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).

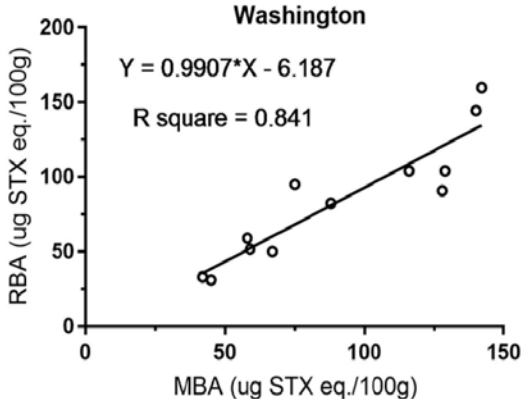
10. Matrix Effects

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)

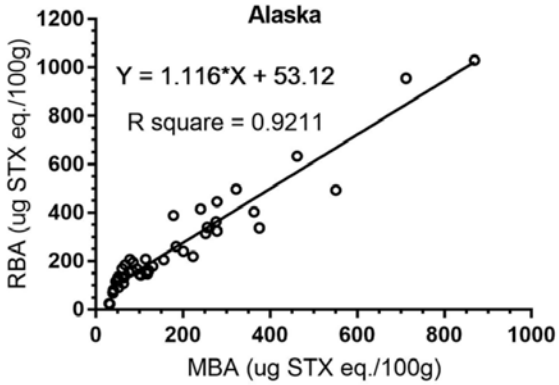
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)

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

© 2012 AOAC INTERNATIONAL

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.

© 2012 AOAC INTERNATIONAL

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

© 2012 AOAC INTERNATIONAL

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

© 2012 AOAC INTERNATIONAL

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.

© 2012 AOAC INTERNATIONAL

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

© 2012 AOAC INTERNATIONAL

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).]

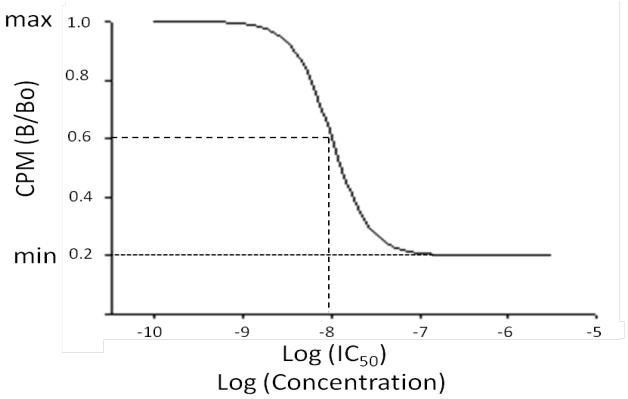


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

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)

© 2012 AOAC INTERNATIONAL

Appendix 2

Proposal No. 17-106

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.

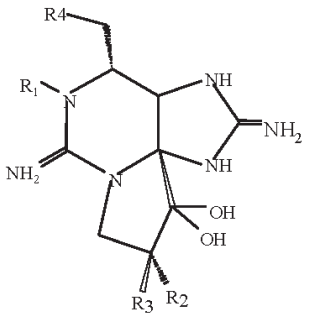
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fran.vandolah@noaa.gov

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.

Proposal No. 17-106

	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 μm pore size type FB glass fiber filter/0.65 μm 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

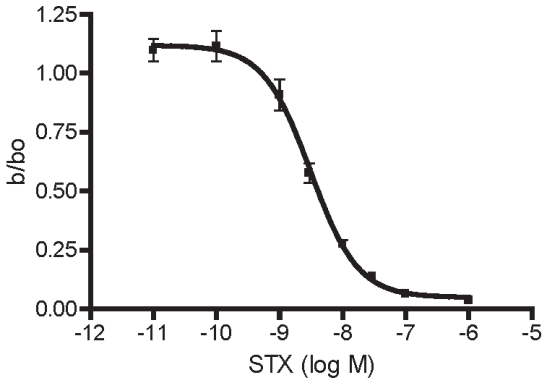


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

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).

