to the MARBIONC ELISA (using PbTx-3 as a standard)** below which we have confidence that oysters and clams would yield <20 MU/100g and above which testing by mouse bioassay (or other future Approved Method) would be required.

Applying these thresholds to the comparative data set presented here would produce no false negatives (no samples testing greater than or equal to 20 MU/100 exceeded these levels by ELISA). Among the subset of samples testing < 20 MU/100g, ELISA results exceeded the thresholds (and would necessitate additional testing by NSP mouse bioassay) for 56% of oyster samples, 22% of hard clam samples, and 68% of sunray venus clam samples. The high proportion of <20 MU sunray venus clams above the threshold is an artifact of our sample set. Because sunray venus clams are relatively new to Florida aquaculture, our sample size is smaller, and collections during and following *K. brevis* blooms have been targeted in recent years to generate quantitative mouse bioassay data for comparisons.

As a first step away from total reliance on the NSP mouse bioassay, the proposed thresholds are conservative, and they may need to be revised in the future when more data and/or other approved methods are available, but they would have eliminated the need for 246 of the 501 bioassays (49%) conducted and represented in this data set. Having this method available as an approved option for NSP testing would greatly benefit all Gulf States. In 2015, a *K. brevis* affected the entire northern Gulf of Mexico, resulting in simultaneous closures of shellfish harvest areas in Florida, Alabama, Mississippi, and Louisiana. Because Alabama, Mississippi, and Louisiana experience these blooms infrequently, they lack the capacity to conduct NSP mouse bioassays. Therefore, sample testing to reopen harvest areas in these states after the bloom had dissipated was coordinated by our lab in Florida with the assistance of Resource Access International in Maine. While this cooperative effort was successful, it was a heavy burden on Florida, taking five weeks following bloom termination to complete and unnecessarily extending closures in these states. In every case, samples submitted by the other states passed by mouse bioassay (contained < 20 MU/100g), and if screening by ELISA had been an approved option, bioassays would not have been necessary in Mississippi or Louisiana, where NSP levels of oyster samples tested by ELISA ranged from 0.16 to 1.22 ppm.

References:

1. Baden DG, Bourdelais AJ, Jacocks H, Michelliza S, Naar J (2005) Natural and derivative brevetoxins: historical background, multiplicity, and effects. Environ Health Perspect 113: 621-625.
2. Baden DG (1989) Brevetoxins: unique polyether dinoflagellate toxins. FASEB J 3: 1807-1817.
3. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW (2004) Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44: 677-685.
4. Abraham A, Plakas SM, Wang Z, Jester ELE, El Said KR, Granade HR, Henry MS, Blum PC, Pierce RH, Dickey RW (2006) Characterization of polar brevetoxin derivatives isolated from Karenia brevis cultures and natural blooms. Toxicon 48: 104-115.
5. Wang Z, Plakas SM, Said KRE, Jester ELE, Granade HR, Dickey RW (2004) LC/MS analysis of brevetoxin metabolites in the Eastern oyster (*Crassostrea virginica*). Toxicon 43: 455-465.
6. Ishida H, Nozawa A, Nukaya H, Rhodes L, McNabb P, Holland PT, Tsuji K (2006) Brevetoxin metabolism in shellfish associated with neurotoxic shellfish poisoning. In: Njapeu H, Trujillo S, van Egmond HP, Park DL, editors. Mycotoxins and Phycotoxins: Advances in Determination, Toxicology, and Exposure Management. The Netherlands Wageningen Academic Publishers. pp. 297-307.
7. Plakas SM, Dickey RW (2010) Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. Toxicon 56: 137-149.
8. Poli MA, Musser SM, Dickey RW, Eilers PP, Hall S (2000) Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. Toxicon 38: 981-993.
9. Morohashi A, Satake M, Naoki H, Kaspar HF, Oshima Y, Yasumoto T (1999) Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat Toxins 7: 45-48.
10. Ishida H, Nozawa A, Totoribe K, Muramatsu N, Nukaya H, Tsuji K, Yamaguchi K, Yasumoto T, Kaspar H, Berkett N, Kosuge T (1995) Brevetoxn B_1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi*. Tetrahedron Lett 36: 725-728.
11. Leighfield TA, Muha N, Ramsdell JS (2014) Tissue distribution of amino acid- and lipid-brevetoxins after intravenous administration to C57BL/6 mice. Chem Res Toxicol 27: 1166-1175.
12. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R (2008) Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (*Crassostrea virginica*). Toxicon 52: 32-38.
13. Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK (2004) Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 300-302.
14. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH (2015) Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (*Mercenaria* sp.) exposed to *Karenia brevis* blooms. Toxicon 96: 82-88.
15. Abraham A, Wang Y, El Said KR, Plakas SM (2012) Characterization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. Toxicon 60: 1030-1040.
16. Trainer VL, Baden DG (1991) An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29: 1387-1394.
17. Melinek R, Rein KS, Schultz DR, Baden DG (1994) Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32: 883-890.

18. Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG (2002) A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110: 179-185.
19. Bottein MY, Fuquay JM, Munday R, Selwood AI, van Ginkel R, Miles CO, Loader JI, Wilkins AL, Ramsdell JS (2010) Bioassay methods for detection of N-palmitoylbrevetoxin-B2 (BTX-B4). Toxicon 55: 497-506.
20. Baden DG, Tomas CR (1988) Variations in major toxin composition for six clones of *Ptychodiscus brevis*. Toxicon 26: 961-963.
21. Corcoran AA, Richardson B, Flewelling LJ (2014) Effects of nutrient-limiting supply ratios on toxin content of *Karenia brevis* grown in continuous culture. Harmful Algae 39: 334-341.
22. Pierce RH, Henry MS (2008) Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. Ecotoxicology. pp. 623-631.
23. APHA (1970) Subcommittee on Laboratory Methods for the Examination of Shellfish. Method for the bioassay of *Gymnodinium breve* toxin(s) in shellfish. Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition. Washington, D.C.: American Public Health Association. pp. 61-66.
24. McFarren EF, Tanabe H, Silva FJ, Wilson WB, Campbell JE, Lewis KH (1965) The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3: 111-123.
25. Naar J, Kubanek J, Weidner AL, Flewelling LJ, Bourdelais A, Steidinger K, Baden DG (2004) Brevetoxin depuration in shellfish via production of non-toxic metabolites: consequences for seafood safety and the environmental fate of brevetoxins. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 488-490.
26. Dickey R, Jester E, Granade R, Mowdy D, Moncreiff C, Rebarchik D, Robl M, Musser S, Poli M (1999) Monitoring brevetoxins during a *Gymnodinium breve* red tide: Comparison of sodium channel specific cytoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. Nat Toxins 7: 157-165.
27. Baden DG, Mende TJ (1982) Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. Toxicon 20: 457-461.

Proposal No. **17-108**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Titan Fan, Ph.D
Affiliation	Beacon Analytical Systems, Inc.
Address Line 1	82 Industrial Park Road
Address Line 2	
City, State, Zip	Saco, Maine 04072
Phone	(207) 571-4302
Fax	(207)602-6502
Email	titan@beaconkits.com, holly@beaconkits.com
Proposal Subject	Detection of ASP biotoxins in *Mytilus edulis* (Blue Mussel) shellfish by ELISA for Domoic Acid
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas, Table 2.
Text of Proposal/ Requested Action	SLV Proposal supporting the use of Beacon Domoic Acid Plate Kit as fit for purpose as an Approved NSSP Method for quantification of ASP toxins in Marine Biotoxin Monitoring Programs.
Public Health Significance	Shellfish consumption can pose a mammal and bird health risk (1) when toxins produced by cyanobacteria present in water and shellfish growing areas, concentrate in shellfish meat due to their filter feeding system. A Closed Status for any growing areas with shellfish tissue levels of ASP of 2 mg/100 g (20 ppm) or more have been established to protect the consumer from exposure (2). The most common clinical signs of acute toxicity are gastrointestinal distress, confusion and neurological symptoms, disorientation, memory loss, coma and death (3). (1). M.Fernanda, F, Mazzillo, C. Pomeroy, J.Kuo, P. Ramondi,R. Prado, M.Silver. 2010. Aquatic Biol. 9:1-12. (2). NSSP Guide for the Control of Molluscan Shellfish: 2015 Rev. Sec.IV Chp. II., p 231. (3). Kathi A. Lefebvre, Alison Robertson, Toxicon, Vol. 56, Issue 2, 15 Aug. 2010, p. 218-230.
Cost Information	The price per sample is eight to nine dollars dependent upon the number of samples tested during one ELISA run, and/or the volume of kits purchased. There is an ELISA Plate Reader requirement. They can range in price from a low cost unit at approximately $2,600 to a higher cost of $15,000 USD unit depending upon complexity.

Single Laboratory Validation (SLV) Submission to the Interstate Shellfish Sanitation Conference (ISSC) in support of Method Approval as an Approved NSSP Method

Justification for New Method

For: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Type of Method: Enzyme Linked Immunosorbent Assay (ELISA) utilizing a polyclonal antibody for detection of the ASP Biotoxin, Domoic Acid.
Dr. Titan Fan, President
Contact Person: Holly Lawton, Director of New Product Development

Beacon Analytical Systems, Inc
82 Industrial Park Rd.
Saco, ME 04072
Phone: 207-571-4302
email: holly@beaconkits.com
Cell: 207-289-6390

Date of submission: June 30, 2017
Purpose of Intended Use of the Method. The purpose and intended use of this method is to provide a laboratory method for quickly establishing a quantified level of the ASP biotoxin, Domoic Acid, in *Mytilus edulis* (Blue Mussel) tissue as required for closing and opening of shellfish growing areas.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods. It appears there is a need for additional approved methods as an alternative to HPLC for the Biotoxin type: Amnesic Shellfish Poisoning (ASP). An ELISA method would enable monitoring laboratories to become proficient in operating a quantification method for ASP toxins levels at a lower equipment and reagent cost using a method that requires less training than HPLC to operate. This proposal demonstrates the equivalency of the HPLC and ELISA when using the same sample extract. This offers an additional benefit in that any confirmation testing could be completed using the same sample extract.

Method Limitations: This proposal offers supporting data for use of the method with *Mytilus edulis (blue mussel)* tissue only.

Method Documentation
Method Title: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Method Scope: The method is a competitive enzyme linked immunosorbent assay (ELISA) for the quantification of domoic acid (DA) residues in *Mytilus edulis* shellfish tissue. Domoic acid is produced by some species of the diatom Pseudo-nitzschia which is the primary toxin associated with amnesic shellfish poisoning (ASP). Current legislation in the NSSP limits the amount of DA allowed in harvested shellfish to 2 mg/100 g (20 ppm) and will close shellfish growing areas to shellfish harvesting to protect consumers from exposure to the toxin. The test kit provides a tool to close and open shellfish growing areas by rapidly monitoring toxin levels as levels can quickly rise and fall.

Beacon Analytical Systems Inc.®

References: (For HPLC Method) M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.

ELISA Principle- The kit is a competitive ELISA method utilizing a plate coated with Rabbit polyclonal antibodies specific to the analyte Domoic Acid. The Domoic Acid–HRP (Horse Radish Peroxidase) Enzyme Conjugate competes with any toxin from the shellfish sample extract for binding with the antibody on the plate. After and incubation period of 30 min, the plate is washed with water to remove excess material and an enzyme substrate is added to react with any HRP Enzyme Conjugate bound to the plate. The amount of bound Conjugate is inversely proportional to the amount of DA toxin in the sample extract and can be visualized by development of a blue color. The ELISA is stopped at 30 min. with a 0.1 N HCl solution, and evaluated by reading the absorbance (OD) at 450 nm wavelength in a plate reader. The OD of the sample is compared to the Calibration Curve and multiplied by the total dilution factor of 4000 to obtain the concentration of toxin in the shellfish tissue sample.

Shellfish Sample Preparation: Fresh shellfish are externally washed and removed from the shell, approximately 15 single animals are combined. Composite sample is washed, drained then homogenized for ~20 seconds using a Waring blender with 16 oz. Mason jar fitted with ice crusher blade. Samples are aliquoted and can be frozen at -20^{o}C until use.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples are used in the ELISA. The extraction and dilution procedure results in a total dilution factor of 4000 to be used in calculation of DA residues present in the original tissue sample.

Proprietary Aspects. Beacon Analytical Systems has developed the kit including antibodies and HRP enzyme conjugate.

Equipment: Microplate Reader with a filter for reading at 450 nm wavelength. Sample Preparation-blender, scale, extraction container with lid (10-20 ml), vortex mixer, microcentrifuge (12,000 rcf), disposable 1.5 ml centrifuge tubes, calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips, timer, and wash bottle.

ELISA Kit Reagents.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

Beacon Analytical Systems Inc.®

ELISA Test Procedures:

1. Allow reagents and sample extracts to reach RT prior to running the test
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader. The OD correlates to a concentration of DA (ppb) based upon the Calibration Curve run with each set of samples.
12. To obtain the concentration of Domoic acid in the sample multiply the concentration results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.

Quality Control:

Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for immunochemical test kit development, manufacturing and supporting activities.

Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment.

Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data.

All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%.

DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control.

The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above.

All CV's must be less than or equal to 5%.

All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

Beacon Analytical Systems Inc.®

Single Laboratory Validation Criteria and Results
Section # 1 - Accuracy/ Trueness & Measurement Uncertainty
Section # 2 – Ruggedness
Section # 3 - Precision & Recovery
Section # 4 - Specificity
Section # 5 - Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity,
Section # 6 - Comparability (If intended as a substitute for an established method accepted by the NSSP).

Materials and Methods

Blank Mussel Tissue: Five different bags of mussels were purchased locally and screened on the ELISA for DA content. The ELISA screening did not find DA residues in these samples. They were used in spiking experiments (Table 1).

Table 1: Sample Type - Mussel (Blank) DA Blank Mussel Tissue used in validation.

ID	Harvest Date	Location	Type	Commercial Name	DA ELISA Screening Results*
A.	10/24/2016	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
58	01/30/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm
59	02/02/2017	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
F.	04/18/2017	P.E.I., Canada	Natural	Cape Cod Shellfish Co.	0 ppm
E.	04/24/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm

*All Blank tissue screening results were below the DA ELISA Level of Quantitation (LOQ).

Domoic Acid Standards

Certified Reference Material (CRM) – Certified Calibration Solution for Domoic Acid (CRM-DA-g, Lot# 20140730) purchased from National Research Council Canada. The certified concentration values and associated uncertainties for Domoic Acid and *epi*-Domoic Acid in this solution is 103.3 $\pm$ 3.4 ug/ml (at 20 ^{o}C). This is used in the manufacturing of Calibrator Solutions supplied with test kit.

Domoic Acid (DA) Standard used for sample spiking at ppm levels - Domoic Acid from *Mytilus edulis*, Calbiochem Catalog # 324378, Lot #2879693, 1 mg/vial. Reconstituted Solution adjusted for purity (98%).

Equivalency of CRM and DA Standards using HPLC Analysis: HPLC was used to confirm the concentration and equivalency of this standard to the reference material prior to the use in spiking experiments. The Calbiochem standard was diluted 1:40 into 10% acetonitrile/DI water (ELISA sample dilution buffer) or 50% methanol/50% DI water (Sample extraction solution) and each run in triplicate on HPLC. The concentration of the Calbiochem solution used for tissue spiking was assigned using the average of 6 replicates as 1.873 mg/ml.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight. Samples were spiked with Calbiochem DA Standard at this time (if

required) and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples were used in the ELISA resulting in a total dilution factor from the sample preparation of 4000.

Mussel Tissue- Certified Reference Material for Domoic Acid in Mussel tissue. - Certified Reference Material for Domoic Acid (CRM-ASP-MUS-d, Lot# 201112) purchased from National Research Council Canada. The concentration of DA and epi-DA is reported to be 49 $\pm$ 3 ug/g.

Extraction efficiency using Mussel Tissue CRM. Evaluation of Test method extraction and recovery was evaluated using this reference material (Table 2). Sample A was prepared by addition of CRM Reference mussel tissue to blank mussel tissue (Table 1) at a 1:1 ratio resulting in a DA tissue concentration of 24.5 ug/g, Sample F was prepared by blending the 4 gm standard with 9 gm of blank mussel tissue to obtain a tissue concentration of 15 ug/g. Both samples were extracted as described above and tested by ELISA. Recovery percentages at two different spiking levels were found to be 107 and 109 percent in mussel tissue by ELISA.

Table 2. – Mussel CRM extraction recovery results.

Sample Blank Mussel Spiked with DA Mussel CRM	DA ELISA Result (ppm)	% Recovery
Sample A spiked at 24.5 ppm DA	26.2 ppm	107 %
Sample F spiked at 15.0 ppm DA	16.3 ppm	109 %

Beacon Analytical Systems Inc.®

Section 1: Accuracy/Trueness & Measurement Uncertainty (Table 3)

Working Range – Twenty samples of DA Blank Mussel Tissue were spiked with a low level (10 ppm), and twenty samples at a high level-20 ppm using the Calbiochem standard and extracted and evaluated by ELISA. Data and results are shown in Table 3.

Data Summary- Accuracy/Trueness

% Accuracy 10 ppm spike = 96.0 %

% Accuracy 20 ppm spike = 95.9 %

Data Summary – Measurement Uncertainty

Measurement uncertainty determined using a two-sided, 95% Confidence interval calculation

10 ppm spike = 0.662

20 ppm spike = 1.224

Table 3. Results of Accuracy/Trueness Testing of Blank and Spiked Mussel Tissue

Sample	Spiked Mussel (10 ppm)	% Accuracy (10 ppm)		Sample	Spiked Mussel (20 ppm)	% Accuracy (20 ppm)
1	10.70	107.0		1	21.06	105.3
2	8.06	80.6		2	23.97	119.8
3	8.07	80.7		3	16.87	84.3
4	10.29	102.9		4	19.07	95.3
5	11.15	111.5		5	16.27	81.4
6	8.18	81.8		6	17.18	85.9
7	8.43	84.3		7	16.80	84.0
8	11.26	112.6		8	18.62	93.1
9	11.42	114.2		9	16.31	81.5
10	8.81	88.1		10	22.74	113.7
11	8.91	89.1		11	20.10	100.5
12	9.51	95.1		12	18.06	90.3
13	97.7	97.7		13	21.85	109.3
14	10.60	106.0		14	17.25	86.2
15	8.63	86.3		15	17.55	87.8
16	12.20	122.0		16	22.39	111.9
17	9.42	94.2		17	19.16	95.8
18	8.39	83.9		18	17.06	85.3
19	8.02	80.2		19	22.27	111.4
20	10.18	101.8		20	18.96	94.8
Average	9.60	96.0 %			19.18	95.9 %
+/- SD	1.31				2.42	
Measurement Uncertainty @ 95% CI		0.662			Measurement Uncertainty @ 95% CI	1.224

Beacon Analytical Systems Inc.®

Section 2: Ruggedness

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The diluted sample extract was evaluated on two different manufactured test kit lots. The data and results can be found in Table 4.

Data Summary:

Value for the test of symmetry of the distribution of Kit Lot 1	**0.153**
Value for the test of symmetry of the distribution of Kit Lot 2	**0.563**
Variance of kit Lot 1	**26.07**
Variance of kit Lot 2	**49.53**
Ratio of the larger to smaller variance of Lot 1 & Lot 2	**1.89**
Significant Difference between Lot 1 & Lot 2 based upon paired t-test	**No**

Table 4

Time of Analysis	Sample	Kit Lot 1	Kit Lot 2
Day 1	1A	8.43	8.81
	1B	6.94	8.91
	2A	11.26	9.51
	2B	11.42	9.77
Day 2	3A	9.51	9.25
	3B	10.6	9.42
	4A	8.63	8.39
	4B	12.2	7.86
Day 3	5A	10.48	8.77
	5B	10.18	9.99
Day 1	6A	17.18	22.74
	6B	16.8	29.36
	7A	18.62	23.97
	7B	16.31	25.41
Day 2	8A	22.39	16.87
	8B	19.16	19.07
Day 3	9A	21.06	16.27
	9B	17.23	24.37
	10A	20.77	17.4
	10B	22.27	19.1
Skewness		0.153	0.563

Variance	**26.07**	**49.53**
Ratio of variances	**1.89**	
P-Value (Paired t-test)	**0.546**	
Significant Difference	**No**	

Results: The data summary indicates the values of symmetry for kit lot 1 and 2 are within the range of -2 to +2, a non-significant degree of skewness in the distribution. The ratio of the variances between lot 1 & 2 is less than 2 indicating homogeneity of variance. A paired t-test used for data analysis results in a p-value of 0.546 which indicates there is no significant difference between Kit 1 and Kit 2.

Ruggedness continued - ELISA parameters

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The ELISA Standard Operating Procedure (SOP) parameters were modified in the ELISA then tested for an evaluation of the critical steps in procedure (Table 5).

1. Incubation time for the initial step of the ELISA is set at 30 min. The incubation time was modified to be a total of 15 minutes or 45 minutes. The spiked sample data was evaluated by Welsh's t-test and found not to be significant at either time point tested.
2. A wash step is required in the ELISA to remove unbound materials. The SOP wash is repeated 5 times with water. This was changed to a 4 time wash. The spiked sample data was evaluated by Welsh's t-test and found to be significant from the SOP data.
3. The SOP for test incubation temperature is that the ELISA should be run at RT (20-28 °C). The incubation temperature was modified to be 4 °C or 30 °C. The spiked sample data was evaluated by a paired t-test and found not to be significant at either temperature tested.
4. The kit reagents should be equilibrated to RT prior to running the ELISA. All kit reagents were removed directly from a 4 °C refrigerator and run in comparison to RT reagents. A t-test on the resulting data indicated no significant difference in the results.

Table 5

	ELISA Standard Operating Procedure	Definition of ELISA SOP		Variation Factor	Significantly different to SOP by t-test.		Variation Factor	Significantly different to SOP by t-test.
1.	**Primary Incubation Time is 30 min.**	**Incubation time for HRP Enzyme Conjugate, Sample Extract or Calibrator on plate**		**Incubation time changed to 15 min.**	**No**		**Incubation time changed to 45 min.**	**No**
2.	**Plate water Wash Step is Repeated 5 times.**	**Water wash step to remove unbound materials prior to**		**Wash Step is changed to repeat 4 times.**	**Yes**			

		Substrate addition						
3.	**Incubation Temperature done at room temperature**	**ELISA incubation steps run at RT (20-28 °C).**		**ELISA Incubation at 4 °C.**	**No**		**ELISA Incubation at 30 °C.**	**No**
4.	**Reagent Temperature**	**Kit reagents are equilibrated to RT prior to running test.**		**Reagent Temperature is cold (4 °C).**	**No**			

Section 3: Precision & Recovery

Precision

Method: Evaluation of mussel tissue spiked with a low (10 ppm), medium (20ppm) and high level (40 ppm) of DA was completed using the method of extract preparation and ELISA analysis previously outlined, to evaluate the method consistency over a range of concentrations.

Data Summary- Precision -The F value obtained in the evaluation between groups was less than the critical value of 2.39 (for 9 and 20 degrees of freedom) at 0.05 significance level indicating the mean values from the samples are not significantly different.

The F value obtained in the evaluation of different concentrations (subgroups within groups) is greater than the critical value of 1.93 (for 20 and 30 degrees of freedom) at the 0.05 significance level indicating the mean values of each concentration are significantly different. This is an expected result since there were three sample concentration used to generate the data (10, 20 and 40 ppm) which are quite different.

From this ANOVA analysis (Table 6) we can conclude that the precision of the method is consistent over the range of sample concentrations tested.

Table 6. **Fully nested/hierarchical random analysis of variance (ANOVA)**

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	30.651802	9	3.405756
Between Subgroups within Groups	9,583.973276	20	479.198664
Residual	323.251852	30	10.775062
Total	9,937.87693	59	

F (VR between groups) = 0.316078 P = 0.9633

F (using group/subgroup msqr) = 0.007107 P > 0.9999

F (VR between subgroups within groups) = 44.472939 P < 0.0001

Beacon Analytical Systems Inc.®

Recovery

Method: Evaluation of mussel tissue spiked with a low, medium and high level of DA was done using the method outlined, to evaluate the method consistency over a range of concentrations. The results are found in Table 7.

Data summary

The variance ratio for the component of concentration in sample is not significant at 95% CI.
Recovery Percentage over the average data set (10, 20 & 40 ppm) using spiked mussel tissue is 99.55%.

Table 7 RECOVERY

	Low Spike 10 ppm		Medium Spike 20 ppm		High Spike 40 ppm	
Sample	Average	Spike minus Average	Average	Spike minus Average	Average	Spike minus Average
1	8.62	1.38	19.96	0.04	37.00	3.00
2	7.93	2.08	23.08	-3.08	39.42	0.58
3	10.39	-0.39	21.30	-1.30	40.02	-0.02
4	10.60	-0.59	20.86	-0.86	37.39	2.61
5	9.38	0.62	19.63	0.37	39.00	1.00
6	10.01	-0.01	19.12	0.88	40.41	-0.41
7	8.51	1.49	18.67	1.34	43.41	-3.41
8	10.03	-0.03	20.80	-0.80	36.52	3.48
9	9.63	0.38	19.09	0.91	43.80	-3.80
10	10.09	-0.09	20.69	-0.69	41.52	-1.52

Anova: Single Factor

Source of variation	*df*	*SS*	*MS*	*F*	*P-value*	*F crit*
Concentration	2	3.24	1.62	0.55	0.59	3.35
Error	27	80.24	2.97			
Total	29	83.48				

Section 4: Specificity

Method: Four compounds were evaluated to challenge the specificity of the ELISA, three were included due to their similarity of structure to DA, glutamine & glutamic acid at 100 ppm and kainic acid at 20 ppm. Saxitoxin (20 ppm) was evaluated due to the potential that it may be present in the shellfish at the same time as DA. Shellfish extracts containing DA from extracted tissue levels of 0 (blank), 10 and 20 ppm DA were run on the ELISA. These same extracts were run in the presence of the suspected interfering compound to evaluate any significant change in the ELISA result.

Beacon Analytical Systems Inc.®

Data Summary:

Using a two sided t-test at a 0.05 significance level it was determined that the average Specificity index (SI_{avg}) for the four compounds tested did not differ from 1 (Table 8).

Table 8

Interfering Compound	Conc. (ppm)	SI_{avg}	Significantly different from control by t-test.
Glutamine	100	1.15	No
Glutamic Acid	100	0.89	No
Saxitoxin	20	1.26	No
Kainic Acid	20	1.15	No

Section #5 Linear Range/Limit of Detection/Limit of Quantitation/Sensitivity

Method: Multiple blank mussel tissue samples were spiked with Calbiochem DA standard at the following levels: 2, 3, 5, 10, 15, 20, 30, 40 ug/g then extracted and evaluated by ELISA. To establish the linear range of response the data was evaluated and expressed in Fig. 1. The line of response falls within the bracketed 0.95-1.05 range with data from tissue concentrations from 3 ug/g to 40 ug/g. The data for 2 ug/g falls outside and is not considered within the linear range of the ELISA. The range of assay detection from 3-40 ppm is inclusive of the current NSSP criteria for closing of shellfish beds at 2 mg DA per 100 grams shellfish tissue.

Figure 2 plots the coefficient of variation for each concentration within the linear range which are all under 10%. We can calculate the limit of detection (LOD) of the method and the limit of quantitation (LOQ) shown below using this data.

Data Summary

Linear range of the method as implemented is 3-40 ppm DA in tissue (Fig.1).

The limit of detection (LOD) of the method as implemented is 0.91 ppm DA in tissue.

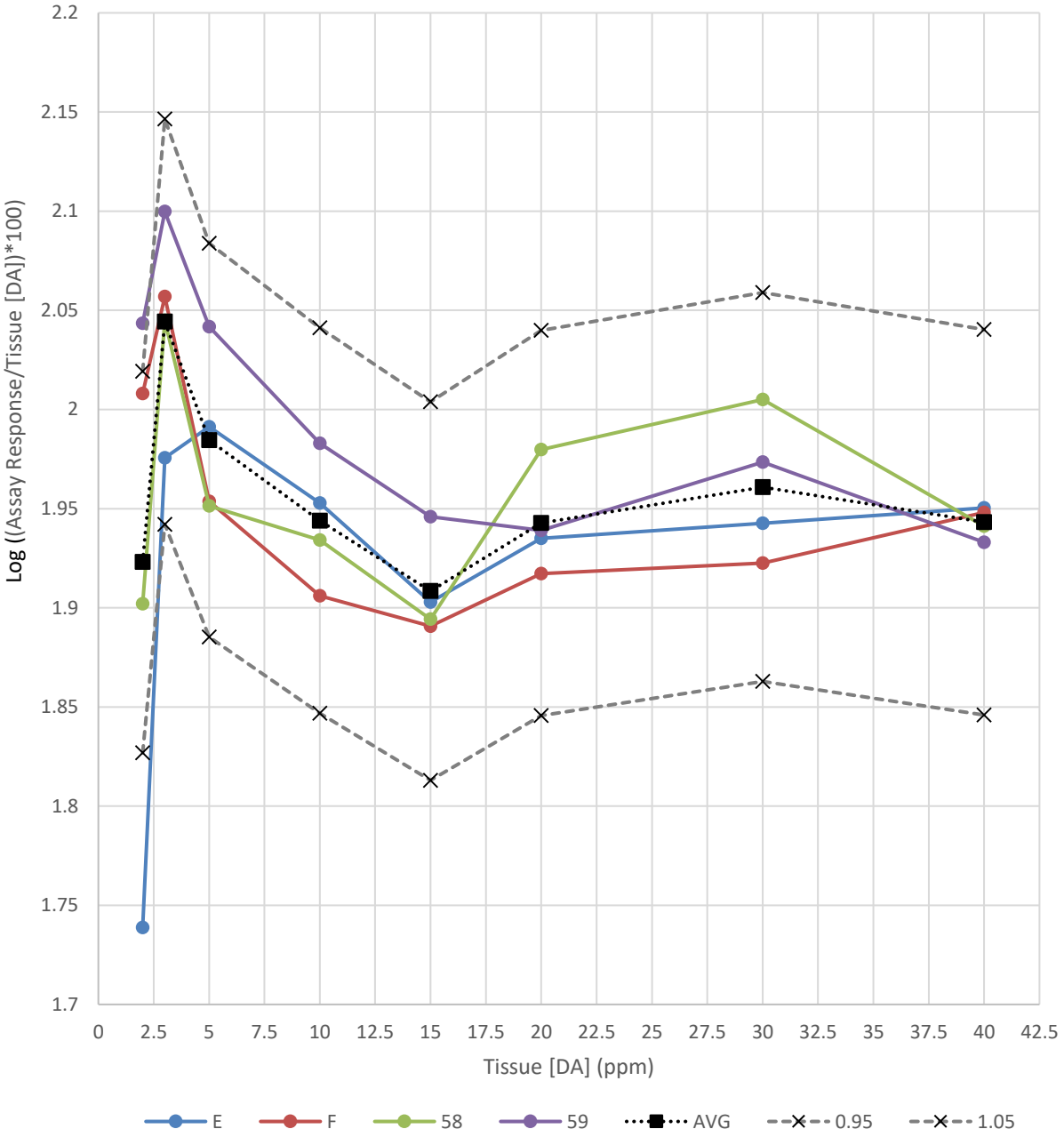
The limit of quantitation (LOQ) of the method as implemented is 3.0 ppm DA in tissue.

Linear Range Plot – Figure 1

The linear range of the ELISA is established to be from 3 to 40 ppm DA in mussel tissue (Figure 1).

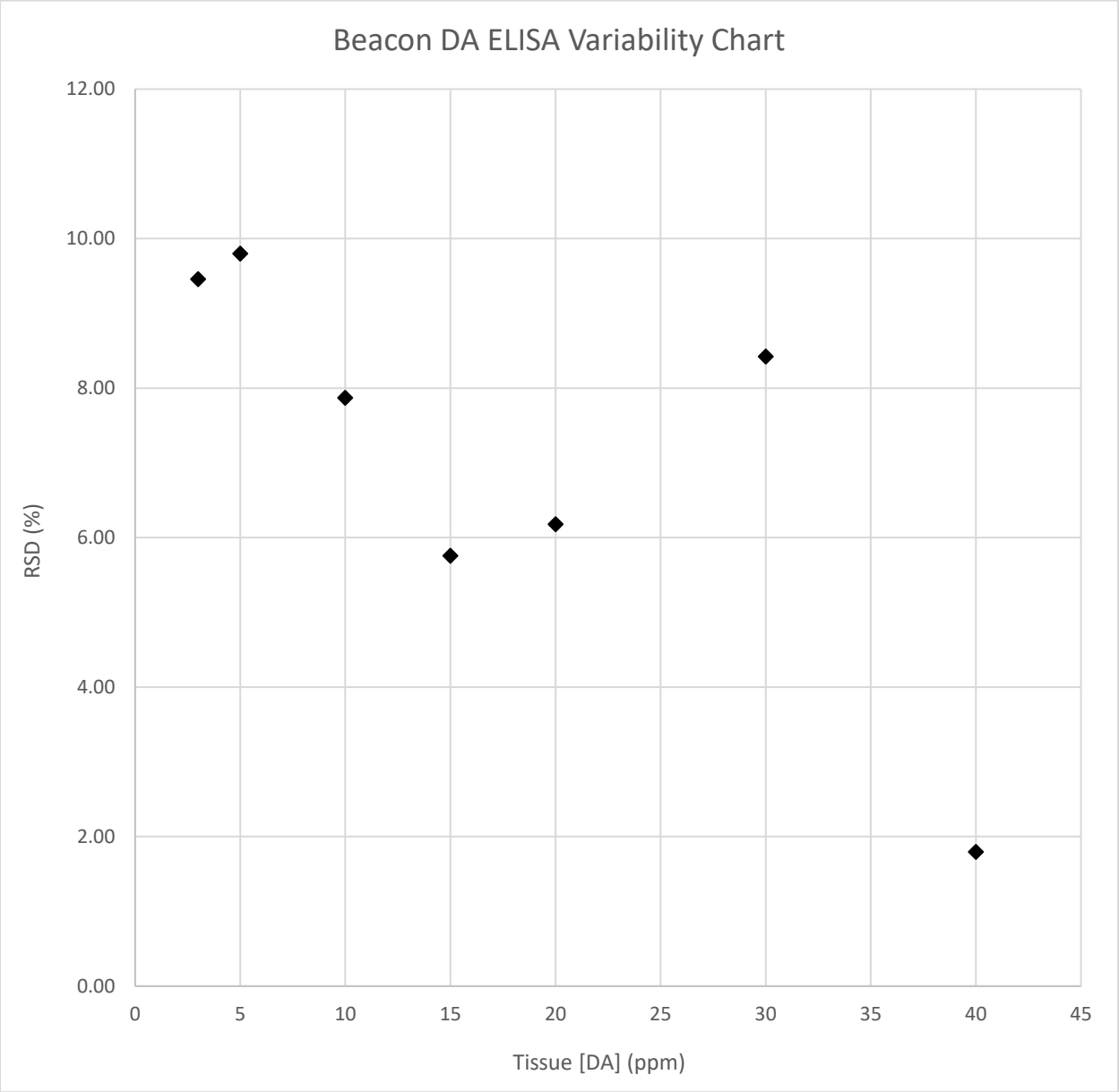
Beacon Analytical Systems Inc.®

Figure 1: Linearity of Beacon DA ELISA



Beacon Analytical Systems Inc.®

Figure 2: Limit of Detection & Limit of Quantitation/Sensitivity



Beacon Analytical Systems Inc.®

Section # 6 - Comparability to NSSP Approved Method for Biotoxin Testing –HPLC

Reference from NSSP Guide for the control of Molluscan Shellfish 2015 Revision.
Table 2- Approved Methods for Marine Biotoxin Testing for ASP: M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.
Method: The HPLC uses a C-18 reverse phase chromatography column with a mobile phase of 10% acetonitrile and 0.1% trifluroacetic acid. The mussel tissue extracts have been prepared using the sample extraction procedure for the ELISA which are then diluted 1:5 with 10% acetonitrile prior to injection into the HPLC system. The 2 procedures use the same sample extracts and the results are compared in Table 9. There were 14 mussel tissue samples tested of which 50% were naturally incurred samples and 50% were spiked with Calbiochem DA standard.

Data Summary for the comparison of the new method to the officially recognized method:
Value for the test of symmetry for the data by HPLC reference method: 0.93
Value for the test of symmetry for the data by the DA ELISA method: 1.45
Symmetry is within the range of -2 to +2 and is not a significant degree of skewness.

Variance of data generated by the HPLC reference method: 166.90
Variance of the data generated by the DA ELISA method: 675.73
Ratio of the larger to smaller of the variances: 4.05
This value indicates a lack of homogeneity of variance and indicates the use of a Welch's t-test for further data analysis to determine if there is a difference between the data means.
Based upon the Welch's t-test there no significant difference between these two analytical methods.

Table 9

Sample	Collection Date	HPLC Data	DA ELISA
Mytilus edulis		DA (ppm)	DA (ppm)
1	9/6/16	9.48	9.50
2	8/30/16	4.78	4.2
3	8/30/16	16.14	19.80
4	01/30/17	4.42	4.80
5	01/30/17	8.77	8.70
6	01/30/17	15.78	22.80
7	01/30/17	28.49	26.20
8	9/20/16	10.64	21.30
9	10/3/16	27.04	51.40
10	9/20/16	1.60	6.90
11	9/28/16	43.11	91.80
12	9/19/16	17.80	36.70
13	9/26/16	39.79	68.70
14	10/3/16	12.10	22.70

	SKEW	0.93	1.45
	VARIANCE	166.90	675.73
	Ratio of Variance		4.05
	Welch's T-test		-1.43
	df =		(19)
	T =		2.09
Conclusion: Means are not different between the 2 methods of analysis.			

