Proposal Subject:	Brevetoxin (NSP) ELISA Kit
Specific NSSP Guide Reference:	Section IV. Guidance Documents, Chapter II Growing Areas, .10 Approved National Shellfish Sanitation Program Laboratory Tests: Microbiological and Biotoxin Analytical Methods Section II. Model Ordinance Chapter III. Laboratory @.02 Methods C. Biotoxin
Text of Proposal/ Requested Action	See attached ISSC Method Application Faster and easier to perform methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples. This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for PSP, DSP, and ASP testing. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.
Public Health Significance:	
Cost Information (if available):	As low as $15 per sample.
Action by 2009 Laboratory Methods Review Committee	Recommended no action on Proposal 09-106. Rationale: Insufficient data.
Action by 2009 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 09-106.
Action by 2009 General Assembly	Adopted recommendation of 2009 Task Force I on Proposal 09-106.
Action by USFDA 02/16/2010	Concurred with Conference action on Proposal 09-106.

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	Brevetoxin (NSP) ELISA Kit	
Name of the Method Developer	Abraxis LLC	
Developer Contact Information	Fernando Rubio 54 Steamwhistle Drive Warminster, PA 18974 Phone: (215) 357-3911 FAX: (215) 357-5232	
Checklist	**Y/N**	**Submitter Comments**
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Shellfish are filter feeders that pump large quantities of water through their bodies when actively feeding. During this process, shellfish can concentrate toxigenic micro-algae and other substances from the water column when they are present. The ability of shellfish to concentrate chemical pollutants from water can lead to accumulation of these toxins to levels that constitute a public health hazard. Red tides containing Brevetoxin have caused mortality events in fish, and sea mammals. In humans, Brevetoxin (NSP) poisoning causes a combination of gastro-intestinal and neurological symptoms. Some of the currently available methods used for the detection and monitoring of brevetoxin in water and shellfish are not conducive for the quick on-site or real time, dockside or ship board monitoring of this toxin. For example: 1) the mouse bioassay is labor intensive, requires the use and destruction of many vertebrate animals, analyses is only performed in a few laboratories with a low turn around time, 2) a research ELISA has been developed by the University of North Carolina, however, this assay requires the user to coat plates with antibodies before analysis, a process that takes at least two days to complete before an analytical result is obtained. Therefore, faster and easier to perform methods are needed to satisfy the needs of the regulatory community and shellfish industry. The proposed ELISA method is a fast and easy to perform method with ready to use reagents i.e. analyst only needs to extract shellfish sample or dilute water sample before analysis. The proposed ELISA also provides a quantitative and/or semi-quantitative screening for shellfish extracts and/or water samples.

		This assay is part of Abraxis platform for marine toxin testing and complements the company's other offering for PSP, DSP, and ASP testing.
2. What is the intended purpose of the method?		The fast analysis of Brevetoxin (NSP) in shellfish extracts and/or water quality monitoring. The proposed ELISA can be used on-site (boat, dock) or established analytical laboratories.
3. Is there an acknowledged need for this method in the NSSP?		Yes. NSSP Guidance Documents, Chapter II Constitution by-laws and procedures of the Interstate Shellfish Sanitation Conference. Procedure XVI. Procedure for acceptance and approval of analytical methods for the NSSP. And: National Shellfish Sanitation Program 2003 Model Ordinance III. Laboratory @ 02 Methods C. Biotoxin. Methods for the analysis of shellfish and shellfish harvest waters shall be: 1) The current APHA method used in bioassay for *Karemia breve* toxin.
4. What type of method? i.e. chemical, molecular, culture, etc.		Immunochemical Method.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Abraxis ELISA Kit for the Screening of Brevetoxin in Shellfish Extract and/or Harvest Waters.
Method Scope		A Method for the screening out negative brevetoxin samples in shellfish regulatory labs, to determine if shellfish are safe to harvest and/or distribute. A method for water classification for brevetoxin around harvest areas and to screen for toxic phytoplankton in seawater to provide early warning.
References		Maucher, J.M., Briggs, L.R, Podmore, C., Ramsdell, J.S. (2007) Optimization of blood collection card method/ELISA for monitoring exposure of bottlenose dolphin to brevetoxin-producing red tides. *Environmental Science & Technology*, 41: 563-567. Inter-lab study data performed by several labs including Ag Research in New Zealand, Cawthron Institute in New Zealand and NOAA is available upon request.
Principle		**The test is a direct competitive ELISA based on the recognition of Brevetoxin by specific antibodies. Brevetoxin, when present in a sample, and a Brevetoxin enzyme-conjugate compete for the binding sites of sheep anti-brevetoxin antibodies that have been immobilized in the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Brevetoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the**

