ISSC Method Application Format for Biotoxin Methods: Method Modification

Critical Information:
Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC Laboratory Committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

Note:
The applicant should provide all information and data identified above as well as the following material, if applicable:

Method Overview
Name of the New Method: Modified Application of Receptor Binding Assay to Mussels Monitoring for Paralytic Shellfish Poison using Porcine Brain Membrane Preparation
Specify the Type of Method (e.g., Chemical, Molecular, or Culture): Molecular. The RBA is a functional assay, whereby toxins present in the standard/sample bind to sodium channel preparations in the assay. Radiolabeled toxins are added to solution to compete with toxins present in the standard/sample for binding sites, and thus a decrease in signal from radiolabeled toxins represents a higher standard/sample concentration. This competitive RBA allows for quantitation that directly relates to the composite toxicity of the sample.
Name of Method Developer / Submitter: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield & Kelly Cusick, NOAA, National Ocean Service, Hollings Marine Lab
Developer / Submitter Contact Information [e.g., Address and Phone Number(s)]: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield: 331 Fort Johnson Rd, Charleston, SC 29412 and 843-580-8154
Date of Submission: September 26th, 2026
Purpose and Intended Use of the Method: The receptor binding assay (RBA) is intended for use as an NSSP Approved Method for the quantitative assessment of paralytic shellfish poison (PSP) in mussels using porcine brain membrane preparation. The application of this project is specifically intended for use in

regulatory laboratory settings. The RBA as described herein serves as an alternative to the rat brain membrane preparation in these applications by utilizing commercially available porcine brain, which is a readily available byproduct of the food industry. The assay achieves a reliable measure of integrated toxicity with high throughput, while successfully eliminating the need for dedicated sourcing of rat brains and the need for local laboratory animal euthanasia, this reduces preparation effort and costs.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:
There is an acknowledged need for this method in the NSSP. Even though the MBA, PCOX, and RBA methods have been respectively NSSP Approved and Approved for Limited Use, there remains a need for the proposed method. The RBA would provide an alternative to (1) the MBA, which uses live animals, (2) the PCOX method, which requires costly equipment and skilled personnel and offers low throughput, (3) the RBA method, which requires direct laboratory animal euthanasia to prepare rat brain receptors and also results in higher preparation costs.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable:
n/a

Other Comments

Method Documentation

Method Title:
Modified Application of a Receptor Binding Assay to Mussels Monitoring for Paralytic Shellfish Poison using Porcine Brain Membrane Preparation

Method Scope:
The RBA provides a high throughput, sensitive, accurate, quantitative assay for PSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Method for the purposes of screening for PSP toxicity using porcine brain homogenate as the receptor source instead of rat brain homogenate.

References:
Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic
shellfish toxins in shellfish. Journal of AOAC International 92(6): 1705-1713. See Appendix II.

Van Dolah et al. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor binding assay: Collaborative study. Journal of AOAC International 95(3): 795-812. See Appendix III.

OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org. See Appendix IV.

Principle:
This PSP receptor binding assay is based on the interaction between the toxins and their native receptor, the voltage-gated sodium channel. All paralytic shellfish toxins (PSTs) bind to site 1 of the voltage-gated sodium channels relative to their affinity to the channel (receptor). These affinities are a primary driver of in vivo toxicity and the read-out of the RBA, results in a measure of integrated potency (independent of knowing which toxin congeners are present) similar to mouse intraperitoneal potency. In the RBA, tritiated saxitoxin (3H-STX) competes with unlabeled PSTs in a shellfish extract for a finite number of available receptor sites in a brain membrane preparation. After a binding equilibrium is reached, unbound 3H-STX is removed by filtration and the remaining 3H-STX is measured with a scintillation counter (measured as counts per minute or CPM). The amount of 3H-STX present is indirectly related to the quantity of unlabeled PSTs in the sample. This output is compared to a standard curve prepared from saxitoxin dihydrochloride and is reported at saxitoxin dihydrochloride equivalents agnostic to the specific PSP congener composition of the extract.

Analytes/Measurands:
The target analytes are paralytic shellfish toxins (PSTs), with NIST Saxitoxin Dihydrochloride (RM 8642a) serving as the certified reference standard for generating calibration curves and spikes for shellfish samples.

The target measurand includes mussels, including blue mussels (*Mytilus edulis*) and California mussels (*Mytilus californianus*)

Proprietary Aspects:
None. All reagents can be prepared or purchased.