Discussion and Summary

The results of this single laboratory validation demonstrate that the Beacon Domoic Acid (ASP) Plate Kit is an effective procedure for quantitative determination of DA residues in *Mytilus edulis* shellfish tissue. Data presented for ELISA performance meets the validation criteria for accuracy/trueness, measurement uncertainty, ruggedness, precision and recovery. The specificity of the test kit was challenged with four compounds of potential interference and was found to perform properly at DA levels of interest. The linear range of the ELISA was determined to be 3 to 40 ppm which brackets the NSSP established criteria of 20 ppm for the ASP biotoxin in shellfish beds. This linear range would allow for the continued use of the method should a lower criteria be established. The comparative data from the ELISA and the officially recognized HPLC method demonstrate good correlative performance.
The ability to use the same sample extract on the ELISA and HPLC confers ease of use for confirmatory testing. The sample throughput is high, while cost and training requirements are minimal. The Beacon Domoic Acid (ASP) Plate Kit is an appropriate tool for quantification of DA residues for use in biotoxin monitoring programs as it allows rapid sample analysis and turnaround time.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**Domoic Acid (ASP) Plate Kit**
Name of the Method Developer	**Dr. Titan Fan**
Developer Contact Information	**Beacon Analytical Systems, Inc.** **82 Industrial Park Road** **Saco, ME 04072** **Tel. (207) 571-4302** **Fax (207) 602 6502** **Email: titan@beaconkits.com**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for an inexpensive laboratory method with the ability to process multiple samples rapidly and quantify the domoic acid levels in mussel tissue.
2. What is the intended purpose of the method?		The method can be used in shellfish bed monitoring programs to document the quantifiable levels of domoic acid in mussel tissue.
3. Is there an acknowledged need for this method in the NSSP?		The method offers higher sample thruput and quantifiable results to monitor increasing or decreasing levels of domoic acid.
4. What type of method? i.e. chemical, molecular, culture, etc.		It is an immunochemical method utilizing an antibody specific to the toxin in an enzyme linked immunosorbent assay (ELISA).
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Domoic Acid (ASP) Plate Kit
Method Scope		Quantitative Analysis of Domoic Acid in mussel tissue
References		Performance Validation Report and Product Insert included with this submission.
Principle		Composite mussel samples are extracted and run on an ELISA Test Kit which quantifies Domoic Acid residues by comparison to an internally run calibration curve.
Any Proprietary Aspects		Yes, this is a commercial test kit.
Equipment Required		Sample Preparation- Blender, scale, extraction container with lid (10-20 ml), vortex mixer, centrifuge (12,000 rcf), Disposable centrifuge tubes. Calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips. Plate Reader, timer, wash bottle.
Reagents Required		Methanol and laboratory grade water in a 1:1 ratio. 10% Acetonitrile in laboratory grade water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2-8°C before testing.
Safety Requirements		Protective safety items are indicated such as safety glasses, gloves and lab coat. Kit reagents including

		calibrators, conjugates and extracts should be handled with caution since they contain a toxic substance. The kit Stop Solution is a strong acidic solution (1 N Hydrochloric Acid) and needs to be safely handled and disposed of appropriately.
Clear and Easy to Follow Step-by-Step Procedure		Product Insert is included in each test kit and included in this submission package.
Quality Control Steps Specific for this Method		The Certificate of Conformity included with each kit documents the performance characteristics of the Test Kit Lot Reagents. This provides the test operators a reference to evaluate the results generated in their laboratory.
C. Validation Criteria		
1. Accuracy / Trueness		SLV - Section 1
2. Measurement Uncertainty		SLV - Section 1
3. Precision Characteristics (repeatability and reproducibility)		SLV - Section 3
4. Recovery		SLV- Section 3
5. Specificity		SLV- Section 4
6. Working and Linear Ranges		SLV - Section 5
7. Limit of Detection		SLV - Section 5
8. Limit of Quantitation / Sensitivity		SLV - Section 5
9. Ruggedness		SLV- Section 2
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		SLV - Section 6
D. Other Information		
1. Cost of the Method		The price per sample is eight to nine dollars dependent upon the number of samples tested during one ELISA run, and/or the volume of kits purchased.
2. Special Technical Skills Required to Perform the Method		Knowledge of GMP laboratory skills as well as proper pipetting technique, and safe handling of solvents.
3. Special Equipment Required and Associated Cost		An ELISA Plate Reader is required which can range in price from a low cost unit at approximately $2,600 to a higher cost of $15,000 USD unit depending upon complexity.
4. Abbreviations and Acronyms Defined		ASP-amnesic shellfish poisoning. DA-domoic acid. ELISA-Enzyme Linked Immunosorbent Assay. OD-Optical Density (Reader Output). HRP-horse radish peroxidase enzyme. % B/B0- percent of measured bound fraction in a test sample divided by the total bound from a blank Calibrator. ppm = parts per million, equivalent to mg/kg, ppb –parts per billion.
5. Details of Turn Around Times (time involved to complete the method)		One assay can be completed in under 90 minutes including sample preparation (12 samples). One plate can be used to test a maximum of 36 samples.
6. Provide Brief Overview of the Quality Systems Used in the Lab		Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for Immunochemical test kit development, manufacturing and supporting activities. Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment. Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data. All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%. -DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control. - The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above. - All CV's must be less than or equal to 5%. - All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

Submitters Signature 	Date: 06/30/2017
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** - the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Beacon Analytical Systems Inc.®

Domoic Acid (ASP)

Plate Kit

Cat. # 20-0249
Product Insert

PLEASE READ COMPLETELY BEFORE USE

INTENDED USE

The Beacon Domoic Acid (ASP) Plate Kit is a competitive ELISA for the quantitative analysis of domoic acid in shellfish samples.

USE PRINCIPLES

The Beacon Domoic Acid (ASP) plate kit is a competitive enzyme-labeled immunoassay for the biotoxin which causes Amnesic Shellfish Poisoning (ASP). Shellfish sample extract(s) or calibrator solution(s) are pipetted into a test well followed by Domoic Acid HRP enzyme conjugate to initiate the reaction. During a 30 minute incubation period, domoic acid from the sample and domoic acid HRP enzyme conjugate compete for binding to the domoic acid antibody coated on the plate wells. Following this incubation, the wells are washed to remove any unbound domoic acid and HRP enzyme conjugate. After washing, a colorless substrate is added to the wells and any bound enzyme conjugate will convert the substrate to a blue color. Following another 30 minute incubation, the reaction is stopped with the addition of stop solution and the amount of color in each well is measured. The color of the unknown sample is compared to the color of the calibrators and the domoic acid concentration of the sample is derived. The color intensity is inversely proportional to the amount of domoic acid present.

MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2 to 8 ºC.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

MATERIALS REQUIRED BUT NOT PROVIDED

Acetonitrile,& Methanol (ACS grade)	Timer
Laboratory quality distilled or deionized water	Wash bottle
Variable volume pipettes with disposable tips capable of dispensing 10-100 microliters (µl), and up to 1000 µl.	Vortex mixer
Multi-channel pipette; 8 channel capable of dispensing 100 µl	Paper towels or equivalent absorbent material
Microwell plate or strip reader with 450 nm filter	Disposable micro centrifuge tubes
Microcentrifuge capable of a speed of 12,000 rcf. (x g)	Kitchen Blender for sample homogenization

SPECIFICITY

Domoic Acid (DA) is an amino acid similar in structure to kainic acid which naturally occurs in some seaweed. The % cross reactivity of several compounds relative to DA is shown in the table below.

Compound	% CR	Compound	% CR
Domoic acid	**100 %**	**Saxitoxin**	**< 0.1 %**
Glutamine	**< 0.1 %**	**Kainic acid**	**0.005 %**
Glutamic acid	**< 0.1 %**		

KIT HANDLING NOTES and PRECAUTIONS

- Store all kit components at 4 °C to 8 °C (39 °F to 46 °F) when not in use.
- Each reagent is optimized for use in the Beacon Domoic Acid (ASP) Plate Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Beacon Plate Kits with different lot numbers.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Do not use reagents after expiration date.
- Reagents should be brought to room temperature (RT), 20 to 28 ºC (62 to 82 ºF) prior to use. Avoid prolonged (> 24 hours) storage at room temperature.
- Domoic acid calibrators contain 10% acetonitrile and should be kept tightly capped to minimize evaporation.
- The Stop Solution is 1N hydrochloric acid, which is corrosive and an irritant. Avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.
- Precise transfer of samples and reagents by using an appropriate and calibrated pipette is critical to obtain proper assay results. Please pipette carefully.
- If running more than two strips at once, the use of a multichannel pipette is required.
- In one assay a maximum of four strips (32 wells) is recommended, for example –4 calibrators in duplicate (8 wells), and 12 sample extracts in duplicate (24 wells).

SAMPLE DILUTION BUFFER PREPARATION- 10% ACETONITRILE/Water

- Mix 1 part ACS grade acetonitrile with 9 parts distilled or deionized (DI) water to make the Sample Dilution Buffer for the shellfish samples.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE EXTRACTION BUFFER PREPARATION- 50% METHANOL/Water

- Mix 1 part ACS grade methanol with 1 part distilled or deionized (DI) water to make the Sample Extration Buffer.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE PREPARATION - Shellfish Tissue Extract –Mytilus edulis (Blue Mussel)

1. Remove shellfish tissue (12-15 animals) from shell, wash, drain dry and homogenize using a kitchen blender.
2. Weigh 2 g of homogenized tissue and add 8 ml of a 50% Methanol/Water solution.
3. Mix for 3 minutes using Vortex mixer (4 X dilution)
4. Transfer 1 ml into a microcentrifuge tube and centrifuge at 12,000 x g for 5 minutes. Extracts can be stored at -20°C.
5. Prepare a 1:1000 dilution of the supernatant with Sample Dilution Buffer using the following procedure:

A. 1:10 dilution - 50 microliters of supernatant layer avoiding any particulates, into 450 microliters Sample Dilution Buffer, Mix.

B. 1:100 dilution – 10 microliters of dilution A. into 990 microliters Sample Dilution Buffer, Mix,

6. Use B. in ELISA. – Total Dilution Factor (TDF) = 4000

Shellfish Analysis:

- ✓ EU Screening Level = 20 ppm (20 mg/kg) Assay Dilution Factors are set to detect 20 ppm Domoic Acid,

Extraction of Shellfish Tissue and Preparation for ELISA	
Dilution of shellfish homogenate in water (2 g homogenized tissue with 8 ml 50% MEOH/DI H_2O)	**4 X** Dilution
*Secondary Dilution into Sample Dilution Buffer	**1000 X Dilution**
Total Dilution Factor (TDF) to obtain Tissue Levels of Domoic Acid	**4000 X**
✓ Assay Range of Detection in Tissue	2 mg / kg to 200 mg / kg
Domoic Acid Plate Kit Calibrators ug / L (ppb)	Predicted Tissue Levels (X 4000 TDF)
Negative Control (Blank)	0
0.5	2 ppm
5.0	20 ppm
50.0	200 ppm

ASSAY PROCEDURE

(Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.)

1. Allow reagents and sample extracts to reach RT prior to running the test.
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader.
12. To obtain the concentration of Domoic acid in the sample multiply the results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

CALCULATE RESULTS

1. Semi-quantitative results can be derived visually by simple comparison of the sample color to the color of the Calibrator wells. Samples containing less color than a Calibrator will have a concentration of Domoic Acid greater than the tissue correlated concentration of the Calibrator. Samples containing more color than a Calibrator will have a concentration less than the tissue correlated concentration of the Calibrator.
2. It is preferred for quantitative results to be determined using commercially available software for ELISA evaluation such using a 4-Parameter curve fit. Alternatively, a semi-log curve fit can be used if 4-Parameter software is not available. Samples with OD's greater than the lowest calibrator, or lower than the highest calibrator will need to be diluted accordingly and repeated with and calibrators in an additional run.
3. Beacon can supply a spreadsheet template which can be used for data reduction. Please contact Beacon for further details.

SAMPLE CALCULATIONS

Well Contents	OD	Average OD ± SD*	%RSD	%B/Bo**
Negative Control	2.033 1.994	2.014 ± 0.027	1.4	100
0.5 ppb Calibrator	1.610 1.671	1.640 ± 0.043	2.7	81
5 ppb Calibrator	1.095 1.155	1.125 ± 0.042	3.8	56
50 ppb Calibrator	0.501 0.482	0.492 ± 0.013	2.7	24

Actual values may vary; this data is for example purposes only.

* Standard deviation

**B/Bo% equals the average sample absorbance divided by the average 0 ppb Calibrator absorbance multiplied by 100.

TECHNICAL ASSISTANCE

For questions regarding this kit or for additional information about Beacon products, call (207) 571-4302 or contact us at info@beaconkits.com.

Safety- To receive complete safety information on this product, contact Beacon Analytical Systems, Inc. and request Safety Data Sheets. Stop Solution is 1N hydrochloric acid. Handle with care.

General Limited Warranty

Beacon Analytical Systems, Inc. ("Beacon") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond a product's printed expiration date. BEACON MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The warranty provided herein and the data, specifications and descriptions of Beacon products appearing in published catalogues and product literature may not be altered except by express written agreement signed by an officer of Beacon. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and, if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Beacon's sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Beacon promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as Beacon is willing and able to repair or replace any nonconforming Beacon product or part. Beacon shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by a customer from the use of its products. However, in some states the purchaser may have rights under state law in addition to those provided by this warranty.

BEACON ANALYTICAL SYSTEMS, INC.®

82 Industrial Park Road

Saco, ME 04072

Tel. (207) 571-4302 Fax (207) 602-6502

www.beaconkits.com

REV.ISSC 06302017HL

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration
Affiliation	U.S. Food and Drug Administration
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Domoic Acid (Amnesic Shellfish Poisoning) HPLC Method Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	The requested action is to adopt the text of the attached checklist for the HPLC method for detecting domoic acid and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Public Health Significance	Currently, there is no checklist adopted by the ISSC for the method approved under the NSSP for domoic acid. The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing the HPLC method for domoic acid to support the NSSP. The checklist documents the number of critical, key or other nonconformities and how overall laboratory status for the method is determined.
Cost Information	

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-2151/2055/4960 FAX 301-436-2601

SHELLFISH LABORATORY EVALUATION CHECKLIST
Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
	REGION:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "√"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply) a. Organization of the laboratory. b. Staff training requirements. c. Standard operating procedures. d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established. e. Laboratory safety. f. Internal performance assessment.
C	5		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	State's Human Resources Department		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	5		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
			1.3 Work Area
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment
K	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]
K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH meter.

			The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded. [Only applicable if using the sample cleanup procedure]
K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4, 15		1.4.10 Freezer temperature is maintained at -10 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	13		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 ºC, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (λ) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4		1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
			1.5 Reagents and Reference Solution Preparation and Storage
C	4, 15		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	8		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	8		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	4, 15		**1.5.4 The mobile phase system used to analyze domoic acid consists of: 10%**

			aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).
O	4		1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.
C	7		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
K	4, 15		1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4		1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	**7**		**1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control:_____**
C	**7**		**1.5.10 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.11 All standards used are within expiration date.**
C	**2**		**1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.**
C	**4, 15**		**1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.**
C	**7**		**1.5.14 Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.15 Quality Control shellfish tissues are stored frozen.**
			1.6 Collection and Transportation of Samples
O	6, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	6, 1		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	14, 2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.

C	2		1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.
PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS			
			2.1 Preparation of Sample
C	6, 1		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	6		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	2, 6		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
			2.2 Sample Extraction
K	4,6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	4		**2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**4**		**2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).**
K	4, 15		2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15		2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.
K	4		2.2.6 When crude samples are injected, dilutions of the crude extracts are used. Dilutions of the crude samples are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 ºC.
			2.3 Sample Cleanup (Optional)
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	**4, 15**		**2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.**
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the

			cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	**4, 15**		**2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.**
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
			2.4 Analysis
C	2		2.4.1 **A standard calibration curve (of at least six concentrations) is performed daily. Results are recorded and records are maintained.**
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	4		2.4.5 **The appropriate analytical column is used: 25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.**
K			2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.
C	**2**		2.4.8 **Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter (<20 cm of 0.01 in id) between the sample injector and the column and between the column and detector.**
			2.5 System Suitability
C	**2**		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for domoic acid.**
	3		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C			**2.5.4 The column is replaced when peak asymmetry becomes <0.9 or >1.3.**
C	2,4		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.**
C	2		**2.5.6 Repeated injections of calibrated standards/samples agree within ±5% (as determined through the use of the coefficient of variation).**
			2.6 Calculation of Sample Toxicity

C	4, 15		2.6.1 **The toxicity of the individual toxins is calculated as follows:** μg/g domoic acid (DA) = DA injected $\times \frac{V}{W} \times (F)$ **where: DA injected = the concentration in µg/ml of the extract injected;** **V = total volume of homogenate and extraction solvent (mL);** **W = weight (g) of tissue homogenate extracted (e.g., 4 g); and** **F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).** **The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.**
C			2.6.2 **Calculated domoic acid concentrations include the sum of domoic acid and isomer/epimer peaks.**
C	12		2.6.3 **Any value at or above 20 ppm (mg/kg or µg/g) domoic acid is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Method 991.26 Domoic Acid in Mussels. Liquid Chromatography Method. First Action 1991. Final Action 1999.
4. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1995. J. AOAC Int. 78(2): 543-554.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
15. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1991. A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Bioscience, Technical Report #64, National Research Council Canada #33001.

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component

1. **Conforms Status: The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥ 4**.
   - b. or the total # of Key nonconformities is **≥ 6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform – Provisionally Conforms – Conforms

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature:_____ Date: _____

Proposal No. **17-110**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	FDA
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Alkaline Phosphatase Probe Method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* Detection in Oysters - Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	The requested action is to adopt the text of the attached checklist for the probe method for detecting *Vibrio vulnificus* (Vv) and *Vibrio parahaemolyticus* (Vp) in oysters and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Public Health Significance	Currently, there is no checklist adopted by the ISSC for the probe method for detecting Vv and Vp in oysters. The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing this method in support of the NSSP. The checklist documents the number of critical, key or other nonconformities and how overall laboratory status for the method is determined.
Cost Information	NA

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: Conformity it noted by a "√"

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

	Alkaline Phosphatase Probe Method for *Vibrio vulnificus* detection in Oysters [PART III]
	Alkaline Phosphatase Probe Method for *Vibrio parahaemolyticus* detection in Oysters [PART II]

PART I – Quality Assurance			
		ITEM	
CODE	**REF**		
		1.1 Quality Assurance (QA) Plan	
K	4, 6	1.1.1 Written Quality Assurance Plan (Check √ those items which apply).	
			a. Organization of the Laboratory.
			b. Staff training requirements.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**4**		**1.1.2 The QA plan is implemented.**
K	6		1.1.3 The Laboratory participates in the *Vibrio* portion of the FDA Shellfish proficiency testing program annually. Specify the program(s): _____
C	**2**		**1.1.4 The Laboratory has and implements a plan to address poor, questionable or unsatisfactory performance in proficiency tests.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area	
O	4,6		1.3.1 Adequate for workload and storage.
K	6		1.3.2 Clean, well lighted.
K	6		1.3.3 Adequate temperature control is maintained.
O	6		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5 Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
K	5		1.4.1 To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of at least 0.1 pH units
K	9		1.4.2 The pH electrodes being used consist of a pH half cell and reference half cell double junction combination electrode, single junction combination electrode or triode. If a single junction electrode is used, it is free of silver/silver chloride or contains an ion exchange barrier to prevent passage of silver ions into the solution (Circle the type of electrode used).
K	6		1.4.3 The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).

K	4		1.4.4 The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.
K	6		1.4.5 A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
K	4		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5, 16		1.4.7 The balances used provide a sensitivity of at least 0.01g at the weights of use.
K	6		1.4.8 Balance calibrations are checked monthly according to manufacturer' specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance calibrations are verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperatures are maintained between 2 and 8°C.
C	1, 7		**1.4.11 Freezer temperature is maintained at -20°C or below.**
K	6, 7		1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**13, 17**		**1.4.13 The temperature of the incubator is maintained at 35+2.0°C**
K	6		1.4. 14 Thermometers used in the air incubators are graduated at no greater than 0.5°C increments.
K	5		1.4.15 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16 Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17 All working thermometers are appropriately immersed.**
C	**2, 18**		**1.4.18 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) possessing the appropriate level of accuracy for the intended monitoring application.**
C	**6, 13, 16**		**1.4.19 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35, 42, 54 and/or 55°C (54°C for *Vibrio parahaemolyticus* and 55°C for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.**
K	3, 5		1.4.20 Standard thermometers are checked annually for accuracy by ice point determination. Any changes are incorporated into all the other calibrated temperature points on the thermometer. These results are recorded and maintained Date of most recent determination:_____
C	**2, 18**		**1.4.21 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05°C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22 All working thermometers are checked annually against the

			standards thermometer at the temperature(s) of use. Results for the in-use temperature checks are recorded and records maintained.
O	6		1.4.23 Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24 Micropipettors are calibrated at appropriate volumes used annually and checked for accuracy quarterly. Results are recorded and records maintained.
K	5		1.4.25 Pipets used to inoculate samples and prepare reagents deliver accurate aliquots and are tested for accuracy with each new lot received.
			1.5 Labware and Glassware Washing
K	5		1.5.1 Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5. 4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method of preparation is used to ensure the appropriate volumes of diluent.
K	5		1.5. 5 In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
			1.6 Sterilization and Decontamination
K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed and the records maintained including calibration of temperature gauges.
C	**6, 18**		**1.6.3 The autoclave provides a sterilizing temperature of 121± 2°C as determined for each load using a calibrated gauge, sensor or thermometer. This measurement is verified weekly with an external maximum registering working thermometer or data logger (if not routinely used). As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	2, 4, 18		1.6.4 An autoclave standards thermometer (data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point is also recommended for mercury autoclave standards thermometers but not required as this allows for in-house checks (by steam point) of the thermometer's accuracy at 121°C .
K	2, 10, 18		1.6.5 The autoclave standards thermometer (data logger) is checked every five years for accuracy at either 121°C by a qualified calibration laboratory or in-house at 100°C (mercury thermometer only), the steam point if the (mercury) thermometer has been previously calibrated by a qualified calibration laboratory at this temperature.

			Date of most recent determination: _____
K	1, 2		1.6.6 Working autoclave thermometers (data loggers) are checked against the autoclave standards thermometer at 121°C yearly. Date of last check:_____
K	6		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the biological effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8 Heat sensitive tape is used with each autoclave load to indicate that the load has been sterilized.
K	6		1.6.9 Autoclave sterilization records are maintained which include the length of the sterilization cycle, total heat exposure time (time in to time out) and maximum chamber temperature Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	9		1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot air sterilizing oven.
K	13		1.6.12 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	11		1.6.13 Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the biological effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	9		1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	9		1.6.15 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**		**1.6.16 The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18 The sterility of pre-sterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	13		1.6.19 Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation	
C	**19, 29**		**1.7.1 TCBS is commercially dehydrated and alkaline peptone water (APW), mCPC, T1N3, CC and VVA agars are prepared from the individual components and pH adjusted appropriately.**
K	11		1.7.2 Media components are properly stored. in a cool dry place.
K	11, 19		1.7.3 Media components are labeled with the analyst's initials, date of receipt, and date opened and date of preparation if applicable (dye solutions).

C	**2**		**1.7.4 Caked or expired media or media components are discarded.**
C	**11**		**1.7.5 Reagent water is tested monthly and exceeds 0.5 megohms-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. Results are recorded and the records maintained. (Circle the appropriate water quality descriptor determined)**
C	**11**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	11		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotropic plate count method. Results are recorded and records maintained.
K	9 19		1.7.8 The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2, 11, 19**		**1.7.9 The total time of exposure of media broths to autoclave temperatures does not exceed 60 minutes.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the when the medium is made from its individual components.** **Positive *Vibrio parahaemolyticus* productivity control_____** **Negative *Vibrio parahaemolyticus* productivity control _____** **Positive *Vibrio vulnificus* productivity control _____** **Negative *Vibrio vulnificus* productivity control _____**
C	**11**		**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
			1.8 Storage of Prepared Culture Media
K	9		1.8.1 Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	13		1.8.2 Stored media are labeled with the storage expiration date. or sterilization date.
K	9		1.8.3 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2, 11 19		1.8.4 Storage of prepared broth media with loose fitting closures and prepared plates stored in sealed plastic bags or containers, to minimize evaporation, does not exceed 1 month.
K	35		1.8.5 Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading.
K	2,17		1.8.6 All prepared broth media stored under refrigeration is warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
			PART II – Oyster Samples

		2.1 Sample Handling and Receipt	
C	**2, 11**		**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	9, 2		2.1.2 Oyster samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	9, 2		2.1.3 Samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**9, 2**		**2.14 Immediately after collection, samples as received have been are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory or rejected. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**9, 35**		**2.1.5 If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.**
C	**1, 9**		**2.1.6 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis	
K	2, 11		2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		2.2.2 Blades of shucking knives are not corroded.
K	9		2.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		2.2.5 Oysters are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	9		2.2.6 Oysters are allowed to drain in a clean container or on clean towels prior to opening
K	9, 30 2		2.2.7 Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**9**		**2.2.8 Oysters are not shucked through the hinge.**
C	**9**		**2.2.9 The contents of the oyster (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**9**		**2.2.10 A representative sample of at least 12 shellfish is used for analysis.**
C	**2, 9**		**2.2.11 A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13, 16, 17		2.2.12 Either a 1:1 dilution is made at this point, or the sample is homogenized without dilution. If a dilution is made, the sample is weighed to the nearest 0.1 gram and an equal amount by weight, of diluent is added.
K	13		2.2.13 Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14 Samples are blended at for 60 to 120 seconds until homogenous.**
PART III- Alkaline Phosphatase Probe method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* detection in Oysters			
		3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method:	

			Direct Plating
K	13, 16		3.1.1 If direct plating, use sterile cell spreaders are used to spread inoculum evenly onto three dry T1N3 agar plates for the analysis of *Vibrio parahaemolyticus*.
C	**13, 16**		**3.1.2 Two tenths (0.2) of a gram of the initial 1:1 diluted oyster homogenate (or 0.1 g of undiluted homogenate) is used as inoculum; one is used to probe for the total (*tlh*) gene and the two remaining are replicate plates used to probe for the pathogenic (*tdh*) gene.**
C	**13**		**3.1.3 Inoculated T1N3 plates are incubated 18-24 h at 35 ±2° C. All plates are used for colony lifts and hybridization, except for those with confluent growth.**
C	**2, 13**		**3.1.4 A tdh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A *V. vulnificus* culture is used as a negative process control. The process control cultures accompany the samples throughout incubation, and hybridization and color development phases of the method. Results are recorded and are maintained.**
			3.2 APW Enrichment
K	13		3.2.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**13, 16, 17**		**3.2.2 The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 mL of sterile PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10g of sample homogenate to 90 mL of sterile PBS.**
C	**14**		**3.2.3 Appropriate sample dilutions are inoculated into sterile APW.** **Specify dilution(s) used** _____ **Specify number of tubes per dilution** _____
C	**2, 16**		**3.2.4 For *V. parahaemolyticus* analysis, a tdh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A *V. vulnificus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A *V. parahaemolyticus* culture is used as a negative process control. The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Results are recorded and records are maintained.**
C	**13**		**3.2.5 Inoculated APW enrichment tubes are incubated at 35±2°C.**
C	**13**		**3.2.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
			3.3 Colony Isolation
K	13		3.3.1 A loopful from the top 1 cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation
C	**13, 15**		**3.3.2 TCBS plates are incubated at 35 ±2°C and mCPC or CC plates are incubated at 35-40°C for 18-24 hours.**
C	**13**		**3.3.4 Presumptive colonies are selected meeting these phenotypic**

			characteristics: ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, 2 to 3 mm in diameter. Interfering large, opaque, and yellow colonies are avoided.** ***V. vulnificus*: appear on mCPC or CC agar, colonies are as round, flat, opaque, yellow colonies, and 1 to 2 mm in diameter. Typical positives have a "fried egg" appearance. Purple/blue colonies are avoided.**
C	**13, 16**		**3.3.5 Colonies are picked and spotted on VVA (*V. vulnificus*) or T1N3 (*V. parahaemolyticus*). For storage and/or ease of replication, colonies are inoculated into a 48 or 96 well plate with APW and incubated for at least 4 and no more than 24 hrs prior to transfer to agar plates.**
		3.4 Filter preparation.	
C	**13, 16**		**3.4.1 VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters (90 mm) for 1 to 30 min.**
K	13, 16, 17		3.4.2 Filters are transferred with colony side up to a plastic or glass Petri dish lid containing 1 ml of lysis solution to wet the filter.
C	**13, 16, 17**		**3.4.3 Filters are microwaved in a vessel or tray for 15-20 sec/filter depending on the wattage of the microwave; filters are dry but not scorched or burned.**
K	13, 16, 17		3.4.4 Filters are neutralized 5 min. in a vessel with ammonium acetate (4 ml/filter) on a shaker at room temperature.
C	**13**		**3.4.5 #541 Whatman filters are briefly rinsed 2 times in 1X SSC buffer (10 ml/filter).**
C	**13, 16, 17**		**3.4.6 Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 min at 42°C. May be conducted in an environmental chamber with shaking (50 rpm) or a water bath.**
K	13		3.4.7 Filters are rinsed 3 times in 1X SSC (10 /filter) for 10 min at room temperature with shaking, at 50 rpm.
		3.5 Hybridization. (May be conducted in an environmental chamber with shaking or a water bath)	
C	**13**		**3.5.1 For *V parahaemolyticus*, the thermolabile hemolysin (*tlh*), AP-labelled probe 5'Xaa agc gga tta tgc aga agc act g 3' is used. For the thermostable direct hemolysin (*tdh*), the AP-labelled probe 5'Xgg ttc tat tcc aag taa aat gta ttt g 3' is used. For *V. vulnificus*, the cytolysin gene (*vvhA*), AP- labelled probe 5'; Xga gct gtc acg gca gtt gga acc a 3' is used.**
C	**13**		**3.5.2 Probes are stored in the refrigerator, not frozen.**
C	**13, 16**		**3.5.3 Filters are presoaked in hybridization buffer for 30 min at 54±0.5°C for *V. parahaemolyticus* or 55±0.5°C for *V. vulnificus.* A maximum of 5 filters with 10ml of buffer is used per bag. Up to 20 filters at a time with buffer at the ratio of 10ml per 5 filters can be combined into a vessel of appropriate size to ensure the solution covers the filters.**
C	**13, 16, 17**		**3.5.4 10 ml fresh pre-warmed buffer per 5 filters is added. Probe (final conc. of 0.5 pmol/ml) is quickly added to bag or vessel with filters and incubated 1-1.5 h at 54±0.5°C for *Vibrio parahaemolyticus* of 55±0.5°C for *Vibrio vulnificus*.**
C	**13**		**3.5.5 Filters are rinsed 2 times for 10 min each in 1X SSC - 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC - 1% SDS (for tdh) (10 ml/filter) at 54°±0.5°C C for *Vibrio parahaemolyticus* or 55±0.5°C for *Vibrio vulnificus*.**

K	13		3.5.6 Filters are rinsed 5 times for 5 min each in 1X SSC (10 ml/filter) at room temperature with shaking, at100 rpm.
		3.6 Color development	
C	**13, 16, 17**		**3.6.1 In petri dish or suitable vessel, containing 20 ml of NBT/BCIP solution filters (5 or fewer) are added to the petri dish/container and incubated with gentle shaking at room temperature, or at 35°C for faster results. The petri dish/container is kept covered to omit light. Color development of the positive control is checked every 30 minutes. Reaction time varies.**
K	13		3.6.2 Rinse in tap water (10 mL/filter) 3 times for 10 min each to stop color development.
C	**2, 13, 16**		**3.6.3 Reactions of test sample colonies are compared to the positive and negative process control cultures. Positive reactions appear as purple or brown spots, yellow spots are considered negative reactions. Filters are stored in the dark.**
C	**13**		**3.6.4 Store probes in the refrigerator; do not freeze.**
		3.7 Computation of Results	
C	**13, 16, 17**		**3.7.1 For direct plating, upon identification of *Vibrio parahaemolyticus* and/or *Vibrio vulnificus*, positive colonies are counted and multiplied by the use dilution factor of the sample to determine the concentration.**
K	16		3.7.2 For direct plating, results are reported as CFU/g of sample.
C	**13, 19**		**3.7.3 For APW enrichment, upon identification of *Vibrio parahaemolyticus* and/or *Vibrio vulnificus*, refer to the original positive APW dilutions and record MPN value as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13, 16, 17		3.7.4 For APW enrichments, results are reported as MPN/g of sample.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C. 7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #1621. PHS, Washington, D.C.
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
5. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition . APHA/AWWA/WEF, Washington, D.C.
7. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study*. U.S. Government Printing, Washington, D.C.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition . APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54 – 60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
12. Adams, W.N. 1974. NETSU. Personal Communication to Dr. Wallace Andrews, FDA.
13. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
14. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
15. Depaola A, Jones JL, Noe KE, Byars RH, Bowers JC. Survey of postharvest-processed oysters in the United States for levels of Vibrio vulnificus and Vibrio parahaemolyticus. J Food Prot. 2009 Oct;72(10):2110-3.
16. David W. Cook, Angelo DePaola, and Susan A. McCarthy. DIRECT PLATING PROCEDURE FOR THE ENUMERATION OF TOTAL AND PATHOGENIC Vibrio parahaemolyticus IN OYSTER MEATS. FDA / Office of Seafood Gulf Coast Seafood Laboratory Dauphin Island, AL 36528-0158.
17. DePaola, A. et al. 1997. Evaluation of an alkaline phosphatase-labeled DNA probe of enumeration of *Vibrio vulnificus* in Gulf Coast oysters. Journal of Microbiological Methods 29:115-120.
18. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988) U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.
19. ISSC Laboratory Committee deliberation. 2015 Biennial Meeting. Committee voted to use BAM calculator to avoid erroneous calculations and ensure consistency.

LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY:		
Page	Item	Observation

Proposal No. **17-111**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	FDA
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	MPN Real-Time PCR Method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* Detection in Oysters - Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	The requested action is to adopt the text of the attached checklist for the MPN real-time PCR method for detecting *Vibrio vulnificus* (Vv) and *Vibrio parahaemolyticus* (Vp) in oysters and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Public Health Significance	Currently, there is no checklist adopted by the ISSC for the MPN real-time PCR method for detecting Vv and Vp in oysters that is approved in the NSSP for Vibrio enumeration. The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing this method in support of the NSSP. The checklist documents the number of critical, key or other nonconformities and how overall laboratory status for the method is determined.
Cost Information	NA

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III]
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III]

Revised 6-17-2017

PART I – Quality Assurance			
		ITEM	
CODE	**REF**		
		1.1 Quality Assurance (QA) Plan	
K	4, 6	1.1.1 Written Plan (Check √ those items which apply).	
			a. Organization of the Laboratory.
			b. Staff training requirements.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**4**		**1.1.2 The QA plan is implemented.**
K	6		1.1.3 The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area	
O	4, 6		1.3.1 Adequate for workload and storage.
K	6		1.3.2 Clean, well lighted.
K	6		1.3.3 Adequate temperature control.
O	6		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5 Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
K	5		1.4.1 To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units
K	9		1.4.2 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	6		1.4.3 The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4 The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.
K	6		1.4.5 A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7).

			The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7 The balances used provide a sensitivity of at least 0.1g at the weights of use.
K	6		1.4.8 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10 Refrigerator temperatures are maintained between 2 and 8°C.
C	**7**		**1.4.11 Freezer temperature is maintained at -20°C or below.**
O	7		1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13 The temperature of the incubator is maintained at 35+2.0°C.**
K	6		1.4.14 Thermometers used in the air incubators are graduated at no greater than 0.5°C increments.
K	5		1.4.15 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16 Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17 All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**6, 20**		**1.4.19 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35, 54 and 55°C (54C for Vp and 55C for Vv). These calibration records are maintained.**
K	3, 5		1.4.20 Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination:_____
C	**2, 20**		**1.4.21 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05°C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22 All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23 Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24 Micropipettors are calibrated at appropriate volumes used annually and checked for accuracy quarterly. Results are recorded and records maintained.
K	5		1.4.25 Pipets used to inoculate samples and prepare reagents deliver

			accurate aliquots and are tested for accuracy with each new lot received.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5 In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3 The autoclave provides a sterilizing temperature of 121± 2°C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4 An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121°C. Calibration at 100°C, the steam point is also recommended but not required.
K	10		1.6.5 The autoclave standards thermometer is checked every five years for accuracy at either 121°C or at 100°C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6 Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check:_____
K	6		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).