		standard curve constructed with each run.
Any Proprietary Aspects		Immunoreagents and sample diluent.
Equipment Required		Pipettes and plate reader. Blender for shellfish extraction.
Reagents Required		Reagents provided in the ELISA kit. In addition methanol is needed.
Sample Collection, Preservation and Storage Requirements		Water samples need to be collected in glass vials and preserved according to users guide (attached). Diluted shellfish extracts should be stored in glass vials. All dilution should be done using provided sample diluent. If not analyzed promptly, samples should be stored refrigerated for up 2 days or frozen if longer periods are required.
Safety Requirements		As with any laboratory procedure, gloves and goggles should be used during the processing and analysis of samples.
Clear and Easy to Follow Step-by-Step Procedure		User's guide and an easy to follow flow chart are provided with each kit (attached).
Quality Control Steps Specific for this Method		As with any analytical procedure laboratory controls (positive and negative) are recommended.
C. Validation Criteria		
1. Accuracy / Trueness		Data ran by AgResearch, New Zealand is provided as an attachment,
2. Measurement Uncertainty		@ 0.042 ng/mL in water SD 0.002 CV 4.8% @ 0.210 ng/mL in water SD 0.010 CV 4.8% @ 0.443 ng/mL in water SD 0.064 CV 14.5%
3. Precision Characteristics (repeatability and reproducibility)		< 15%
4. Recovery		Average water recovery 86%, shellfish extract recovery 104%
5. Specificity		PbTx-3 100% Deoxy PbTx-2 133% PbTx-5 127% PbTx-2 102% PbTx-9 83% PbTx-6 13% PbTx-1 5%
6. Working and Linear Ranges		0.01-2 ng/mL water or 0.5-100 ng/gm or in shellfish extract or higher depending on dilution.
7. Limit of Detection		0.05 ng/mL
8. Limit of Quantitation / Sensitivity		0.01 ng/mL in water; 4.5 ng/gm in shellfish extract
9. Ruggedness		Since and analytical curve is run with each assay and the samples are compared to the standard curve, the proposed ELISA is rugged.
10. Matrix Effects		If used according to instructions (dilutions), none detected

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Method is intended as a screening method to complement other accepted NSPP methods: i.e. mouse bioassay.
D. Other Information		
1. Cost of the Method		As low as $15 per sample
2. Special Technical Skills Required to Perform the Method		Some technical skills are required. Familiarity with laboratory setting is adequate. Kit Manufacturer's on-site training is available.
3. Special Equipment Required and Associated Cost		As low as $1,800. Strip reader and pipette
4. Abbreviations and Acronyms Defined		ELISA: Enzyme linked immuno sorbent assay NSP: neurotoxic shellfish poisoning
5. Details of Turn Around Times (time involved to complete the method)		40 samples can be run in duplicate in approximately 2 hours. Shellfish sample extraction requires approximately 15 minutes
6. Provide Brief Overview of the Quality Systems Used in the Lab		The ELISA kits are manufactured following GMP and GLP procedures.

Submitters Signature	Date:
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

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

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.

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

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Testing for Assay Ruggedness

The performance of the brevetoxin ELISA was tested when small changes in the operating conditions were made similar to those likely to arise in different laboratories. This was undertaken not only to demonstrate that the assay is resistant to small departures from the conditions specified in the protocol but also to identify conditions requiring critical control and to set limits for steps in the protocol.