Proposal No. 17-106

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:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM equiv. STX in extract}$$

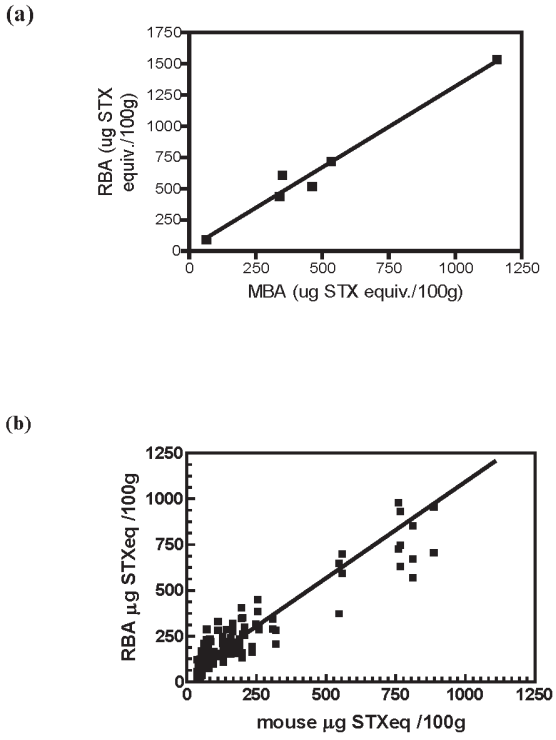


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with 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.

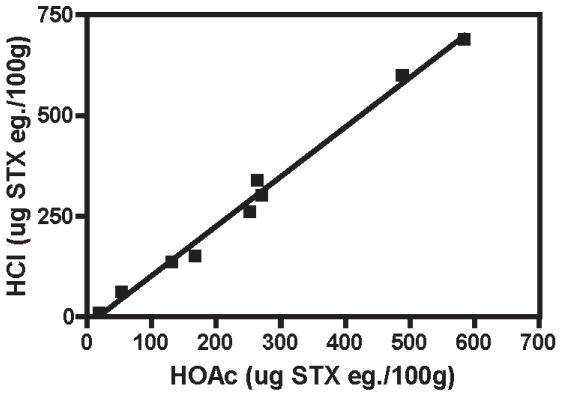


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Proposal No. 17-106

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.

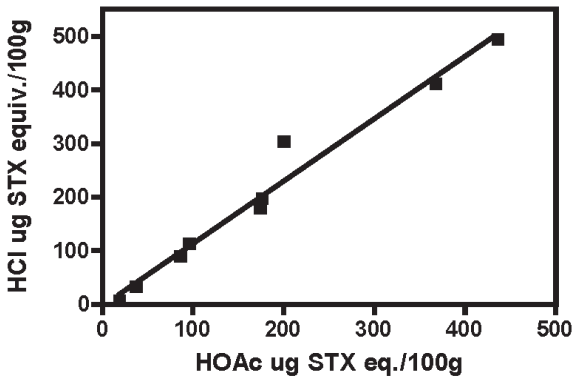


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

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.

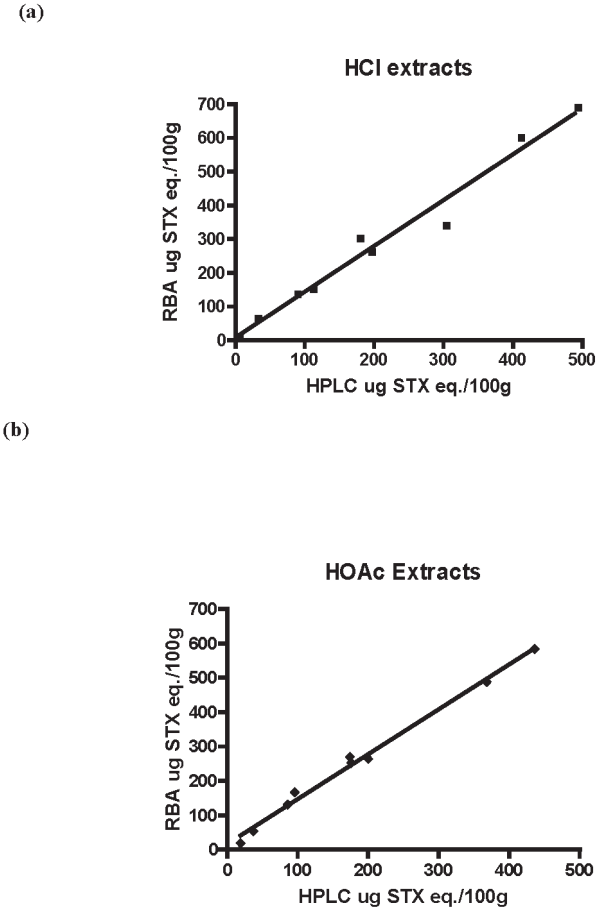


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

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

Proposal No. 17-106

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

Appendix 3

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.