K	6		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180°C.
K	5		1.6.11 A thermometer capable of determining temperatures accurately in the range of 160 to 180°C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13 Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170°C for 2 hours.
C	**2**		**1.6.16 The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18 The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19 Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation	
K	13, 14		1.7.1 Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2 Media components are properly stored in a cool dry place.
O	6		1.7.3 Media components are labeled with the analyst's initials, date of receipt and date opened.
C	**6**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotropic plate count method. Results are recorded and records maintained.
K	5		1.7.8 The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9 The total time of exposure of media broths to autoclave temperatures does not exceed 60 minutes.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media	

K	5		1.8.1 Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2 Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3 Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4 Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5 Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6 All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II –Samples			
		2.1 Collection and Transportation of Samples	
C	**2, 6**		**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2 Oyster samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3 Oyster samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.14 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis	
K	2, 6		2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		2.2.2 Blades of shucking knives are not corroded.
K	5		2.2.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5 Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6 Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7 Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8 Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9 The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10 A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11 A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**

K	2, 13		2.2.12 Either a 1:1 dilution can be made at this point, or proceed directly to If a dilution is made, the sample is weighed to the nearest 0.1 gram and an equal amount, by weight, of diluent is added.
K	13		2.2.13 Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14 Samples are blended for 60 to 120 seconds until homogenous.**
PART III- PCR method for Vibrio vulnificus and Vibrio parahaemolyticus detection in Oysters			
		3.1 APW Enrichment	
K	5		3.1.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**3.1.2 The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 mL of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10g of sample homogenate to 90 ml of PBS.**
C	**17**		**3.1.3 Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution _____**
C	**2, 15**		**3.1.4 For V. parahaemolyticus analysis, a tdh+ V. parahaemolyticus culture diluted to <10^3 per ml is used as a positive process control. A V. vulnificus culture is used as a negative process control.** **For V. vulnificus analysis, a V. vulnificus culture diluted to <10^3 per ml is used as a positive process control. A V. parahaemolyticus culture is used as a negative process control.** **The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Records are maintained.**
C	**13**		**3.1.5 Inoculated APW enrichment tubes are incubated at 35+2°C.**
C	**13**		**3.1.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
		3.2 PCR Reagents	
C	**14, 15**		**3.2.1 Lyophilized primers and probes are stored according to manufacturers instructions**
K	14, 15		3.2.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.2.3 The PCR forward and reverse primers used target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT -3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG -3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F:5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3'

			<u>For Vv Real-time PCR Method</u> vvhF 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR 5'-TTCTTTATCTAGGCCCCAAACTTG-3'
C	**14, 18**		**3.2.4 Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.2.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.2.6 Storage of reconstituted primers and probes in -20°C manual defrost freezer does not exceed 1 year.**
C	**16**		**3.2.7 Platinum *Taq* DNA is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**16**		**3.2.8 PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		3.3 DNA Extraction	
C	**14, 18**		**3.3.1 All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.3.2 Pipet tips have aerosol barriers.**
K	14, 18		3.3.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.3.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.3.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.3.6 One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.3.7 Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 100+/-5°C for 10 minutes.**.
K	14, 18		3.3.8 A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.3.9 After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis.**
K	14, 18		3.3.10 Frozen extracts are analyzed within 6 months of frozen storage.
		3.4 Preparation of the Master Mix for PCR	
C	**14, 16, 18**		**3.4.1 Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.4.2 For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 16, 18		3.4.3 The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18**		**3.4.4 Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.4.5 Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		3.5 PCR	
C	**14, 19**		**3.5.1 Immediately prior to use, DNA extracts are centrifuged at >5,000xg for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.5.2 Two (2) µL of DNA template is added to each reaction tube or plate well containing 23µL of Master Mix for a total PCR reaction volume of 25µL.**

K	14, 19		3.5.3 Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19**		**3.5.4 Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23µL of Master Mix.**
C	**14, 19**		**3.5.5 Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23µL of Master Mix**.
O	14, 19		3.5.6 Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23µL of Master Mix as the positive PCR control.
K	14, 19		3.5.7 Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.5.8 After centrifugation, tubes or plates are inserted into the instrument.**
		3.6 PCR Amplification	
C	**14, 19**		**3.6.1 The appropriate instrument platform is used for the protocol.**
K	16		3.6.2 Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.6.3 The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.6.4 Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.6.5 The analysis settings are adjusted as specified in the protocol.**
		3.7 Computation of Results	
K	14, 19		3.7.1 All runs in which the positive control generates a Ct value for the target(s) of interest and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C			**3.7.2 Data is quality checked by the analyst.**
C	**14, 19**		**3.7.2 All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.7.3 Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.7.4 All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.7.5 Any reaction in which no Ct value is generated for the target(s) of interest or the internal control is considered invalid and should be re-tested.**
C	**13**		**3.7.6 Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.7.7 For APW enrichment, results are reported as MPN/g of sample.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C.
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
5. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition . APHA/AWWA/WEF, Washington, D.C.
7. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study*. U.S. Government Printing, Washington, D.C.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition . APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54 – 60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
12. Adams, W.N. 1974. NETSU. Personal Communication to Dr. Wallace Andrews, FDA.
13. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
14. Campbell, Mark, S. and Wright, Anita, C. Real-time PCR analysis of *Vibrio vulnificus* from oysters, Appl Environ Microbiol. 69, 12 (2003).
15. Wright, Anita, C., Garrido, V, Debuex, G, Farrell-Evans, M, Mudbidri, A, A. and Otwell, W, S. Appl Environ Microbiol. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. 73, 22 (2007).
16. Cepheid© product information.
17. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
18. Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray, and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic V. parahaemolyticus bacteria in oysters. Appl. Environ. Microbiol. 73(18):5840-5847.
19. Kinsey, T.P., K.A. Lydon, J.C. Bowers, J.L. Jones. 2015. Effects of Dry Storage and Resubmersion of Oysters on Total Vibrio vulnificus and Total and Pathogenic (tdh+/trh+) Vibrio parahaemolyticus Levels. J. Food. Prot. 78(8): 1574-1580.
20. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988) U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.

LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical, Key and Other (O) Nonconformities in Parts I-III	

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or** _____

   b. The total # of Key nonconformities is **≥ 13 or** _____

   c. The total # of Critical, Key and Other is **≥ 18** _____

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1**

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory
Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

Revised 6-17-2017

LABORATORY:		
Page	Item	Observation

Proposal No. 17-112

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Requirements for certification of State Shellfish Laboratory Evaluation Officers (LEOs).
Specific NSSP Guide Reference	Section IV Guidance Documents – Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	Section IV Guidance Documents – Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists amend language. General Provisions 1. If the State Shellfish Control Authority (Authority) uses the analytical services of private/commercial/fee for services laboratories to support the NSSP, then he/she should select a qualified individual to become certified as a State Shellfish Laboratory Evaluation Officer (State Shellfish LEO). 2. If the Authority uses the analytical services of multiple public laboratories (state, county, parish town, etc.) to support the NSSP, then he/she may select a qualified individual to become a State Shellfish LEO. 3. If the Authority chooses not to participate in the certification process, FDA can evaluate the state's public laboratories. FDA, however, does not normally evaluate private/commercial/fee for services laboratories. FDA may, under certain circumstances as resources permit, evaluate these laboratories on a case-by-case basis at the request of the Authority. This request must be in writing and made through the FDA ~~Regional~~ Shellfish Specialist. 4. State Shellfish LEOs will perform official NSSP evaluations of laboratories which have been previously evaluated by FDA and been found to fully conform to NSSP laboratory requirements. 5. State Shellfish LEOs may evaluate laboratories in a different state under a memorandum of understanding between the states involved and FDA, consistent with NSSP requirements. 6. State Shellfish LEOs may not evaluate laboratories in which they are employed or which they supervise or laboratories within the same supervisory chain of command to ensure complete objectivity in the evaluation process and avoid the appearance of a conflict of interest. 7. To qualify for certification, the prospective State Shellfish LEO ~~should~~ must ~~be~~: a. ~~A~~ Be a state employee; b. Have a minimum of two years of shellfish laboratory experience or a laboratory background~~;~~ with three to five years of bench level experience with the specific methods that will be evaluated;

c. ~~Preferably h~~ Have ~~laboratory evaluation~~ experience performing laboratory evaluations or supervising a laboratory; and,

d. Be free from any commercial, financial or other pressures or conflicts of interest that might cause or appear to cause the prospective State Shellfish LEO to act in other than an impartial or non-discriminatory manner.

8. If the prospective or current State Shellfish LEO is employed by the laboratory supporting the NSSP, that laboratory must be fully conforming to NSSP requirements or the individual will not be certified and if currently certified, certification will be revoked.

Responsibilities of the FDA National Laboratory Standard

1. The FDA National Laboratory Standard/s will be responsible for standardizing all LEOs.
2. The FDA National Laboratory Standard will conduct certifications/recertifications. The Standardization evaluation process will consist of a minimum of two (2) practice evaluations in areas under consideration for certification and one (1) formal standardization evaluation. The evaluation will be checklist specific and the State Shellfish LEO will be standardized to evaluate the methods only for which they have been certified.
3. FDA Standard Operating Procedure for Laboratory Evaluations will be provided to every LEO candidate for the purpose of evaluation standardization.

Responsibilities of the State Shellfish Control Authority

1. The Authority must ensure that appropriate written documentation is provided to FDA to demonstrate that a prospective State Shellfish LEO is adequately qualified to assume the responsibilities of a State Shellfish LEO as described above.
2. The Authority must provide or ensure that adequate time, resources and support are made available to the State Shellfish LEO to fully participate in the certification process and to fulfill his/her obligation as a State Shellfish LEO.
3. The Authority will provide, or ensure adequate opportunity for, State Shellfish LEOs to maintain communication with FDA LEOs, as needed, to provide guidance and updates relevant to the NSSP laboratory evaluation program and any changes to their State programs.

FDA's Responsibilities

1. FDA is responsible for the certification/recertification of State Shellfish LEOs.
2. As a result FDA must:

a. Select qualified individuals to receive training based upon the documentation supplied by the Authority;

b. Develop and provide training that will enable prospective and current State Shellfish LEOs to consistently and uniformly apply evaluation criteria in determining the competence of laboratories to support or continue to support the NSSP;

c. Certify prospective State Shellfish LEOs that successfully complete the certification process;

d. Maintain communication with State Shellfish LEOs as needed to provide guidance and updates relevant to the NSSP laboratory evaluation program;

e. Recertify current State Shellfish LEOs pursuant to the criteria established

for satisfactory performance below;
f. Monitor the performance of State Shellfish LEOs to ensure that the evaluation process is being performed consistent with NSSP requirements as described in the current NSSP Guide for the Control of Molluscan Shellfish and this guidance;
g. Maintain communication as needed with the Authority and other pertinent state officials, prospective and current State Shellfish LEOs and FDA Shellfish Specialists relevant to the certification/recertification process;
h. Revoke certification of State Shellfish LEOs for cause; and,
i. Void certification when the need for a State Shellfish LEO no longer exists within the state shellfish sanitation program or when the State Shellfish LEO is no longer employed by the state.

State Shellfish Laboratory Evaluation Officer's Responsibilities

1. Conduct on-site laboratory evaluations at least every three (3) years. However, more frequent evaluations are strongly encouraged and may be necessary with marginally performing laboratories, or when major changes in workloads or priorities have occurred or when there has been a substantial turnover of personnel, or, at the specific request of the Authority.
2. Provide appropriate post-evaluation follow-up for each laboratory evaluated, (i.e., monitoring corrective actions and resolutions of all nonconformities).
3. Prepare ~~timely~~ narrative evaluation reports within 30 days for all laboratories evaluated. The report should consist of the completed FDA Shellfish Laboratory Evaluation Checklist for the component(s) evaluated and a narrative discussion that accurately and concisely describes the overall operation of the laboratory. All nonconformities noted should be described in this narrative~~;~~, and, where relevant, an explanation provided relating the potential impact of the deficiency to ~~on~~ the analytical results. Completed corrective actions should be included in the narrative report only if they were completed on-site. Recommendations for corrective action or, if applicable, suggestions to enhance laboratory operations should also be included in the narrative report.
4. Distribute completed evaluation reports with checklists to FDA LEOs and to the appropriate FDA ~~Regional~~ Shellfish Specialist.
5. Inform FDA Shellfish ~~Laboratory Evaluation Officers~~LEOs when a laboratory has been found to be in nonconforming status immediately upon closeout. A letter informing FDA National Laboratory Standard of upgraded status by way of a separate Completed Corrective Action Memo will be sent, should one be necessary.
6. Coordinate proficiency testing at least yearly for all laboratories in the State supporting the microbiology component of the NSSP.
7. Prepare annually (in December) a summary list of all laboratories, ~~and~~ qualified analysts, and methods performed in each NSSP laboratory and transmit it to the FDA Shellfish LEOs.

Certification Process
Certification is designed to be accomplished through individualized training and field standardization. Individuals are certified for evaluating either the microbiological and/or ~~post-harvest processing (PHP)~~ vibrio detection and/or marine ~~B~~biotoxin components of the NSSP depending on their qualifications and

the needs of the state shellfish sanitation program. ~~and at the discretion of FDA.~~ Certification is dependent upon the perspective State Shellfish LEO satisfying all the following performance criteria.

a. Demonstration of good familiarity with evaluation requirements.
b. Demonstration of a thorough knowledge of the evaluation methods and documents.
c. Demonstration of the technical knowledge/familiarity with the analytical procedures being used.
d. Ability to communicate effectively both orally and in writing.
e. Successful completion of both training course and field standardization.

Field Standardization

1. Field Standardization is designed to evaluate the prospective State Shellfish LEO's ability to determine the competence of the laboratory to meet NSSP laboratory requirements,~~;~~ recognize laboratory practices inconsistent with NSSP requirements when they occur,~~;~~ make appropriate recommendations for corrective action,~~;~~ and~~;~~ provide the necessary follow-up activity to bring the laboratory into conformity with the NSSP.

2. Field standardization consists of ~~one or several joint but independent~~ a minimum of two practice and one ~~final~~ onsite evaluations ~~with an~~ the FDA National Laboratory Standard. ~~Shellfish Laboratory Evaluation Officer and preparation of the corresponding narrative evaluation reports.~~ For the final standardization assessment, the onsite evaluation, all "Critical" nonconformities cited, or lack thereof, must be in agreement between the FDA National Laboratory Standard and the State LEO candidate. Additionally, for "Key" and "Other" nonconformities, the evaluation checklists completed by the prospective State Shellfish LEO candidate and the FDA National Laboratory Standard should be in 90% agreement.

~~2.~~3. During all joint field evaluations the State Shellfish LEO Candidate will be the lead evaluator. He or she will be responsible for requesting documents, assessing records, and conducting the evaluation. FDA Standard Operating Procedure for inspection will be followed regarding assessment requests. The Candidate shall also conduct the "exit" interview and discuss all significant findings with management.

~~3.~~4. The narrative evaluation report must be prepared by the State Shellfish LEO candidate for each joint but independent evaluation conducted. The report(s) should consist of the completed FDA Shellfish Laboratory Evaluation Checklist(s) and a narrative discussion that accurately and concisely describes the overall operation of the laboratory. All nonconformities noted should be described in the narrative, and where relevant, an explanation provided relating the potential impact of the deficiency ~~on~~ to the analytical results. Recommendations for corrective action, or if applicable, suggestions to enhance laboratory operations should be included in this narrative report(s).

~~4.~~5. Final ~~F~~field standardization should be performed in NSSP laboratories within the prospective State Shellfish LEO's home state to provide realistic evaluation scenarios. ~~The narrative evaluation report detailing the evaluation findings must be prepared.~~ The draft narrative report(s) with accompanying checklist(s) must be submitted to the certifying FDA Shellfish Laboratory Evaluation Officer within 30 ~~60~~ days of the evaluation(s). All documents submitted will be reviewed for appropriate content, accuracy, and uniformity of approach by the certifying FDA ~~Shellfish Laboratory Evaluation Officer~~ National Laboratory Standard.

~~5.~~6. Field standardization is ~~based on a~~ pass/fail ~~system~~.

~~6.~~7. After successfully completing the Field Standardization Exercise, the State Shellfish LEO Candidate will be granted the title of Laboratory Evaluation Officer. A certificate recognizing that accomplishment will be forwarded to the State Shellfish LEO Candidate, along with formal notification to the State Shellfish LEO Candidate's supervisor, within thirty (30) days.

~~Certification~~

~~1. Certification is dependent upon the perspective State Shellfish LEO satisfying~~

~~all the following performance criteria.~~

~~a. Demonstration of good familiarity with evaluation requirements.~~

~~b. Demonstration of a thorough knowledge of the evaluation methods and documents.~~

~~c. Demonstration of the technical knowledge/familiarity with the analytical procedures being used.~~

~~d. Ability to communicate effectively both orally and in writing.~~

~~e. Successful completion of both training and field standardization.~~

~~2. Upon successful completion of the certification process, a letter of certification will be issued by the FDA Shellfish Laboratory Evaluation Officer and a copy will be sent to both the requesting Authority and the FDA Regional Shellfish Specialist.~~

~~3. Certification is normally valid for up to five (5) years unless revoked or voided.~~

Failure to be Certified

1. If a prospective State Shellfish LEO fails to satisfy any of the performance criteria listed above, he/she will not be certified.
2. As resources permit ~~and at the discretion of FDA~~, the prospective State Shellfish LEO may receive additional training to better prepare him/her to be certified; including attending the Shellfish Program Laboratory Methods and Evaluation Procedures Course. If the LEO candidate is unsuccessful in his/ her final standardization attempt he/ she must repeat the two (2) practice evaluations and one (1) final standardization evaluation. If failure continues after the second attempt, the candidate will not be eligible for a third attempt at standardization without the expressed permission of the National Laboratory Standard.
3. The requesting Authority may withdraw the prospective State Shellfish LEO from consideration.

Recertification

1. Recertification normally occurs every ~~five (5)~~ six (6) years and is contingent upon the continuing need in the state shellfish sanitation program for the services of a State Shellfish LEO.
2. Recertification is based on the State Shellfish LEO satisfactorily meeting the following employment and performance criteria.
   a. The individual must continue to be employed by the state and be free of any commercial, financial or other pressures or conflicts of interest real or perceived that may cause the State Shellfish LEO to act in other than an impartial and non-discriminatory manner.
   b. The individual must demonstrate continued competence in the evaluation of NSSP laboratories by performing ~~one to several joint~~ evaluations with an FDA Shellfish Laboratory Evaluation Officer and providing an appropriate narrative evaluation report to the FDA National Laboratory Standard. ~~co-~~

	~~evaluator for review and comment for each of the laboratories jointly evaluated.~~ c. The individual must have performed laboratory evaluations at the minimum frequency prescribed in the current edition of the Guide for the Control of Molluscan Shellfish and have all Narrative evaluation reports up to date. 3. State Shellfish LEOs who successfully complete recertification will be issued a letter of recertification by FDA and be cleared to distribute the completed report(s) to the appropriate ~~Regional~~ Shellfish Specialist. A copy of this letter will be sent to the State Shellfish Control Authority and appropriate ~~Regional~~ Shellfish Specialist. 4. If FDA is unable to conduct a recertification visit by the expiration of the individual's certification, his/her certification may be extended until such time as recertification can be completed. If requested, a letter extending the certification can be provided as appropriate. Standardization Maintenance 1. Maintenance will be provided in the form of updated Laboratory Evaluation Officer courses, updated field standardization guides, and other guidance/technical assistance activities on an as needed basis. 2. State Shellfish LEOs will be required to attend the Shellfish Program Laboratory Methods and Evaluation Procedures Course every three years or when it is offered by FDA Revocation of Certification 1. State Shellfish LEOs who fail to meet any of the certification/recertification, employment, or performance criteria listed above will have their certification revoked. 2. Certification may be voided when state shellfish sanitation programs no longer have a need for the services of a State Shellfish LEO. 3. Voided certifications may be reactivated at the discretion of FDA if the need for the analytical services of additional laboratories by the state shellfish sanitation program recurs. 4. Revoked certifications will not normally be restored. 5. The National Laboratory Standard will document the reason(s) for revocation of the LEO certification. This information shall be forwarded to the Candidate's supervisor and a copy shall be placed in the FDA file. All evidence and conclusions reached by the FDA shall be documented in writing by the Standard and shall be retained for three (3) years in accordance with the Freedom of Information Act.
Public Health Significance	The updated/revised requirements for certifying State Shellfish LEOs will help to ensure a more objective, standardized approach to the certification process.
Cost Information	Costs associated with activities for certification of State Shellfish LEOs are the responsibility of the State Shellfish Control Authority. However, it is anticipated that costs specifically associated with attendance at the Shellfish Program Laboratory Methods and Evaluation Procedures Course would be funded by FDA.

Proposal No. **17-113**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	ISSC Male-Specific Coliphage Committee
Affiliation	Interstate Shellfish Sanitation Conference
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Classification of Shellfish Growing Areas Adjacent to Waste Water Treatment Plants
Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II. Growing Areas .19 Determining Appropriately Sized Prohibited Areas Associated with Wastewater Treatment Plants
Text of Proposal/ Requested Action	**~~19. Determining Appropriately Sized Prohibited Areas Associated with Wastewater Treatment Plants~~** ~~A.Introduction~~ ~~The original National Shellfish Sanitation Program (NSSP) principles have proved effective in controlling bacterial illness associated with shellfish harvested from polluted waters. These principles, namely a robust sanitary survey, regular water and shellfish monitoring using bacterial indicators, controlled harvest times and labelling the origin of shell stock remain applicable as the primary preventative food safety control measures for growing areas.~~ ~~However, there is now ample scientific evidence to show that the current bacterial indicators are inadequate to predict the risk of viral illness for the following reasons:~~ ~~(1) Enteric viruses are resistant to treatment and disinfection processes in a Waste Water System Discharge (WWSD) and are frequently detected in the WWTP's final effluent under normal operating conditions (Baggi et al. 2001; Burkhardt et al. 2005, Pouillot et al. 2015).~~ ~~(2) Shellfish can bioaccumulate enteric viruses up to 100 fold from surrounding water (Seraichekas et al. 1968; Maalouf et al. 2011).~~ ~~(3) Certain enteric viruses are retained by molluscan shellfish to a greater extent and for longer than the indicator bacteria currently used to classify shellfish growing areas (Sobsey et al. 1987; Dore~~

& Lees 1995; Love et al. 2010). It has been well documented that enteric virus detection is not indexed by levels of conventional indicator bacteria.

For several decades now viral illnesses, in particular norovirus (NoV) and Hepatitis A (HAV), have been the most common food safety problem associated with bivalve molluscan shellfish (Woods 2010; Iwamoto et al 2010; Scallan et al. 2011; Batz et al. 2012; Hall et al 2012). NoV genogroups I, II and IV and HAV are typically associated with ill individuals and transferred by the fecal-oral route. Because WWTPs do not completely remove infectious enteric viruses emphasis should be placed on the importance of ensuring there is adequate dilution between a sewage source and a shellfish growing area.

In addition to the risk of enteric viruses WWTP effluents may also contain other chemicals and deleterious substances including pharmaceuticals, nanoparticles, and other contaminants of emerging concern. Establishment of a prohibitive area in proximity to WWTP discharges is an effective strategy to reduce the risk posed by both enteric viruses and other contaminants found in WWTP effluents. This guide provides information on the recommended dilution rates with respect to enteric viruses to ensure WWTP effluent does not cause a significant viral food safety risk within shellfish growing areas. The guide also considers the factors that should be used to assess a WWTP.

B.Delineation of the Prohibited Area around a Waste Water System Discharge (WWSD)

The NSSP Model Ordinance Section II, Chapter IV. @.03 (2) (b) and @.03 E(5) states that all growing areas which have a sewage treatment plant outfall or other point source outfall of public health significance within or adjacent to the shellfish growing area must have a prohibited classification established adjacent to the outfall taking account of the following factors:

(1) The volume flow rate, location of discharge, performance of the Waste Water System Discharge (WWSD) and the microbiological quality of the effluent;

(2) The decay rate of the contaminants of public health significance in the wastewater discharged;

(3) The wastewater's dispersion and dilution and the time of waste transport to the area where shellstock may be harvested; and

(4) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.

C.Establishing the Size of Each Prohibited Area There are several important considerations for the shellfish authority to consider when establishing the size of each prohibited area:

~~(1) The area to ensure that there is adequate dilution when the WWTP is operating as normal. "Normal" means that the WWTP is operating fully within the plant's design specifications, including design flows; treatment stages; disinfection; as well as compliance with all permit conditions that relate to the WWTPs effectiveness in reducing enteric viruses in sewage.~~

~~Below is not an exhaustive list but serves as examples of situations that could occur and are critical for Shellfish Control Authorities (SCAs) on evaluating each WWTP when developing Conditional Area Management Plan (CAMP):~~

~~(a)Bypassing stage of treatment~~

~~A plant may be considered operating outside of normal operation if a treatment stage such as primary or secondary treatment is bypassed which may result in an increased load of solids in the disinfection step and reduce the effectiveness of disinfection. An additional example would be when a WWTP experiences a loss in disinfection and thus the ability to effectively treat the final effluent. SCAs should determine the significance of these types of events and make appropriate provisions in the CAMP.~~

~~(b)Operating outside design specifications/other types of failures or events~~

~~It is not uncommon for a WWTP to periodically experience mechanical failures of equipment that could alter the treatment of sewage. Additionally, a WWTP may also need to periodically perform routine maintenance to the various stages of treatment and may need to temporarily take a portion of a treatment stage off-line for cleaning. Other unexpected maintenance may need to occur for example bio-fouling of filters or membranes used in treatment. SCAs should be informed by WWTP operators of these events to determine if any additional temporary action is needed if not addressed in the CAMP.~~

~~(c)Operating above design flow~~

~~Some WWTPs may operate above its design flow and not necessarily bypass any particular stage of treatment. During these events it is typical for WWTP operators to adjust the operation of the WWTP which may include reducing the treatment time in the aeration stage and/or solids separation/settling stage of treatment. Under some circumstances this could lead to a significant reduction in the effectiveness of disinfection. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these type of events and if additional provisions should be~~

made in the CAMP.

(d)WWTP permit violations

If a WWTP is exceeding the permitted bacterial indicator levels in the final effluent this indicates that effectiveness of the disinfection step has been reduced. Other measured parameters in the effluent (e.g. Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD)) may also indicate a reduction in treatment efficiency as occurred. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these type of events and if additional provisions should be made in the CAMP.

Situations where compliance with permit but risk to shellfish growing area.

There could be situations in which a particular WWTP could be in compliance with a permit, and could still pose a risk to the shellfish harvest area. For example, a WWTP may have permit conditions to allow for flow blending during high flow periods where a portion of the sewage may receive full treatment but a portion of the sewage may only be partially treated and "blended" in the final disinfection step. Although this may be an acceptable practice under a permit it could result in conditions in which the efficiency of the WWTP to remove enteric viruses is considerably reduced. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these type of events and if additional provisions should be made in the CAMP.

(2) That the collection system has no malfunctions, bypasses or other factors that would lead to significant leakages of untreated sewage to the marine environment.

(3) That there is adequate detection and response time when any malfunction occurs to ensure that all harvesting ceases and closures are enforced, so that contaminated product does not reach the market.

Additional considerations

It is critical for SCAs to communicate with WWTP operators and ensure that there is no confusion over how SCAs define "outside of normal operation" in a Conditional Area Management Plan (CAMP) which may differ from how "malfunctions" or "violations" are defined in a permit. The SCAs also need to ensure that the WWTP operators understand the CAMP and that shellfish growing areas may close based on conditions of the CAMP even though the WWTP is operating in compliance within permitted conditions. Thus, it is important to communicate with WWTP operators to ensure that when shellfish closures occur and are reported that SCAs are using terminology that is understood by both parties.

~~D.Guidelines for Dilution, Dispersion, and Time of Travel of Effluent~~

~~Dilution refers to the dilution of effluent that occurs when the effluent is subjected to a number of physical processes in the receiving waters including turbulent mixing of the effluent in the vicinity of the outfall and at further distances primarily through tidal action, wind, and density stratification. Dispersion refers to the spread, location, and shape of the effluent discharge plume with time as it leaves the WWTP outfall. Time of travel refers to the time it takes effluent to reach the shellfish harvest site starting from the point of discharge.~~

~~It is essential to recognize that water samples collected near discharge outfalls are not useful for determining the size of prohibited areas because normal operating conditions in WWTPs can effectively reduce or even eliminate the fecal and total coliforms which are the current indicator microorganisms used to assess treatment efficiency. In contrast, many human enteric viruses are not inactivated by functioning WWTP treatment and disinfection systems, hence the need for an adequate dilution zone between the outfall and the shellfish resource.~~

~~It is important to consider not only the WWTP discharge, but also overflow points on the collection system such as those from pumping stations. While a malfunctioning WWTP may provide partial treatment, the discharge from a collection system is untreated and may be a more common failure point in the overall system.~~

~~When determining if a WWTP or collection system discharge within the watershed or catchment area draining to a shellfish estuary potentially impacts a shellfish growing area, in the absence of a performance history of the treatment and collection system, and a database of influent and effluent quality, the NSSP recommends that a worst case raw sewage discharge be assumed. In this circumstance, if a level of 1.4×10^6 FC/100ml is assumed for a raw sewage release, a 100,000:1 dilution would be required to dilute the sewage sufficient to meet the approved area standard of 14 FC/100ml. If dilution analysis determines that the location of the discharge is such that the dilution of effluent would be greater than 100,000:1 then the WWTP could be considered located outside the zone of influence to the shellfish growing area. Different dilution ratios may be applied depending on the known concentration of sewage, provided that the water quality objective of the downstream harvest area is met.~~

~~In areas where the required WWTP discharge dilution is less than 100,000:1 and/or a raw sewage release results in FC levels in the growing area of >14 FC/100 ml a conditional management may be considered. However, conditional management is only recommended for, highly efficient WWTPs that are well monitored to detect malfunctions and changes in effluent quality and when the shellfish authority has the resources to effectively administrate and patrol the conditions of the growing area management plan.~~

~~In all cases the FDA recommends the minimum of a 1000:1 dilution around~~

~~a WWTP outfall to mitigate the impact of viruses on shellfish growing areas.~~

~~A dye study can be used to measure the dilution and dispersion of the effluent during specific discharge conditions. Computer modeling programs can also be used to estimate the dispersion and dilution of the effluent plume from WWTPs and collection system overflows.~~

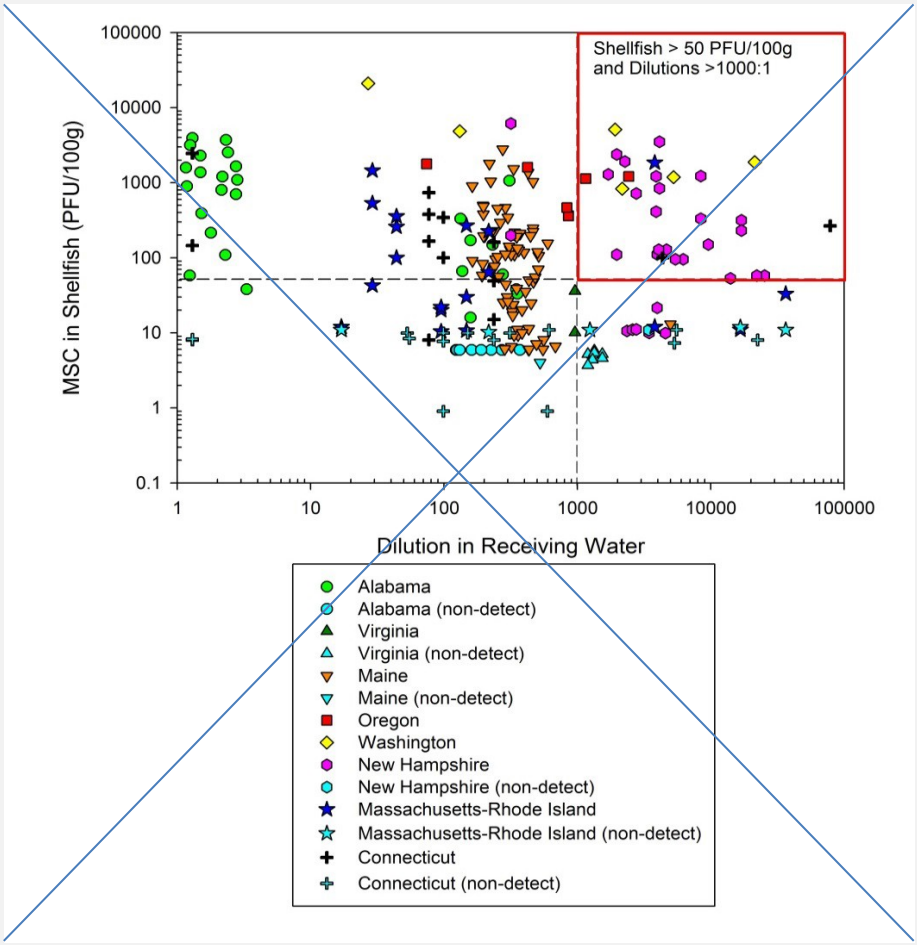
~~E.Scientific Rationale for 1000:1 Dilution Guidance~~

~~In 1995 the FDA determined the 1000:1 dilution was necessary using the most relevant the scientific literature available at that time (Kohn, et al. 1995; Havelaar et al. 1993; Kapikian et al. 1990; Liu et al. 1966). In 2008 FDA performed an investigation in the upper portion of Mobile Bay, Alabama, the results of which were published in the Journal of Shellfish Research (Goblick, et al., 2011). The article describes how FDA used technical advances to assess the 1995 1000:1 dilution recommendation. The Mobile Bay study confirmed that this level of dilution was appropriate to mitigate the risk of viruses discharged in treated wastewater effluent.~~

~~Since the 2008 Mobile Bay study there have been major advances in the detection and enumeration of NoV in wastewater and shellfish and fluorometer technologies have enabled more sophisticated hydrographic dye study methods. Using these advances, FDA has now conducted numerous dye studies supplemented with the testing of shellfish sentinels for enteric viruses and their surrogates. The findings from these studies demonstrate that achieving a steady-state 1000:1 dilution level in the requisite Prohibited area appears to be adequate for mitigating the impacts of viruses on shellfish when WWTPs have typical treatment and disinfection practices, such as secondary treatment and chlorination, and when operating under normal conditions.~~

~~While evaluating the 1000:1 dilution level Male Specific Coliphage (MSC) results in shellfish from the 2008-2015 studies were evaluated. These collaborative studies with State Shellfish Control Authorities and Industry were conducted in the Gulf, Mid-Atlantic, East and West Coast, and under varying hydrographic and meteorological conditions. Various additional factors were considered such as type of wastewater treatment and disinfection technology, seasonal conditions, and shellfish species etc. and are represented in the data collected. In some cases, data was collected during a period of which the WWTP was considered to be operating outside of "normal" operating conditions. In other cases, the WWTP was considered not suitable for conditional area management due to design/poor performance even during routine/normal operation. Focus was given to the MSC threshold of 50 PFU/100 grams of shellfish tissue which is the level used for re-opening harvest areas after an emergency closure due to raw untreated sewage discharged from a large community sewage collection system or a WWTP (Model Ordinance (Section II, Chapter IV, @.03 A(5)(C)(ii))). From the 2008-2015 studies, a total 216 samples were assessed including conditions when the WWTPs were considered operating normally as well as under a bypass or degraded operation conditions. In summary, 216 samples were analyzed for MSC of which 176 samples (81%) were~~

~~positive for MSC; 118 samples (67%) contained MSC levels > than 50 PFU/100 grams; and 43 samples (20%) had MSC levels > 50 PFU/100 grams and wastewater effluent dilution was greater than 1000:1. These results are shown in Figure 1 and Table 1 below.~~

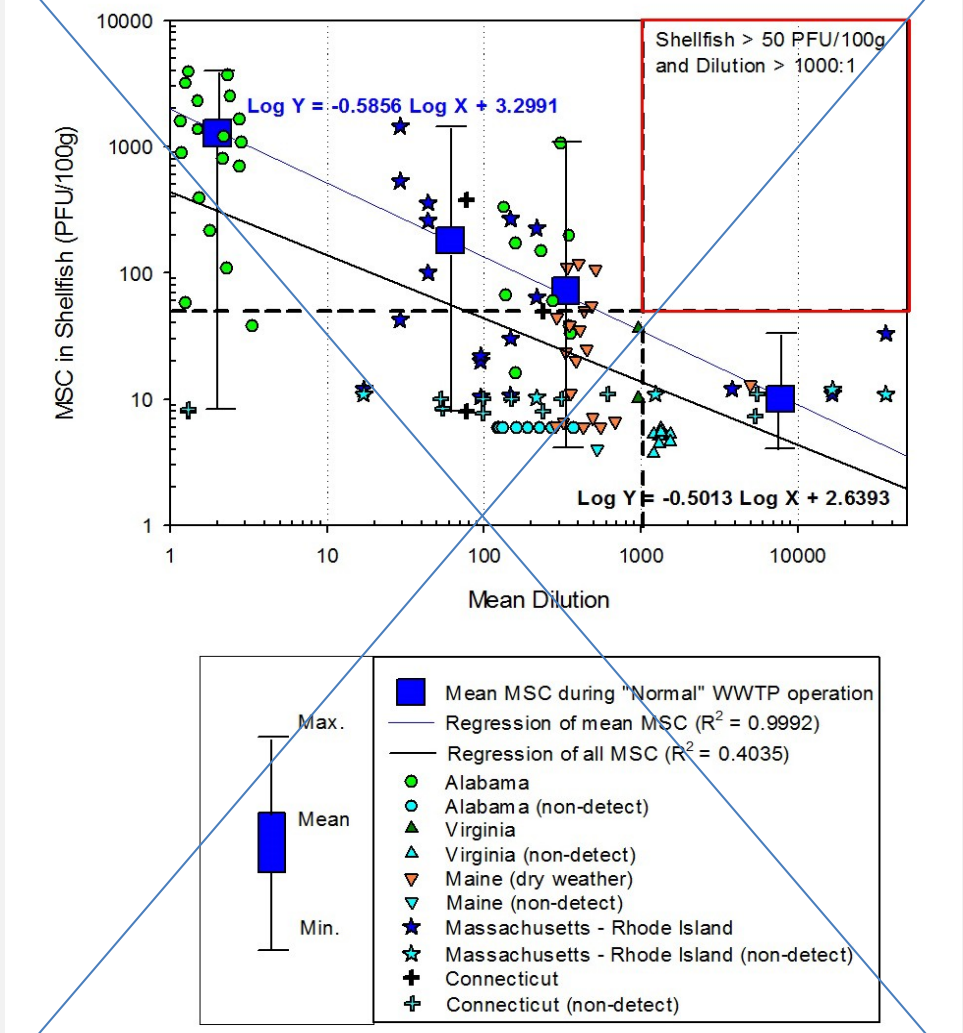


~~Figure 1: Comparison of dilution in receiving water and MSC levels in shellfish – all conditions Table 1: MSC in shellfish operating under "normal" and outside of normal operation~~

~~**MSC Results**~~	~~**All Conditions (n=216)**~~	~~**Normal Operating Conditions (n=129)**~~
~~MSC detectable~~	~~81% (176)~~	~~62% (80)~~
~~MSC levels >50 pfu/100g~~	~~67% (118)~~	~~36% (46)~~
~~MSC levels >50 pfu/100g and Dilution in Growing Area >1000:1~~	~~20% (43)~~	~~0% (0)~~

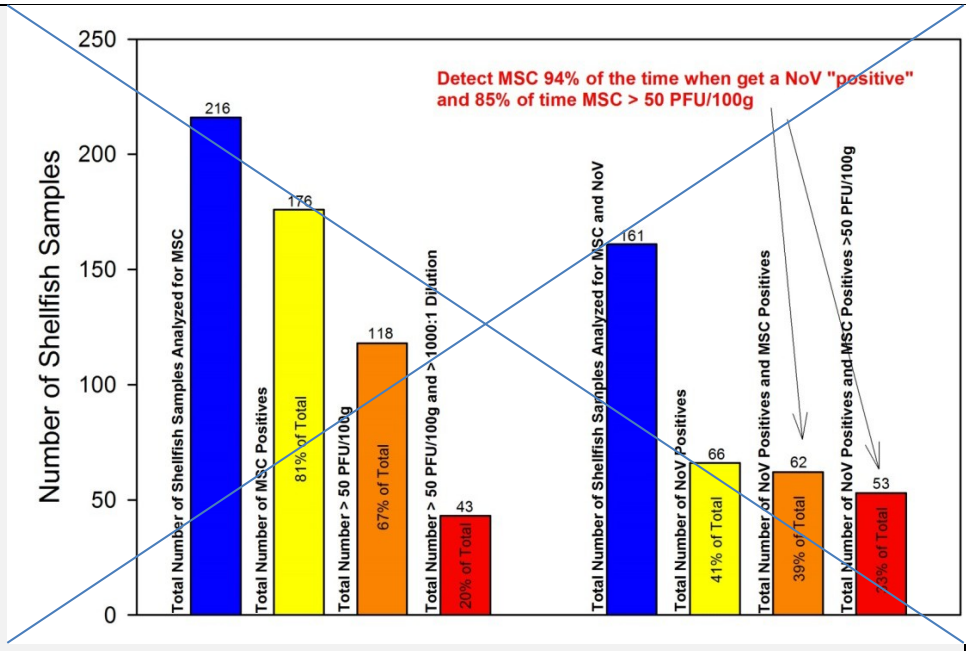
~~In separating the data attributed to "normal" operation from other conditions, 129 of the 216 total samples were considered to be attributed to~~