Based on a fractional factorial design (Table 1), as described by Wernimont *et al.* (1996), seven different experimental factors were selected. Two levels for each factor in eight assay runs were used in the study, i.e., under optimal and sub-optimal conditions. The effect of the sub-optimal condition was determined as the percentage difference between assay parameters (I_{20}, I_{50}, I_{80}) and sample concentrations measured using the optimal and sub-optimal assay conditions (Table 2). The study was carried out on eight plates each with a standard curve for the analysis of three shellfish extracts at two dilutions that fell within the working range of the standard curve. Contaminated shellfish extracts were used as samples for the test because contaminated dolphin blood extracts were not available. Each standard or dilution of shellfish extract was analysed in duplicate.

Table 1. Test design

Factor Value	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8
A or a	A	A	A	A	a	a	a	a
B or b	B	B	b	b	B	B	b	b
C or c	C	c	C	c	C	C	C	c
D or d	D	D	d	d	d	d	D	D
E or e	E	e	E	e	e	E	e	E
F or f	F	f	f	F	F	f	f	F
G or g	G	g	g	G	g	G	G	g
Observed result:	s	t	u	v	w	x	y	z

Summary:
Percentage differences in the quantification of samples determined using both optimal and sub-optimal conditions were, except for one point (16%), below 15%. This indicates that overall the assay is robust in terms of incubation times, temperature and plate shaking during TMB development.

The ruggedness test has, however, identified the incubation *temperature* during the competitive binding step with sample or standard, as a critical factor in effecting assay parameters (I_{20}, I_{50}, I_{80}) and to a lesser extent the incubation *time* for this step.

Reference:
Wernimont, G.T. (1996) Use of statistics to develop and evaluate analytical methods, Fifth Edition. AOAC International, Gaitersburg, MD, USA.

Table 2. Ruggedness test for the brevetoxin ELISA

	Experimental factor	**Optimal** or sub-optimal condition	Differences determined using optimal and sub-optimal conditions (%)					
			I_{20}	I_{50}	I_{80}	Sample A	Sample B	Sample C
1	Fridge temp. during capture Ab binding step	A: **4°C**						
		a: 10°C	+14	+7	0	+3	+3	0
2	Temp during specific Ab binding step	B: **22°C**						
		b: 30°C	-5	-2	+1	+1	-3	0
3	Incubation time with sample or standard	C: **1 hr**						
		c: 1hr 15min	**+24**	**+16**	+10	+4	+11	**+16**
4	Incubation temp with sample or standard	D: **22°C**						
		d: 30°C	**+43**	**+43**	**+43**	+3	+5	+8
5	Temp. for TMB development	E: **22°C**						
		e: 4°C	+1	+3	+4	+2	+5	-2
6	Time for TMB development	F: **15 min**						
		f: 20 min	+1	-1	+1	+1	+6	-1
7	Shaking for TMB development	G: **Yes**						
		g: No	-11	-3	+6	-5	-6	-4

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Abraxis Comments: Although a difference is seen in curve parameters when the assay is incubated at different temperatures (this is to be expected with any immunoasay), due to the fact that a standard curve is run with each batch of samples, the result obtained on those unknown samples should not change.

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Within-run and Between-run Variability Studies
Brevetoxin ELISA

1. Within-run Variability

Five methanolic extracts of Green-lipped mussels (*Perna canaliculus*) were analysed on ten different days to determine within-run variability for the ELISA. Four replicates of each sample were analysed and the extracts were stored at -20°C between assays.

The within-run variability (RSD_R, i.e., residual standard deviation repeatability) was calculated for each concentration measurement and an overall mean RSD_R was calculated for each run. Finally, an overall mean for the 10 days was calculated. The within-day repeatability for all analyses of the extracts, ranged from 4.0 to 11.6% with an overall mean RSDr value of 6.6%.