This publication does not constitute an endorsement of any commercial product or intend to be an opinion beyond scientific or other results obtained by the National Oceanic and Atmospheric Administration (NOAA). No reference shall be made to NOAA, or this publication furnished by NOAA, to any advertising or sales promotion which would indicate or imply that NOAA recommends or endorses any proprietary product mentioned herein, or which has as its purpose an interest to cause the advertised product to be used or purchased because of this publication.

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:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \cdot EC50\ \text{Hill slope})}}$$

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 (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%.

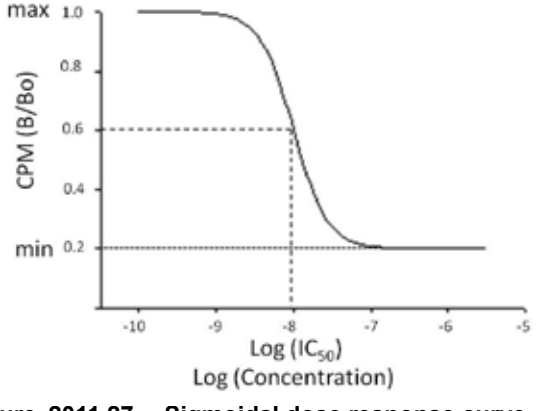


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

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.

Proposal No. 17-106

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

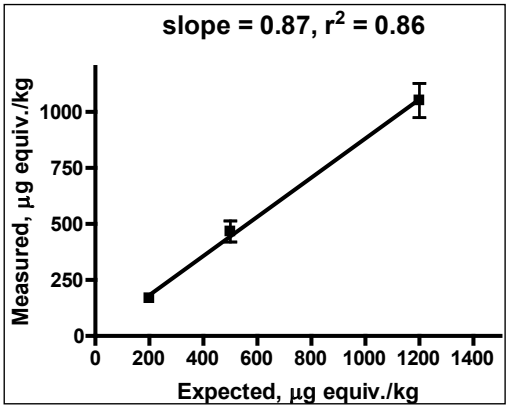


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

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

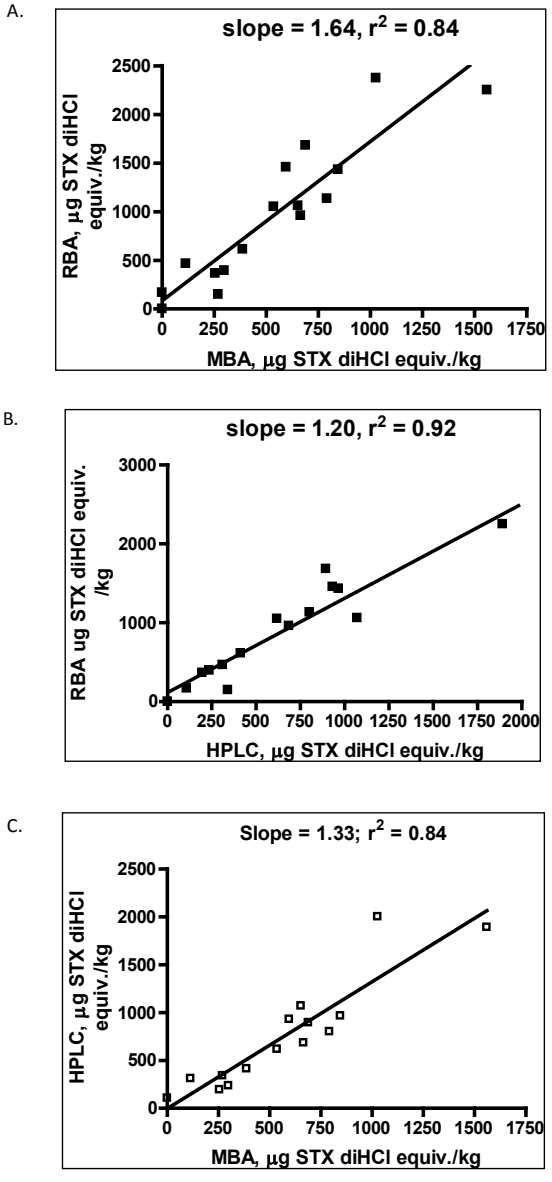


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

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.

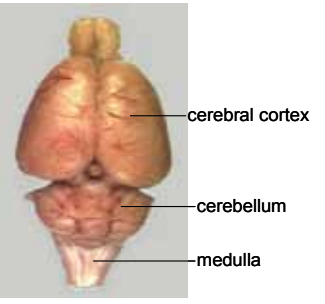


Figure 1. Rat brain.