	~~"normal" WWTP operation, also shown on Table 1. Eighty seven (87) samples were removed as they were attributed to conditions of WWTP malfunction or situations considered not suitable for conditional area management. From the 87 samples, 80 were associated with degraded WWTP performance or malfunction of which 6 were associated with a primary bypass, 13 were associated within a period of a WWTP upgrade during which the WWTP reportedly was operating an extended period (weeks) without disinfection, 31 were associated with degraded treatment quality because of rainfall/flows exceeding the WWTP design capacity, and 30 were attributed to a WWTP with no secondary treatment and operated frequently with flows exceeding the design capacity. Of the remaining 7 samples, 6 were associated with a WWTP utilizing unconventional disinfection technology (membrane filtration) and demonstrated poor performance in removing viruses compared to other conventional technologies during normal operating conditions, and 1 sample was attributed to a potential point source sewage discharge other than the WWTP.~~ ~~When considering the remaining 129 samples attributed to "normal" WWTP operating conditions there were no samples that were above 50 PFU/100 grams when dilution was greater than 1000:1. In comparison, of the 87 samples attributed to malfunction or unsuitable conditions, 43 samples exceeded 50 PFU/100 grams when dilution was greater than 1000:1. These results are shown in Figure 2 below.~~ ~~Figure 2: Comparison of dilution in receiving water and MSC levels in shellfish under normal operation~~



~~Comparing MSC with NoV sample results, out of the 216 samples analyzed for MSC, 161 samples were also analyzed for NoV. Of the 161 samples tested for NoV, 66 were positive (41% of total) were positive for NoV. Out of the 66 NoV positive samples, 62 (94% of total) were also positive for MSC and 53 (85% of total) had levels greater than 50 PFU/100 grams. There were only 4 cases where NoV was positive but MSC was not detected. However, in these cases, 3 of the sample results were near the Limit of Detection (LOD) for NoV enumeration. In one case it is suspected that both MSC and NoV may have been present but not likely viable as the WWTP utilized UV disinfection and was operating under normal conditions. These results are shown in Figure 3 and Table 2 below:~~

~~Figure 3: Comparison of MSC and NoV results~~



~~Table 2: Comparison of MSC and NoV Results in shellfish~~

~~**MSC and NoV Results**~~	
~~NoV detected in shellfish~~	~~41% (66 of 161)~~
~~MSC detectable~~	~~39% (62 of 161)~~
~~MSC negative when NoV detected (MSC<10 pfu/100g)~~	~~7% (4 of 66)*~~
~~MSC present when NoV detected (MSC>10 pfu/100g)~~	~~94% (62 of 66)~~
~~MSC present when NoV detected (MSC>50 pfu/100g)~~	~~85% (53 of 66)~~

~~*NoV detected at LOD of Assay~~

~~The overall results of FDA's field studies demonstrate a strong relationship between increased levels of enteric viruses and MSC and decreased levels of dilution. This trend was observed in all of the studies conducted by FDA at conventional WWTPs. These results also emphasize the critical need for sufficient notification time, meaning travel time from the WWTP discharge in the prohibited area is long enough to close the shellfish growing area in the event of a malfunction. This preventative measure may necessitate the Prohibited Area be larger than the zone necessary to achieve 1000:1 dilution. Furthermore, this analysis demonstrates the need to individually assess each WWTP, to assess their performance to remove enteric viruses.~~

~~In addition to the FDA field studies, as part of a Joint United States-Canada Norovirus in Bivalve Molluscan Shellfish Risk Assessment, a Meta-Analysis of the Reduction of NoV and MSC Concentrations by~~

~~Wastewater Treatment was conducted (Pouillot, 2015). The meta-analysis included previously unpublished surveillance data from the United States and Canada and relevant data reported in the literature (2,943 measurements in total).~~

~~For WWTPs with mechanical systems and chlorine disinfection, mean log10 reductions were 2.4 log10 gc/liter, for NoV GI, 2.7 log10 gc/liter, for NoV GII, and 2.9 log10 PFU per liter for MSCs. Comparable values for~~

~~WWTPs with lagoon systems and chlorine disinfection were 1.4 log10 gc/liter for NoV GI, 1.7 log10 gc/liter for NoV GII, and 3.6 log10 PFU per liter for MSCs. WWTPs with ultra violet (UV) disinfection demonstrated slightly higher mean log10 reductions with 3.0 log10 gc/liter, for NoV GI, 3.3 log10 gc/liter, for NoV GII, and 4.3 log10 PFU per liter for MSCs. The results of the reduction of NoV and MSC are shown in Table 3 below:~~

~~Table 3: Log reduction in NoV and MSC in treated wastewater with disinfection~~

~~**Wastewater Treatment and Disinfection**~~	~~**Log_{10} NoV GI Reduction**~~	~~**Log_{10} NoV GII Reduction**~~	~~**Log_{10} M Reducti**~~
~~Mechanical with Chlorine Disinfection~~	~~2.4~~	~~2.7~~	~~2.9~~
~~Lagoon with Chlorine Disinfection~~	~~1.4~~	~~1.7~~	~~3.6~~
~~Mechanical with UV Disinfection~~	~~3.0~~	~~3.3~~	~~4.3~~

~~This meta analysis also demonstrated that Chlorine Disinfection had little effect on the mean reductions of the NoV and MSC. The mean log10 reduction that occur due to mechanical and biological treatment of the facility (prior to disinfection) were 2.2 log10 gc/liter, for NoV GI, 2.5 log10 gc/liter, for NoV GII, and 2.4 log10 PFU per liter for MSCs which varied little from mean log reduction after disinfection. In addition, a strong correlation, 0.8, existed between the reductions of NoV GII and MSC that occurred following treatment at the same WWTP indicating that MSCs could be useful in evaluating the efficiency of a WWTP.~~

~~F.~~ ~~Alternate Options~~

~~The FDA studies also suggested that certain factors, such as the quality of sewage treatment or the time of year, may exert influences on the levels of viruses discharged. However, at this time FDA does not have reliable data to justify specific dilution levels associated with environmental variables. It is recognized that such criteria could be determined by SCAs on a case by case basis, where factors of WWTP performance, disinfection method, tidal flushing, shellfish species and seasonal impacts may vary.~~

~~For example, in consideration of a raw sewage discharge, a lower dilution level than a 100,000:1 could be justified provided that specific data to that particular WWTP demonstrates that a lower bacteriological level associated with a potential raw sewage discharge is supported. Additional or other site specific information also can be used to justify alternative approaches that take into account other factors (such as no prior history of raw sewage discharges or containment structures sufficiently sized to accommodate a raw sewage event preventing a discharge).~~

~~Alternative options for calculating the size of the prohibited area to mitigate the virological effects of WWTP discharges at the shellfish growing area may be used provided that they are based on sound scientific principles that can be verified. For example, it is reasonable to~~

~~expect a potentially higher reduction in viral load from a properly maintained wastewater treatment system employing ultraviolet (UV) disinfection, tertiary treatment and operating under optimum design flow conditions. Regardless of the technology employed any proposed alternative minimum level of dilution for conditional management other than 1000:1 would need validation. MSC could potentially be used on a case by case basis as the validation process (for example to validate treatment efficiency) if demonstrated it is a successful/feasible strategy for the given location/situation. However, when there is insufficient information available for a growing area to support the use of a lower level of dilution, the 1000:1 dilution should be employed. If MSC is selected as an alternative option for calculating the size of the prohibited area of a WWTP discharge, the authority should select an MSC criteria that adequately protects shellfish growing areas from virological effects and should be based on the most recent data and regional studies.~~

~~References~~

~~Baggi, F., A. Demarta, and R. Peduzzi. (2001) Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. Res. Microbiol. 152, 743–751~~

~~Batz, M. B., Hoffman, S., Morris, G.J. Ranking the Disease Burden of 14 Pathogens in Food Sources in the United States Using Attribution Data from Outbreak Investigations and Expert Elicitation. Journal of Food Protection, Vol 75 (7):1278-1291~~

~~Burkhardt, W. III, J.W. Woods, and K.R. Calci. 2005. Evaluation of Waste Water System Discharge (WWSD) Efficiency to Reduce Bacterial and Viral Loading Using Real-time RT-PCR. Poster Presentation, ASM, Atlanta, GA, Annual Educational Conference.~~

~~Dore, W.J. and D.N. Lees. 1995. Behavior of *Escherichia coli* and male-specific bacteriophage in environmentally contaminated bivalve molluscs before and after depuration. Appl. Environ. Microbiol. 61:2830-2834.~~

~~Goblick, G.N., Anbarchian J M,. Woods J.,, Burkhardt W. and Calci K. 2011. Evaluating the Dilution of Wastewater Treatment Plant Effluent and Viral Impacts on Shellfish Growing Areas in Mobile Bay, Alabama. Journal of Shellfish Research, Vol. 30 (3), 1-9.~~

~~Hall AJ, Eisenbart VG, Etingue AL, Gould LH, Lopman BA, Parashar UD. 2012. Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. Emerg Infect Dis 18:1566-1573.~~

~~Havelaar, AH, M. van Olphen, and Y.C. Drost. 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. Appl. Environ. Microbiol. 59(9):2956-2962.~~

~~Iwamoto, M., Ayers, T., Mahon, B and Swerdlow, D.L 2010. Epidemiology of Seafood-Associated Infections in the USA. Clinical Microbiology Reviews. April,2010 . p399-411.~~

~~Kapikian, AZ and Chanock RM. 1990. Norwalk Group of Virus in Virology. New York, NY: Raven Press Ltd. pp. 671-693.~~

~~Kohn, et al. 1995. An Outbreak of Norwalk Virus Gastroenteritis Associated with Eating Raw Oysters, Implications of Maintaining Safe Oyster Beds. JAMA.~~

~~Liu, OC, Seraichekas, HR, Murphy, BL. 1966. Viral Pollution of Shellfish, I: Some Basic Facts of Uptake. Proc. Soc. Exp. Biol. Med. 123:481-487.~~

~~Love, D.C., Lovelace, G.L., & Sobsey, M.D. 2010. Removal of *Escherichia coli*, *Enterococcus fecalis*, coliphage MS2, poliovirus, and hepatitis A virus from oysters (*Crassostrea virginica*) and hard shell clams (*Mercinaria mercinaria*) by depuration. *Int.J.Food Microbiol.*, 143, (3) 211-217~~

~~Maalouf, F. Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R., Crawford, S.E. & Le Guyader, F.S. (2011) Strain-dependent Norovirus bioaccumulation in oysters. *Applied and Environmental Microbiology* 77(10): 3189~~

~~Pouillot, R., Van Doren, J.M., Woods, J., Smith, M., Plante, D., Goblick, G., Roberts, C., Locas, A., Hajen, W., Stobo, J., White, J., Holtzman, J., Buenaventura, E., Burkhardt III, W., Catford, C., Edwards, R., DePaola, A., Calci, K.R. 2015. Meta-Analysis of the Reduction of Norovirus and Male-Specific Coliphage Concentrations in Wastewater Treatment Plants. J. Appl. Environ Microbiol. 81: 4669-4681~~

~~Scallan, E., Hoekstra, R.M. Tauxe, R. V et al. Foodborne Illness Acquired in the United States – Major Pathogens. Emerging Infectious Diseases Vol17. No1, January 2011.~~

~~Seraichekas, H. R., D. A. Brashear, J. A. Barnick, P. F. Carey & O. C. Liu. 1968. Viral deputation by assaying individual shellfish. Appl. Microbiol. 16:1865-1871.~~

~~Sobsey, M.D., A.L. Davis, and V.A. Rullman. 1987. Persistence of hepatitis A virus and other viruses in depurated eastern oysters. In: NOAA, editor. Proceedings, Oceans '87. Halifax, Nova Scotia: NOAA. 5:1740-1745.~~

~~Woods, J. S. 2010. Determining the relationship of human enteric viruses in clinical, wastewater, and environmental samples utilizing molecular and cell culture techniques. PhD diss., University of Southern Mississippi. 145 pp.~~

Note: When the above document is removed from the NSSP Guide, it will be available on the ISSC website at www.issc.org/document-library.

.19 Classification of Shellfish Growing Waters Adjacent to Waste Water Treatment Plants

Note: NSSP Model Ordinance excerpts are listed in italics.

I. Introduction

One of the goals of the NSSP is to control the safety of shellfish for human consumption by preventing its harvest from contaminated growing areas. The positive relationship between sewage pollution of shellfish growing areas and disease has been demonstrated many times. Shellfish-borne infectious diseases are generally transmitted via a fecal-oral route. The pathway can become quite circuitous. The cycle usually begins with fecal contamination of the growing waters.

The primary responsibility of the State Shellfish Control Authority is to ensure the public health safety of the shellfish growing areas through compliance with the NSSP Model Ordinance. The Authority must perform a sanitary survey that collects and evaluates information concerning actual and potential pollution sources that may adversely affect the water quality in each growing area. Based on the sanitary survey information, the authority determines what use can be made of the shellstock from the growing area and assigns growing areas one (1) of five (5) classifications.

The shoreline survey (also known as the pollution source survey) is the sanitary survey component in which the actual and potential pollution sources that may adversely affect the growing area are identified. These sources may introduce infectious disease agents or poisonous and deleterious substances to the growing waters where they may be taken up and concentrated by shellfish. Detailed and accurate information concerning the pollution sources is necessary for a proper growing area classification.

The key to the accurate classification of shellfish growing areas is the sanitary survey. The principal components of a sanitary survey include: (1) an evaluation of the pollution sources that may affect the areas; (2) an evaluation of the meteorological factors; (3) a review of hydrographic factors that may affect distribution of pollutants throughout the area; and (4) an assessment of water quality.

A pollution source survey must be conducted of the shoreline area and watershed to locate direct discharges (e.g., municipal and industrial waste discharges and package treatment units) and non-point sources of pollution (e.g., septic tanks, storm water runoff and agricultural and wildlife area runoff). Municipal and industrial wastewater treatment facilities should be evaluated in terms of design capacity versus actual loading, type and concentration of pollutants discharged, and the type and effectiveness of pollution control devices.

Water samples are collected to determine if the water quality meets the water quality standards for this growing area classification. The NSSP recognizes two (2) water quality-monitoring strategies: adverse pollution condition and systematic random sampling. Presence of point sources of pollution requires the use of the adverse pollution condition monitoring

system to collect data for the application of the water quality standard. In growing areas not affected by point sources, the Authority may elect to use either system. The presence or absence of point sources of pollution and the monitoring system used dictate the frequency of samples that must be collected for application of the water quality standards.

The original National Shellfish Sanitation Program (NSSP) principles have historically proved effective in controlling bacterial illness associated with shellfish harvested from polluted waters. These principles, namely a robust sanitary survey, regular water and shellfish monitoring using bacterial indicators, controlled harvest times and labelling the origin of shell stock remain applicable as the primary preventative food safety control measures for growing areas.

However, there is now ample scientific evidence to show that the current bacterial indicators are inadequate to predict the risk of viral illness for the following reasons:

(1) Enteric viruses are resistant to treatment and disinfection processes in a Waste Water Treatment Plant (WWTP) and are frequently detected in the WWTP's final effluent under normal operating conditions (Baggi et al. 2001; Burkhardt et al. 2005, Pouillot et al. 2015).

(2) Shellfish can bioaccumulate enteric viruses up to 100-fold from surrounding water (Seraichekas et al. 1968; Maalouf et al. 2011).

(3) Certain enteric viruses are retained by molluscan shellfish to a greater extent and for longer than the indicator bacteria currently used to classify shellfish growing areas (Sobsey et al. 1987; Dore & Lees 1995; Love et al. 2010). It has been well documented that enteric virus detection is not indexed by levels of conventional indicator bacteria.

For several decades now viral illnesses, in particular norovirus (NoV) and hepatitis A (HAV), have been identified as common food safety problems associated with the consumption of bivalve molluscan shellfish (Woods 2010; Iwamoto et al 2010; Scallan et al. 2011; Batz et al. 2012; Hall et al 2012). NoV genogroups I, II and IV and HAV are typically associated with ill-individuals and transferred by the fecal-oral route. Because WWTPs do not completely remove infectious enteric viruses emphasis should be placed on the importance of ensuring there is adequate dilution between a sewage source and a shellfish growing area.

In addition to the risk of enteric viruses present in wastewater, WWTP effluents may also contain chemicals and other deleterious substances including pharmaceuticals, nanoparticles, and other contaminants of emerging concern. Establishment of appropriate classification based upon virus removal efficacy and proximity and

source strength of WWTP discharges is an effective strategy to reduce the risk posed by both enteric viruses and other contaminants found in WWTP effluents. NSSP requires that shellfish growing waters be classified into one of five classifications. They include:

(1) Prohibited – A classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering of seed for aquaculture, is not permitted.
(2) Restricted – A classification used to identify a growing area where harvesting shall be by special license and the shellstock, following harvest, is subjected to a suitable and effective treatment process through relaying or depuration.
(3) Conditionally Restricted - A classification used to identify a growing area that meets the criteria for the restricted classification except under certain conditions described in a management plan.
(4) Conditionally Approved - A classification used to identify a growing area which meets the criteria for the approved classification except under certain conditions described in a management plan.
(5) Approved - A classification used to identify a growing area where harvest for direct marketing is allowed.

This guidance document provides information on the five shellfish harvest classifications and the appropriate use of these classifications impacted by WWTP effluents. A sanitary survey report is required prior to the establishment of the classifications listed above with the exception of areas classified as prohibited.

II. General Requirements for Growing Area Classification

A. Chapter IV. Shellstock Growing Areas

@.01 Sanitary Survey

A. General.
(1) The sanitary survey is the written evaluation report of all environmental factors, including actual and potential pollution sources, which have a bearing on water quality in a shellfish growing area. The sanitary survey shall include the data and results of:
(a) A shoreline survey;
(b) A survey of the microbiological quality of the water. In growing areas adjacent to wastewater system discharges the State

Shellfish Control Authority may utilize MSC results from analysis of shellfish meat samples and the analysis of the data will be included in the sanitary survey report;

(c) An evaluation of the effect of any meteorological, hydrodynamic, and geographic characteristics on the growing area; and

(d) A determination of the appropriate growing area classification.

(2) The sanitary survey shall be periodically updated through the triennial reevaluation and the annual review in accordance with Section C. to assure that data is current and that conditions are unchanged.

(3) The documentation supporting each sanitary survey shall be maintained by the Authority. For each growing area, the central file shall include all data, results, and analyses from:

(a) The sanitary survey;

(b) The triennial reevaluation; and

(c) The annual review.

(4) Wherever possible, the Authority shall provide the necessary information to Federal, State, or local agencies which have the responsibility to minimize or eliminate pollution sources identified in the sanitary survey.

(5) The Authority shall maintain a current comprehensive, itemized list of all growing areas, including maps showing the boundaries and classification of each shellstock growing area.

B. Sanitary Survey Required.

(1) A sanitary survey shall not be required to classify growing areas as prohibited. The findings of a sanitary survey, however, may result in a growing area being classified as prohibited.

(2) A sanitary survey, including the triennial reevaluation, when available, of each growing area shall be required prior to:

(a) The harvest of shellstock for human consumption; and

(b) The classification of a growing area as approved, conditionally approved, restricted, or conditionally restricted.

C. Sanitary Survey Performance.

(1) A sanitary survey of each growing area shall be performed at least once every twelve (12) years and shall include the components in Section A. (1).

(2) When a written sanitary survey report is not

	completed, the area shall be placed in the closed status. *(3) The growing area classification and the supporting data from the sanitary survey shall be reviewed at least every three (3) years.* *(a) This triennial reevaluation shall include:* *(i) A review in accordance with Section C. (5) and (6) of the water quality samples;* *(ii) Documentation of any new pollution sources and an evaluation of their effect on the growing area;* *(iii) Reevaluation of all pollution sources, including the sources previously identified in the sanitary survey, as necessary to fully evaluate any changes in the sanitary conditions of the growing area. The reevaluation may or may not include a site visit;* *(iv) A comprehensive report which analyzes the sanitary survey data and makes a determination that the existing growing area classification is correct or needs to be revised; and* *(v) If the triennial reevaluation determines that conditions have changed based on the information and data collected during the triennial review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.* *(b) When a written triennial reevaluation report is not completed, the Authority shall place the growing area in the closed status.* *(4) The triennial reevaluation may include:* *(a) Inspection of waste water system discharges (WWSD) or collection of additional effluent samples to determine their impact on the growing area;* *(b) Hydrodynamic studies;* *(c) Additional field work to determine the actual impact of pollution sources; and* *(d) Collection of additional water samples.* *(5) On an annual basis, the sanitary survey shall be updated to reflect changes in the conditions in the growing area. The annual*

reevaluation shall include:

(a) A field observation of the pollution sources which may include:

(i) A drive-through survey;

(ii) Observations made during sample collection; and

(iii) Information from other sources.

(b) Review, at a minimum, of the past year's water quality sample results by adding the year's sample results to the data base collected in accordance with the requirements for the bacteriological standards and sample collection required in Section @.02;

(c) Review of available inspection reports and effluent samples collected from pollution sources;

(d) Review of available performance standards for various types of discharges that impact the growing area; and

(e) A brief report which documents the findings of the annual reevaluation.

(f) The SSCA may use MSC meat sampling data and/or MSC waste water sampling data in the annual revaluation of (5) (b), (c), and (d) above to evaluate the viral contributions of the performance standards of waste water system discharge (WWSD) impacts on shellfish growing areas. If MSC meat and/or water data is being used, the SSCA shall conduct annual sample collection and analysis in determining performance standards.

(6) If the annual reevaluation determines that conditions have changed based on the information and data collected during the annual review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.

D. Shoreline Survey

Requirements.

(1) In the shoreline survey for each growing area, the Authority shall:

(a) Identify and evaluate all actual and potential sources of pollution which may affect the growing area;

(b) Determine the distance from the

pollution sources to the growing area and the impact of each source on the growing area;

(c) Assess the reliability and effectiveness of sewage or other waste treatment systems;

(d) Determine if poisonous or deleterious substances adversely affect the growing area; and

(e) Consider the presence of domestic, wild animal or resident and migrating bird populations for possible adverse effects on growing areas.

(2) The Authority shall assure that the shoreline survey meets the following minimum requirements:

(a) The boundaries, based on the area topography, of each shoreline survey area are determined by an in-field investigation which identifies only the properties with the potential to impact the shellfish waters;

(b) Each shoreline survey area is identified by a unique designation which results in identification of all data associated with each shoreline survey by the unique designation;

(c) Each shoreline survey area is investigated and pollution sources evaluated by qualified, trained personnel; and

(d) Documentation for each pollution source identified by the Authority as affecting a growing area includes:

(i) The location of the site on a comprehensive map of the survey area; and

(ii) The determination that the pollution source has a direct or indirect impact on shellfish waters: and

(e) A written summary of the survey findings.

III. Guidance for Growing Area Classification

As a result of the information gathered during the sanitary survey, the Authority is responsible for distinguishing those growing areas suitable for harvest of shellstock for direct human consumption, those growing areas where the shellfish will require treatment prior to consumption, and those

growing areas unsuitable to harvest for human consumption. The probable presence or absence of pathogenic microorganisms, marine Biotoxin or other poisonous or deleterious substances in growing area waters is important to the Authority in deciding how the shellfish obtained from the growing area should be used. The Authority's decision, based on the sanitary survey information, will place all actual and potential growing areas in one of the five possible NSSP growing area classifications.

The five (5) growing area classifications are approved, conditionally approved, restricted, conditionally restricted and prohibited. Except for an emergency situation such as conditions following a hurricane when a growing area in the approved classification may be placed temporarily in the closed status, a growing area in the approved classification is always in the open status. The remaining four growing area classifications all place some type of restriction on shellstock harvesting. For more information concerning the enforcement of these restrictions, see the NSSP Guidance Document, Growing Area Patrol and Enforcement of Growing Area Restrictions (ISSC/FDA, 2015).

IV. Prohibited Classification

A. Definition

A classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering of seed for aquaculture, is not permitted.

B. Requirements for a Prohibited Area Adjacent to a Waste Water Treatment Plant (WWTP)

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

A. General. Each growing area shall be correctly classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, as provided by this Ordinance.

(1) Emergency Conditions…

(2) Classification of All Growing Areas. All growing areas which:

(a) Are not subjected to a sanitary survey every twelve (12) years shall be classified as prohibited;

(b) Have a sewage treatment plant outfall or other point source outfall of public health significance within or adjacent to the growing area shall have an area in the prohibited

classification established adjacent to the outfall in accordance with Section E. Prohibited Classification; and

(c) Are subjected to…

(3) Boundaries…

(4) Revision of Classifications…

(5) Status of Growing Areas…

(2) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification

E. Prohibited Classification.

(1) Exception…

(2) General…

(3) Sanitary Survey. A growing area shall be classified as prohibited if:

(a) No current sanitary survey exists;

(b) A sanitary survey determines:

(i) The growing area is adjacent to a sewage treatment plant outfall or other point source outfall with public health significance;

(ii) Pollution sources may unpredictably contaminate the growing area;

(iii) The growing area is contaminated with fecal waste so that the shellfish may be vectors of disease microorganisms;

(iv) The concentration of…

(v) The area is contaminated with poisonous or deleterious substances causing the shellfish to be adulterated.

(4) Risk Assessment. A growing area shall be classified as prohibited if a risk assessment performed in accordance with Chapter II. Risk Assessment and Risk Management indicates the shellstock are not safe for human consumption.

(5) Wastewater Discharges.

(a) An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance.

(b) The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria:

(i) The volume flow rate, location of discharge, performance of the

wastewater treatment plant and the microbiological quality of the effluent. The SSCA may utilize MSC waste water sample data in the determination of the performance of the sewage treatment plant;

(ii) The decay rate of the contaminants of public health significance in the wastewater discharged;

(iii) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and

(iv) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.

C. Allowable Uses of Shellfish from a Prohibited Growing Area

(1) Depletion

Depletion means the removal, under the direct control of the Authority, of shellstock from a growing area classified as prohibited.

(2) Seed

Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Depletion and Gathering of Seed

(1) Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification

E. Prohibited Classification

(1) Exception…

(2) General. The Authority shall:

(a) Not permit the harvest of shellstock from any area classified as prohibited, except for the harvest of shellstock for the gathering of seed for aquaculture or the depletion of the areas classified as prohibited; and

(b) Ensure that shellstock removed from any growing area classified as prohibited is effectively excluded from human consumption unless it is seed to be cultured as outlined in the NSSP Model Ordinance Chapter VI. Shellfish

Aquaculture @.02 Seed Shellstock.

(3) Sanitary Survey…

(4) Risk Assessment…

(5) Wastewater Discharges…

(2) Chapter VI. Shellfish Aquaculture

Requirements for the Harvester/Dealer

.03 Seed Shellstock

Seed may come from any growing area, or from any growing area in any classification, provided that:

A. The source of the seed is sanctioned by the Authority; and

B. Seed from growing areas or growing areas in the prohibited classification are cultured for a minimum of six (6) months.

E. Guidance for Determining the Size of Each Prohibited Are Adjacent to a Waste Water System Treatment Plant (WWTP)

There are several important considerations for the shellfish authority to consider when establishing the size of each prohibited area adjacent to a WWTP discharge:

(1) The area is large enough to ensure that there is adequate dilution for the type of classification that will be used adjacent to the prohibited area. If a conditional classification (either conditionally restricted or conditionally approved) is established adjacent to the prohibited area, adequate dilution should be determined when the WWTP is operating as normal. "Normal" means that the WWTP is operating fully within the plant's design specifications, including design flows; treatment stages; disinfection; as well as compliance with all permit conditions that relate to the WWTPs effectiveness in reducing enteric viruses in discharged wastewater.

Should a restricted area for the purposes of relaying or depuration be established adjacent to the prohibited area, establishing the size of the prohibited area should be based on worst case plant operating conditions. This same consideration would apply for an approved area adjacent to the prohibited area.

Below are several scenarios that could occur and are critical for Shellfish Control Authorities (SCAs) on

evaluating each WWTP when determining appropriate classifications:

(a) Bypassing stage of treatment

A treatment plant should be considered operating outside of normal operation if a treatment stage such as primary or secondary treatment is bypassed which may result in an increased load of solids in the disinfection step and reduce the effectiveness of disinfection. An additional example would be when a WWTP experiences a loss in disinfection and thus the ability to effectively treat the final effluent. SCAs should determine the significance of these types of events and determine appropriate classification for the growing waters adjacent to the prohibited area.

(b) Operating outside design specifications/other types of failures or events

It is not uncommon for a WWTP to periodically experience mechanical failures of equipment that could alter the treatment of sewage. Additionally, a WWTP may also need to periodically perform routine maintenance to the various stages of treatment and may need to temporarily take a portion of a treatment stage off-line for cleaning. Other unexpected maintenance may need to occur. For example cleaning of filters or membranes that have become bio-fouled.

(c) Operating above design flow

Some WWTPs may operate above its design flow and not necessarily bypass any particular stage of treatment. During these events it is typical for WWTP operators to adjust the operation of the WWTP which may include reducing the treatment time in the aeration stage and/or solids separation/settling stage of treatment. Under some circumstances this could lead to a significant reduction in the effectiveness of disinfection. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these type of events.

(d) WWTP permit violations

If a WWTP is exceeding the permitted bacterial indicator levels in the final effluent this indicates that effectiveness of the disinfection step has been reduced. Other

measured parameters in the effluent (e.g. Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD)) may also indicate a reduction in treatment efficiency has occurred. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these types of events.

Compliance of WWTP operation permit compliance does not necessarily eliminate the potential transmission of pathogens present in wastewater effluent to contaminating shellfish in the impacted area.

There could be situations in which a particular WWTP could be in compliance with a permit, and could still pose a risk to the shellfish harvest area. For example, a WWTP may have permit conditions to allow for flow blending during high flow periods where a portion of the sewage may receive full treatment but a portion of the sewage may only be partially treated and "blended" in the final disinfection step. Although this may be an acceptable practice under a permit it could result in conditions in which the efficiency of the WWTP to remove enteric viruses is considerably reduced. SCAs may consider assessing the efficiency of WWTPs to determine the significance of these events.

(2) The integrity of the collection system. Collection system malfunctions, bypasses or other factors can lead to significant leakages of untreated sewage to the marine environment.

(3) That there is adequate detection and response time when a malfunction occurs to ensure that all harvesting ceases and closures are enforced, so that contaminated product does not reach the market.

F. Guidance for the Use of MSC in Shellfish Meats in determining the size of the prohibited area impacted by WWTP discharge.

MSC has been demonstrated to accurately assess enteric virus dynamics through contaminant mitigation strategies such as relay. MSC levels in shellstock from growing areas adjacent to WWTP discharge are a function of WTTP performance, seasonal persistence of viruses in the environment and the shellfish, species-specific anomalies, and distance from the outfall. The regulatory level of 50 PFU/100gm is a conservative value used for

re-opening approved growing areas (after 7 days) after a sewage spill and end point target values for viral relay. Before using MSC for these purposes, the Authority should perform preliminary studies to familiarize themselves with the seasonal viral persistence patterns, regional and species-specific anomalies.

Seasonal persistence of MSC in shellfish meats can vary greatly from warm summer months to the cooler fall, winter, and spring months. MSC levels can be 2 to 3 logs (100 to 1000) higher in the late fall, winter, and early spring months demonstrated by multiple studies from conducted in northern temperate latitudes using both MSC and molecular enumeration using PCR for enteric viruses. This dramatic tendency to accumulate virus particles by 2 to 3 logs over the winter months has species-specific implications for warm-water adapted species such as American oysters and northern quahogs, which tend to shut down as cooling water temperatures approach 10°C. Viruses and bacteria bio-accumulated in shellfish behave very differently; FC is prone to die-off in a week or two over colder months while viruses can persist at high levels under these cold water conditions for months. Cold-water adapted species such as soft-shelled clams, Pacific oysters, European oysters, and mussel all demonstrate the tendency to increase by 2 to 3 log values over the colder months.

If the Authority is interested in using MSC in shellfish meats, it is recommended that monthly samples be taken over the course of a year in multiple growing areas inside the 1000:1 line to understand these seasonal, spatial, and species-specific variations. This data can be very useful to assess the feasibility of using the conditionally restricted classification for the purpose of relay.

G. Use of MSC in Evaluating WWTP Efficiency

At a minimum, MSC may be used in conjunction with conventional bacterial indicators to conduct a comprehensive assessment of WWTP microbiological performance. The differences between influent, pre-disinfection effluent, and final effluent samples taken under normal and challenged conditions can be used to assess the viral deactivation efficiency of a specific waste water treatment process. The analysis is somewhat similar to the determination of WWTP efficacy using bacterial indicators such as E. coli, which is currently used to comply with EPA's National Pollution Discharge Elimination System (NPDES) permit requirements for municipal wastewater treatment plant discharge. Many studies have shown that deactivation of bacterial and viral indicators (and pathogens) can be significantly different in different treatment processes and under challenged conditions. There are several case studies showing that under

certain conditions, differential bacterial indicators may indicate highly effective treatment of wastewater while differential MSC samples show little deactivation efficiency.

By collecting differential wastewater samples including influent, pre-disinfection effluent, and final effluent and evaluating these samples for MSC, the viral performance of the wastewater treatment process can be determined. If a comprehensive sampling program includes sufficient samples to assess the WWTP under typical operating conditions as well as challenged condition such as high flow, the viral efficiency of the WWTP can be determined. A comprehensive assessment of WWTP microbiological performance using MSC as well as the conventional bacterial indicators can inform the SSCA on the risk associated with a growing area adjacent to a WWTP outfall. An assessment of a WWTP must demonstrate the range of effluent quality during routine operation through an appropriate sampling study and the ability to accurately predict those times when effluent microbiological quality is detrimentally impacted by challenged conditions.

H. Public Health Significance

The positive relationship between disease and consuming contaminated shellfish has been clearly established. Prevention of consumption of contaminated shellfish is the primary objective of the NSSP. The prohibited area classification is the most restrictive growing area classification and is used for areas subject to gross pollution. The use of this classification is also required for all growing areas immediately adjacent to a wastewater treatment plant and where the shellfish authority has not performed a sanitary survey. The harvesting of shellstock is not allowed for any human food use. For additional information concerning the classification of growing waters and the sanitary survey, see the NSSP Model Ordinance. Depletion and Gathering of Seed (Chapter IV @.03 E. Prohibited Classification (2) (a) & (b) and Chapter VI .03 Seed Shellstock A. & B.)

A growing area is placed in the prohibited classification when the sanitary survey or marine Biotoxin surveillance program indicates that fecal material, pathogenic microorganisms, poisonous or deleterious substances, marine Biotoxin, or radionuclides may reach the harvest area in excessive concentrations. The NSSP Model Ordinance also requires that a growing area for which there is no sanitary survey be placed in the prohibited classification as a precautionary measure. Taking shellstock from a prohibited area

for any human food purpose is not allowed.

The NSSP Model Ordinance also requires that an area classified as prohibited must be established between any sewage treatment plants or other waste discharge of public health significance and any growing area placed in the approved, conditionally approved, restricted, or conditionally restricted classification. The size of the prohibited area should be based on the effectiveness and level of sewage treatment; the location of the shellstock resource that would be affected; the classification of adjacent waters, the total time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority; the time it would take the Authority to issue a notice to stop shellstock harvesting, and the degree of effluent dilution. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night.

I. Establishment of Boundaries for the Prohibited Area

The establishment of the boundary for the prohibited area is dependent upon other classification which may be adjacent to the prohibited area. Examples could include water bodies in which the Authority chose to use all five (5) classifications or a situation where the Authority only uses prohibited and approved. The decision of adjacent classifications is often based on shellfish uses for the water body or environmental control and protection efforts by State Water Control Agencies. The requirements of the classification adjacent to the prohibited area and the allowable uses in the areas will often dictate the distance the boundary line for the prohibited area is from the outfall.

Guidance for Dilution Ratios

To determine the impact of a WWTP on adjacent waters, it is imperative that the Shellfish Control Authority assess the waste water dispersal and dilution and the time of transport to the area where shellstock may be harvested. In determining the appropriate dilution for establishing the size of the prohibited area, the Shellfish Control Authority must determine the classification which will be adjacent to the prohibited area. The dilutions below outlines recommended dilution for the boundary line between prohibited and other possible classifications based on dilutions of WWTP effluent, based on initial FC values of 1.4 x 106 FC/100ml.. Each of these dilutions will be discussed in more detail in the context of each classification.

(1) Prohibited to Restricted Boundary
Minimum dilution – The SCA should determine the effluent quality based on a worst case scenario and should establish a dilution ratio that would accomplish a dilution equivalent to a MPN of 88 (or 163) which is the upper limit restricted standard for depuration and relaying without a contaminant reduction study.

(2) Prohibited to Conditionally Restricted Boundary
Minimum dilution of 320:1 based on "Critical Dilution for Toxics to Ambient (Background)" from the Clean Water Act and EPA's Regulatory Mixing Zone (RMZ).

(3) Prohibited to Conditionally Approved Boundary
Minimum dilution 1000:1 or justified by other data.

(4) Prohibited to Approved Boundary
Minimum dilution >100,000:1 dilution based on worst case scenario or justified by other data.

V. Restricted Classification.

A. Definition

A classification used to identify a growing area where harvesting shall be by special license and the shellstock, following harvest, is subjected to a suitable and effective treatment process through relaying or depuration.

B. Requirements for Use of the Restricted Classification

(1) Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification

A. General…
B. Approved Classification…
C. Conditional Classifications…
D. Restricted Classification.
(1) General
(a) A growing area may be classified as restricted when:
(i) A sanitary survey indicates a limited degree of pollution; and
(ii) Levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such

levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid-canned food processing.