Table 1. Within-run variability for the analysis of shellfish extracts

# of runs	Replicates (n)	Sample ID	Mean PbTx-3 equivalents (ng/mL)	SD	RSD_R (%)	Mean RSD_R (%)	Overal RSD_R (%)
1	4	1	1967	75	4		
	4	2	1307	72	6		
	4	3	1217	61	5		
	4	4	180	12	7		
	4	5	25	1	5		
						5.2	
2	4	1	2145	142	7		
	4	2	2019	191	10		
	4	3	1324	86	7		
	4	4	211	33	16		
	4	5	25	2	7		
						9.0	
3	4	1	1813	136	20		
	4	2	1368	117	9		
	4	3	1251	51	4		
	4	4	159	30	19		
	4	5	23	2	7		
						11.6	
4	4	1	1897	106	6		
	4	2	1639	49	3		
	4	3	1441	39	3		
	4	4	244	14	6		
	4	5	27	1	3		
						4.0	

Table 1. Contd.

# of runs	Replicates (n)	Sample ID	Mean PbTx-3 equivalents (ng/mL)	SD	RSD_R (%)	Mean RSD_R (%)	Overall RSD_R (%)
5	4	1	2153	95	4		
	4	2	1608	66	4		
	4	3	1368	66	5		
	4	4	226	14	6		
	4	5	28	1	3		
						4.6	
6	4	1	2686	112	4		
	4	2	2224	75	3		
	4	3	1934	32	2		
	4	4	294	18	6		
	4	5	23	3	13		
						5.7	
7	4	1	2573	81	3		
	4	2	1997	99	5		
	4	3	2030	120	6		
	4	4	326	25	8		
	4	5	38	3	8		
						5.9	
8	4	1	2462	201	8		
	4	2	1816	124	7		
	4	3	1757	171	10		
	4	4	311	32	10		
	4	5	36	3	8		
						8.7	
9	4	1	2277	145	6		
	4	2	1599	93	6		
	4	3	1518	106	7		
	4	4	261	18	7		
	4	5	29	1	3		
						5.9	
10	4	1	2199	132	6		
	4	2	1624	103	6		
	4	3	1496	48	3		
	4	4	222	10	5		
	4	5	35	1	3		
						4.6	**6.6**

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2. Between-run Variability

The data in Table 1 was combined to calculate the between-day variability for each sample analysed on ten different occasions over a three month period.

Table 2. Between-run variability for the analysis of shellfish extracts

# of runs	Replicates (n)	Sample ID	Overall mean (ng/mL)	SD	RSD_R (%)	Overall RSD_R (%)
10	40	1	2217	288	**13.0**	
10	40	2	1720	292	**17.0**	
10	40	3	1534	283	**18.4**	
10	40	4	243	55	**22.6**	
10	40	5	29	6	**19.1**	**18.0**

The between-day variability (RSD_R, i.e., residual standard deviation reproducibility) for the analysis of brevetoxin concentrations in shellfish ranging from 26 to 1500 ng/mL, was below 18.0 %.

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Importance of Brevetoxin Determination

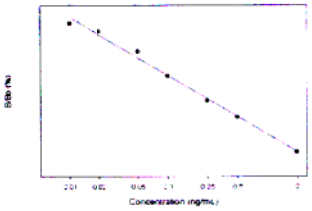
Neurotoxic shellfish poisoning (NSP) is caused by polyether toxins known as Brevetoxins. Brevetoxins (PbTxs) are produced by the dinoflagellate *Karenia brevis*, which causes harmful algal blooms (HABs) know as red tides. The Brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish. Contamination of shellfish with Brevetoxin has been associated with the presence of harmful algal blooms in various parts of the world.

Mortality events attributed to HABs have been documented for fish, manatee, dolphins, and seabirds. In man, NSP causes dose-dependent symptoms of diarrhea, nausea, and vomiting, chills, sweats, reversal of temperature, hypotension, arrhythmias, numbness, tingling, bronchoconstriction, paralysis, seizures, and coma.

The Brevetoxin ELISA allows the determination of 40 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of detection for Brevetoxin is calculated as: Xn +/- 3SD (n=20) and is equal to 0.005 ng/ml in water and 22.5 ng/gm in diluted shellfish (when using a dilution factor of 450). The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.16 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%, CVs for samples: <15%.