Equipment:
(Provide a list of specialized equipment needed to perform the method.)
The following list identifies the equipment and supplies needed for conducting the RBA.

<u>For the assay:</u>
(a) Microplate Scintillation counter suitable for measuring low counts of tritium (<1000dpm) (e.g. MicroBeta from Revvity)
(b) An 8-channel pipettor (5-200 µl variable volume and disposable tips)
(c) Micropipettors (1-1000 µl variable volumes and disposable tips)
(d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (MilliporeSigma; Cat. No. MSFB N6B 50)
(e) MultiScreen vacuum manifold (MilliporeSigma; 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) (for buffer preparation)

(n) -80 °C freezer
(o) Refrigerator
(p) pH meter (for buffer preparation)

For sample extraction:
(q) Blender or homogenizer for sample homogenization
(r) Pipets
(s) Centrifuge tubes (15 ml, conical, plastic)
(t) pH meter or pH paper
(u) Hot plate or water bath
(v) Graduated centrifuge tubes (15 ml)
(w) Centrifuge and rotor for 15 ml tubes

Reagents:
(Provide a list of specialized chemicals, reagents, etc. needed to perform the method.)
For the assay:
(a) STX diHCl standards (NIST RM 8642a; National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA]
(b) 3H-STX (0.05 mCi per ml, ≥10 Ci per mmol, ≥90% radiochemical purity; American Radiolabeled Chemicals, St. Louis, MO)
(c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent])
(d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent])
(e) ultrapure water (MilliQ or equivalent)

For microplate counter only:
(f) Ultima Gold liquid scintillation cocktail (Revvity.; Waltham, MA; Cat. No. 6013321 [or equivalent])

For sample extraction:
(g) Hydrochloric acid (HCl; 1.0 and 0.1 N)
(h) Sodium hydroxide (0.1 N)
(i) Water (distilled or deionized [18 μΩ])

Media:
None.

Matrix or Matrices of Interest:
Mussels: Blue mussel (*Mytilus edulis*) and California mussel (*Mytilus californianus*)

Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
A representative shellfish sample should include 12 market size organisms pooled together (should be at least 100 g). Clean the outside of shellfish with running tap water. Open the shell by cutting into the adductor muscle, being careful to not cut or damage the viscera.
Rinse the inside to remove sand and dirt and remove tissue from ~12 organisms. Collect the tissue on a number 10 sieve and allow to drain for ~5 minutes. Remove any obvious pieces of

shell or debris. Transfer meat to blender or homogenizer and blend until homogeneous. This homogenate is then extracted for toxins. For the detailed sample extraction procedure see Sample Extraction in Appendix A. Shellfish homogenates must be tested immediately or stored frozen prior to analysis. Saxitoxin standards must be stored refrigerated and 3H-STX must be stored at -70 °C. The rat brain preparation can be produced in bulk, partitioned into aliquots, and stored long-term at -70 °C until use.

Safety Requirements:
(Describe the safety measures, beyond those of routine laboratory practices, required to perform the method, including personal protective equipment, fume hoods, etc.)
General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and low-level radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Although low levels of radiation are used for this assay, sometimes under an NRC exemption clause for the ARC sourced 3HSTX, users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of radioisotopes. Please see Appendix B for further safety requirements.

Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]):

The estimated cost per 96-well plate assay is ~$235. 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 ~$35. If running multiple plates or in screening mode, sample costs would be reduced. A microplate scintillation counter is needed and the cost is ~$70-250K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K.

Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turnaround time for data in approximately 3 hours. Up to four plates per analyst are possible in one day, yielding a throughput of at least 36 samples per day.

Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.):

The protocol is very clear and easy to follow. Please see the detailed protocol below in Appendix A.

Quality Control (Provide Specific Steps):
Quality control steps are in place to determine if assay results are acceptable:

(a) The slope of the standard curve must be between - 0.8 and -1.2 (theoretical slope is -1). If the slope of a standard curve from a given assay falls outside of this range, the data should be considered unacceptable and the assay must be rerun.

(b) The RSDs of triplicate counts per minute (CPMs) for the standards must be below 30%.

(c) If the IC50 (inhibitory concentration at which CPM is 50% max) is out of the acceptable range (1.75 nM ± 30%), the data should be considered unacceptable and the assay should be rerun.