(b) The Authority shall have effective controls to assure that shellfish are harvested from restricted areas only:

(i) By special license; and

(ii) Under the supervision of the Authority.

(2) Water Quality. Water quality in the growing area shall meet the bacteriological standards in Section @.02 for a growing area in the restricted classification if the growing area is used for depuration. (These standards are included later in this section.)

(3) Shellstock Quality Criteria. The Authority shall establish shellstock quality criteria for use in placing an area in the restricted classification. Depending on the treatment process to be applied to the shellstock, the criteria shall be established in accordance with:

(a) Chapter V. Shellstock Relaying; or

(b) Chapter XV. Depuration

E. Prohibited Classification…

C. Allowable Uses of Shellfish from a Restricted Growing Area

(1) Relay with a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(2) Relay without a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the

treatment process.

(3) Depuration
Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(4) Seed
Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Relaying with a Contaminate Reduction Study

(1) Chapter V. Shellstock Relaying

@.01 General

The Authority shall assure that:

A. The shellstock used in relaying activities is harvested from growing areas classified as conditionally approved, restricted, or conditionally restricted;
B. The level of contamination in the shellstock can be reduced to levels safe for human consumption;
C. The contaminated shellstock are held in growing areas classified as approved or conditionally approved for a sufficient time under adequate environmental conditions so as to allow reduction of pathogens as measured by total coliform or fecal coliform or poisonous or deleterious substances that may be present in shellstock. For shellstock harvested from areas impacted by waste water system discharge, MSC may be used as a measure for viral reduction.
D. If shellstock are relayed in containers:
(1) The containers are:
(a) Designed and constructed so that they allow free flow of water to the shellstock; and
(b) Located so as to assure the contaminant reduction required in Section C.; and
(2) The shellstock are washed and culled prior to placement in the containers.

@.02 Contaminant Reduction.

A. The Authority shall establish species-specific critical values for water temperature, salinity, and other environmental factors which may affect the natural treatment process in the growing area to which shellstock will be relayed. The growing area to be used for the treatment process shall be monitored with sufficient frequency to identify when limiting critical values may be approached.

B. The effectiveness of species-specific contaminant reduction shall be determined based on a study. The study report shall demonstrate that, after the completion of the relay activity:

(1) The microbiological quality of each shellfish species is the same microbiological quality as that of the same species already present in the approved or conditionally approved area; or

(2) Contaminant levels of poisonous or deleterious substances in shellstock do not exceed FDA tolerance levels; or

(3) When the source growing area is impacted by waste water system discharge, the viral quality of each shellfish species meets the male-specific coliphage standard of 50 PFU/100 gm or predetermined levels established by the Authority based on studies conducted on regional species under regional conditions.

C. The authority may waive the requirements for a contaminant reduction study if:

(1) Only microbial contaminants need to be reduced; and

(2) The shellstock are relayed from a conditionally approved, restricted, or conditionally restricted area meeting the bacteriological water quality for restricted areas used for shellstock depuration per Chapter IV. @.02 G. and Chapter IV. @.02 H.; and

(3) The treatment period exceeds sixty (60) days.

D. The time period shall be at least fourteen (14) consecutive days when environmental conditions are suitable for shellfish feeding and cleansing unless shorter time periods are demonstrated to be adequate.

E. When container relaying is used and the Authority allows a treatment time of less than fourteen (14) days, the Authority shall require more intensive sampling including:

(1) Product sampling before and after relay; and

(2) Monitoring of critical environmental parameters such as temperature and salinity; and

(3) For SSCAs using male-specific coliphage, monitoring before and after relay for shellstock relayed from areas impacted by waste water system discharge.

F. The Authority shall establish the time period during the year when relaying may be conducted.

In addition to the requirements of Chapter IV. @.02 G. & H., restricted growing waters used for relaying without a contaminant reduction study must meet the requirements of Chapter IV. @.03 D.

E. Guidance for Restricted Classification for Relaying with a Contaminant Reduction Study

Model Ordinance Chapter IV and V do not include microbial standards for classifying growing areas as restricted that are the source of shellstock for relaying when a contaminant reduction study is required. In establishing of the boundary between prohibited and restricted classifications, the Authority must ensure that levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid-canned food processing.

In determining an appropriate boundary, the Authority shall consider the following factors associated with the wastewater discharge:

(1) The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent.

The Authority may utilize MSC waste water sample data in the determination of the performance of the sewage treatment plant;

(2) The decay rate of the contaminants of public health significance in the wastewater discharged;

(3) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and

(4) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.

A growing area may be placed in the restricted classification instead of the prohibited classification when the sanitary survey indicates a limited degree of pollution. This option may be used when the sanitary survey for the growing area indicates that the microbiological quality or poisonous or deleterious substances in the growing area are such that additional treatment through relay can render the shellstock safe for human consumption. The Authority should use the restricted classification only when sufficient relay studies have been conducted to establish raw product quality requirements at the harvest level; and when the Authority has sufficient administrative and technical resources to properly administer this classification. These resources include monitoring of pollution sources; providing coordination between state, local and industry officials; issuing special harvesting permits; and supervising the harvesting and transport of shellstock to relay sites. For a complete discussion of the supervision requirements at the harvest level, see the NSSP Guidance Document, Shellstock Relay (ISSC/FDA, 2015).

Use of the restricted classification for relaying with a contaminant reduction study requires the Authority to develop the controls necessary to assure that the shellfish are relayed prior to consumption. The criteria may vary according to the use to be made of the shellstock and the effectiveness of the relay process used to cleanse the shellstock. Process effectiveness is determined through a study, which establishes the levels of microbilogical quality indicators in shellstock at the time of harvest, and the density that can be achieved at the completion of the process. Effectiveness of the process is likely to vary between growing areas used for natural cleansing treatment in relay operations. The species of shellstock may also affect the effectiveness of the relay. For a complete discussion of relay, see the NSSP Guidance Document, Shellstock Relay (ISSC/FDA, 2015).

F. Guidance for Conducting a Contaminant Reduction Study for Relay

The use of the restricted classification for the purpose of relaying with a contaminant reduction study does not require the authority to demonstrate that the growing area meets a microbiological water quality standard. However, in determining the boundary between the prohibited area and the restricted area for relaying with a contaminant reduction study, the authority shall give consideration to the types of contamination that may be in the growing area prior to allowing the area to be in the source of shellfish for relaying. The contaminants may include:

- Pathogenic Organisms
- Poisonous or Deleterious Substances
- Marine Biotoxins
- Physical and Chemical Contaminants

Contaminant Reduction is a specified activity defined in Chapter V. Shellstock Relaying @.02. The authority shall establish species-specific critical values for water temperature, salinity and other environmental factors such as dissolved oxygen and turbidity which may affect the natural treatment process (e.g. relay process). These critical values must be monitored and the Authority shall establish the time of year when relay may be conducted. The relay process requires that shellstock are held in the receiving growing area for a sufficient time under adequate environmental conditions to allow reduction of pathogens as measured by total coliform or fecal coliform. To verify the effectiveness of a relay process, contaminant reduction studies are required. The only exception to this requirement is when water quality in the restricted growing area meets Chapter IV.@.02 G-H, only microbial contaminants need to be reduced, and the treatment period exceeds sixty (60) days. For all other relay operations, the Authority shall establish species-specific critical values for water temperature, salinity, and other environmental factors which may affect the natural treatment process in the receiving growing area. The receiving waters shall be monitored with sufficient frequency to identify when limiting critical values may be approached. The effectiveness of species-specific contaminant reduction shall be determined based on a study. The study shall demonstrates that after the completion of the relay activity, the microbiological quality of each shellfish species is the same microbiological quality as that of the same species already present in the approved or conditionally approved area or contaminants levels of poisonous or deleterious substances in shellstock do not exceed NSSP tolerance levels. Based on the study, the Authority shall establish the time period during the year when relaying may be

conducted. Shellstock shall be relayed for at least fourteen 14 consecutive days when environmental conditions are suitable for shellfish feeding and cleansing unless shorter time periods are demonstrated with the contaminant reduction study to be adequate. If the shellstock are container relayed and the treatment times are less than 14 days, intensive sampling is required. This intensive sampling includes lot sampling before and after relay as well as monitoring of critical environmental parameters such as seawater temperature and salinity.

Although minimum requirements for contaminant reduction studies have not been specified in the Model Ordinance, there are certain principles of process verification studies that should be considering including; study design, sampling replicates, and data analysis providing statistical reliability. Shellstock and water samples collected during a contaminant reduction study must be analyzed in NSSP-conforming laboratories using NSSP-approved methods. Shellfish samples should be collected at regular intervals from both source and receiving growing areas over the time period of the relay and the natural cleansing process that is proposed. It is important to produce a sufficiently robust database to demonstrate the process is consistently working and the variables affecting the cleansing process are understood. All shellfish samples of 10 to 12 animals should be collected in triplicate so that the mean as well as standard deviation or standard error can be calculated. Water temperature and salinity should be measured at both source and receiving waters at the time of shellstock collection. Fecal coliform levels of shellstock already present in the receiving growing area should be collected in triplicate and evaluated for comparison to relayed shellstock microbial levels. Contaminant reduction studies are specific to species, source growing area, and receiving growing area. In states with extensive experience with relay practices, the Authority may approach contaminant reduction studies on a more regionally basis covering multiple source and receiving growing areas.

When the source growing area is adjacent to a WWTP outfall, the authority may utilize MSC in conducting the contaminant reduction study. Should the Authority utilize MSC sampling, the MSC levels in each shellfish species after the relay process must be assessed. The male-specific coliphage (MSC) standard of 50 PFU/100gm or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions are both approved for these assessments. Relay dynamics for bacterial and viral pathogens can be very different and assessing both offers more insight into the potential health risk. Seasonal persistence of bio-accumulated viral particles in

shellfish can range 1,000 times higher in the winter months verses the summer months. Depuration rates can vary from 1 log in 44 hours at receiving water temperature above 18°C to 1 log in 25 days when receiving water temperature fall below 10°C. Understanding these dynamics for each species and region is paramount to successful relay from restricted or conditionally restricted growing areas adjacent to WWTP outfall. When container relaying is considered and treatment times of less than 14 days are planned, an intensive MSC sampling program based on before and after relay samples can be utilized to assure relayed shellstock are less than the 50PFU/100gm standard or pre-determined levels established by the authority based on studies conducted on regional species under regional conditions.

G. Guidance for the use of MSC in Contaminant Reduction Studies and Process Control for Shellstock Relay

MSC has been shown to be an appropriate modeling organism for contaminant reduction studies and process verification for shellstock from growing areas impacted by a WWTP outfall. The ability of MSC to model enteric viral dynamics in relay and depuration has been demonstrated in several studies using different species in different parts of the northern temperate zone. The MSC standard of 50 PFU/100gm used in process end-point samples was shown to be conservative with respect to public health outcomes.

The conditionally restricted classification recommended for relay adjacent to WWTP outfalls where contaminant studies will be used, should have limits such as zero-hour maximum MSC limits in the shellstock from the source growing areas, seasonal limits, and receiving water temperature and salinity limits as determined by comprehensive contaminant reduction studies. This is in addition to controls to assure the continued operation of the adjacent WWTP under the management plan to keep the source growing area in the restricted status. MSC data from sampling shellstock from the source growing area may help determine those times when viral loading and/or viral persistence in the shellstock are low and viral mitigation strategies are feasible. In both viral depuration and viral relay pilot studies using soft-shelled clams in Maine, periods of time were identified using bi-weekly MSC assays of the target species to understand those times when bio-accumulated MSC levels in the shellstock were at a seasonal low (low viral persistence). Receiving waters temperatures were correspondingly high in those summer months resulting in significantly higher depuration rates, especially when water temperature exceeded 64.4°F (18°C). Studies showed the

depuration rate approached a single log reduction in 44 hours when water temperatures were above 64.4°F (18°C). In contrast, those studies also determined that as water temperature approached 41°F (5°C), it would take approximately 20 days to see a comparable single log reduction in MSC levels. The combination of seasonally low MSC levels in the soft-shelled clams and higher summertime depuration rates resulted in successful depuration consistently meeting a shellfish end-point of 50 PFU/100gm.

Species-specific and regional anomalies in persistence and relay and depuration dynamics require that comprehensive contaminant reduction studies be performed for each growing area for each relay or depuration process being considered. In planning a comprehensive contaminant reduction study, sufficient quantities of target specie(s) from the source area should be collected on a regular basis and evaluated for fecal coliform and MSC (triplicate samples of 10-12 animals), during that period of time when the restricted harvest is being considered. Background levels of MSC are not known in a new species or region, the Authority might consider collecting samples year round in the first year to understand the range of viral persistence throughout the year to understand those times when viral mitigation strategies are feasible.

Trial lots of shellstock should be evaluated monthly during the period of time when the relay is being considered. One to two bushels are adequate for relay trials. Triplicate shellfish samples of 10 to 12 animals from the approved relay site should be collected at appropriate intervals and analyzed for fecal coliform and MSC. Contaminant reduction studies should use triplicate samples so that variation as well as mean value can be assessed yielding improved statistical reliability for the contaminant reduction studies. If little is known about the depuration rates of the target species, it may be necessary to conduct a separate study using shellfish that are highly contaminated with MSC to assess the viral depuration rate in that region. The goal of contaminant reduction studies is to show those periods of time and the conditions when relay is effective.

The Authority may permit an end-point value other than 50 PFU/100gm based if pre-determined levels established by the Authority based on studies conducted on regional species in regional conditions are known.

H. Model Ordinance Requirements for Relaying without a Contaminant Reduction Study

(1) Chapter V. Shellstock Relaying

@.01 General

The Authority shall assure that:

A. The shellstock used in relaying activities is harvested from growing areas classified as conditionally approved, restricted, or conditionally restricted.

B. The level of contamination in the shellstock can be reduced to levels safe for human consumption;

C. The contaminated shellstock are held in growing areas classified as approved or conditionally approved for a sufficient time under adequate environmental conditions so as to allow reduction of pathogens as measured by total coliform or fecal coliform. For shellstock harvested from areas impacted by waste water system discharge, MSC may be used as a measure for viral reduction, or poisonous or deleterious substances that may be present in shellstock.

D. If shellstock are relayed in containers:

(1) The containers are:

(a) Designed and constructed so that they allow free flow of water to the shellstock; and

(b) Located so as to assure the contaminant reduction required in Section C.; and

(2) The shellstock are washed and culled prior to placement in the containers.

(2) Chapter V. Shellstock Relaying

@.02 Contaminant Reduction

C. The Authority may waive the requirements for a contaminant reduction study if:

(1) Only microbial contaminants need to be reduced; and

(2) The shellstock are relayed from a conditionally approved, restricted, or

conditionally restricted area meeting the bacteriological water quality for restricted areas used for shellstock depuration per Chapter IV. @.02 G. and Chapter IV. @.02 H.; and

(3) The treatment period exceeds sixty (60) days

(3) Chapter IV. Shellstock Growing Areas

@.02 Microbiological Standards

G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration.

(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section G. (2).

(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of:

(a) 300 MPN per 100 ml for a three-tube decimal dilution test;

(b) 173 MPN per 100 ml for a twelve-tube single dilution test; or

(c) 163 CFU per 100 ml for a MF (mTEC) test.

(3) Required Sample Collection. Samples shall be collected in accordance with Section E. (3).

H. Standard for the Restricted Classification of Growing Areas Affected by Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration.

(1) Exception. If the tidal stage increases the fecal coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.

(2) Pollution Sources. Growing areas shall meet the requirements in Section F. (2).

(3) Water Quality. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Section G. (2) or Section H. (4).

(4) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed a MPN or MF (mTEC) of:

(a) 260 MPN per 100 ml for a five-tube decimal dilution test;

(b) 300 MPN per 100 ml for a three-tube decimal dilution test; or

(c) 163 CFU per 100 ml for a MF (mTEC) test.

(5) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by the same method described in Section F. (5).

(6) Required Sample Collection.

(a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section G. (2).

(b) Systematic Random Sampling Standard. The Authority shall collect samples in the same intensity and frequency, and shall apply the sample results in the manner described in Section F. (6) for the application of the standard under Section H. (4).

In addition to the requirements of Chapter IV @.02 G & H., restricted growing waters used for relaying without a contaminant study must meet the requirements of Chapter IV @.03 D. (Page 12)

I. Guidance for Restricted Classification for Relay Without a Contaminant Reduction Study

The NSSP Model Ordinance provides state Authorities the option to allow relaying from a restricted area affected by a point source without a contaminant reduction study. The requirement for establishing the restricted classification for this use is different than the requirements for relay with a contaminant reduction study. The Authority must assure that the bacteriological quality of every station meets Chapter IV @.02 G (2). Additionally, the treatment period must exceed sixty (60) days. Should the Authority have viral concerns, the use of MSC sampling of the shellfish would be appropriate. The Authority could use the 50 PFU/100gm level or predetermined levels established by the Authority based on studies conducted in the area.

J. Model Ordinance Requirements for Depuration

(1) Chapter IV. Shellstock Growing Areas

@.02 Microbiological Standards.

G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration.

(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section G. (2).

(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of:

(a) 300 MPN per 100 ml for a three-tube decimal dilution test;

(b) 173 MPN per 100 ml for a twelve-tube single dilution test; or

(c) 163 CFU per 100 ml for a MF (mTEC) test.

(3) Required Sample Collection. Samples shall be collected in accordance with Section E. (3).

H. Standard for the Restricted Classification of

	Growing Areas Affected by Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration. *(1) Exception. If the tidal stage increases the fecal coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.* *(2) Pollution Sources. Growing areas shall meet the requirements in Section F. (2).* *(3) Water Quality. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Section G. (2) or Section H. (4).* *(4) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median or geometric mean MPN or MF(mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed a MPN or MF (mTEC) of:* *(a) 260 MPN per 100 ml for a five-tube decimal dilution test;* *(b) 300 MPN per 100 ml for a three-tube decimal dilution test; or* *(c) 163 CFU per 100 ml for a MF (mTEC) test.* *(5) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by the same method described in Section F. (5).* *(6) Required Sample Collection.* *(a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section G. (2).* *(b) Systematic Random Sampling Standard. The Authority shall collect samples in the same intensity and frequency, and shall apply the sample results in the manner described in Section F. (6) for the application of the*

standard under Section H. (4).

(2) Chapter XV. Depuration

.01 Critical Control Points.

A. Receiving Critical Control Point - Critical Limits.

(1) The dealer shall…

(2) The dealer shall…

(3) Should a dealer…

(4) The dealer shall receive and depurate only shellstock obtained from a special licensed harvester who has:

(a) Harvested or supervised the harvest of shellstock from a Restricted or Conditionally Restricted area in the open status.

(b) Identified the shellstock…

K. Guidance for Restricted Classification for Depuration

Use of the restricted classification for depuration requires the Authority to conduct a sanitary survey of the growing area as required in Chapter IV @ 01 and establish a monitoring program to ensure the water quality requirements of Chapter IV @ 02 G & H and @03 D.

Depuration process verification described in Chapter XV. @.03 Section J. is based on conditional and approved protocols. The protocol is conditional when statistical analysis of the database containing the 10 most recent FC end point samples fails to meet prescribed species-specific indices. The intent of which is to ensure an appropriate level of testing and quality assurance, including release criteria, during those periods of time when the depuration process is being challenged. These process verification protocols are based on fecal coliform assays of shellfish meats. The requirement for adverse case sampling of the restricted growing area is to assure that water quality in the restricted harvest growing areas does not exceed a median FC score of 88/100ml (or 163 FC.100ml) and P90 requirements.

Water quality requirements for the restricted growing area used for depuration were put in place to prevent grossly contaminated shellfish from being processed. It was not the inability to depurate high FC levels from contaminated shellstock, but rather that viruses associated with grossly contaminated shellstock were

thought to not effectively depurate viruses in 44 hours. In contrast, restricted growing areas adjacent to WWTP discharges used for relay with contamination reduction studies are considered effective for viral reductions and do not require a water quality sampling program based on 14 consecutive days of relay. The inability to detect viruses using fecal coliform based process verification and the lack of any suitable viral indicator assays was the original rationale behind restricted growing areas for depuration requiring water quality limits.

L. Model Ordinance Requirements for Use of a Restricted Area as the Source of Seed

(1) Chapter VI. Shellfish Aquaculture

.03 Seed Shellstock

Seed may come from any growing area, or from any growing area in any classification, provided that:

A. The source of the seed is sanctioned by the Authority; and

B. Seed from growing areas or growing areas in the prohibited classification are cultured for a minimum of six (6) months.

M. Seed

If a restricted growing area is used as a source for seed and the Authority requires that the shellfish must be cultured in the approved growing area for a minimum of six (6) months, the classification requirements for relay and depuration are adequate for this use.

N. Determination of the Boundaries Between Prohibited and Restricted Areas

The establishment of boundaries separating prohibited and restricted growing areas is dependent upon the uses to be allowed within the restricted growing area. MO Chapters IV and V address the classification requirements for allowable shellfish uses in the restricted classification. These uses include the following:

(1) Relay with a contaminant reduction study
(2) Relay without a contaminant reduction study
(3) Depuration

If harvesting for relay with a contaminant reduction study, the

boundary line should be based on an acceptable dilution ratio. If harvesting for relay without a contaminant reduction study or depuration, the boundary line must be based on a fecal coliform sampling program. The SCA has the option to utilize MSC.

Guidance for Dilution Ratios

Restricted areas that are the source for shellstock relaying with a contaminant reduction study are not required to meet a microbiological standard. Shellstock from restricted areas used for relaying without a contaminant reduction study or for depuration do have to meet a microbiological standard. In the absence of a microbiological standard, dilution ratios become very important to protect public health. A Shellfish Control Authority should not allow relay with a contaminant reduction study from any portion of a restricted area that does not meet a minimum dilution. The SCA should determine the effluent quality based on a worst case scenario and should establish a dilution ratio that would accomplish a dilution equivalent to a MPN of 88 (or 163) which is the upper limit restricted standard for relaying without a contaminant reduction study and for depuration. This dilution is 16,000:1. Should the Shellfish Control Authority choose to classify waters not meeting a dilution ratio equivalent to the upper limit MPN standard of 88 (or 163), the classification should be supported by fecal or MSC sampling of WWTP effluent to demonstrate a wastewater quality level less than 1.4×10^6 or the results of the contaminate reduction studies conducted over worst-case scenarios at the upstream WWTP discharge.

VI. Establishment of Conditional Classifications

The basic concept of the NSSP is to control the safety of shellfish by preventing their harvest from contaminated growing areas. In reviewing growing area classifications and sanitary surveys conducted by Shellfish Control Authorities, it appears that a common misinterpretation is the classification of an area as approved when in fact the area should have been classified as conditional. Critical investigations usually reveal that the area is subject to intermittent pollution events. Careful consideration of an intermittent pollution event, development and application of a management plan, and cooperation and compliance by all parties may also allow upgrading of an area to a conditionally approved or conditionally restricted classification instead of requiring the area to be restricted or prohibited at all times.

Intermittent pollution to shellfish growing waters has been a significant cause of shellfish-borne infectious disease outbreaks worldwide. In 1978, at least 20,000 persons were involved in an outbreak of oyster-associated gastroenteritis attributed to Norwalk virus. The investigation of the outbreak indicated that a combination of meteorological and hydrographic

events had caused inadequately treated and diluted sewage from a nearby municipal facility to reach the area. In an incident in 1982, at least 471 persons developed gastroenteritis after consumption of sewage contaminated oysters when a combination of raw sewage bypasses, high rainfall, strong winds, and abnormally low tides caused contamination of an area that was classified as approved. In both of these instances, application of the conditionally approved area concept probably could have prevented the outbreaks.

A common situation where this classification might be appropriate is when water quality is, to some degree, dependent upon the operation of a Waste Water Treatment Plant (WWTP). For example, the boundaries of an approved shellfish area might be improperly determined during a period when a WWTPSD is operating at a satisfactory level. If there is some interruption in treatment, it follows that there will be some degradation of water quality in the growing area which may require a relocation of the boundaries. The degree of relocation would depend upon such items as the distance between the pollution source and the growing area, hydrography, the amount of water, and the amount of pollution.

The first step in determining whether an area should be classified as conditionally approved or conditionally restricted is to determine whether sufficient State resources are available to manage, survey, monitor, control harvesting, affect closures, and reopen the area as required. It should be noted that sources of pollution must be routinely monitored; coordination between State, local and industry officials must be timely; performance standards must be monitored; and closures must be immediate and effective. States electing to classify areas as conditionally approved have found the public resource investment to be substantial.

The second step in determining whether an area should be placed in the conditionally approved or conditionally restricted classification is to evaluate the potential sources of pollution in terms of their effect on water quality in the area. Potential sources of pollution involving a WWTP include: bypasses and overflows within a sewage collection and treatment system.

The third step in establishing a conditionally approved or conditionally restricted area is to evaluate the source of pollution in terms of the water quality standards to be maintained, and to formulate performance standards for each pollution source having a significant effect on the sanitary quality of the area. The following is an example of performance standards that might be developed:

Performance standards or closure criteria may be based upon the bacteriological quality of effluent from sewage treatment plants. This

might be stated in terms of chlorine residual if the bacteriological quality of the effluent can be positively related to chlorine residual. The following is an example of a performance standard for an effluent discharge: "The median coliform MPN, in any one (1) month, shall not exceed 500 per 100 ml, based on not less than sixteen (16) composite samples per month, and not more than ten (10) percent of the samples shall have an MPN in excess of 10,000 per 100 ml. Determinations of the chlorine residual of the effluent should be made hourly and recorded in the permanent plant records."
A performance standard may be based upon total quality of sewage, which can be discharged from any given unit, or from a combination of units, without causing the basic water quality standards to be exceeded.

The design of a waste treatment plant and the plant effluent specifications may be critical to the designation of an area classified as conditionally approved or conditionally restricted. Design criteria which may be useful in determining the quality of sewage which can be discharged into an area without exceeding the desired water quality standards include: population equivalent (coliform) of sewage, predicted survival of coliform in seawater, effectiveness of chlorination and the total quality of clean dilution water in an area. Results of many studies on the survival of bacteria in seawater have been published.

The mechanical equipment at critical sewage treatment or pumping units should be such that interruptions will be minimized. Wherever possible, operations should be automatically recorded on charts. Requirements that might be imposed depend upon the importance of the unit's relationship to water quality. Important design features of a sanitary waste collection system that should be considered include:
Storm water should be excluded from the sanitary system. There should be stand-by equipment to insure that treatment or pumping will not be interrupted. It should be taken into account that interruptions may occur because of damage to a single unit or a power failure.

The pumps and critical units should be fitted with meters or gauges so the regulatory agency can monitor performance standards.

Installation of recording scales to indicate rate of chlorine use is helpful. Chlorine flow meters are available that integrate hydraulic flow with chlorine demand.

Liquid level recording gauges fitted with alarms and located in overflow channels of sewage treatment plants and wet wells of lift stations are useful. They can be set to indicate when overflow takes place. It is good operating procedure to date recording charts. Gauges should be calibrated and maintained so that indicated discharge rates are accurate.

Automatic devices to warn of failure or malfunctioning at self-operated pumping stations or treatment plants can be an important control.

Another factor to consider in developing a conditionally approved or conditionally restricted area is that a prohibited area must be interposed between the conditionally approved or restricted area and the source of pollution. The size of such area should be based on the total time it would take for the operating agency to detect a failure, notify the State Shellfish Control Agency, and for the latter agency to issue a notice to stop shellfish harvesting. It is recommended that the area be of such size that the flow time through the safety area is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that closure actions might be necessary on holidays or at night.

The length of time a conditionally approved or conditionally restricted area should be closed following a temporary closure will depend upon several factors including the species of shellfish, water temperature, shellfish activity and cleansing rates, presence of silt or other chemicals that might interfere with the physiological activity of the shellfish, and the degree of pollution of the area.

The conditional classifications are designed to address growing areas that are subject to intermittent microbiological pollution. These optional classifications offer the Authority an alternative to placing the area in the restricted or prohibited classification year round when during certain times of the year or under certain conditions, the shellstock from the growing area may be safely harvested. Public health protection and the control of shellfish safety in the use of the conditional classifications are afforded through the use of a management plan. The management plan for each growing area placed in a conditional classification is based on the information gathered during the sanitary survey. The plan establishes a strict set of criteria that must be met for the growing area to remain in the open status. Failure to meet the criteria automatically places the growing area in the closed status, with immediate notice to the public, the affected industry, and the plan's participants. Two (2) of the most important components of the management plan are: the acceptance of and the agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, State and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved; and the annual reevaluation of compliance with the plan to assure public health protection. Use of the conditional classification requires more intense monitoring and more frequent reevaluation because of the intermittent nature of the pollution event.

When the Authority has sufficient resources to manage a conditional

classification, the use of the conditional classification could allow the safe use of growing areas that might otherwise not be available to the shellfish industry. For a complete discussion of the conditional classification, see the NSSP Model Ordinance Guidance Documents: Management Plans for Growing Areas in the Conditional Classification (ISSC/FDA, 2015). For additional information concerning the classification of growing waters and the sanitary survey, see the NSSP Model Ordinance Guidance Documents: Sanitary Survey and the Classification of Growing Waters (ISSC/FDA, 2015).

A. Requirements for Conditional Area Adjacent to a Waste Water Treatment Plant (WWTP)

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(1) Survey Required. The sanitary survey meets the following criteria:

(a) The area will be in the open status of the conditional classification for a reasonable period of time. The factors determining this period are known, are predictable, and are not so complex as to preclude a reasonable management approach;

(b) Each potential source of pollution that may adversely affect the growing area is evaluated;

(c) Microbiological water quality correlates with environmental conditions or other factors affecting the distribution of pollutants into the growing area; and

(d) For SSCAs utilizing MSC meat sample data, this data correlates with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.

	(2) Management Plan Required. For each growing area, a written management plan shall be developed and shall include: *(a) For management plans based on wastewater treatment plant function, performance standards that include:* *(i) Peak effluent flow, average flow, and infiltration flow;* *(ii) Microbiological quality of the effluent;* *(iii) Physical and chemical quality of the effluent;* *(iv) Conditions which cause plant failure;* *(v) Plant or collection system bypasses;* *(vi) Design, construction, and maintenance to minimize mechanical failure, or overloading;* *(vii) Provisions for monitoring and inspecting the waste water treatment plant; and* *(viii) Establishment of an area in the prohibited classification adjacent to a wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;* *(b) For management plans based on pollution sources other than waste water treatment plants:* *(i) Performance standards that reliably predict when criteria for conditional classification are met; and* *(ii) Discussion and data supporting the performance standards.* *(c) For management plans based on waste water system discharge function or pollution sources other than waste water system discharge*

	criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum criteria are: *(i) Performance standards of the plan are fully met;* *(ii)Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;* *(iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. The study may establish criteria for reopening based on coliform levels in the water. The SSCA may utilize MSC in growing areas adjacent to waste water system discharge. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. Analytical sample results shall not exceed a level of 50 MSC per 100 grams or pre-determined levels established by the Authority based on studies conducted on regional species under regional*

conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed; and

(iv) Shellstock feeding activity is sufficient to achieve microbial reduction.

(d) For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;

(f) Procedures for immediate notification to the Authority when performance standards or criteria are not met;

(g) Provisions for patrol to prevent illegal harvest; and

(h) Procedures to immediately place the growing area in the closed status in 24 hours or less when the criteria established in the management plan are not met.

(3) Reevaluation of Conditional Classification.

(a) The classification shall be reevaluated at least once each year. The reevaluation shall include:

(i) Evaluation of compliance with the management plan;

(ii)Determination of adequacy of reporting of failure to meet performance standards;

(iii) Review of the cooperation of the persons involved;

(iv) Evaluation of water

	quality in the growing area with respect to the bacteriological standards for its classification; *(v) Field inspection of critical pollution sources, where necessary; and* *(vi) Written findings, evaluations and recommendations.* *(b) Water Sample Collection.* *(i) When the conditional management plan is based on the absence of pollution from marinas for certain times of the year, monthly water samples are not required when the growing area is in the open status of its conditional classification provided that at least three of the water samples collected to satisfy the bacteriological standard for the open status are collected when the growing area is in the open status.* *(ii) When the conditional management plan is based on the operation and performance of a Waste Water System Discharge (WWSD) (s); combined sewer overflow(s); or other point sources of pollution, monthly water samples are required when the growing area is in the open status of its conditional classification.* *(iii) If a monthly sample cannot be collected due to environmental constraints, the monthly sampling requirement will be satisfied if an additional water sampling run is conducted the following month.*

	(iv) When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events, storm water runoff, and seasonal variations, a minimum of five (5) sets of water samples (when the Adverse Pollution Condition sampling regimen is used) or six (6) sets of water samples (when the Systematic Random Sampling regimen is used) are required. The samples shall be collected when the growing area is in the open status. *(v) When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events or storm water runoff, and the area is in the open status for less than six (6) months a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). At least one (1) sample shall be collected each month the area is placed in the open status. This sample shall be collected while the area is open. If closed status samples are used to meet the minimum sample requirements only two (2) sets of samples may be utilized and they must have been taken within five (5) days of when the Authority anticipates that the area will*

	be placed in the open status. For growing areas in the open status less than two (2) months, at least one (1) sample must be collected while the area is in the open status. Samples collected during the closed status to meet the minimum five (5) sets of water samples shall be applied to annual and triennial reevaluations of the area. *(vi) When the conditional management plan is based on the seasonal opening and closing of the area, and the area is in the open status for a predetermined period of less than six (6) months, a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). All samples shall be collected while the area is in the open status unless the Authority has historical water quality data to demonstrate that the area meets open status criteria while in the closed status. If closed status samples are used to meet the minimum sample requirements they must be collected within thirty (30) days prior to the area being placed in the open status.* *(4) Understanding of and Agreement With the Purpose of the Conditional Classification and Conditions of Its Management Plan by All Parties Involved.* *(a) The management plan shall be*

developed by the Authority in coordination with:

(i) The local shellfish industry;

(ii) The individuals responsible for the operation of any Waste Water System Discharge (WWSD)s involved; and

(iii) Any local or State agencies; and

(b) Failure of any one party to agree shall constitute sufficient justification to deny the application of the conditional classification to a growing area.

(5) Conditional Area Types. There are two (2) types of conditional areas:

(a) Conditionally approved; and

(b) Conditionally restricted

B. Guidance for a Conditional Area Management Plan

The management plan for a growing area in the conditionally approved or conditionally restricted classification must meet certain minimum requirements to ensure that the safety of the shellfish for human consumption is maintained. The use and success of the conditional classification depends upon a thorough and accurate management plan. Therefore, it is important that all aspects of the management plan be fully considered and implemented. The minimum requirements to be addressed are:

(1) An understanding of and an agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, state and federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved;

(2) A written management plan for the growing area being placed in the conditional classification, which includes a general description of the growing area with a map showing the area's boundaries, and which addresses all items in C. through H..

(3) A sanitary survey that shows the growing area will be in the open status of its conditional classification for reasonable periods of time. The survey must provide a description of the factors determining the growing area's suitability for being classified conditionally approved or conditionally restricted, and the supporting information and data.

(4) A description of the predictable pollution event or events that are being managed and the performance standards established for each pollution source contributing to the pollution event including:

(a) For a wastewater treatment facility, the performance standard should be based on:

(i) Peak effluent flow

(ii) Bacteriological quality of the effluent

(iii) Physical and chemical quality of the effluent

(iv) Bypasses from the treatment plant or its collection system

(v) Design, construction, and maintenance to minimize mechanical failure or overloading (i.e., the reliability of the treatment system and collection system components)

(vi) Provisions for verifying and monitoring efficiency of the wastewater treatment plant and the feedback system for addressing inadequate treatment.

(vii) Identification of conditions that lead to Waste Water Treatment Plant (WWTP) failure and closure of the conditionally approved area.

(b) For meteorological or hydrological events, the performance standard should be based on:

(i) Identification of the specific meteorological and/or hydrologic event that will cause the growing area to be placed in the closed status;

(ii) Discussion and data analyses concluding that effects on water quality from these specific meteorological and/or hydrologic events are predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for the growing area placed in the conditional classification; and

(iii) The predicted number of times, based on historical findings, that the pollution event will occur within one (1) year.

(c) For seasonal events, such as marina operation, seasonal rainfall, and waterfowl migration, the performance standard should be based on:

i. Identification of the seasonal event that will cause the growing area to be placed in the closed status, including its estimated duration; and

(ii) Discussion and data concluding that the seasonal event is predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for a growing area placed in the conditional classification;

(5) A description of the plan for monitoring water quality including numbers and frequency;

(6) A description of how the closed status for the conditional classification will be implemented, which must include:

(a) A clear statement that when the performance standards are not met, the growing area will immediately be placed in the closed status;

(b) A requirement to notify the Authority or Authorities that the

management plan performance standards have not been met, including:

(i) The name of the agency or other party responsible for notifying the Authority;

(ii) The anticipated response time between the performance standards not being met and notification of the Authority; and

(iii) The procedures for prompt notification including contingencies such as night, weekend and absences of key personnel;

(c) A description of the implementation and enforcement, including:

(i) The response time between the notification to the Authority of the failure to meet performance standards and activation of the legal closure of the growing area by the Authority;

(ii) The procedures and methods to be used to notify the shellfish industry; and

(iii) The procedures and methods to be used to notify the patrol agency (enforcement agency) including:

- The name of the responsible patrol agency;
- The anticipated response time between the Authority's legal closure of the growing area and notification of closure to the patrol agency; and
- A description of the patrol agencies anticipated activities to enforce the closed status.

(7) A description of the criteria that must be met prior to reopening a growing area in the closed status, including the need to determine that:

(a) The performance standards established in the management plan are again fully met;

(b) The flushing time for pollution dissipation is adequate;

(c) A time interval has elapsed which is sufficient to permit reduction of human pathogens as measured by the coliform indicator group in the shellstock;

(d) Where necessary, the bacteriological quality of the water must be verified; and

(e) Shellstock feeding activity is sufficient to achieve reduction of pathogens to levels present prior to the pollution event.

(8) A commitment to a reevaluation of the management plan at least annually using, at a minimum, the reevaluation requirements in the NSSP Model Ordinance

VII. Conditionally Restricted

A. Definition

A classification used to identify a growing area that meets the criteria for the restricted classification except under certain conditions described in a management plan.