Selectivity: This ELISA recognizes Brevetoxin and other NSP toxins to varying degrees:

Cross-reactivities:

PbTx-3	100%
Deoxy PbTx-2	133%
PbTx-5	127%
PbTx-2	102%
PbTx-9	83%
PbTx-6	13%
PbTx-1	5%

No cross-reactivity was shown with any of the following common PSP shellfish toxins: saxitoxin, neosaxitoxin, dc-STX, gonyautoxins-1/4, gonyautoxins-2/3, B-2; B-1; C-1/2 and domoic acid.

Samples: Salt Water and shellfish samples (after recommended dilution) were tested for matrix effects in the ELISA. No matrix effects were determined.

General Limited Warranty: Abraxis LLC warrants the products manufactured by the Company, against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. **Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose**

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R031808

Brevetoxin (NSP) ELISA, Microtiter Plate

ABRAXIS

Enzyme-Linked Immunosorbent Assay for the Determination of Brevetoxin (NSP) in Water and Contaminated Samples

Product No. 520026

1. General Description

The Brevetoxin ELISA is an immunoassay for the quantitative and sensitive detection of Brevetoxin. Brevetoxin is one of the toxins associated with neurotoxic shellfish poisoning (NSP). This test is suitable for the quantitative and/or qualitative detection of Brevetoxin in water samples as well as shellfish samples. For shellfish samples a sample preparation is required. If necessary, positive samples can be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Brevetoxin (PbTx-3). In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Brevetoxin ELISA should to be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Brevetoxin by specific antibodies. Brevetoxin, when present in a sample, and a Brevetoxin enzyme-conjugate compete for the binding sites of sheep anti-brevetoxin antibodies that have been immobilized in the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Brevetoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Brevetoxin ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Brevetoxin ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.) positive samples requiring some action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated with sheep anti-Brevetoxin
2. Standards PbTx-3 (8): 0, 0.010, 0.025, 0.05, 0.1, 0.25, 0.5, 2.0 ng/mL
3. Brevetoxin-HRP Conjugate, 6 mL
4. Sample Diluent (1X), 2 X 30 mL. Use to dilute samples
5. Wash Solution (5X) Concentrate, 100 mL
6. Color Solution (TMB), 12 mL
7. Stop Solution, 2 X 6 mL
8. Sea Water Pre-treatment Solution, 25 mL

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the conjugate, the substrate solution and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard solutions, enzyme conjugate, substrate and stop solution are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
5. The stop solution should be handled with care as it contains diluted H_2SO_4.

C. Assay Procedure

1. Add 50 µL of the **standard solutions or the samples** (water) or **sample extracts** (shellfish) into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of **enzyme conjugate** solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop for about 30 seconds. Be careful not to spill contents. Incubate for 60 minutes.
3. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips three times using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
4. Add 100 µL of **substrate solution** to the wells. The strips are incubated for 30 min at room temperature. Protect the strips from direct sunlight.
5. Add 100 µL of **stop solution** to the wells in the same sequence as for the substrate solution.
6. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the %B/B$_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the %B/B$_0$ for each standard on the vertical linear (y) axis versus the corresponding Brevetoxin concentration on the horizontal logarithmic (x) axis on graph paper. %B/B$_0$ for samples will then yield levels in ppb of Brevetoxin by interpolation using the standard curve. Samples showing lower concentrations of Brevetoxin compared to standard 1 (0.01 ng/mL) are considered as negative. Samples showing a higher concentration than standard 7 (2.0 ng/mL) must be diluted further to obtain more accurate results.