(d) A QC sample should always be included on each plate and found to be in range. Typically, a 1.8 x 10-8 M STX concentration (3 nM STX in-well concentration) is run as a QC and the measured value should be within 30% of the expected concentration. Results outside of this range should trigger consideration of assay acceptance. The following criteria must be met to accept sample measurement:

(e) For sample measurement, quantitation should only be done on sample dilutions that fall within the linear range. As such, binding (B, measured as counts per minute) scaled by the maximum binding (B_0) should be between 0.2-0.7 for sample quantitation to be performed (any sample falling outside of this range is considered out of the dynamic range). If $B/B_0 > 0.7$, the concentration is too low to be quantified and should be reported as below the limit of detection (LOD). If $B/B_0 < 0.2$, the sample should be diluted and rerun if quantitation is needed.

(f) The RSDs for the sample CPMs should be ≤ 30%. These quality control criteria are also stated in section H in Appendix IV.

(g) The calculated 'top' value (0 nM STX in-well concentration) must be greater than 1,000 CPM. This threshold is required to maintain an adequate signal-to-noise ratio and a sufficient dynamic range, both of which are essential for accurate curve fitting and reliable IC_{50} determination.

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

Please see below for Specificity, Ruggedness, and Comparability results.

Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.).

Please see below for SOP 1, 2, and 3 for data and statistical analyses completed for Specificity, Ruggedness, and Comparability, respectively.

Calculations and Formulas Used for Each Validation Criterion Tested.

Please see below for calculations and formulas used for Specificity, Ruggedness, and Comparability criterions.

Results for Each Validation Criterion Tested. Please see below for results for Specificity, Ruggedness, and Comparability criterions.
Discussion of Each Validation Criterion Tested. Please see below for discussion for Specificity, Ruggedness, and Comparability criterions.
Summary of Results. This validation study evaluates replacing the standard rat brain membrane preparation with a commercially prepared porcine brain membrane preparation in the Receptor Binding Assay (RBA) for Paralytic Shellfish Toxins (PSTs). Across 120 paired naturally incurred mussel samples, the porcine preparation demonstrated near-perfect correlation with the rat preparation ($R^2 = 0.99$). Although a standard paired t-test detected a minor systematic offset where the rat preparation read 5.7% higher on average (+0.024 log units), a Two One-Sided Test (TOST) confirmed that this difference falls cleanly within the predefined biological equivalence bounds of ±20%. Furthermore, the assay proved highly rugged against routine batch-to-batch reagent variation ($t(9)= -0.254$, $p > 0.05$) and demonstrated high specificity, showing no significant interference in the presence of co-occurring domoic acid ($SI_{avg} = 1.01$, $p > 0.05$).

Additional Requirement:
If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval as a modified version of the existing checklist.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2017, Guidance Documents, Chapter II – Growing Areas, .15 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation
Checklists.)

Background:

This submission follows the proposal for extending the Receptor Binding Assay (RBA) to multiple shellfish matrices. The current RBA method uses a rat brain membrane preparation; the proposed method modification replaces the rat brain membrane with a porcine brain membrane preparation. The use of porcine brain membrane homogenate also offers both economic and operational advantages over the current rat brain preparation, provided the porcine preparation demonstrates comparable assay performance. Based on the current testing volume of approximately 1,500 samples over six months at the Washington State Department of Health (WADoH), the porcine preparation costs approximately $0.50 per sample compared with approximately $1.00 per sample for the rat preparation, resulting in an estimated direct savings of $750 over six months. Importantly, the $1.00 per-sample cost for the rat preparation accounts only for direct material costs, including MOPS, rat brains, cryovials, PMSF, and choline chloride, and does not include additional labor, equipment use, consumables, or time required to prepare the rat brain homogenate. In contrast, the commercially prepared porcine brain membrane homogenate substantially reduces the preparation burden and has the potential to provide greater consistency and standardization while reducing dependence on animal sourcing and in-house tissue preparation. Therefore, if the porcine preparation demonstrates comparable receptor-binding performance, sensitivity, precision, and QC results to the rat preparation, its use represents a more cost-effective, deployable, and operationally efficient approach for routine PSP testing while maintaining the analytical purpose and performance of the RBA.