B. Requirements for Conditionally Restricted Area Adjacent to a Waste Water Treatment Plant (WWTP)

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(7) Conditionally Restricted Classification. Any growing area in the conditionally restricted classification shall:

(a) Meet the requirements for:

(i) A restricted classification when the conditionally restricted classification is in the open status; and

(ii) A prohibited classification when the conditionally restricted classification is in the closed status; and

(b) Designate in its management plan whether the harvested shellstock are to be relayed or

depurated.

(2) Use of the conditionally restricted classifications by the Authority is optional. The conditionally restricted classification is designed to address growing areas that are subject to intermittent microbiological pollution. These classifications offer the Authority an alternative to placing the area in the prohibited classification year round when, under certain conditions, the shellstock from the growing area may be safely harvested for restricted purposes. The concept also applies to situations where conditions are acceptable for harvest when wastewater treatment plant operation is satisfactory, but not when a malfunction occurs. A management plan is required that describes the controls to provide public health protection in the use of the conditionally restricted classification. For a full explanation of the conditional classifications and their use, see the NSSP Guidance Document, *Management Plans for Growing Areas in the Conditional Classifications* (ISSC/FDA, 2015).

State Control Authorities that allow relaying or depuration may utilize the conditionally restricted classification adjacent to prohibited areas established as a result of a WWTP outfall. The use of the conditionally restricted classification is dependent upon the predictable factors associated with the WWTP discharge. These factors may include volume, treatment efficient, seasonality or other factors which affect the quality of the WWTP effluent. The quality concerns are bacterial, viral, toxic chemical and poisonous deleterious substances. Portions of the prohibited area that are less impacted by the WWTP outfall during predictable time periods can be classified conditional and used as a source of shellfish for relaying and depuration.

The conditionally restricted classification management plan must establish a strict set of criteria, which must be met for the growing area to remain in the restricted status. The following are examples of different types of performance standards that could be used:

(a) Performance standards might stipulate the bacteriological quality of effluent from sewage treatment plants. The

microbiological quality can be monitored in terms of disinfection residual or dosage for ultraviolet light disinfection. An example of a performance standard for an effluent discharge is:

"The median fecal coliform MPN, in any one (1) month, shall not exceed 200 per 100 ml, based on not less than sixteen (16) samples per month, and not more than ten (10) percent of the samples shall have an MPN in excess of 1,000 per 100 ml. This fecal coliform limit shall be presumed to be met if the chlorine residual in the effluent is at least 1.0 ppm and the chlorine residual in the effluent is continuously recorded on a chart by chlorine residual analyzer or is measured hourly and recorded in the daily monitoring records as required for the plant's NPDES permit."

(b) For disinfection by ultraviolet (UV) light, the disinfection is based on dosage. An example of a performance standard is, "A minimum UV dose of 37 mW-Sec/cm^2 is to be maintained. The calculation of intensity of the UV light is to include factors for effluent quality, including turbidity, suspended solids, and transmittance. The effluent factors contributing to the dose, including turbidity, suspended solids, transmittance, and flow will be continuously measured and recorded. An alarm will be activated if any of the factors are above design limits."

A detailed discussion of ways to increase the reliability of sewage treatment plants can be found in *Protection of Shellfish Waters* (USEPA, 1974) and *Design Criteria for Mechanical, Electric and Fluid System Component Reliability* (USEPA, 1974).

The fourth step is to determine the water quality, which will occur in the growing area when the performance standards are not met, and what portion of the growing area will be affected. Once these determinations are made, the Authority can select the appropriate management strategy for the portion of the growing area

that will be placed in the closed status when performance standards are not met, and can select the boundaries for the closed status. The boundaries of that portion of the growing area to be placed in the closed status would depend upon such items as the distance and travel time from the pollution source to the area, the concentration of pollutants in the discharge during the breakdown condition, amount of effluent and hydrographic factors including dilution available in the receiving water.

The use of the conditional classification where a sewage treatment plant is the pollution source being managed requires a fifth step. An area in the prohibited classification must be established between the sewage treatment plant and the growing area placed in the conditionally approved or conditionally restricted classification. The size of the prohibited area should be based on the level of sewage treatment; the total time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority; and the time it would take the Authority to issue a notice to stop shellstock harvesting. The size of the area in the prohibited classification should allow for an effluent travel time through the prohibited area that is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night. A minimum effluent dilution is to be determined at the prohibited boundary and can be the controlling factor in situations where there is efficient detection and notification of breakdowns.

The length of time that a growing area should be in the closed status of its conditional classification will depend upon several factors. These factors include the degree of pollution in the growing area and flushing capacity of the estuary, the species of shellfish, water temperature, shellstock activity and cleansing rates, and presence of silt or other chemicals that might interfere with the physiological activity of the shellstock. Additional information on the natural cleansing of shellstock is provided in the NSSP Guidance Document, *Shellstock Relay* (ISSC/FDA, 2015).

C. Allowable Uses of Shellfish from a Conditionally Restricted Growing Area

(1) Allowable Uses When Area is in Restricted Status

(a) Relay without a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or

conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(b) Relay with a Contaminant Reduction Study
Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(c) Depuration
Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(d) Seed
Seed means shellstock which is less than market size.

(2) Allowable Uses When Area is in Prohibited Status

(a) Seed
Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Relay with a Contaminant Study
The Requirements for Relay with a Contaminant Study are defined in Section V. D.

E. Model Ordinance Requirements for Relay without a Contaminant Study
The Requirements for Relay without a Contaminant Study are defined in Section V. H.

F. Model Ordinance Requirements for Depuration
The Requirements for Depuration are defined in Section V.J.

G. Model Ordinance Requirements for Seed
The Requirements for Seed are defined in Section V.L.

H. Determining Boundaries for Conditionally Restricted Growing Areas

Should the Authority utilize the conditionally restricted

classification to allow relay or depuration, the area classified as conditionally restricted would be established within the portion of the prohibited area established adjacent to the WWTP. Shellfish uses allowed in the restricted classification would be allowed in the conditionally restricted area when the plant is operating within the satisfactory conditions outlined in the conditionally restricted management plan. (Chapter IV@ .03 C (2). Use of the conditionally restricted classification for relay without contaminant reductions studies and depuration requires the Authority to determine whether the growing area is impacted by additional point and non-point sources of pollution in addition to the management plan which is intended to address all potential problems with the adjacent WWTP. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Chapter IV.@.02 Section G. (2) or Section H. (3) depending upon whether there is an additional point source or just non-point sources of contamination impacting the conditionally restricted growing area. Sufficient water quality samples shall be collected in accordance with Chapter IV.@.02 Section E. (3) at representative water quality sampling stations throughout the impacted restricted growing area.

The establishment of boundaries separating prohibited and conditionally restricted growing areas is dependent upon the uses to be allowed within the restricted growing area. MO Chapters IV and V address the classification requirements for allowable shellfish uses in the restricted classification. These uses include the following:

(1) Relay with a contaminant reduction study
(2) Relay without a contaminant reduction study
(3) Depuration

If harvesting for relay with a contaminant reduction study, the boundary line should be based on an acceptable dilution ratio. If harvesting for relay without a contaminant reduction study or depuration, the boundary line must be based on a fecal coliform sampling program. The SCA has the option to utilize MSC.

The use of the conditionally restricted classification should not affect other adjacent classifications such as restricted, conditionally approved or approved. The area will be considered in the prohibited status when the management plan criteria are not met.

Guidance for Dilution Ratios

For Shellfish Control Authorities that choose to establish conditionally restricted areas, the operating efficiency of the plant must be a primary consideration. A portion of what might be the standard prohibited area could be classified as conditionally restricted when the WWTP is operating efficiently. An explanation for operating efficiency is included in Section VI paged 26 of this document. Conditionally restricted areas, when meeting the NSSP requirement for the restricted classification, can be used for a source for shellstock relaying with a contaminant reduction study. These areas are not required to meet a microbiological standard. Shellstock from restricted areas used for relaying without a contaminant reduction study or for depuration do have to meet a microbiological standard. In the absence of a microbiological standard, dilution ratios become very important to protect public health.

A Shellfish Control Authority should not consider any portion of a growing area that does not meet a 320:1 dilution ratio as a source for relaying with a contaminant reduction study. The concept of a 320:1 dilution ratio was first documented in a technical paper written by Virgil Carr of FDA. The technical paper was based on studies conducted at WWTP utilizing UV for disinfection.

This study proposed that the prohibited area, could approach the size requirements for Critical Dilution for Toxics to Ambient (Background) from the Clean Water Act. Similarly, the EPA's Regulatory Mixing Zone (RMZ) is 300:1, which is approximately the transition line from near field dilution zone to far field dilution zone where most mixing has already occurred. The 320:1 dilution ratio is needed to assure that poisonous and deleterious substances are not present in high enough concentrations to present a public health concern.

From a pragmatic point of view, dilution from the outfall to the 320:1 line is a dilution factor of 320 while dilution from 320:1 to 1000:1 is a dilution factor of 3.1. This roughly equates to 100 times more dilution of the originate effluent occurring within the 320:1 dilution line than occurs from the 320:1 dilution line to the 1000:1 dilution line. This is an important factor to consider when one is attempting to understand the viral density in growing waters overlying growing areas adjacent to WWTP discharge and the associated risk.

VIII. Conditionally Approved

A. Definition

A classification used to identify a growing area which meets the criteria for the approved classification except under certain conditions described in a management plan.

B. Requirements for Conditionally Approved Area Adjacent to a Waste Water Treatment Plant (WWTP)

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(6) Conditionally Approved Classification. Any growing area in the conditionally approved classification shall:

(a) Meet the requirements for:

(i) An approved area classification when the conditionally approved classification is in the open status; and

(ii) A restricted or prohibited classification when the conditionally approved classification is in the closed status; and

(b) If the closed status meets the criteria for the restricted classification, designate in its management plan whether the shellstock may be harvested for relaying or depuration.

Growing areas are placed in the approved classification when the sanitary survey information and marine Biotoxin surveillance data indicate that fecal material, pathogenic microorganisms, poisonous, or deleterious substances are not present in the growing area in unacceptable concentrations. Shellstock harvested from these growing areas may be sold directly to the public for consumption raw or cooked.

C. Allowable Uses of Shellfish in a Conditionally Approved Growing Area

(1) Allowable Uses when the Conditionally Approved Area is in the Open Status

(a) Direct Marketing

Direct Marketing means the sale for human consumption of shellfish which:

(i) Does not require depuration or relaying prior to sale; or

(ii) Has been subjected to depuration or relaying activities

(b) Relay

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(c) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(d) Seed

Seed means shellstock which is less than market size.

(e) Post-Harvest Processing

Post-Harvest Processing means any process which has been validated using NSSP validation procedures which reduces the levels of pathogenic hazards to below the appropriate FDA action level or in the absence of such a level, below the appropriate level as determined by the ISSC.

(2) Allowable Uses when the Conditionally Approved Area is in the Closed Status

(a) Relay

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(b) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(c) Seed
Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Direct Marketing
There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for direct market.

E. Model Ordinance Requirements for Relay
The Requirements for Relay are defined in Section V. H.
There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for relay.

F. Model Ordinance Requirements for Depuration
There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for depuration.

(1) Model Ordinance Chapter XV. Depuration

.01 Critical Control Points.

A. Receiving Critical Control Point - Critical Limits.

(1) The dealer shall receive and depurate only shellstock which is obtained from a licensed harvester who has:

(a) Harvested the shellstock from an Approved or Conditionally Approved area in the open status as indicated by the tag; [C] and

(b) Identified the shellstock with a tag on each container or transaction record on each bulk shipment; [C] and

(c) Harvested the shellstock in compliance with the time/temperature requirements of Chapter VIII. @.02 A. (1), (2) or (3) as determined from records supplied by the harvester described in Chapter VIII. .02 G. (2) [C].

(2) The dealer shall…
(3) Should a dealer…
(4) The dealer shall…

The Requirements for Depuration of shellfish harvested from conditionally approved areas in the closed status are defined in Section V.J.

G. Model Ordinance Requirements for Seed

The Requirements for Seed are defined in Section V.L.
There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for seed.

H. Model Ordinance Requirements for Post-Harvest Processing

There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for post-harvest processing.

I. Model Ordinance Requirements for Relay with a Contaminant Reduction Study

The Requirements for Relay with a Contaminant Reduction Study are defined in Section V.D.

J. Model Ordinance Requirements for Relay without a Contaminant Reduction Study

The Requirements for Relay without a Contaminant Reduction Study are defined in Section V.H.

K. Determining Boundaries for Conditionally Approved Growing Areas

Should the Authority utilize the conditionally approved classification to allow harvest for direct marketing, the area classified as conditionally approved would be established within the portion of the prohibited or restricted area established adjacent to the WWTP. Shellfish uses allowed in the approved classification would be allowed in the prohibited or restricted area when the plant is operating within the satisfactory conditions outlined in the conditionally approved management plan. (Chapter IV@ .03 C (2).

In addition to meeting the satisfactory conditions outline in the conditionally approved management plan, the area must also conduct a sanitary survey of the growing area as required in Chapter IV @ 01 and establish a monitoring program to ensure the water quality requirements of Chapter IV @ 02 E. The area will

be considered in the prohibited or restricted status when the management plan criteria is not met.

Guidance for Dilution Ratios

For Shellfish Control Authorities that choose to establish conditionally approved areas for harvest uses allowable within the approved classification, the operating efficiency of the plant must be a primary consideration. A portion of the prohibited or restricted area could be classified as conditionally approved when the WWTP is operating efficiently. An explanation for operating efficiency is included in Section VI page 26 of this document. The minimum dilution of 1000:1 is recommended for establishing a conditionally approved area adjacent to a WWTP. The rationale for the 1000:1 dilution rate was included in Section IV: Guidance Document Chapter II 19., which was adopted by the ISSC in 2015. Conditionally approved areas, when not in the approved status, can be used for a source for shellstock relaying with a contaminant reduction study, shellstock relaying without a contaminant reduction study and depuration. To utilize shellfish for these purposes, these areas are required to meet the Model Ordinance requirements associated with those uses (e.g. restricted water quality standard).

IX. Approved Classification

A. Definition

A classification used to identify a growing area where harvest for direct marketing is allowed.

B. Requirements for Use of the Approved Classification

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

B. Approved Classification. Growing areas shall be classified as approved when the following criteria are met.

(1) Survey Required. A sanitary survey finds that the area is:

(a) Safe for the direct marketing of shellfish;

(b) Not subject to contamination from human or animal fecal matter at levels that, in the judgment of the Authority, presents an actual or potential public health hazard; and

(c) Not contaminated with:

	(i) Pathogenic organisms; *(ii) Poisonous or deleterious substances;* *(iii) Marine Biotoxins; or* *(iv) Bacteria concentrations exceeding the bacteriological standards for a growing area in this classification.* *(2) Water Quality. The water quality in the growing area shall meet the bacteriological standards for an approved classification in Section @.02.* ***@.02 Microbiological Standards*** *E. Standard for the Approved Classification of Growing Areas Affected By Point Sources.* *(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2).* *(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml, and not more than ten (10) percent of the samples shall exceed an MPN or MF (mTEC) of:* *(a) 43 MPN per 100 ml for a five-tube decimal dilution test;* *(b) 49 MPN per 100 ml for a three-tube decimal dilution test;* *(c) 28 MPN per 100 ml for a twelve-tube single dilution test; or* *(d) 31 CFU per 100 ml for a MF (mTEC) test.* *(3) Required Sample Collection.* *(a) A minimum of five (5) samples shall be collected annually under adverse pollution conditions from each sample station in the growing area.* *(b) A minimum of the most recent fifteen (15) samples collected under adverse pollution conditions from each sample station shall be used to calculate the median or geometric mean and percentage to determine compliance with this standard.* *(c) Sample station locations shall be*

adjacent to actual or potential sources of pollution.

C. Allowable Uses of Shellfish in an Approved Growing Area

(1) Direct Marketing

Direct Marketing means the sale for human consumption of shellfish which:

(a) Does not require depuration or relaying prior to sale; or

(b) Has been subjected to depuration or relaying activities

(2) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(3) Seed

Seed means shellstock which is less than market size.

(4) Post-Harvest Processing

Post-Harvest Processing means any process which has been validated using NSSP validation procedures which reduces the levels of pathogenic hazards to below the appropriate FDA action level or in the absence of such a level, below the appropriate level as determined by the ISSC.

D. Model Ordinance Requirements for Direct Marketing

There are no classification restrictions on shellfish harvested from approved areas for direct market.

E. Model Ordinance Requirements for Depuration

The Requirements for Depuration are defined in Section XIII.F.

There are no classification restrictions on shellfish harvested from approved areas for depuration.

F. Model Ordinance Requirements for Seed

The Requirements for Seed are defined in Section V.L.

There are no classification restrictions on shellfish harvested from approved areas for seed.

G. Model Ordinance Requirements for Post-Harvest Processing

There are no classification restrictions on shellfish harvested from approved areas for post-harvest processing.

H. Determining Boundaries for Conditionally Approved Growing Areas

	In establishing boundaries between approved areas and other classifications adjacent to a WWTP, the SCA should consider dilution ratios and the approved area must meet the microbiological standards for approved growing areas. Guidance for Dilution Ratios When determining if a WWTP or collection system discharge within the watershed or catchment area draining to a shellfish estuary potentially impacts a shellfish growing area, the NSSP recommends that a worst case raw sewage discharge be assumed. In this circumstance, if a level of 1.4×10^6 FC/100ml is assumed for a raw sewage release, a 100,000:1 dilution would be required to dilute the sewage sufficient to meet the approved area standard of 14 FC/100ml. If dilution analysis determines that the location of the discharge is such that the dilution of effluent would be greater than 100,000:1 then the WWTP could be considered located outside the zone of influence to the shellfish growing area. Different dilution ratios may be applied depending on the known concentration of sewage, a performance history of the treatment and collection system and a database of influent and effluent quality, provided that the water quality objective of the downstream harvest area is met.
Public Health Significance	In 2015, the ISSC adopted proposal 15-102 which incorporated the use of Male Specific Coliphage into the NSSP. The ISSC voting delegates directed the development of a guidance document to provide clarification for the use of MSC. This guidance document provides guidance regarding the use of MSC in the classification of shellfish growing areas adjacent to waste-water treatment plants. The classification guidance provides details and clarification that shellfish Authorities should find very helpful.
Cost Information	

Proposal No. **17-114**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	FDA
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	National Shellfish Sanitation Program Quality System - Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section II Model Ordinance - Chapter I Shellfish Sanitation Program @.03 Evaluation of Shellfish Sanitation Program Elements And Section IV Guidance Documents Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	**Section II Model Ordinance - Chapter I Shellfish Sanitation Program @.03 Evaluation of Shellfish Sanitation Program Elements** B. Criteria for evaluation of shellfish sanitation program elements shall be as follows: 1. Laboratory a. Requirements for evaluation of shellfish laboratories shall include at a minimum: i. Records audit of laboratory operations~~:~~ both Quality Systems and Technical methods; ii. Direct observation of current laboratory operating conditions; and iii. Information collection from the Authority and other pertinent sources concerning laboratory operations. b. Laboratory status is determined by the number and types of nonconformities found in the evaluation using NSSP standardized criteria contained in the FDA Shellfish Laboratory Evaluation Checklists found in the Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists. i. Quality System Evaluation. (a) This checklist includes a conforming and nonconforming status only. All nonconformities must be reconciled prior to scheduling an onsite evaluation of technical methods in NSSP laboratories. As this part of the evaluation specifically refers to the Quality manual and SOPs and other documentation considered the basis for data defensibility, this documentation must be in order prior to further LEO scheduling. The Quality Systems evaluation is performed as a desk audit and is in accordance with checklist found in Chapter II. ~~i.~~ ii. Technical Evaluation: Conforms. In order to achieve or maintain conform~~s~~ing status under the NSSP, a laboratory must

meet the following laboratory evaluation criteria:

~~ii~~(a) No critical nonconformities in the microbiological or marine Biotoxin component under evaluation have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist; and

~~iii~~(b) Not more than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine Biotoxin components have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist; and

~~iv~~(c) Not more than eighteen (18) critical, key, and other nonconformities in total in the microbiological component, twelve (12) critical, key and other nonconformities in total for the PSP component, or ten (10) critical, key and other nonconformities in total for the NSP component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist. This number must not exceed the numerical limits established for either the critical or key criteria; and

~~v~~(d) No repeat key nonconformities have been identified in the microbiological or marine Biotoxin component under evaluation in consecutive evaluations using the appropriate FDA Shellfish Laboratory Evaluation Checklist.

~~c.~~iii. Technical Evaluation: Provisionally Conforms. In order to be deemed provisionally conforming under the NSSP, a laboratory must meet the following laboratory evaluation criteria:

~~i.~~(a) Not more than three (3) critical nonconformities in the microbiological component, four (4) in the PSP component, or three (3) in the NSP component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist; and

~~ii~~(b) Not more than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine Biotoxin component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist; and

~~iii~~(c) Not more than eighteen (18) critical, key and other nonconformities in total in the microbiological component, or twelve (12) critical, key and other nonconformities in total in the PSP component or ten (10) critical, key and other nonconformities in total in the NSP component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist. This number must not exceed the numerical limits established for either the critical or key criteria; and

~~iv~~(d) Not more than one (1) repeat key nonconformity has been identified in the microbiological or marine Biotoxin component under evaluation in consecutive evaluations using the appropriate FDA Shellfish Laboratory Checklist.

~~d.~~iv. Technical Evaluation: Nonconformance. When a laboratory exceeds the following criteria, it will be determined to be in nonconformance:

~~i.~~(a) More than three (3) critical nonconformities in the microbiological component or four (4) in the PSP component, or three (3) in the NSP component have been identified using the appropriate FDA Shellfish Laboratory Checklist; or

~~ii.~~(b) More than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine Biotoxin component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist;

~~iii.~~(c) More than eighteen (18) critical, key, and other nonconformities in total in the microbiological component, or more than twelve (12) critical, key and other nonconformities in total in the PSP component, or more than ten (10) critical, key, and other nonconformities in total in the NSP component have been identified using the appropriate FDA Shellfish Laboratory Evaluation Checklist; or

~~iv.~~(d) One (1) or more repeat critical or two (2) or more repeat key nonconformities have been identified in consecutive evaluations in either the microbiological or marine Biotoxin components using the appropriate FDA Shellfish Laboratory Evaluation Checklist.

~~e.~~c. Corrective Actions for Conforming Status. A laboratory found to be in conforming status for ~~either the microbiological or marine Biotoxin component or for both components~~ technical checklists, other than the Quality Systems checklist, has up to ninety (90) days to successfully correct all nonconformities noted in each component evaluated or has an approved action plan in place to deal with the nonconformities noted. After this period, the laboratory's status will be downgraded to nonconforming if any key nonconformities remain to be successfully corrected. As a result, data being generated by the laboratory will no longer be acceptable for use in support of the NSSP for the laboratory component in question.

~~f.~~ d. Corrective Actions for Provisionally Conform~~s~~ing Status. A laboratory found to be in provisionally conforming status for ~~either the microbiological or marine Biotoxin component or for both components~~ technical methods checklists has up to sixty (60) days to successfully correct all nonconformities found in each provisionally conforming component evaluated or has an approved action plan in place to deal with the nonconformities noted. After this period, the laboratory will be assigned the following status for the laboratory component(s) in question:

i. Conforms if all the critical and key nonconformities have been successfully corrected in each provisionally conforming component evaluated; or

ii. Nonconforming if any critical or key nonconformities remain to be successfully corrected in each provisionally conforming component evaluate, or if the lab is not able to be evaluated because of a nonconforming Quality System. As a result, data being generated by the laboratory will no longer be acceptable for use in support of the NSSP for the laboratory component in question.

~~g~~ e. Nonconformance.

i. Upon a determination of nonconforming status in any of the ~~either the microbiological or marine Biotoxin component or in both~~ technical

	method components, the laboratory has up to thirty (30) days to demonstrate successful correction of all nonconformities found. After this period, if all critical and key nonconformities have been successfully corrected, the status of the laboratory will be upgraded to conforming for the laboratory component(s) in question. However, if any critical or key nonconformities remain to be successfully corrected, the status of the laboratory for the laboratory component(s) in question will continue to be nonconforming; and as a result, data being generated by the laboratory for this/these laboratory components will continue to be unacceptable for use in support of the NSSP. ii. Upon a determination of nonconformance for the Quality Systems component, the laboratory will have to successfully implement a quality system prior to the onsite technical evaluation. Once all nonconformities are reconciled successfully, a technical evaluation for NSSP methods using the appropriate method specific FDA Shellfish Laboratory Evaluation Checklist will be scheduled with the laboratory. ~~ii~~iii. When a laboratory is found to be nonconforming in either the ~~microbiological or marine Biotoxin~~ technical or quality component or in both components for failure to successfully implement the required corrective action, or for having repeated critical or key nonconformities in consecutive evaluations, the Authority will ensure that an action plan is developed to correct the situation in an acceptable and expeditious manner or discontinue use of the laboratory to support the NSSP. iii. For each laboratory component evaluated, the laboratory will be reevaluated either on-site or through a thorough desk audit as determined by the FDA Shellfish Laboratory Evaluation Officer and the FDA certified State Shellfish Laboratory Evaluation Officer if one is utilized by the State. Only a finding of fully conforming in laboratories whose data has ceased to be acceptable to the NSSP will restore its acceptability for use in the NSSP for the laboratory components in question. **Section IV Guidance Documents Chapter II Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists** The requested action is to adopt the text of the attached checklist for the Quality System of NSSP Laboratories and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.
Public Health Significance	A Quality System is critical to the successful defense of laboratory data. A defensible laboratory quality results in data accuracy, reliability, and minimization of laboratory errors. Laboratory quality assurance operations must be reliable, and quality control well documented. The management of the system is critical to its success to ensure it is maintained. Without oversight and documentation of the steps a laboratory takes to ensure the highest level of laboratory quality management, the data generates is indefensible. Whether the data is challenged in a court of law or during an audit for customer or quality, a Quality System provides a level of assurance upon which data can be relied. Additionally, with time and resources for State and Federal Programs at premium, Quality Systems are an element that can successfully be evaluated remotely and ensure laboratories have

	continued contact with Federal partners. Once quality system essentials are in place, an onsite audit may proceed; thus, resources are conserved and laboratories are fully prepared. NSSP laboratories are producing excellent data and must be as defensible as laboratories held to accreditation standards. Currently, there is no checklist adopted by the ISSC and no standardized evaluation method for the NSSP to determine defensibility of the Quality System adopted by the NSSP. The attached checklist provides the metric by which laboratory evaluation officers will evaluate quality management, quality assurance and quality control elements of NSSP laboratory Quality Systems. The checklist documents whether items are present or not present, noting the labs conformance or nonconformity. If the lab fails to maintain a quality system an onsite evaluation will not be scheduled until such time as the nonconformities are rectified.
Cost Information	There will not be an additional immediate cost as this would be the first step in the routine triennial evaluation cycle.

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Conformity is noted by a (Y), no (N), or not applicable (N/A) for each checklist item. Please note that for all N/A indications, you must document the reason why this requirement is N/A on a separate record. Record comments related to any requirement on the space provided in the summary of nonconformities. All nonconformities must be identified and explained. Quality System must be in place for onsite laboratory evaluation to be scheduled.

Parts of the Quality Checklist	
Part I	Quality Management: Laboratory Operations and Responsibilities for Quality Systems
Part II	Quality Assurance: The Process of Documenting and Maintaining a Quality System
Part III	Quality Control: Documentation for Quality System Defensibility

PART I – Quality Management: Laboratory Operations and Responsibilities for National Shellfish Sanitation Program Laboratory Quality Systems		
		ITEM
Conformance Comments	**Ref**	
		1.1 Components of the Laboratory Quality System
	1,3,6,9	1.1.1 The laboratory has an overall Quality System supported by quality management structure, quality assurance processes and quality control functions.
	1,3,6,9	1.1.2 Management and technical structure exist to support the Quality System.
	1,3,6,9	1.1.3 Quality documentation is required by the laboratory. These include a Quality Assurance (QA) Manual (or otherwise named) and Standard Operating Procedures (SOPs) to support the quality assurance process of the laboratory.
	1, 9	1.1.4 The documents used to implement the quality assurance process and records used to verify quality control (QC) function of the laboratory are reviewed and controlled.
	9	1.1.5 An established process of Quality System assessment and technical proficiency are documented with results retained until the next review.
	9	1.1.6 Resolution, management review and prevention of nonconformities are a documented component of the Quality System.
		1.2 Laboratory Management Structure and Quality Systems
	1,3,6,9	1.2.1 The laboratory's structure is clearly organized with supervisory chain delineated.
	9	1.2.2 The laboratory has ensured that its management and personnel are free from any undue internal and external commercial, financial and other pressures and influences that may adversely affect the quality of their work.
	9	1.2.3 The laboratory has documentation of dedicated personnel with the authority and resources required to carry out their duties, including implementing and maintaining the Quality System of the laboratory.
	1, 9	1.2.4 The laboratory's designated quality personnel ensure adherence to the quality system, including SOPs and QC. These staff have clear documented authority to initiate actions to prevent or minimize departures from quality system and monitor the corrective action process.
	9	1.2.5 The laboratory has documentation of a designated quality system manager, responsible for monitoring all aspects of the quality system to assure defensibility. This person shall have unrestricted access to FDA Shellfish Laboratory Evaluation Officers (LEOs) and the highest levels of the laboratories management. In the case of a single person laboratory, FDA LEOs will assist with developing a monitoring plan.
	1, 9	1.2.6 A documented system is in place to ensure that appropriate review of and communication regarding the elements of the quality system are established among the laboratory staff and laboratory management.
		1.3 Laboratory Personnel and Roles in a Quality System
	1,3, 9	1.3.1 The roles and responsibilities of all personnel are defined in the QA

			manual, read by all staff and the acknowledgments of these responsibilities are retained.
	9	1.3.2	The laboratory policy and the training procedures for personnel are documented and relevant to the scope of the current activities in the laboratory. If the laboratory intends to add methods to their scope, training SOPs must also be added with successful completion by the analyst(s) that will perform the method(s). In the case of a single person laboratory, method proficiency verification must be retained during the life of the methods use in the laboratory.
	9	1.3.3	The laboratory shall maintain a personnel file/ record of any relevant authorization(s), qualifications, trainings, and/or proficiencies for each analyst. This information shall be available upon request as verification of staff training and shall be retained for all staff until two years after they are no longer employed by the laboratory.
	1, 3, 9	1.3.4	The laboratory has documented that all personnel involved in testing have read and understand the applicable SOPs and associated quality documentation and implement the policies and procedures required for the performance of their technical function.
PART II – Quality Assurance: The Process of Documenting and Maintaining a Quality System			
		2.1 Quality Assurance Process: QA Manual, SOPs and Document Control	
	1, 9	2.1.1	The QA manual shall include or make reference to all laboratory SOPs and any supporting procedures, including technical procedures.
	1, 9	2.1.2	SOPs are controlled documents and include detailed, written instructions to achieve uniformity of test methods and quality control procedures, such that items that might affect the quality of the outcome are mitigated.
	1, 9	2.1.3	SOPs and the QA Manual are controlled documents, such that specific individuals are designated within the laboratory with editorial control. These individuals are identified in the QA Manual.
	1, 9	2.1.4	Each time an SOP or the QA manual has changed, the new version will be marked as such and will be distributed to the laboratory with older versions removed from circulation.
	1, 9	2.1.5	Staff training requirements are documented in the QA manual and the training procedure is included.
		2.2 Quality Manual Items	
	1, 9	2.2.1	Quality Assurance Manual contains: □ Table of Contents; □ Organizational chart; □ A description of the Quality System and procedure for implementation and maintenance; □ Policy and procedure for resource management (human resources, competence and training, work environment and safety), description of responsibilities; □ Policy and procedures for rejection criteria; □ Policy and procedures for calibration of equipment and Equipment file items such as maintenance; □ Policy and procedure for traceability and required documentation, □ Policy and procedure for internal audits; □ Policy and Procedure for data analysis and control of nonconforming work; and □ Policy for corrective actions (CAs) and preventative actions (PAs).
	1,3,6,9	2.2.2	The organizational chart clearly depicts laboratory structure with quality and technical personnel listed.

	1, 9	2.2.3	The policy for human resources provisions includes hiring and assignment of staff, competence and responsibilities for positions, and a procedure of training for each technical competence, including proficiencies required.
	1, 3, 4, 6, 9	2.2.4	Policies for work environment and safety protocols, analytical methods, and quality control performed for the National Shellfish Sanitation Program (NSSP) are included or referenced in the QA Manual and shall be provided upon request.
	1, 9	2.2.5	The policy for sample rejection criteria includes what the laboratory will accept and reject based on NSSP requirements and chain of custody.
	1, 3, 4, 6, 9	2.2.6	The laboratory shall have sample acceptance procedures that include safe handling, transport, and storage to prevent contamination or deterioration and to protect the sample integrity. These procedures are provided to customers.
	1, 3, 4, 6, 9	2.2.7	The laboratory has procedures for handling nonconforming samples and who will be contacted in the case of sample rejection.
	1, 9	2.2.8	A policy regarding appropriate equipment file maintenance and retention (e.g., calibration records, maintenance documentation, manuals of operation) is included in the QA Manual.
	1, 9	2.2.9	The SOP for calibration and maintenance of equipment is kept or referenced in the QA Manual and shall be provided upon request.
	1, 9	2.2.10	The SOP for traceability of analytical results is included or referenced in the QA Manual and shall be provided upon request. This traceability procedure includes a documented procedure for the unique identification of samples and the process for chain of custody verification.
	1, 9	2.2.11	The QA Manual has a policy and a procedure for internal quality audits. These audits are planned and scheduled annually or as needed. The policy states auditors do not audit their own work. In the case of a single person laboratory, FDA LEOs will assist with an audit plan.
	1, 9	2.2.12	The QA Manual contains a policy for data analysis to require that all analyses performed have been carried out correctly, documented, controls were used accurately and the results meet specified requirements.
	1, 9	2.2.13	The QA Manual contains a procedure for the control of nonconforming work in the case of : □ identification, documentation, evaluation, segregation (where practical), disposition of nonconforming sample/analyte/result and customer notification; □ assigning responsibility for the review and the authority for disposition of nonconforming sample/analyte/result; □ a nonconforming result correction and the re-verification/calibration of the affected equipment after the correction to demonstrate conformity (if necessary); and □ handling a nonconforming result when it is detected, after delivery to the customer.
	1, 9	2.2.14	The QA manual contains a procedure for preventative actions in which laboratory staff identify potential nonconformities in audit results, quality records, or customer complaints through a review process. Steps are then determined to identify preventive actions to implement. The necessary changes are made to SOPs and this exercise is recorded, and records maintained.
	1, 3, 6, 9	2.2.15	The QA manual has a policy and a procedure for developing

			corrective action(s) to eliminate the cause of identified nonconformities in order to prevent recurrence. Corrective actions describe the nonconformities, define the process for evaluating the need for actions to ensure that nonconformities do not recur (root cause analysis), explain the process to implement the corrective action(s) needed, and the resultant outcome. There is also a procedure to monitor progress of any ongoing corrective actions and the resolution.
	1, 3, 4, 6, 9	2.2.16	The QA Manual contains a policy stating laboratory management shall ensure and document the competence of staff independently operating equipment resulting in a documented measurement, analysis result, quality control value/result, determination of data value for sample result, and review/closure of corrective action for efficacy.
PART III- Quality Control: Documentation for Quality System Defensibility			
		3.1 Documentation	
	1, 9	3.1.1	The laboratory investigates proficiency testing (PT) programs for areas of continual improvement and actively addresses problematic results through the prescribed corrective action process.
	1, 9, 10	3.1.2	The laboratory personnel performing sampling and testing participate in PT programs and exercises when available. If no PT exists, participation in interlaboratory comparisons is considered.
	1, 3, 6, 9, 10	3.1.3	Corrections to quality control records, bench sheets and reports follow the requirements below: □ A single line is drawn through the incorrect information; □ The correct information is written next to the incorrect information; □ The person responsible for the correction initialed the information; □ If not obvious, the reason for correction has been included; and □ If corrections are necessary in an electronic document, old information must be retained in some form, the person making the change must be identified, the date of the change noted, and the reason for the change noted.
	1, 3, 6, 9, 10	3.1.4	All records, required to be retained for two years (or length of time as dictated by State law), shall be legible and shall be stored in such a way that they are readily retrievable to prevent damage or loss.
	1	3.1.5	All records and documents must be written in indelible ink.
		3.2 Method Performance Validation	
	1, 3, 6, 9	3.2.1	The laboratory will internally validate new methods to confirm with objective evidence that the intended protocols are demonstrated and outcomes are fulfilled.
	1, 9	3.2.2	Methodologies do not deviate from the validated method and the laboratory's internal validation shall remain on file in the laboratory.
	1, 3, 6, 9, 10	3.2.3	The laboratory shall report the method chosen in writing to the customer.
	1, 4, 9	3.2.4	Methodologies and protocols are selected based on NSSP requirements and samples are processed as per the citation in the current Model Ordinance.
	1, 4, 9	3.2.5	Methodologies and protocols are selected based on NSSP requirements, and samples are processed as per the citation in the current Guide for the Control of Molluscan Shellfish.
		3.3 Environmental Conditions	
	1, 3, 4, 5, 6, 9, 10	3.3.1	Laboratory facilities for analysis, including lighting and environmental conditions such as temperature and humidity, shall support accurate performance of the tests.
	1, 3, 4,	3.3.2	The laboratory shall monitor, control, and record environmental

	5, 6, 9, 10		conditions as required by the relevant specifications, methods and procedures, or where they influence the outcome of results (e.g., biological sterility, dust, humidity, electrical supply, temperature, vibration).
	1, 3, 4, 6, 9, 10	3.3.3	Laboratory personnel shall stop testing when the environmental conditions jeopardize the results of analyses.
	1, 3, 4, 6, 9, 10	3.3.4	Personnel shall ensure good housekeeping in the laboratory.
		3.4 Equipment	
	1, 3, 4, 6, 9, 10	3.4.1	The laboratory shall have instructions and/ or SOPs on the use and operation of all relevant equipment, and on the handling and preparation of items for testing, where the absence of such could jeopardize the outcome of analysis or influence results.
	1, 9, 10	3.4.2	All equipment in the laboratory is labelled with the manufacturer's name, identification number, and serial number or other unique identification that is traceable.
	1, 9, 10	3.4.3	Equipment files contain reports and certificates of all calibrations, the due date of next calibration, dates and results of any maintenance, adjustments, damage, malfunction, and modification or repair to the equipment.
	1, 9, 10	3.4.4	If equipment (e.g., thermometer, balance) was sent out of the laboratory for service, performance has been verified prior to use again in the laboratory.
		3.5 Temperature Measuring Devices	
	1, 8, 9, 10	3.5.1	Serial number, ice point date (if applicable) and any correction factor is recorded on in use temperature measuring device (TMD).
	1, 8, 9, 10	3.5.2	TMDs are calibrated as per the NSSP requirements and ice points/steam points are performed annually on Standards thermometers.
	1, 8,	3.5.3	TMDs calibration certificates are retained for three consecutive calibration cycles.
	1, 8, 9, 10	3.5.4	Where calibrations give rise to a set of correction factors, the laboratory shall have procedures to ensure these records are retained until the next check is performed.
	1, 8, 9, 10	3.5.5	Range and graduations of all TMDs are appropriate for the designated use. Dial thermometers are not used in the laboratory.
	1, 8, 9, 10	3.5.6	Temperature Monitoring Systems (wired/wireless) must record temperature reading from each sensor/probe in the piece of equipment being monitored at the same or greater frequency and accuracy as stipulated for mercury in glass thermometers, as per manufacturer specifications.
		3.6 Disposables and Pipettors	
	1, 3, 4, 6, 9, 10	3.6.1	Pipettors, accuracy checked, fixed volume or electronic are calibrated according to NSSP requirements.
	1, 3, 10	3.6.2	Pipettors are etched with identification (imprinted serial numbers acceptable) and tagged with last date of accuracy check.
	1, 2, 3, 4, 6, 9, 10	3.6.3	Appropriate pipettor tips are used and sterility checks are performed on an appropriate quantity.
	1, 2, 3, 4, 6, 9, 10	3.6.4	Sterility checks on disposables are performed according to a cited QC practice, within a designated SOP. (e.g., laboratory may cite and implement a recognized standard of sterility testing, they may test 10% of a “lot” or any 3 in a box.)
		3.7 Test Record/Bench Sheet Requirements	
	1, 3, 4,	3.7.1	Test records/bench sheets shall contain information to facilitate

	6, 9, 10		repeatability under conditions as close as possible to the original including QC information (or reference) for media and supplies used.
	1, 9, 10	3.7.2	Test records/bench sheets must show date, time and temperature of samples at the start of analysis and contain the name or initials of the analyst performing the test for each group of samples.
	1, 4, 9, 10	3.7.3	Test records/bench sheets must include sterility controls or a reference to the document containing sterility controls for disposables and dilution buffer.
	1, 4, 9, 10	3.7.4	Test records/bench sheets must include media productivity (positive and negative) controls or a reference to the document containing media productivity controls.