E. Additional Materials (not delivered with the test kit)

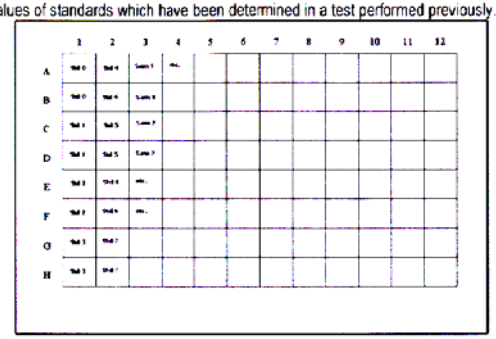
1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wave length 450 nm)
5. Shaker for microtiter plates (optional)

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 7: Standards
0; 0.010; 0.025; 0.05; 0.1; 0.25
0.5; 2.0 (ng/mL) or ppb

Sam1, Sam2, etc.: Samples



G. Preparation of Samples

I. Mussels

NOTE: For regulatory purposes, a 100g sample needs to be used. Extraction solution volume needs to be adjusted accordingly.

1. Mussels are removed from their shells, washed with deionized water, thoroughly dried and homogenized (Waring blender, Polytron or equivalent).
2. A 1.0 gm portion of the homogenized mussels is then placed in a 40 mL glass vial.
3. Add 9.0 mL of a methanol/deionized water solution (9:1 v/v).
4. Vial is capped and hand shaken vigorously for 2 minutes.
5. Centrifuge mixture for 10 minutes at 3000 g. Collect the supernatant.
6. Remove 20 uL of collected extract and dilute to 1.0 mL with Sample Diluent (equals a 1:50 dilution).
7. Analyze diluted extracts as samples (Assay Procedure step 1)

The Brevetoxin concentration contained in the samples is determined by multiplying the concentration of the diluted extract by a factor of 450. Highly contaminated samples outside the range of the curve should be diluted further and re-analyzed.

II. Sea Water

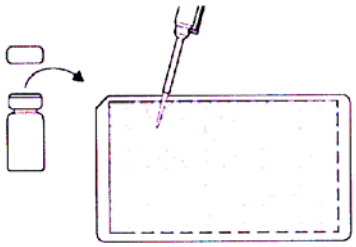
1. Collect 2 mL of sea water sample in a glass container.
2. To prevent loss of Brevetoxin to the glass surface, immediately add 0.5 mL of Sea Water Pretreatment Solution, mix by hand.
3. Analyze preserved sample as samples (Assay Procedure step 1)

The Brevetoxin concentration contained in the sea water sample is determined by multiplying the concentration of the diluted sample by a factor of 1.25. Highly contaminated samples outside the range of the curve should be diluted in Sample Diluent (PN 205226), and re-analyzed. Additional Sample Diluent or Sea Water-Pretreatment Solution (PN 205227) can be purchased from Abraxis.

Brevetoxin (NSP) Plate, Detailed ELISA Procedure

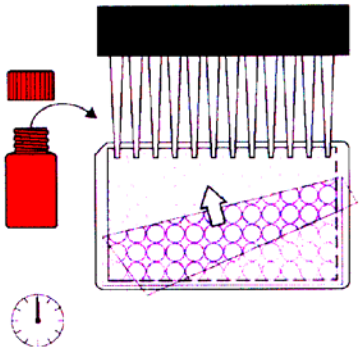
1. Addition of Standards, Samples

Add 50 uL of the standard solutions or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.



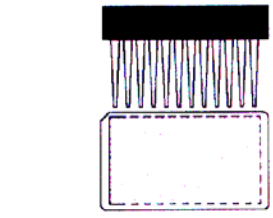
2. Addition of Enzyme Conjugate

Add 50 uL of the enzyme conjugate to the individual wells successively using a multi- channel pipette or a stepping pipette. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 60 minutes at room temperature.

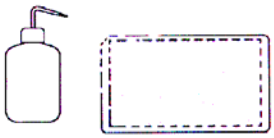


3. Washing of Plates

After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips three times with a multi-channel pipette or wash bottle using the diluted 1X washing buffer solution. Please use at least a volume of 250 uL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.