(*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

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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
© 2012 Taylor & Francis
http://dx.doi.org/10.1080/19440049.2012.712063
http://www.tandfonline.com

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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).

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

- 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).

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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

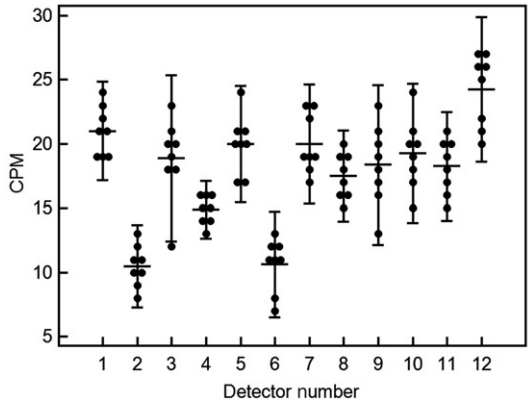


Figure 2. Plot of the instrument background plate second count cycle depicting the randomness of CPMs in the 96 wells. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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.

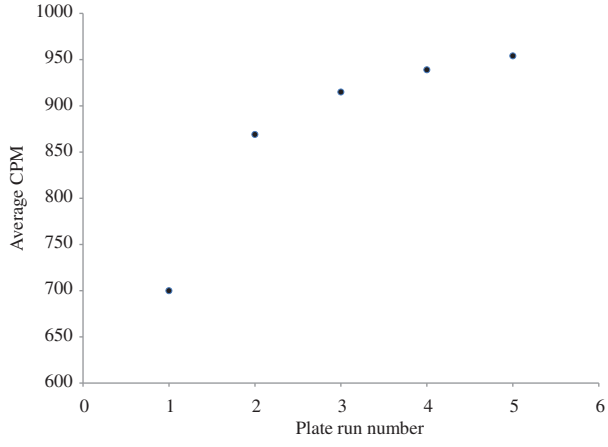


Figure 3. Graph of the blank plate five sequential readings showing a gradual increase in average CPM.

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

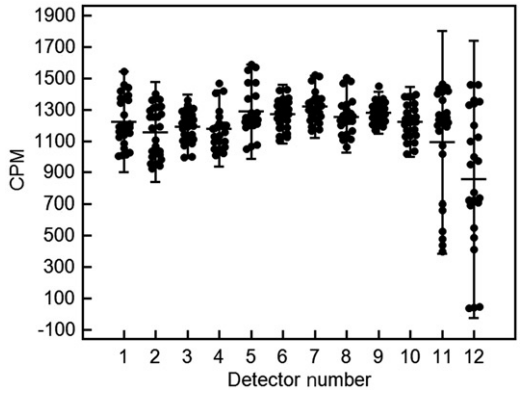


Figure 4. Scatter plot of the reference plate, first reading showing detector variability. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

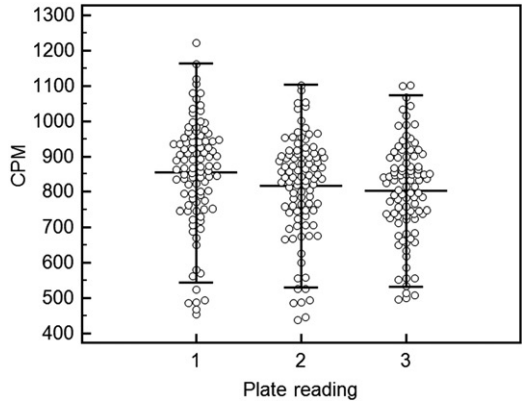


Figure 5. Results of the QC plate showing the variability of CPM in the three successive readings. For each run the mean and error bars at 2 SDs per plate are shown.