A total of 641 mussel samples were collected for this project, including both samples with little to no detectable PSP-toxins and with PSP-toxins detected using the mouse bioassay (MBA). The ‘blank’ materials used in the study contained very low, but still detectable, PSP-like activity, ranging from less than 2 µg STX/100 g of shellfish tissue to approximately 10 µg STX/100 g of shellfish tissue. Initial screening using the MBA (limit of detection ~40 µg STX/100g of shellfish tissue) identified negative samples, which were subsequently re-screened using a modified RBA with a lower limit of detection of ~2 µg STX/100 g. To achieve the required quantity for the validation study, the identified ‘blank’ materials were combined to produce the 11 homogenates with 41 sub-aliquots needed for spikes at 1/2x 1x, and 2x the regulatory limit (80 ug STX/100 g of shellfish tissue) for the shellfish matrix as shown in the study design (Figure 1). The ‘blank’ sample for each homogenate served as a reference blank to be subtracted from the spiked sub-aliquots after performing RBA to ensure accuracy in measured spiked samples (RBA-Blank)

In total, 403 of 641 PSP samples were below the MBA’s limit of detection from Washington state and were re-screened using a modified extraction procedure (0.5 g sample + 0.5 mL 0.1 N HCl) (Figure 2). For the study, 17 samples were selected for inclusion. While 396 samples were not used for the study, but they are currently being stored in the NOAA Hollings Marine Laboratory’s freezer inventory for future studies (Charleston, SC).

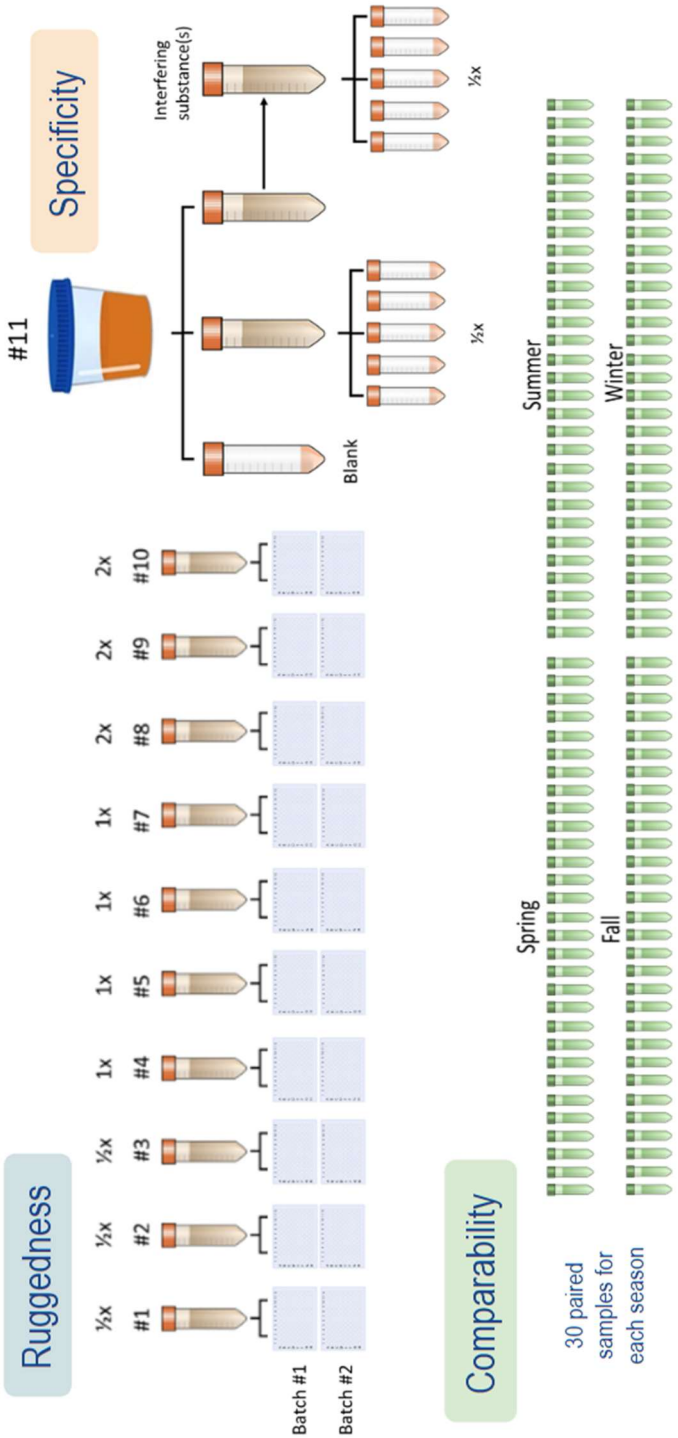


Figure 1. Method Modification study design showing allocation of 121 samples for mussels to meet three validation data criteria: ruggedness (blue), specificity (orange), and comparability (green).