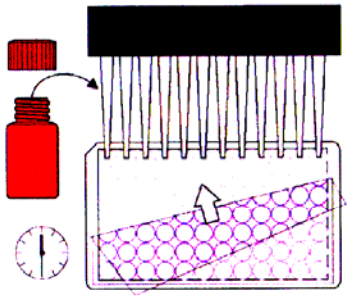


OR



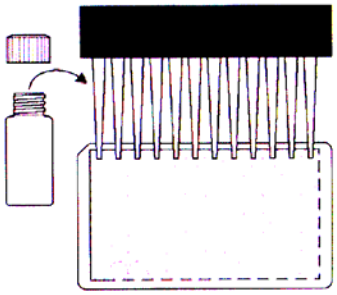
4. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop. Be careful not to spill contents. Incubate the strips for 30 min at room temperature.



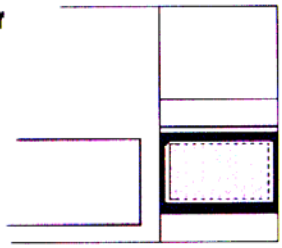
5. Addition of Stopping Solution

Add 100 uL of stop solution to the wells in the same sequence as for the substrate solution using a multi- channel pipette or a stepping pipette.



6. Measurement of Color

Read the absorbance at 450 nm using a microplate ELISA reader. Calculate results.



For Ordering or Technical Assistance Contact:
ABRAXIS, LLC
54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

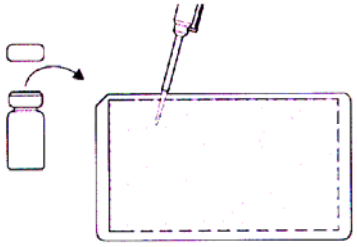
ABRAXIS

Brevetoxin (NSP) Plate Kit Part # 520026

Brevetoxin (NSP) Plate, Concise ELISA Procedure

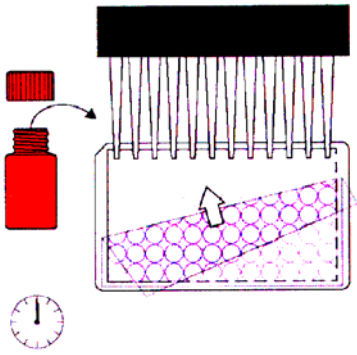
1. Addition of Standards, Samples

Add 50 uL of standard solutions or samples.



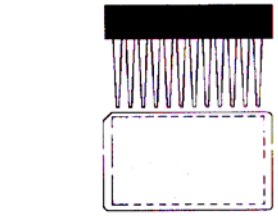
2. Addition of Enyzme Conjugate

Add 50 uL of enzyme conjugate. Cover and mix for 30 seconds by rotating on benchtop. Incubate for 60 minutes at room temperature.

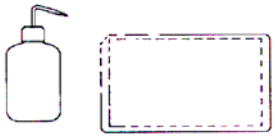


3. Washing of Plates

Wash the plates three times with 250 uL of diluted 1X washing buffer.

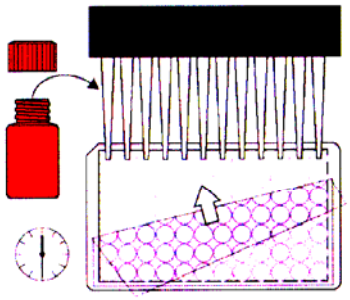


OR



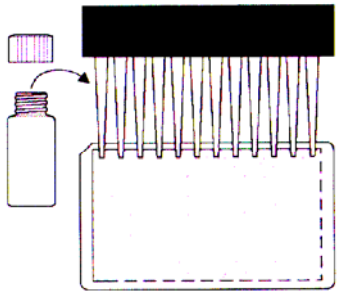
4. Addition of Substrate/Color Solution

Add 100 uL of substrate/color solution. Incubate 30 minutes at room temperature and away from direct sunlight.



5. Addition of Stopping Solution

Add 100 uL of stop solution.



6. Measurement of Color

Measure color at 450 nm. Calculate results.

For Ordering or Technical Assistance Contact:
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54 Steamwhistle Drive, Warminster, PA 18974
Phone: 215-357-3911 Fax: 215-357-5232
www.abraxiskits.com

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Brevetoxin (NSP) Plate Kit Part # 520026