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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.

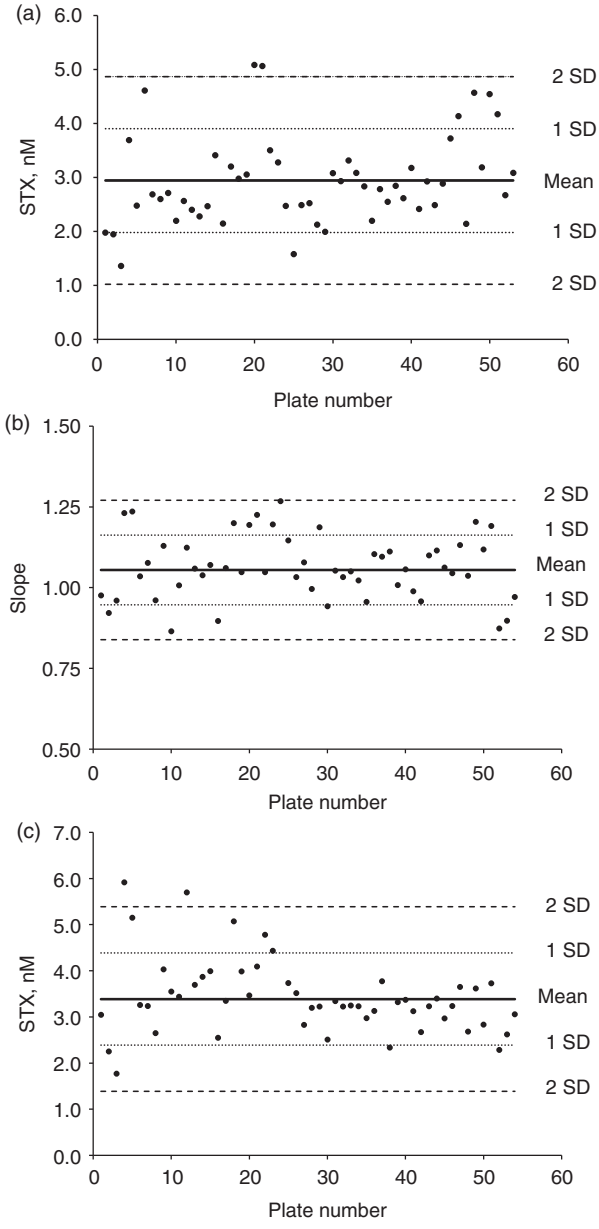


Figure 6. Control charts for (a) averaged daily QC samples on a plate, (b) slope of the binding curve and (c) EC_{50} per plate.

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012

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.

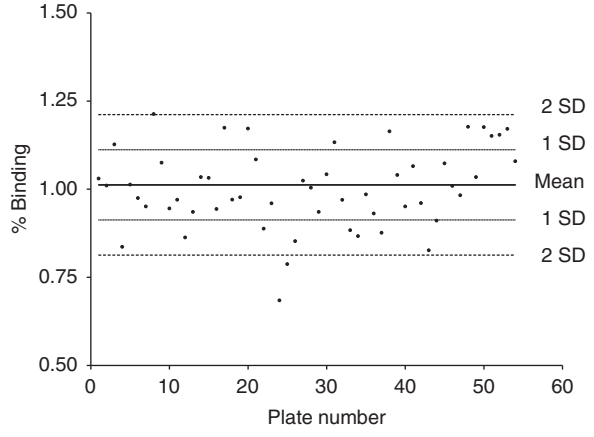


Figure 7. Control chart for the top plateau, maximum binding (B_{max}), of the calibration curve.

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.

Downloaded by [S.R. Ruberu] at 11:46 30 August 2012