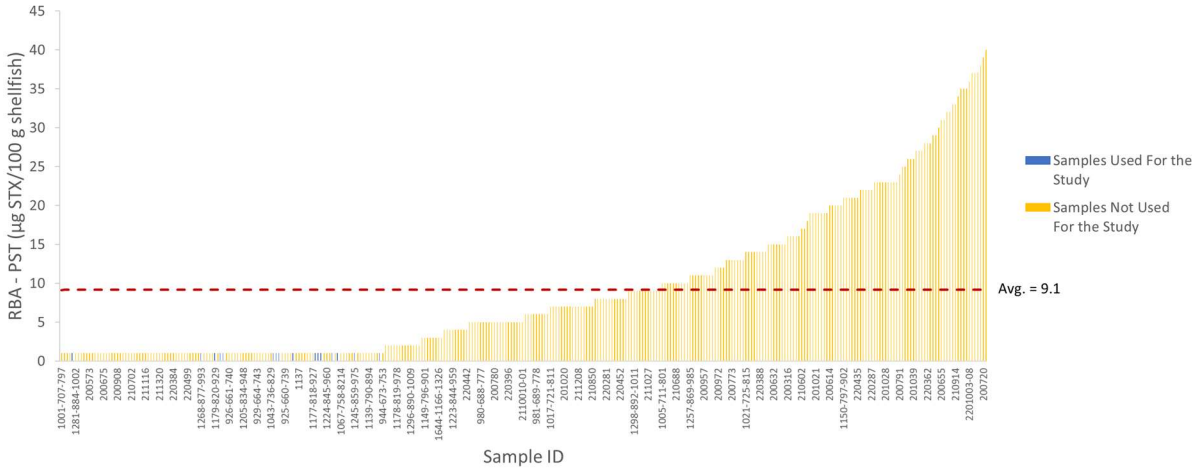


Figure 2. A total of 403 samples from mussel matrix that tested negative for toxins using the MBA (limit = 45 µg STX/100 g shellfish tissue) were re-screened with a modified RBA to identify "blank" samples for spiking in homogenates for the Method Modification study. Average PST values (indicated by the red dashed line) for mussels is 9.1 µg STX/100 g shellfish tissue. Only 17 samples were selected and used for the study (blue), while the remaining samples (n= 396; yellow) are stored at the Hollings Marine Laboratory, Charleston, SC.

Samples were collected across Washington State over various harvest days, locations, and calendar seasons. Seasons were defined as follows: Spring (March 20–June 20), Summer (June 21–September 21), Fall (September 22–December 20), and Winter (December 21–March 19). To obtain the required tissue weight for each homogenate, multiple individual samples collected within 30-days in the same season were combined prior to spiking. Meeting the requested sample size for each season to satisfy the comparability criteria for naturally incurred PSP positive samples was challenging because PSP levels during the winter season were consistently below the MBA limit of detection and therefore unavailable to use for this study. For this study, 120 samples were collected and analyzed, including 87 summer, 20 fall, 13 spring, and 0 winter samples. Although the target of 30 paired samples per season was not achieved, the overall sample-size requirement was met. The seasonal imbalance was attributable to the naturally occurring distribution of PSP and the associated limitations in obtaining spring, fall, and winter samples, rather than insufficient sample effort. The available samples provide broad representation across harvest dates, location, and seasons and are considered adequate to evaluate the conditions relevant to the comparability criteria (Table 1).

Table 1. Summary of mussel tissue samples (blue mussels and California mussels) collected across Washington sites from 2019 to 2025, categorized by group, location, and season. Group letter represents the spiked samples while group number represents the naturally incurred samples.