REFERENCES

1. Good Laboratory Practice.
2. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C.
3. Association of Official Analytical Chemists (AOAC). 1991. Quality Assurance Principles for Analytical Laboratories. AOAC, Arlington, VA.
4. Interstate Shellfish Sanitation Conference (ISSC). 2017. ISSC, Columbia, SC.
5. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study*. U.S. Government Printing, Washington, D.C. *Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
6. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
7. U S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
8. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988)U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.
9. The International Organization for Standardization and the International Electrotechnical Commission. Online: https://www.iso.org/obp/ui/#iso:std:iso-iec:17025:ed-2:v1:en accessed June 6, 2017.
10. National Conference on Interstate Milk Shipments. Cultural Procedures, 2400 Form. Online: http://ncims.org/programs/ accessed June 6, 2017.

LABORATORY:			**DATE of EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY of NONCONFORMITIES**			
Page	Item	Observation	Documentation Required

Revised 6-16-2017

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE/POINT OF CONTACT:	

NSSP Quality System Evaluation: (Part I-III)

A. Criteria for Determining Laboratory Status of the Quality System Component:

1. **Laboratory must satisfy all sections of the Quality System prior to onsite evaluation**:

a. The total # of nonconformities in Part I _____

b. The total # of nonconformities in Part II _____

c. The total # of nonconformities in Part III _____

B. Laboratory Status (*circle appropriate*)

Does Not Conform **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All Corrective Actions will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____ so onsite evaluation can be scheduled.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

Revised 6-16-2017

LABORATORY:

Page	Item	Observation

Proposal No. **17-115**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	☐ Growing Area ☒ Harvesting/Handling/Distribution ☐ Administrative

Submitter	J. Michael Hickey Margaret Barette David Fyfe
Affiliation	Massachusetts Division of Marine Fisheries Pacific Coast Shellfish Growers Association NWIFC Treaty Tribes
Address Line 1	1213 Purchase Street 120 State Avenue NE, #142 19472 Powder Hill Place NE, Suite 210
Address Line 2	
City, State, Zip	New Bedford, MA 02740 Olympia, WA 98501 Poulsbo, WA 98370
Phone	508-965-2273 360-754-2744 360-397-6502
Fax	508-990-0449 360-754-2743
Email	Michael.hickey@state.ma.us margaretbarrette@pcsga.org dfyfe@nwifc.org
Proposal Subject	Reconditioning of Recalled Shellfish Implicated in a Norovirus Outbreak
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment & Risk Management @.01 Outbreaks of Shellfish Related Illness.
Text of Proposal/ Requested Action	J. Molluscan shellfish product that is recalled as a result of an illness outbreak associated with *V.v.*, *V.p.*, or Norovirus may be reconditioned. 1. Validated reconditioning processes for *V.v.* and *V.p.* include subjecting product to validated PHPs or placing into approved, conditionally approved, conditionally restricted, or restricted growing areas for an appropriate period of time, not less than fourteen (14) days, with appropriate controls and documentation to be determined by the State Shellfish Control Authority (SSCA). 2. Product associated with a Norovirus outbreak may be reconditioned by returning the product, within three (3) days of the recall, to the growing area from which it was harvested for an appropriate period of time. The period of time shall not be less than twenty-one (21) days. The Authority shall ensure appropriate controls and provide documentation of the activity.
Public Health Significance	A twenty-one (21) day submergence period is consistent with the amount of time required at Section II. Chapter IV. A. (5) (b) (ii) and C. (2) (c) (iii), Shellstock Growing Areas.
Cost Information	No substantial increased cost to SSCAs and to the shellfish industry. would constitute a cost saving

Proposal No. **17-116**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	U.S. Food and Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Sanitary Control of Molluscan Shellfish Harvested From Federal Waters
Specific NSSP Guide Reference	Section I Purposes & Definitions Section II Model Ordinance Chapter IV Shellstock Growing Areas Section II Model Ordinance Chapter VI Shellfish Aquaculture
Text of Proposal/ Requested Action	Insert the following definition for Federal Waters in Section I Purposes & Definitions as follows: **Federal Waters** means the waters that fall outside of State and local jurisdiction but within U.S. sovereignty (typically 3-200 nautical miles offshore). Federal waters include the territorial sea and exclusive economic zone. Insert the language below for Section II Model Ordinance Chapter IV Shellstock Growing Areas @.01 Sanitary Survey. E. Sanitary surveys for Federal waters will be the responsibility of FDA. Sanitary surveys will be conducted in accordance with Chapter IV @.01, as applicable. @.03 Growing Area Classification. F. FDA is responsible for the classification of growing areas in Federal waters. Federal waters are classified as Approved for shellfish harvesting unless such areas are known to be polluted (i.e., microbiological, chemical, and marine biotoxin hazards) and involve commercial shellfish resources . Insert the language below for Section II Model Ordinance Chapter VI Shellfish Aquaculture just after the text in @.03and prior to Shellfish Gardening @.04 Aquaculture in Federal Waters A. Federal Agency Responsibilities. Once the appropriate permits for the construction of the aquaculture facility have been obtained, (1) NOAA is responsible for establishing a contract, in consultation with FDA, with the aquaculture facility describing requirements of the NSSP including (a) the frequency with which NOAA will audit the aquaculture facility and vessels, (b) testing requirements of the aquaculture facility, and (c) the generation of product identification for traceability (i.e., tag numbers); and (2) FDA is responsible for reviewing the aquaculture facility operational

	plan prior to the start of operations, as well as the annual inspection of records, to ensure adherence to NSSP requirements. FDA is also responsible for the classification of the growing area(s) associated with the aquaculture facility. @.~~04~~05 Shellfish Gardening Insert the language below for Section II Model Ordinance Chapter VI Shellfish Aquaculture just after .07 .08 Requirements for the Harvester in Aquaculture in Federal Waters A. Prior to beginning any aquaculture activities, the person who performs aquaculture or operates an aquaculture facility to raise shellfish in Federal waters for human consumption shall obtain the appropriate permission(s) from Federal agencies as described in @.04. B. Operational Plan. Each aquaculture facility shall have a written operational plan as described for Land Based Aquaculture in Section II Chapter VI .05(A). The operational plan shall also include: (1) Description of harvest, tagging, handling, storage, transportation, and landing procedures; (2) Description of a marine biotoxin management and contingency plan (Section II Chapter IV @.04) to include marine biotoxin sampling consistent with Section II Chapter IV @.04(a)(5) and ensure product segregation and control until biotoxin results confirm the shellfish do not contain biotoxins equal to or exceeding criteria established in Section IV Chapter II .08.; (3) Description of a contingency in the event of an emergency situation or condition (e.g., sewage or oil spills); and (4) Procedures for implementing product recalls. C. Each aquaculture facility obtain review from the FDA to ensure adherence to NSSP requirements prior to its implementation. If the aquaculture facility makes changes to the operational plan, they shall obtain a new review from the FDA to ensure adherence to the NSSP requirements.
Public Health Significance	Currently, the NSSP Guide does not explicitly cover requirements for the sanitary control of molluscan shellfish harvested from U.S. Federal waters. The lack of standards for this activity has impeded the harvest of shellfish, notably aquaculture, from Federal waters to date. FDA's policy on the classification of growing areas in offshore Federal waters as described in Verber 1977 was followed in drafting the Proposal. Adding specific language to the Model Ordinance on the appropriate requirements for this activity will facilitate safe and sanitary access to additional shellfish resources.
Cost Information	N/A

Proposal No. **17-117**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	ISSC Male-Specific Coliphage Committee
Affiliation	Interstate Shellfish Sanitation Conference
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Utilizing Male-Specific Coliphage in Growing Areas
Specific NSSP Guide Reference	Section I. Purpose and Definitions Section II. Model Ordinance Chapter IV. Shellstock Growing Area and Chapter V. Shellstock Relaying
Text of Proposal/ Requested Action	**Section I. Purpose and Definitions** Add new definitions: **Wastewater Treatment Plant (WWTP)** means a facility that treats or removes contaminants from sanitary and industrial sewage through a combination of processes to a point where it can be discharged to the environment or reclaimed for other purposes. **Wastewater Collection System** means a collection system which may comprise of sanitary sewer pipes, or a combination of sanitary sewer pipes and stormwater pipes, and pump stations to ensure that disposed wastewater is delivered to the wastewater treatment plant to be treated. **Wastewater Treatment Plant Design Flow** means the flow that the WWTP is designed to discharge over a specified time period (such as hourly, daily, monthly, or annually) and typically expressed as a daily or hourly average with the expectation of meeting permit requirements **Section II. Model Ordinance** **Chapter IV. Shellstock Growing Areas** **@.02 Microbiological Standards.** A. General… B. Water Sample Stations... C. Exceptions... D. Standard for the Approved…. E. Standard for the Approved Classification of Growing Areas Affected By Point Sources. (1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2).

(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml, and not more than ten (10) percent of the samples shall exceed an MPN or MF(mTEC) of:

(a) 43 MPN per 100 ml for a five-tube decimal dilution test; (b) 49 MPN per 100 ml for a three-tube decimal dilution test;

(c) 28 MPN per 100 ml for a twelve-tube single dilution test; or

(d) 31 CFU per 100 ml for a MF (mTEC) test.

(e) For SSCA utilizing MSC data in conjunction with bacteriological data to evaluate waste water system discharge (WWSD) impacts, the MSC level shall not exceed fifty (50) MSC per hundred (100) grams.

(3) Required Sample Collection.

(a) A minimum of five (5) samples shall be collected annually under adverse pollution conditions from each sample station in the growing area.

(b) A minimum of the most recent fifteen (15) samples collected under adverse pollution conditions from each sample station shall be used to calculate the median or geometric mean and percentage to determine compliance with this standard.

(c) Sample station locations shall be adjacent to actual or potential sources of pollution.

F. Standard for the Approved…

G. Standard for the Restricted…

H. Standard for the Restricted…

@.03 Growing Area Classification.

A. General. Each growing area shall be correctly classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, as provided by this Ordinance.

(1) Emergency Conditions..

(2) Classification of All Growing Areas…

(3) Boundaries…

(4) Revision of Classifications...

(5) Status of Growing Areas... The status of a growing area is separate and distinct from its classification and may be open, closed or inactive for the harvesting of shellstock.

(a) Open Status...

(b) Closed Status...

(c) Reopened Status. A growing area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:

(i) The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of contaminant levels in the shellstock to pre-closure levels.

In addressing pathogen concerns, the study may establish criteria for reopening based on coliform levels in the water; or

(ii) For emergency closures of harvest areas caused by the occurrence of raw untreated sewage discharged from a large community sewage collection system or Waste Water System Discharge (WWSD), the analytical sample results shall not exceed the ~~a~~ levels established in Chapter IV @ 02. E ~~of fifty (50) male specific coliphage per 100 grams~~ or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions from shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted or until the event is over and 21 days have passed; or

(iii) The requirements for Biotoxins or conditional area management plans as established in Section .04 and Section .03, respectively, are met; and

(iv) Supporting information is documented by a written record in the central file.

(d) Inactive Status...

(e) Remote Status...

(f) Seasonally Remote/Approved Status…

B. Approved Classification...

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(1) Survey Required…

(2) Management Plan Required. For each growing area, a written management plan shall be developed and shall include:

(a) For management plans based on wastewater treatment plant function, performance standards that include:

(i) Peak effluent flow, average flow, and infiltration flow;

(ii) Microbiological quality of the effluent;

(iii) Physical and chemical quality of the effluent;

(iv) Conditions which cause plant failure;

(v) Plant or collection system bypasses;

(vi) Design, construction, and maintenance to minimize mechanical failure, or overloading;

(vii) Provisions for monitoring and inspecting the waste water treatment plant; and

(viii) Establishment of an area in the prohibited classification adjacent to a wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;

(b) For management plans based on pollution sources other than waste water treatment plants:

(i) Performance standards that reliably predict when criteria for

(ii) Discussion and data supporting the performance standards.

(c) For management plans based on waste water system discharge function or pollution sources other than waste

water system discharge criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum criteria are:

(i) Performance standards of the plan are fully met;

(ii) Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;

(iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. The study may establish criteria for reopening based on coliform levels in the water. The SSCA may utilize MSC levels to establish that sufficient time has elapsed to allow the water quality to return to acceptable levels in growing areas adjacent to waste water system discharge. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. Analytical sample results shall not exceed the MSC levels established in Chapter IV @02 E.~~a level of 50 MSC per 100 grams~~ or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed; and

(iv) Shellstock feeding activity is sufficient to achieve microbial reduction.

(d) For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;

(e) For management systems based on marine Biotoxins, the procedures and criteria that
reliably determine when the growing area may be placed in the open status;

(f) Procedures for immediate notification to the Authority when performance standards or criteria are not met;

(g) Provisions for patrol to prevent illegal harvest; and

(h) Procedures to immediately place the growing area in the closed status in 24 hours or less when the criteria established in the management plan are not met.

(3) Reevaluation of Conditional Classification...

(4) Understanding of and Agreement With…

(5) Conditional Area Types...

(6) Conditionally Approved Classification…

(7) Conditionally Restricted Classification...

D. Restricted Classification…

	E. Prohibited Classification… **Chapter V. Shellstock Relaying** **@.02 Contaminant Reduction.** A. The Authority shall … B. The effectiveness of species-specific contaminant reduction shall be determined based on a study. The study report shall demonstrate that, after the completion of the relay activity: (1) The microbiological quality of each shellfish species is the same microbiological quality as that of the same species already present in the approved or conditionally approved area; or (2) Contaminant levels of poisonous or deleterious substances in shellstock do not exceed FDA tolerance levels; or (3) When the source growing area is impacted by waste water system discharge, the viral quality of each shellfish species meets the male-specific coliphage(MSC) levels established in Chapter IV @02.E. ~~standard of 50 PFU/100 gm~~ or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions. C. The authority may… D. The time period… E. When container relaying… F. The Authority shall…
Public Health Significance	In 2015, the ISSC adopted proposal 15-102 which incorporated the use of Male Specific Coliphage into the NSSP. The ISSC voting delegates directed the development of a guidance document to provide clarification for the use of MSC. In the development of the guidance document, the MSC Committee concluded to changes were needed in Chapter IV for clarification and consistency. The proposed changes do not change the requirements of Chapter IV.
Cost Information	

Proposal No. **17-118**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Thomas Dameron BK Rastogi Chris Shriver
Affiliation	Surfside Foods Atlantic Capes Fisheries LaMonica Fine Foods Bumble Bee Foods
Address Line 1	1733 Main Street
Address Line 2	
City, State, Zip	Port Norris, NJ 08349
Phone	856-785-2115
Fax	856-785-0975
Email	tdameron@surfsidefoods.com brastogi@surfsidefoods.com cshriver@atlanticcapes.com
Proposal Subject	Marine Biotoxin Control / Memorandums of Understanding
Specific NSSP Guide Reference	Section II. Model Ordinance, Chapter IV. Shellstock Growing Areas,@.04 Marine Biotoxin Control A. Contingency Plan (5)
Text of Proposal/ Requested Action	(5) Prior to allowing the landing of shellfish harvested from federal waters closed due to periodic toxic algal blooms associated with PSP, and where routine monitoring of saxitoxin levels is not conducted, the State Authority in the landing State, in cooperation with appropriate Federal agencies, shall develop agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. Any properly permitted shellfish harvester or individual shellfish dealer may request an agreement or memoranda of understanding and the Authority shall provide the requirements for the application for an agreement or memoranda of understanding within 10 business days. The Authority will respond to all applications, originals and resubmittals, for agreements or memoranda of understandings within 30 business days of receipt with either an approval of the application for an agreement or memoranda of understanding or a denial complete with the rational for the denial. The agreements or memoranda of understanding shall provide strict safety assurances. At a minimum agreements or memoranda of understanding shall include provisions for:
Public Health Significance	**The Problem** – State Shellfish Control Authorities are under no obligation to enter agreements with properly permitted, out of state shellfish harvesters within any specific time. An Authorities' refusals to enter discussions or agreements with out of State firms is improperly burdening or discriminating against interstate commerce and has public health ramifications as indicated below. The MOU 225-84-2003 between the FDA and ISSC states, "The purpose of the ISSC is to provide a formal structure wherein State regulatory authorities can establish updated guidelines, and *procedures for the uniform application of those guidelines*, for sanitary control of the shellfish industry." The use of timeframes where agreements or memoranda of understanding must move forward will provide regulatory uniformity and cooperation for **all harvesters or individual shellfish**

	dealers wanting to land shellfish harvested from the open portion of Georges Bank. Significant amounts of time and energy is being needlessly wasted when an Authority can wait indefinitely to respond to requests. This proposed update to the Model Ordinance will streamline an unnecessarily burdensome requirement and allow industry to work in as efficient a manner as possible, to maintain product quality and protect public health. **Public Health Significance** – The current NSSP Guidelines allow the indefinite delay of an agreement. This prohibits organizations from offloading shellfish in the closest port to the open portion of Georges Bank, when a state doesn't respond to requests for agreements. As an example – a Surfside Foods harvest vessel has been seeking an Agreement with Massachusetts for 14 months. The harvest vessel will experience an additional 13 hours of travel to New Jersey, a State where a written Agreement had been established in a timely manner, to harvest from Georges Bank. Additional travel time by the harvest vessel increases the time until the shellfish are under continuous cooling and it adds to the degradation of the product and the bacterial load.
Cost Information	As an example: the cost to Surfside Foods, LLC due to the refusal of the Massachusetts SSCA to act on our request for an agreement or memoranda of understanding has been significant. We submitted all documentation requested to the MA SSCA more than 13 months prior to this proposal submittal and we have yet to receive a response to our request, in the affirmative or negative. Since then we have submitted additional requests, one more than two months prior to this writing by certified mail and have gotten no response. We have secured dockage and then lost it to other vessels because we were not able to utilize it. We have missed a full season fishing Georges Bank and it appears we will miss another one.

Proposal No. **17-119**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	U.S. Food and Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Update the Control of Marine Biotoxins in Federal Waters
Specific NSSP Guide Reference	Section II Model Ordinance Chapter IV Shellstock Growing Areas @.04 Marine Biotoxin Control A(5) Section IV Guidance Documents Chapter II Growing Areas .06 Protocol for the Landing of Shellfish from Federally Closed Waters Due to PSP
Text of Proposal/ Requested Action	Update the language as indicated below for Section II Model Ordinance Chapter IV Shellstock Growing Areas @.04 Marine Biotoxin Control A. Contingency Plan (5) Prior to allowing the landing of shellfish harvested from ~~f~~Federal waters ~~closed due to periodic toxic algal blooms associated with PSP, and~~ where routine monitoring of ~~saxi~~toxin levels is not conducted, in addition to following all other requirements in the Model Ordinance, the State Authority in the landing State, in cooperation with appropriate Federal agencies, shall develop agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. The agreements or memoranda of understanding shall provide strict safety assurances. At a minimum agreements or memoranda of understanding shall include provisions for: (a) Harvest permit requirements. (b) Training for individuals conducting onboard toxicity screening using NSSP methods. (c) Vessel monitoring; (d) Identification of shellfish for each harvesting trip to include: (i) Vessel name and owner (ii) Captain's name (iii) Person conducting onboard screening tests (iv) Port of departure name and date (v) Port of landing name and date (vi) Latitude and longitude coordinates of designated harvest area (vii) Onboard screening test results (viii)Volume and species of shellfish harvested (ix) Intended processing facility name, address and certification number (x) Captain's signature and date (e) Pre-harvested (onboard) sampling that includes a minimum of five (5) samples from the intended harvest area be tested for

~~saxi~~toxins that are likely to be present. Harvesting shall not be permitted if any of the pre-harvested samples contain ~~saxi~~toxin levels in excess of half of the established criteria listen in Chapter IV @.04©(1) (e.g., 44 µg/l00 g when using a quantitative test or a positive at a limit of detection of 40 µg/100 g for the qualitative screening test for PSP toxins).

(f) Submittal of onboard screening homogenates and test results to the authority in the state of landing.

(g) The collection ~~and saxitoxin level testing~~ of a minimum of seven (7) dockside samples by the SSCA or designee and the testing of those samples for toxins using an NSSP method by an NSSP conforming Laboratory.

The SSCA may require more samples based on the size of the vessel and the volume of shellfish harvested.

(h) Holding and providing separation until dockside samples verify that ~~saxi~~toxin levels are below the established criteria (e.g., 80 µg/100 g for PSP toxins).

(i) Disposal of shellfish when ~~should~~ dockside test results meet or exceed the established criteria in Chapter IV@.04(c)(1) (e.g., 2 mg domoic acid ~~80 µg~~ /100 g for ASP toxins.

(j) Notification prior to unloading.

(k) Unloading Schedule.

(l) Access for Dockside Sampling.

(m) Record Keeping.

(n) Early Warning/Alert System.

NOTE: The plan may include other requirements, as deemed necessary by the authority in the state of landing, to ensure adequate public health protection under the NSSP.

Update the language as indicated below for Section IV Guidance Documents Chapter II Growing Areas
.06 Protocol for the Landing of Shellfish from Federal~~ly Closed~~ Waters ~~Due to PSP~~

~~When the h~~Harvest of molluscan shellfish ~~is closed~~ in Federal Waters not routinely monitored for toxins in shellfish (such as the Federal waters on Georges Bank closed due to ~~Paralytic Shellfish Poison (~~PSP~~)~~ risks)~~, exceptions to the prohibitions~~ may be authorized provided the Authority in the State of landing in cooperation with appropriate Federal agencies shall develop agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. The~~is~~ following guidance provides descriptions of the specific information to be included in the protocol.

A. Harvest Permit Requirements

~~The Authority in the landing state will only allow the landing of shellfish~~ If harvesting from ~~f~~Federal waters closed due to ~~PSP~~ toxins, the Authority in the landing state will only allow the landing of shellfish from vessels in possession of an appropriate Exempted Fishing Permit (EFP) issued by the National Marine Fisheries Service (NMFS) by vessels participating in the Federal Vessel Monitoring Systems (VMS). The NMFS shall receive concurrence from the SSCA in the State of landing. Vessels operating in open

Federal waters will also need applicable permits.

B. Training

The Authority shall ensure that all shipboard persons conducting onboard ~~sampling~~ testing have been trained by a U.S. Food and Durg Administration (FDA) ~~National Shellfish Sanitation Program (NSSP)~~ Laboratory Evaluation Officer (LEO) or an ~~US Food and Drug Administration (~~FDA~~)~~ marine ~~B~~biotoxin expert to conduct onboard ~~PSP~~ toxin screening using an NSSP recognized method(s). Shipboard persons conducting onboard toxin testing must receive refresher training every 3 years. A designee of the FDA LEO or FDA marine biotoxin expert may be appointed in writing to provide the training and/or refresher training.

C. Vessel Monitoring

The Authority shall ~~ensure that~~ monitor the harvesting location(s) of each landing vessel. ~~has been appropriately monitored. This requirement may be met by the vessel participating in the Federal Vessel Monitoring System (VMS).~~

D. Identification of Shellfish

Prior to landing, each vessel Captain or Mate shall provide the Authority with a Harvest Record, which may be electronic provided that it is made available to the authorized individual at dockside, for each harvesting trip ~~record~~ identifying each lot of shellfish as follows: ~~For each harvesting trip the Captain or Mate shall record the following information on a "Harvest Record." Electronic logging of this information may be permitted provided it is made available to the authorized individual at dockside~~

1. Vessel name and Federal Fishing Permit number
2. Name and telephone number of the vessel Captain and vessel owner
3. Date(s) of harvest
4. Number of lots and volume of catch per lot or number of containers per lot
5. Location(s) of harvest (GPS coordinates or latitude/longitude coordinates in degrees:minutes:seconds)
6. Identification of each harvest lot, including cage tag numbers for surf clams and ocean quahogs, and container numbers or identification codes for other shellfish species
7. Location (GPS coordinates or latitude/longitude coordinates in degrees: minutes: seconds) of each ~~PSP~~ toxin screening sample
8. Results of each ~~PSP~~ toxin screening test
9. Destination(s) and purchaser(s) of each lot and amount of each lot to each destination

The Captain or Mate shall sign the "Harvest Record." The "Harvest

Record" shall be checked by the individual authorized to sample the harvested shellfish. Failure to provide complete and accurate information will result in revocation or suspension of the NMFS EFP and rejection of the entire lot(s) of harvested shellfish. Four (4) copies of the "Harvest Record" shall be prepared. One (1) copy shall remain with the vessel, one (1) copy shall be provided to the SSCA in the state of landing, one (1) copy shall accompany the catch to the processing firm(s), and one (1) copy shall be retained by the laboratory authorized to conduct lot sample analyses.

Container Labeling:

Each container of shellfish shall be clearly labeled (indelible and legible) with the following NSSP required information at the time of harvest:

1. For sSurf clams and ocean quahogs existing NMFS tagging requirements.
2. For aAll other molluscan shellfish (including Stimpson clams also known as Arctic surf clams) using durable, waterproof, Authority sanctioned prior to use Tyvek tags:
   a. Vessel name;
   b. Type and quantity of shellfish;
   c. Date of harvest; and
   d. Harvest lot area defined by GPS coordinates or latitude/longitude coordinates in degrees:minutes:seconds.

E. Pre-Harvest Sampling

Prior to commercial harvesting of molluscan shellfish, a minimum of five (5) screening samples shall be collected within each area of intended harvest (lot area) and tested for PSP marine biotoxins that are likely to occur in accordance with an NSSP recognized screening method. Each screening sample shall be collected during a separate and distinct gear tow. Screening sample tows shall be conducted in a manner that evenly distributes the five (5) samples throughout the intended harvest area for each area of intended harvest (see Section H.). Only shipboard officials trained by an FDA LEI or FDA marine biotoxin expert (or their designee as expressly indicated in writing)in the use of the designated NSSP screening method may conduct these tests. Each of the five (5) samples must test negative for PSP toxins (i.e., below half of the established criteria in Chpater IV). A positive result from any one (1) sample shall render the "lot area" unacceptable for harvest. The harvest vessel eCaptain shall immediately report all positive screening test results, by telephone or email, to the SSCA within the intended state of landing, the FDA Shellfish Specialist, and the processor NMFS. The FDA shall notify the NMFS. The NMFS shall notify permitted harvesters to advise them to cease fishing in the affected area (s). The Captain should also notify other permitted harvest vessels of the positive screening test and advise them to avoid the questionable area.

For each screening test, whether positive and or negative, the remaining sample material (homogenate) shall be maintained under refrigeration for

later use should the SSCA in the State of landing request confirmatory testing using an NSSP recognized ~~test~~ method.

Each screening sample shall be comprised of at least twelve (12) whole animals with the exception of mussels and "whole" or "roe-on" scallops. For mussels each sample shall be comprised of thirty (30) animals. For "whole" scallops each sample shall be comprised of twenty (20) scallop viscera and gonads. For "roe-on" scallops each sample shall be comprised of twenty (20) scallop gonads.

F. Submittal of Onboard Screening Homogenates and Test Results

All screening results shall be recorded on the ~~"~~Harvest Record~~"~~ as stipulated in Section D. of this Protocol. Upon landing of the harvest vessel, the ~~"~~Harvest Record~~"~~ and screening homogenates shall be provided to the SSCA or designee and the testing of those samples for toxins using an NSSP method by an NSSP conforming laboratory ~~authority~~ in the State of landing ~~authorized to sample the harvested shellfish~~ as described in Section G. of this Protocol.

G. Dockside Sampling

After dockside samples are collected by the SSCA or designee, molluscan shellfish may be processed while awaiting ~~PSP analytical~~ toxin results. Each lot must be identified and segregated during storage while awaiting dockside sample test results. Under no circumstances will product be released from the processor prior to receiving satisfactory ~~paralytic shellfish~~ toxin ~~test~~ results that demonstrate that toxin levels are below the established criteria in Chapter IV@.04(c)(1).

The dockside sampling protocol for molluscan shellfish shall be as follows:

1. For each lot of molluscan shellfish, a minimum of seven (7) composite samples, each comprised of at least twelve (12) whole animals, shall be taken at random by the individual authorized by the SSCA to sample, with the following exceptions:
   a. For each lot of mussels, a minimum of seven (7) composite samples, each comprised of at least thirty (30) whole animals, shall be taken at random by the individual authorized to sample.
   b. For each lot of "whole" scallops, a minimum of seven (7) composite samples, each comprised of twenty (20) scallop viscera and gonads, shall be taken at random by the individual authorized to sample.
   c. For each lot of "roe-on" scallops, a minimum of seven (7) composite samples, each comprised of twenty (20) scallop gonads, shall be taken at random by the individual authorized to sample.
2. Shellfish samples collected in accordance with G.1 shall be tested for the presence of ~~paralytic shellfish~~ toxins using an NSSP recognized methods~~.~~
3. Laboratory test results for each lot of shellfish shall be forwarded to

the SSCA in the state in which the shellfish is being held prior to the product being released by the SSCA in the state of landing, or if processed in another state, the SSCA in the state of processing.

H. Holding and Lot Separation

A harvest lot is defined as all molluscan shellfish harvested during a single period of uninterrupted harvest activity within a geographic area not to exceed three (3) square miles. Once harvesting has ceased and the harvest vessel moves to another location, regardless of the distance, a new harvest lot will be established. Any harvest vessel containing more than one lot shall clearly mark and segregate each lot while at sea, during off loading, and during transportation to a processing facility. Prior to harvesting in Federal waters, each harvest vessel shall submit to the NMFS a written onboard lot segregation plan. The SSCA in the intended state of landing and the FDA ~~Regional~~ Shellfish Specialist must approve the proposed lot segregation plan.

I. Disposal of Shellfish

If test results of any one (1) of the seven (7) samples collected in accordance with G.1 equal or exceed the established criteria in Chapter IV@.04(c)(1) (e.g., 80 µg ~~of paralytic shellfish toxins~~/100 g for PSP toxins) ~~of shellfish tissue~~ (n=7, c=0), the entire lot must be discarded or destroyed at the cost of the harvester under the supervision of the SSCA in accordance with state laws and regulations except when:

A lot of "whole" or "roe-on" scallops equals or exceeds the established criteria in Chapter IV @.04©(1) ~~80 µg paralytic shellfish toxins/100 g of tissue~~, the adductor muscle may be shucked from the viscera and/or gonad and marketed. The remaining materials (viscera and/or gonad) must be discarded or destroyed under supervision of the SSCA in accordance with state laws and regulations.

Dockside toxin testing ~~Confirmatory PSP analyses~~ shall be according to NSSP recognized methods and shall be conducted by laboratories ~~certified~~ evaluated in accordance with NSSP guidelines. Private laboratories may be used if ~~certified~~ evaluated by an ~~Federal or state shellfish Laboratory Evaluation Officer (~~LEO~~)~~ in accordance with NSSP guidelines.

J. Notification Prior to Unloading

Prior to the issuance of an EFP, the harvester shall be responsible for notifying the SSCA in the state of landing and in a manner approved by the SSCA that molluscan shellfish is being harvested for delivery to the intended receiving processor.

Each vessel shall give at least twelve (12) hours' notice to the individual authorized to sample prior to unloading shellfish. Notice of less than twelve (12) hours may be approved by the authorized

individual at his/her discretion. SSCAs may ~~approve industry~~ appoint a designee in writing for sampling and sample transport to the NSSP certified testing laboratory in accordance with the practices and procedures used by the SSCA under the NSSP. The procedures, as well as training and certification records, must be available for evaluation. ~~Such procedures may be approved by the SSCA only when sample collection and sample transport training is provided by the SSCA.~~

Shellfish from a ~~federally closed~~ Federal water harvest area(s) must be kept separate and not sold until so authorized by the SSCA in the state of landing or, if processed in another state, the SSCA in the state of processing.

Failure to comply with the provisions of this Protocol will result in the suspension or revocation of the vessel's ~~EFP~~ permits through the NMFS.

K. Unloading Schedule

Unloading shall take place between 7:00 A.M. and 5:00 P.M. Monday through Friday, unless otherwise mutually agreed upon by the individual authorized to sample, the processing plant manager, the harvest vessel captain, and the SSCA in the state of landing~~, sample testing, and processing~~.

L. Access for Dockside Sampling

Individuals authorized to sample shall be provided access to the catch of shellfish.

M. Record Keeping

Record keeping requirements shall be as follows:

1. The vessel shall maintain Harvest Records for at least one (1) year.
2. The processor(s) shall maintain Harvest Records for at least one (1) year or two (2) years if the product is frozen.
3. The SSCA in the State of landing shall retain Harvest Records for at least two (2) years.

N. Early Warning/Alert System

~~PSP sample~~ Toxin data acquired as a result of onboard screening and dockside testing shall be transmitted to ~~a central data register to be maintained by~~ the FDA. These data, both screening and ~~confirmatory~~dockside, shall be transmitted to the FDA by the NSSP certified laboratory conducting ~~PSP analyses~~ toxin testing of the sampled lot(s) within one (1) week of the completion of the ~~PSP~~ toxin analyses. The data provided shall include the following:

1. Shellfish species;
2. Harvest location name and coordinates (GPS or latitude/longitude);

	3. Harvest date; 4. Onboard screening test method, date, and results; and 5. Laboratory test date, test method, and test results for dockside samples. Results of all samples having acceptable levels of ~~paralytic shellfish~~ toxins (e.g., <80 µg/100 g for PSP toxins) shall immediately be reported to the SSCA in the state of landing. If the results of any one (1) sample equal or exceed the estabished criteria in Chapter IV @.04(c)(1) ~~80 µg/100 g~~ the testing laboratory shall immediately notify the FDA ~~Regional~~ Shellfish Specialist, the SSCA, and the processor by telephone. The FDA shall notify the NMFS. The NMFS shall notify permitted harvesters to advise them to cease fishing in the affected area(s). NOTE: Due to the resources necessary to meet the requirements of this Protocol, State Shellfish Control Authorities (SSCAs) may find it necessary to require industry to fund associated costs. These costs may include sample collection, screening, transportation, analysis, inspection, enforcement, and other related expenses.
Public Health Significance	The protocol adopted by the ISSC in 2011 to allow the harvest of surf clams and ocean quahogs from Federal waters closed due to the risk of paralytic shellfish poisoning (PSP) toxins has granted access to valuable shellfish resources with measures in place to protect public health. While the protocol, referred to as onboard screening dockside testing, was designed for surf clam and ocean quahog harvests on Georges Bank, its success has demonstrated its applicability to other Federal waters where routine monitoring for marine biotoxins is not feasible. The goal of this proposal and the requested updates to the language in the Model Ordinance and Guidance Documents is to broaden the application of this successful protocol to other regions and for other toxins as they emerge into the regions of interest, thereby safely expanding access to shellfish resources in Federal waters.
Cost Information	N/A

Proposal No. **17-120**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Paul D. Golden
Affiliation	PacRim
Address Line 1	375 Hudson Street
Address Line 2	
City, State, Zip	Port Townsend, WA 98368
Phone	360-302-3030 ext 306
Fax	n./a
Email	paul.golden@dfw.wa.gov
Proposal Subject	Risk Category Reductions for Monitoring and Control of Surveillance Activities
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VIII. Control of Shellfish Harvesting, @.01 Control of Shellstock Growing Areas, B. Patrol of Growing Areas (4)(e)
Text of Proposal/ Requested Action	(e) The following criteria should be used to adjust the rating, if warranted: (i) If a community-policing program is in place, the subtotal may be reduced by up to 0.25 points. If such a program leads to frequent citations, the subtotal may be reduced by up to 0.5 points. Community policing may include but is not limited to telephone hot lines, out-reach programs, financial incentives, local law enforcement activities not covered by B. (5), or private security arrangements. (ii) If specialized equipment is available to the patrol agency, the subtotal may be reduced by up to 0.40 points. The actual reduction should be dependent upon the type of equipment that is available and its frequency of use. For example, frequent use of an aircraft can warrant a 0.4 point reduction, and frequent use of night vision or periodic use of aircraft can warrant a 0.2 point reduction. (iii) If the patrol agency implements a strategy for comprehensive monitoring and control of surveillance activities, the subtotal may be reduced by up to 1 point. Activities include airport, dock, border, truck, wholesale and retail inspections. The actual reduction should be dependent on the frequency and extent of the activities. ~~(iii)~~(iv) If a growing area is conditionally managed or is poorly marked, the subtotal may be increased by up to 0.2 point. Adding or subtracting the appropriate adjustment(s) calculates the total score.
Public Health Significance	Agencies with units responsible for patrol activities vary throughout the country with respect to their statutory authority and primary mission. While some agencies operations are primarily limited to surveillance of growing areas, others extend beyond the harvest area to include shippers and additional receivers and buyers. Patrol agencies that implement broad monitoring, control, and surveillance strategies monitor variations in fishing effort, control harvest and sales through regulatory restrictions, and conduct surveillance and enforcement activities through the various stages of seafood transfer. Agencies with units responsible for patrol activity that conduct inspections and investigations of seafood both on the harvest grounds and beyond have opportunities to intercept illegal product at chokepoints where seafood is transferred, processed, shipped, and sold. Additionally, health authorities and natural resource agencies throughout the country are more frequently facing expanding responsibilities and competing priorities, while at the same time they are facing shrinking budgets and funding that is earmarked for narrowly defined activities. Agency managers and officers must prioritize their limited resources to make the most impact to deter illegal harvest. Widespread presence in the seafood harvest and supply chain protects seafood consumers and legitimate seafood businesses.

Cost Information	none

Proposal No. **17-121**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Disposal of Human Sewage and Bodily Fluids
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VIII. Control of Shellfish Harvesting Requirements for Harvesters .02 Shellstock Harvesting and Handling. Section II. Model Ordinance Chapter IX. Transportation Requirements for Harvesters .01 Conveyances Used to Transport Shellstock to the Original Dealer and .02 Conveyances Used to Transport Shellstock from Dealer to Dealer
Text of Proposal/ Requested Action	Chapter VIII. .02 Shellstock Harvesting and Handling D. Disposal of Human Sewage and Bodily Fluids~~from Vessels~~. (1) Human sewage and bodily fluids shall not be discharged overboard from ~~a~~any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. (3) Portable toilets shall: (a) Be used only for the purpose intended; (b) Be secured while on board and located to prevent contamination of shellstock by spillage or leakage; (c) Be emptied only into a sewage disposal system; (d) Be cleaned before being returned to the vehicle or vessel~~boat~~; and (e) Not be cleaned in equipment used for washing or processing food. (4) Use of other receptacles for sewage disposal may be approved by the Authority if the receptacles are: (a) Constructed of impervious, cleanable materials and have tight fitting lids; (b) Indelibly labeled “Human Waste” in contrasting letters at least three (3) inches in height; and (c) Meet the requirements in Section D. (3). Chapter IX. .01 Conveyances Used to Transport Shellstock to the Original Dealer G. Disposal of Human Sewage and Bodily Fluids (1) Human sewage and bodily fluids shall not be discharged overboard from any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle

	shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. Portable toilets shall meet the requirements of VIII. .02. D. (3). Chapter IX. 02 Conveyances Used to Transport Shellstock from Dealer to Dealer C. Disposal of Human Sewage and Bodily Fluids (1) Human sewage and bodily fluids shall not be discharged overboard from any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. Portable toilets shall meet the requirements of VIII. .02. D. (3).
Public Health Significance	During evaluations, harvesters and certified dealers buying trucks are observed within harvesting areas and aquaculture lease site areas. The vehicles are often there for hours while harvesting, husbandry, and purchasing activities are taking place. In many areas, there are no nearby toilet facilities to accommodate emergency (or non-emergency) needs for toilet facilities to accept human digestive waste or vomit, putting the area at risk of foodborne illness, e.g. norovirus, hepatitis A, etc. The requirement for marine sanitation devices should not only pertain to vessels in order to protect the public health.
Cost Information	~$5.00 for a five (5) gallon bucket with a lid.

Proposal No. **17-122**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	ISSC Executive Office
Affiliation	Interstate Shellfish Sanitation Conference
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Marine Biotoxin Control
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment and Risk Management @.01 A. Chapter IV. Shellstock Growing Area @.04
Text of Proposal/ Requested Action	**Section II. Model Ordinance** **Chapter II. Risk Assessment and Risk Management** **@.01 Outbreaks of Shellfish-Related Illness.** A. When shellfish are implicated in an illness outbreak involving two (2) or more persons not from the same household (or one or more persons in the case of ~~paralytic shellfish~~shellfish toxicity poisoning associated with marine biotoxins ~~[PSP])~~, the Authority shall determine whether an epidemiological association exists between the illness and the shellfish consumption by reviewing: (1) Each consumer's food history; (2) Shellfish handling practices by the consumer and/or retailer; (3) Whether the disease has the potential or is known to be transmitted by shellfish; and (4) Whether the symptoms and incubation period of the illnesses are consistent with the suspected etiologic agent. **Chapter IV. Shellstock Growing Areas Management** **@.04 Marine Biotoxin Control.** A. Contingency Plan. (1) The Authority shall develop and adopt a marine Biotoxin contingency plan for all marine and estuarine shellfish growing areas addressing the management of PSP, ASP, NSP, DSP and AZP in the event of the emergence of a toxin-producing phytoplankton that has not historically occurred or an illness outbreak caused by marine biotoxins. (2) The plan shall define the administrative procedures and resources necessary to accomplish the following: (a) Initiate an emergency shellfish sampling and assay program; (b) Close growing areas and embargo shellfish; (c) Prevent harvesting of contaminated species; (d) Provide for product recall;

(e) Disseminate information on the occurrences of toxic algal blooms and/or toxicity in shellfish meats to adjacent states, shellfish industry, and local health agencies; ~~and~~

(f) Coordinate control actions taken by Authorities and federal agencies; and~~.~~

(g) Establish reopening criteria including the number of samples over what period of time.

~~(3) Except that the Authority shall classify as prohibited any growing areas where shellfish are so highly or frequently affected by marine Biotoxins that the situation cannot be safety managed, the presence of marine Biotoxins shall not affect the classification of the shellfish growing area under Section @ .03. The Authority may use the conditionally approved classification for areas affected by marine Biotoxins.~~

~~(4) The plan may include agreements or memoranda of understanding, between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting in designated parts of a State growing area while other parts of the same growing area are placed in the closed status. Such controlled harvesting shall be conducted with strict assurances of safety. In State growing areas or designated portions of State growing waters that are closed, the Authority may allow for harvesting if an end product testing program is developed and samples of each lot are tested and found to be below the action levels specified in Section C. The program must include at a minimum:~~

~~(a) Establishment of appropriate pre-harvest screening levels;~~
~~(b) Establishment of appropriate screening and end product testing methods;~~
~~(c) Establishment of appropriate laboratories/analysts to conduct screening and end product testing methods;~~
~~(d) Establishment of representative sampling plan for both (a) and (b) above; and~~
~~(e) Other controls as necessary to ensure that shellstock are not released prior to meeting all requirements of the program.~~

~~(5) Prior to allowing the landing of shellfish harvested from federal waters closed due to periodic toxic algal blooms associated with PSP, and where routine monitoring of saxitoxin levels is not conducted, the State Authority in the landing State, in cooperation with appropriate Federal agencies, shall develop agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. The agreements or memoranda of understanding shall provide strict safety assurances. At a minimum agreements or memoranda of understanding shall include provisions for:~~

~~(a) Harvest permit requirements.~~
~~(b) Training for individuals conducting onboard toxicity screening using NSSP methods.~~
~~(c) Vessel monitoring;~~
~~(d) Identification of shellfish for each harvesting trip to include:~~

~~(i) Vessel name and owner~~
~~(ii) Captain's name~~
~~(iii) Person conducting onboard screening tests~~
~~(iv) Port of departure name and date~~
~~(v) Port of landing name and date~~
~~(vi) Latitude and longitude coordinates of designated harvest area~~
~~(vii) Onboard screening test results~~
~~(viii)Volume and species of shellfish harvested~~
~~(ix) Intended processing facility name, address and certification number~~
~~(x) Captain's signature and date~~
~~(e) Pre-harvested (onboard) sampling that includes a minimum of five (5) samples from the intended harvest area be tested for saxitoxins. Harvesting shall not be permitted if any of the pre-harvested samples contain saxitoxin levels in excess of 44 µg/100 g when using a quantitative test or a positive at a limit of detection of 40 µg/100 g for the qualitative screening test.~~
~~(f) Submittal of onboard screening homogenates and test results to the authority in the state of landing.~~
~~(g) The collection and saxitoxin level testing of a minimum of seven (7) dockside samples.~~
~~The SSCA may require more samples based on the size of the vessel and the volume of shellfish harvested.~~
~~(h) Holding and providing separation until dockside samples verify that saxitoxin levels are below 80 µg/100 g.~~
~~(i) Disposal of shellfish should dockside test results exceed 80 µg /100 g.~~
~~(j) Notification prior to unloading.~~
~~(k) Unloading schedule.~~
~~(l) Access for Dockside Sampling. (m) Record Keeping.~~
~~(n) Early Warning/Alert System.~~

NOTE: The plan may include other requirements, as deemed necessary by the authority in the state of landing, to ensure adequate public health protection under the NSSP.

B. Marine Biotoxin ~~Monitoring~~Management Plan .

In those areas that have been implicated in an illness outbreak or where toxin-producing~~forming~~ phytoplankton ~~organisms~~ are known to occur ~~periodically~~ and the toxins are prone to accumulate in shellfish, and when appropriate at those times when marine ~~B~~biotoxins can be reasonably predicted to occur, representative samples of the water may be collected and~~/or~~ shellfish shall be collected during harvest periods. The samples shall be collected from indicator stations at intervals determined by the Authority. Water samples ~~will~~may be assayed for the presence of toxin-producing~~forming organisms~~ phytoplankton and shellfish meat samples shall be assayed for the presence of toxins.

(1) The Authority shall develop and adopt a marine biotoxin management plan for all marine and estuarine shellfish growing areas if there is a history of biotoxin closures related to PSP, ASP, NSP, DSP, or AZP; if toxin-producing phytoplankton are known to occur in the growing area; or a reasonable likelihood that biotoxin closures could occur.

(2) The plan shall define the administrative procedures and resources necessary to accomplish the following:

(a) Maintain a routine shellfish sampling and assay program including;

i. Establishment of appropriate shellfish screening levels;

ii. Establishment of appropriate shellfish screening and testing methods;

iii. Establishment of appropriate laboratories/analysts to conduct shellfish screening and testing methods;

iv. Establishment of a sampling plan for both (i) and (ii) above; and

v. Other controls as necessary to ensure that shellstock are not harvested when levels of marine biotoxins meet or exceed the established criteria in Section C.

(b) Close growing areas and embargo shellfish;

(c) Prevent harvesting of contaminated species;

(d) Provide for product recall;

(e) Disseminate information on the occurrences of toxic algal blooms and/or toxicity in shellfish meats to adjacent states, shellfish industry, and local health agencies;

(f) Coordinate control actions taken by Authorities and federal agencies; and

(g) Establish reopening criteria.

(3) The Authority may use precautionary closures based on screening or water sample results as defined in their marine biotoxin management program. Precautionary closures may be lifted immediately if confirmatory testing using an approved method shows toxin-producing phytoplankton in the growing waters and/or the level of biotoxin present in shellfish meats are not equal to or above established criteria in Section C.

(4) Except that the Authority shall classify as prohibited any growing areas where shellfish are so highly or frequently affected by marine biotoxins or so remote that adequate sampling cannot be achieved and thus the situation cannot be safety managed, the presence of marine biotoxins shall not affect the classification of the shellfish growing area under Section @ .03. The Authority may use the conditionally approved classification for areas affected by marine biotoxins.

(5) The plan may include agreements or memoranda of understanding, between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting in designated parts of a State growing area while other parts of the

same growing area are placed in the closed status. Such controlled harvesting shall be conducted with strict assurances of safety. In State growing areas or designated portions of State growing waters that are closed, the Authority may allow for harvesting if an end product testing program is developed and samples of each lot are tested and found to be below the action levels specified in Section C. The program must include at a minimum:

(a) Establishment of appropriate pre-harvest screening levels;
(b) Establishment of appropriate screening and end product testing methods;
(c) Establishment of appropriate laboratories/analysts to conduct screening and end product testing methods;
(d) Establishment of representative sampling plan for both (a) and (b) above;
(e) Disposal of shellfish should end product test results meet or exceed established criteria specified in Section C.
(f) Other controls as necessary to ensure that shellstock are not released prior to meeting all requirements of the program.

(6) Prior to allowing the landing of shellfish harvested from federal waters closed due to periodic toxic algal blooms associated with PSP, and where routine monitoring of saxitoxin levels is not conducted, the State Authority in the landing State, in cooperation with appropriate Federal agencies, shall develop agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers. The agreements or memoranda of understanding shall provide strict safety assurances. At a minimum agreements or memoranda of understanding shall include provisions for:

(a) Harvest permit requirements.
(b) Training for individuals conducting onboard toxicity screening using NSSP methods.
(c) Vessel monitoring;
(d) Identification of shellfish for each harvesting trip to include:
(i) Vessel name and owner
(ii) Captain's name
(iii) Person conducting onboard screening tests
(iv) Port of departure name and date
(v) Port of landing name and date
(vi) Latitude and longitude coordinates of designated harvest area
(vii) Onboard screening test results
(viii)Volume and species of shellfish harvested
(ix) Intended processing facility name, address and certification number
(x) Captain's signature and date
(e) Pre-harvested (onboard) sampling that includes a minimum of five (5) samples from the intended harvest area be tested for saxitoxins. Harvesting shall not be permitted if any of the pre-harvested samples contain saxitoxin levels in excess of 44 µg/l00 g when using a quantitative test or a positive at a limit of detection of 40 µg/100 g for the qualitative screening test.

(f) Submittal of onboard screening homogenates and test results to the authority in the state of landing.
(g) The collection and saxitoxin level testing of a minimum of seven (7) dockside samples.
The SSCA may require more samples based on the size of the vessel and the volume of shellfish harvested.
(h) Holding and providing separation until dockside samples verify that saxitoxin levels are below 80 µg/100 g.
(i) Disposal of shellfish should dockside test results exceed 80 µg /100 g.
(j) Notification prior to unloading.
(k) Unloading schedule.
(l) Access for Dockside Sampling.
(m) Record Keeping.
(n) Early Warning/Alert System.

NOTE: The plan may include other requirements, as deemed necessary by the authority in the state of landing, to ensure adequate public health protection under the NSSP.

C. Closed Status of Growing Areas.
(1) A growing area, or portion(s) thereof as provided in Section A.(4), shall be placed in the closed status for the taking of shellstock when the Authority determines that the number of toxin-forming organisms in the growing waters and/or the level of Biotoxin present in shellfish meats is sufficient to cause a health risk. The closed status shall be established based on the following criteria:
(a) PSP - ~~cells/L n/a;~~ 80 µg saxitoxin equivalents/100 grams
(b) NSP - 5,000 cells/L or 20 MU/100 grams (0.8 mg brevetoxin-2 equivalents/kg)
(c) AZP - ~~cells/L n/a;~~ 0.16 mg azaspiracid-1 (AZA-1) equivalents/kg (0.16 ppm)
(d) DSP – ~~cells/L n/a;~~ 0.16 mg okadaic acid (OA) equivalents/kg (0.16 ppm)
(e) ASP - ~~cells/L n/a;~~ 2 mg domoic acid/100 grams (20 ppm)
~~(f) The concentration of paralytic shellfish poison (PSP) equals or exceeds 80 µg per 100 g of edible portion of raw shellfish; or~~
~~(g) For neurotoxic shellfish poisoning (NSP), the harvesting of shellstock shall not be allowed when:~~
~~(i) The concentration of NSP equals or exceeds 20 mouse units per 100 grams of edible portion of raw shellfish; or~~
~~(ii) The cell counts for *Karenia brevis* organisms in the water column exceed 5,000 per liter; or~~
~~(h) For domoic acid, the toxin concentration shall not be equal to or exceed 20 ppm in the edible portion of raw shellfish.~~
~~(i) For azaspiracid shellfish poisoning (AZP), the concentration of azaspiracids shall not be equal to or exceed 0.16 mg/kg (AZA 1 equiv.) in the edible portion of raw shellfish.~~

~~(j) For diarrhetic shellfish poisoning (DSP), the concentration of DSP toxins shall not be equal~~
t~~o or exceed 0.16 mg/kg (OA equiv.) in the edible portion of raw shellfish.~~

(2) For any marine Biotoxin producing organism for which criteria have not been established under this Ordinance, either cell counts in the water column or Biotoxin meat concentrations may be used by the Authority as the criteria for not allowing the harvest of shellstock.

(3) When sufficient data exist to establish that certain shellfish species can be safely exempted from the marine ~~B~~biotoxin management~~contingency~~ plan, the closed status for harvesting may be applied selectively to some shellfish species and not others.

(4) The closed status shall remain in effect until the Authority has data to show that the toxin content of the shellfish in the growing area is below the level established for closing the area.

(5) The determination to return a growing area to the open status shall consider whether toxin levels in the shellfish from adjacent areas are declining.

(6) The analysis upon which a decision to return a growing area to the open status is based shall be adequately documented.

D. Heat Processing. If heat processing is practiced, a control procedure shall be developed. This procedure shall define the following:
(1) Toxicity limits for processing;

(2) Controls for harvesting and transporting the shellstock to processor;
(3) Special marking for unprocessed shellstock;

(4) Scheduled processes; and

(5) End product controls on the processed shellfish.

E. Records. The Authority shall maintain a copy of all of the following records.

(1) All information, including monitoring data, relating to the levels of marine Biotoxins in the
shellfish growing areas;

(2) Copies of notices placing growing areas in the closed status;

(3) Evaluation reports; and

(4) Copies of notices returning growing areas to the open status.

Public Health Significance	In response to the ISSC 2015 Summary of Actions, the USFDA requested the ISSC and FDA begin discussion regarding establishment of minimum requirements for sample collection and analysis for safely reopening areas following Biotoxin closures. This effort should include examination of existing practices and the level of safety they provide. In response to this request, the ISSC Executive Board agreed to host a Biotoxin meeting to discuss the Biotoxin issues listed above. States that are frequently involved in Biotoxin closures and reopenings were invited to discuss present state efforts to implement the NSSP Model Ordinance requirements for biotoxin management. The participants agreed that changes should be made to the Model Ordinance and existing biotoxin guidance. These proposed changes were provided to the Biotoxin Committee for comments. This proposal reflects the recommendation developed from that review process.
Cost Information	

Proposal No. **17-123**

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	ISSC Executive Office
Affiliation	Interstate Shellfish Sanitation Conference
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Marine Biotoxin Control Guidance
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II .02
Text of Proposal/ Requested Action	**Chapter II. Growing Areas** **.02 Guidance for Developing Marine Biotoxin ~~Contingency~~ Plans.** NSSP guidance documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, which includes the requirements of the program ~~and summaries of the requirements for that component~~. NSSP *Model Ordinance* requirements apply only to interstate commerce although most states apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance. **Introduction** Shellfish are filter feeders and, therefore, they have the ability to concentrate ~~toxigenic dinoflagellates~~toxic phytoplankton from the water column when present in shellfish growing waters. The toxins produced by ~~these dinoflagellates~~ certain species of phytoplankton can cause illness and death in humans. Toxins are accumulated in the viscera and/or other tissues of shellfish and ~~are transferred to~~ human~~s~~ exposure occurs when the shellfish are eaten (Gordan *et al.*, 1973). These toxins are not normally destroyed by cooking or processing and cannot be detected by taste. ~~Most of these toxins are detected through animal testing. However, some involve the use of instrument based or biochemical analyses for detection. Since the dinoflagellates are naturally occurring, their~~The presence of toxic phytoplankton in the water column or traces of their toxin in shellfish meat does not necessarily constitute a health risk, as toxicity is dependent on concentration (dose) in the shellfish. To protect the consumer, the Authority must evaluate the concentration of toxin present in the shellfish or the ~~dinoflagellate~~toxic phytoplankton concentration in the water column against the levels established in the NSSP Model Ordinance to determine what action, if any, should be taken. There are a wide range of methodologies developed for screening and confirmation of toxic phytoplankton and their toxins. Only methods adopted into the NSSP can be implemented for the purpose of confirming toxin concentration levels and making decisions to close or reopen growing areas. Additionally, some screening methods

have been evaluated by the ISSC and found fit for purpose for the NSSP, thereby providing confidence in their use for specific screening purposes. Toxin methods fall into two categories in the NSSP: Approved Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 2.) and Approved Limited Use Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 4.). These methods range from mouse bioassays to immunochromatography and other antibody based platforms to chemical analytical methods such as high performance liquid chromatography (HPLC). Information available in the referenced Tables above provides references for the methods and, as applicable, what limitations are placed on the use of the method within the NSSP. For toxins that have no method adopted into the NSSP, best available science is employed.

There are ~~three (3)~~five (5) types of shellfish poisonings which are specifically addressed in the NSSP Model Ordinance: Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), ~~and~~ Amnesic Shellfish Poisoning (ASP), also known as Domoic Acid poisoning, Diarrhetic Shellfish Poisoning (DSP) and Azaspiracid Shellfish Poisoning (AZP). ~~All three (3)~~Of these five (5) types of shellfish poisoning, PSP, NSP and ASP are the most dangerous. ~~toxins, and~~ PSP and ASP ~~or domoic acid~~ can cause death at sufficiently high exposure~~concentrations~~. In addition, ASP can cause lasting neurological damage. PSP is caused by saxitoxins produced by the dinoflagellates of the genus *Alexandrium* (formerly *Gonyaulax*). The dinoflagellate *Pyrodinium bahamense* is also a producer of saxitoxins. NSP is caused by brevetoxins produced by the dinoflagellates of the genus *Karenia* (formerly *Gymnodinium*). ASP is caused by domoic acid and is produced by diatoms of the genus Pseudonitzchia. Certain *Dinophysis* spp. and *Prorocentrum* spp. produce okadaic acid and dinophysis toxins that cause DSP. *Azadinium* spp. is the producer of azaspiracids, which cause AZP.

Both *Alexandrium* and *Karenia* can produce "red tides", i.e. discolorations of seawater caused by blooms of the algae; however, they may also reach concentrations that cause toxic shellfish without imparting any water discoloration. Toxic blooms of these dinoflagellates can occur unexpectedly or follow predictable patterns. The unpredictability in occurrence of toxic blooms was demonstrated in New England in 1972 when shellfish suddenly became toxic in a previously unaffected portion of the coastline and resulted in many illnesses (Schwalm, 1973). Historically, *Alexandrium* blooms have occurred between April and October along the Pacific coasts from Alaska to California and in the Northeast from the Canadian Provinces to Long Island Sound (U.S. Public Health Service, 1958); but these patterns may be changing. The blooms generally last only a few weeks and most shellfish (with the exception of some species of clams and scallops which retain the toxin for longer periods) clear themselves rapidly of the toxin once the bloom dissipates. Occurance of *Karenia* blooms ~~NSP, which is less common, has occurred~~ extends from the Carolinas south ~~and extends~~ throughout the Gulf Coast states. ~~It shows no indication of regular recurrence and shellfish generally take longer to eliminate the toxin (Liston, 1994).~~DSP and AZP cause similar symptoms mostly related to diarrhea and abdominal pain. DSP toxin-producing phytoplankton have been documented to occur off the coasts of Washington (Trainer et al. 2013) and

Texas (Deeds et al. 2010) as well as off the coast in the Northeast (e.g., Massachusetts [Tong et al. 2015]).While AZP has occurred in the U.S., the contaminated shellfish was imported (Klontz et al. 2009). Harvesting closures in the U.S. have not been documented due to AZP toxins.

The minimum concentration of PSP toxin that will cause intoxication in susceptible persons is not known. Epidemiological investigations of PSP in Canada, however, have indicated 200 to 600 micrograms of PSP toxin will produce symptoms in susceptible persons. A death has been attributed to the ingestion of a probable 480 micrograms of PSP toxin. Investigations indicate that lesser amounts of the toxin have no deleterious effects on humans. Shellfish growing areas should be closed at a PSP toxin level, which provides an adequate margin of safety, since in many instances PSP toxicity levels can change rapidly.

The NSSP Model Ordinance requires that growing areas be placed in the closed status when the PSP toxin concentration is equal to or exceeds the action level of 80 micrograms per 100 grams of ~~edible portion of~~ raw shellfish (FDA, 1977; FDA, 1985).

~~I~~n shellfish growing areas where low levels of PSP toxin routinely occur, harvesting for thermal processing purposes may be an alternative to consider. Thermal processing as defined by applicable FDA regulations (21 CFR 113) will reduce ~~but not entirely destroy~~ the PSP toxin concentration ~~content~~ of the shellfish via dilution, not destruction. If thermal processing is practiced, the Authority must develop and implement procedures to control the harvesting and transportation of the affected shellfish to the processing plant.

In Gulf coast areas, toxicity in shellfish has been associated with red tide outbreaks caused by massive blooms of the toxic dinoflagellate, *Karenia brevis*. The most common public health problem associated with *Karenia* blooms is respiratory irritation; however, neurotoxic shellfish poisonings associated with *Karenia brevis* blooms have been reported in Florida (Center for Disease Control, 1973 [a] and [b]). Uncooked clams from a batch eaten by a patient with neurotoxic symptoms were found to contain 118 mouse units per 100 grams of shellfish meat. The NSSP Model Ordinance mandates that growing areas be placed in the closed status when any NSP toxin is found in shellfish meat at or above 20 MU per 100 grams of shellfish, or when the cell counts for members of the genus *Karenia* in the water column equal or exceed 5,000 cells per liter of water.

ASP is caused by domoic acid, which is produced by diatoms of the genus *Pseudo-nitzs~~a~~chia*. Blooms of *Pseudo-nitzs~~a~~chia* are of ~~relatively short duration~~varying intensity, duration and extent.. ~~However, d~~During ~~the~~a 1991-1992 incident in Washington and a 2015 event on the west coast from Washington to California, high toxin levels persisted for several months (Liston, 1994; McCabe et al. 2016). There was also an extensive event in the Northeast from Maine to Rhode Island in 2016, with different regions showing varying toxicity and species dominance within the bloom. The event started in late September in eastern Maine and ended in October; however, Rhode Island experienced another bloom in February of 2017.The NSSP Model Ordinance requires that growing

areas be placed in the closed status when the domoic acid concentration is equal to or exceeds 20 parts per million in ~~the edible portion of~~ raw shellfish.

The suitability of some growing areas for shellfish harvesting is periodically influenced by the presence of marine biotoxins such as those responsible for PSP, NSP, ~~domoic acid~~ASP, DSP and AZP ~~or other marine Biotoxins~~. The occurrence of these toxins is often unpredictable, and the potential for them to occur exists along most coastlines of the United States and other countries having shellfish sanitation Memoranda of Understanding (MOU) agreements with the United States. As a result, states or countries with MOUs with the U.S. need to have management plans and/or ~~make~~ contingency plans to address shellfish-borne intoxications.

Controlling Marine Biotoxins in Shellfish

There are two types of plans defined in the NSSP MO for the control of marine biotoxins. A contingency plan is developed by an Authority that has no history or reason to expect toxin-producing phytoplankton in their growing areas. A marine biotoxin management plan is developed by an Authority that has historic occurrence of toxin-producing phytoplankton and toxicity in shellfish from their growing areas.

The Contingency Plan

The contingency plan is primarily for reactive management to an illness outbreak or an emergence of a toxin-producing phytoplankton in a growing area that has not historically occurred before. The contingency plan must describe administrative procedures, laboratory support, sample collection procedures, ~~and~~ patrol procedures to be implemented on an emergency basis and reopening criteria ~~in the event of the occurrence of shellfish toxicity~~ (Wilt, 1974). The contingency plan is only appropriate for a shellfish Authority that has no history or reason to expect toxin-producing phytoplankton in their growing areas. The primary goal of ~~this planning~~the contingency plan should be to ensure that maximum public health protection is provided. To achieve this goal the following ~~objectives~~elements should be ~~met~~included:

- A process for immediate precautionary closures;
- A sampling plan that considers water samples to evaluate the extent and intensity of the toxic phytoplankton distribution;
- A sampling plan that considers species-specific shellfish sampling;
- Access to biotoxin tests: both screening and approved methods;
- Trained staff to carry out sample collection and testing if necessary; and
- A reopening criteria.

~~*An early warning system should be developed and implemented.~~
~~*Procedures should be established to define the severity of occurrences.~~
~~*The state or MOU country should be able to respond effectively to minimize illness.~~
~~*Adequate intelligence and surveillance information should be gathered~~

~~and evaluated by the Authority.~~
~~*Procedures should be instituted to return the Biotoxin contaminated areas to the open status of their~~
~~growing area classification.~~

Under the certification provisions of the NSSP, FDA and receiver states should have the assurance that shellfish producing states or MOU countries are taking and can take adequate measures to prevent harvesting, shipping, and consumption of toxic shellfish. To provide this assurance, the NSSP requires the Authority to develop and adopt a marine Biotoxin contingency plan for all marine and estuarine shellfish growing areas. The Authority's plan should specify how each of the objectives listed above will be accomplished. This document provides recommended guidelines to be used in preparing a plan to meet these objectives.

The Marine Biotoxin Management Plan

The marine biotoxin management plan is primarily for proactive management of marine biotoxins for growing areas with a history of toxin-producing phytoplankton and toxicity in shellfish and/or a previous illness event or outbreak. The management plan must describe an early warning system, administrative procedures, laboratory support, sample collection procedures, patrol procedures to be implemented and reopening criteria (Wilt, 1974). A management plan is required for a shellfish Authority that has a history of toxin-producing phytoplankton, toxicity in shellfish and/or an illness event or outbreak attributed to their growing areas. A shellfish Authority might have a management plan for certain marine biotoxins like PSP toxins but a contingency plan for toxins like AZP toxins. The primary goal of the management plan should be to prevent illnesses from toxic shellfish and ensure that maximum public health protection is provided. To achieve this goal the following elements should be included:

- An early warning system should be developed and implemented.
- Procedures should be established to define the severity of occurrences.
- The Authority should be able to respond effectively to minimize risk of illness.
- Adequate intelligence and surveillance information should be gathered and evaluated by the Authority.
- Procedures should be instituted to return the biotoxin contaminated areas to the open status of their growing area classification.

Recommended Contingency Plan Guidelines

** Provide an early warning system:*

1. Communication procedures should be established with other

appropriate agencies to rapidly report to the Authority any abnormal environmental phenomenon that might be associated with shellfish growing areas such as bird or fish kills, water discoloration or abnormal behavior of shellfish or marine scavengers.

2. The Authorities should establish procedures for health agencies to report any toxin-like illnesses.

3. An early warning phytoplankton and/or shellfish-monitoring program should be implemented.

These monitoring programs should use the "~~key~~primary station" (for both phytoplankton and shellfish monitoring) and "critical species" concepts (for shellfish monitoring).

* Sampling stations (primary stations) should be located at sites where past experience has shown toxin is most likely to appear first.
* When monitoring shellfish, samples should be collected of species which are most likely to
reveal the early presence of toxin and which are most likely to show the highest toxin levels (critical species). For example, mussels have been found to be useful for early ~~PSP~~ detection. Sampling design should always consider what species are present in the growing area and commercially harvested.
* The frequencies and ~~periods~~geographic distribution for collection of samples should be established recognizing the randomness of ~~PSP~~toxic algal blooms. This assumes several years of baseline data in order to establish stations and sampling plans.
* Frequency and geographic distribution of sampling should be adequate to monitor for fluctuations in coastal phytoplankton populations and the influence of meteorological and hydrographic events. For example, a large rain storm may cause nutrient loading in coastal waters and trigger a toxic phytoplankton bloom or a hurricane may drive offshore phytoplankton blooms onshore. .

4. Channels of communication concerning shellfish toxicity should be established with other states, countries (in the case of MOU countries), FDA, and other responsible officials. A marine Biotoxin control official should be designated by the Authority to receive and distribute all marine
Biotoxin related information. Consultation with adjacent jurisdictions, marine biologists and
other environmental officials ~~might also be~~is also useful (Felsing, 1966; Quayle, 1969; Prakash *et al.*, 1971).

** Define the severity of the problem:*

1. A procedure should be established to promptly expand the sampling program for marine Biotoxins in the event of increased toxicity/cell counts at any indicator monitoring stations identified within the plan. Sampling stations and frequencies of sampling should be increased when monitoring data or other information suggests that toxin levels are increasing. The procedure should include plans for obtaining the additional resources necessary to implement the expanded sampling and laboratory analysis program.

2. Information should be available concerning the location of commercial shellfish resource areas and species present in the state.
3. Criteria should be developed to define the circumstances under which growing areas will be placed in the closed status because of marine Biotoxin contamination. The criteria should integrate public health, conservation, and economic considerations. Principal items of concern include consideration of the rapidity with which toxin levels can increase to excessive levels, the inherent delays in sample collection and results, the number of samples required to initiate action, the size of the area to be closed (including a safety zone), and the type of harvesting restrictions to be invoked (all species or specific species). It may be appropriate to close harvesting areas adjacent to known toxic areas until increased sampling can establish which areas are toxin free and that toxin levels have stabilized.
4. Procedures should be established to promptly identify which shellfish products or lots might be potentially contaminated, and to determine the distribution of these products or lots.

** Respond effectively to minimize illness:*

1. A summary should be provided citing the laws and regulations in the state (or MOU country) that promptly and effectively allow the Authority to restrict harvesting, withdraw interstate shipping permits, and to embargo/recall any potentially toxic shellfish already on the market in the event of a marine ~~B~~biotoxin ~~episode~~event. The plan should clearly define the timeframe involved in taking appropriate legal action.
2. The administrative procedures necessary to place growing areas in the closed status, to withdraw interstate certification of dealers, and to embargo and recall shellfish should be delineated. The timeframe necessary to accomplish these actions should also be specified.
3. A plan should be developed which will define what type of patrol program is necessary to properly control harvesting in toxin contaminated growing areas. The program should be tested to ensure prompt implementation in the event it is needed.
4. Procedures should be developed to promptly disseminate information on the occurrences of toxic phytoplankton blooms to the industry and local health agencies. It is helpful to establish relationships and procedures with other agencies such as the state CDC and Poison Control and authorities in advance of any serious biotoxin event.
5. Procedures should be established to coordinate control activities taken by state and federal agencies or departments and district, regional, or local health authorities.

** Gather follow-up data:*

1. Appropriate records of illnesses should be compiled and maintained by the Authority. These records should include data on the incidence of illness and appropriate case history data. This information may be

important in defining the severity of the problem, as well as for a retrospective evaluation of the adequacy of the entire control program.

2. Records of shellfish sample results from toxin testing should include analysis of trends, detoxification curves, phytoplankton and water sample analyses, and pertinent environmental observations.

3. Whenever possible the Authority should archive shellfish homogenates for additional analysis.

** Return growing areas to the open status of their NSSP classification:*

1. Once a growing area is placed in the closed status because of marine Biotoxin contamination, a procedure should be instituted to gather data necessary to decide when the area can be returned to the open status of its classification. A system of representative samples to establish detoxification curves should be part of this procedure.
2. The Authority should develop a set of criteria that must be met before a growing area can be returned to the open status. These criteria should integrate public health, conservation, and economic considerations, and employ a sufficient number of samples and other environmental indices, if used, to establish that the level of toxin or cell counts are below the closure level. For example, experience has shown that appropriate reopening criteria for PSP include a minimum of three (3) samples collected over a period of at least fourteen (14) days. These samples should show the absence of PSP or levels below 80 micrograms per 100 grams of shellfish tissue.
3. A program of consumer education should be continued as long as any area remains in the closed status because of marine Biotoxin contamination.

References

1. Center for Disease Control (a). 1973. Shellfish Poisoning - Florida. *Morbid. Mortal. Weekly Rep*.22(48):397-398.
2. Center For Disease Control (b). 1973. Neurotoxic Shellfish Poisoning - Florida. *Morbid. Mortal.Weekly Rep*. 22(48):397-398.
3. Felsing, W.A., Jr. 1966. Proceedings of Joint Seminar on North Pacific Clams, September 24-25,1965. U.S. Public Health Service, Washington, D.C.
4. Food and Drug Administration. 1977. Poisonous or Deleterious Substances in Food. *FederalRegister* 42(190):52814-52819.
5. Food and Drug Administration. 1985. Action Levels For Poisonous or Deleterious Substances in Human Food and Animal Feed. U.S. Department of Health and Human Services, Public Health Service, Washington, D.C. 20204. 13 pages.
6. Gordon, K., M.D., *et al*. 1973. Shellfish Poisoning. *Morbid. Mortal. Weekly Rep*. 22, (48):397-398.
7. Liston, J. 1994. Association of *Vibrionaceae*, natural toxins, and parasites with fecal indicators, p.215-216. In Hackney, C.R. and M.D. Pierson (eds.), *Environmental Indicators and Shellfish Safety*. Chapman and Hall, New York, NY.

	8. Prakash, A., J.C. Medcof, and A. D. Tennant. 1971. Paralytic shellfish poisoning in easternCanada. Bulletin 177, Fisheries Research Board of Canada. Ottawa, Canada. 9. Quayle, D.B. 1969. Paralytic shellfish poisoning in British Columbia. Bulletin 168, FisheriesResearch Board of Canada. Ottawa, Canada. 10. Schwalm, D.J. 1973. The 1972 PSP outbreak in New England. FDA Report, Boston, MA. U.S.Food and Drug Administration, Washington, D.C. 11. U.S. Public Health Service (PHS). 1958. Proceedings: 1957 Conference on Shellfish Poison. U.S.PHS, Washington, D.C. 125 pages. 12. Wilt, D.S. (ed). 1974. Proceedings of Eighth National Shellfish Sanitation Workshop. January 16-18. New Orleans, LA. National Technical Information Services (PB8 6 236916/AS), U.S. Dept. of Commerce, Springfield, VA. 158 p.
Public Health Significance	This proposal includes modifications to Guidance Document .02 Guidance for Developing Marine Biotoxin Contingency Plans. This proposal includes guidance document modifications which support Proposal 17-122.
Cost Information	