Group Letter/ Number	Shellfish Matrix	Location	Collection Date	Season
A	blue mussel	South King County, King	6/23/2023	Summer
B	blue mussel	Port Ludlow, Jefferson	5 & 6/2023	Spring
C	blue mussel	Possession Sound, Snohomish	5 & 6/2023	Spring
D	blue mussel	Nisqually Reach, Thurston	5 & 6/2023	Spring
E	blue mussel	Narrows, Pierce	6/1//2023	Spring
F	blue mussel	West Bainbridge, Kitsap	6/22/2023	Summer
G	blue mussel	Sinclair Inlet, Kitsap	6/22/2023	Summer
H	blue mussel	Budd Inlet, Thurston	6/23//2023	Summer
I	blue mussel	North King County, King	6/23/2023	Summer
J	blue mussel	Liberty Bay, Kitsap	6/21/2023	Summer
K	blue mussel	Case Inlet, Mason	5-6/2023	Spring
21-1	blue mussel	Kingston Mariana, Kitsap	8/4/2025	Summer
62-1	blue mussel	Discovery Bay, Jefferson	7/30/2025	Summer
67-1	blue mussel	Ediz Hook, Clallam	7/16/2025	Summer
71-1	blue mussel	Fossil Bay, San Juan	8/7/2024	Summer
72-1	blue mussel	Semiahmoo Mariana, Whatcom	5/21/2024	Spring
73-1	California mussel	Freshwater Bay, Clallam	8/6/2024	Summer
74-1	California mussel	Grays Harbor Coast, Grays Harbor	5/22/2024	Spring
75-1	blue mussel	Fossil Bay, San Juan	7/23/2024	Summer
76-1	California mussel	Clallam Bay, Clallam	7/8/2024	Summer
77-1	blue mussel	Port Ludlow, Jefferson	7/1/2024	Summer
78-1	blue mussel	Sequim Bay, Clallam	8/26/2024	Summer
79-1	blue mussel	Cape George, Clallam	6/25/2024	Summer
80-1	blue mussel	Anacortes Marina, Skagit	7/17/2024	Summer
81-1	blue mussel	Bellingham Bay, Whatcom	6/19/2024	Spring
82-1	California mussel	Long Beach, Pacific	6/10/2024	Spring
83-1	blue mussel	Silverdale, Kitsap	10/21/2024	Fall
84-1	California mussel	South Jetty, Grays Harbor	6/6/2024	Spring
85-1	California mussel	Mocrocks Area, Grays Harbor	6/2/2024	Spring
86-1	blue mussel	Squalicum Harbor, Whatcom	10/21/2024	Fall
87-1	blue mussel	Sequim Bay, Clallam	10/28/2024	Fall
88-1	blue mussel	Ediz Hook, Clallam	11/6/2024	Fall
89-1	California mussel	Neah Bay, Clallam	10/28/2024	Fall
90-1	California mussel	South Jetty, Grays Harbor	5/30/2024	Spring
91-1	blue mussel	Sequim Bay, Clallam	11/4/2024	Fall
92-1	California mussel	Willapa Bay, Pacific	6/5/2024	Spring
93-1	California mussel	Makah Bay, Clallam	6/11/2024	Spring

94-1	blue mussel	Rosario Point, San Juan	10/16/2024	Fall
95-1	California mussel	Neah Bay, Clallam	11/12/2024	Fall
96-1	blue mussel	Rosario Point, San Juan	12/2/2024	Fall
97-1	blue mussel	Squalicum Harbor, Whatcom	6/10/2024	Spring
98-1	blue mussel	Poulsbo Marina, Kitsap	8/4/2025	Summer
99-1	blue mussel	EPA Lab Dock, Kitsap	8/4/2025	Summer
100-1	blue mussel	Penn Cove, Island	7/28/2025	Summer
101-1	blue mussel	Utsalady Bay, Island	7/27/2025	Summer
102-1	blue mussel	Brownsville, Kitsap	7/14/2025	Summer
103-1	blue mussel	South Egler Bay, Island	7/16/2025	Summer
104-1	blue mussel	Dockton, King	7/28/2025	Summer
105-1	blue mussel	Ediz Hook, Clallam	7/31/2025	Summer
106-1	blue mussel	Dockton, King	7/2/2025	Summer
107-1	blue mussel	Port Orchard Marina, Kitsap	8/11/2025	Summer
108-1	blue mussel	Silverdale, Kitsap	8/4/2025	Summer
109-1	blue mussel	Utsalady Bay, Island	8/6/2025	Summer
110-1	blue mussel	Quartermaster Harbor, King	8/4/2025	Summer
111-1	blue mussel	Port Washington Marina, Kitsap	8/11/2025	Summer
112-1	blue mussel	Kingston Marina, Kitsap	7/15/2025	Summer
113-1	blue mussel	Gig Harbor, Pierce	8/7/2025	Summer
114-1	blue mussel	Poulsbo Marina, Kitsap	7/14/2025	Summer
115-1	blue mussel	Poulsbo Marina, Kitsap	7/28/2025	Summer
116-1	blue mussel	South Egler Bay, Island	7/28/2025	Summer
117-1	blue mussel	Brownsville, Kitsap	7/28/2025	Summer
118-1	blue mussel	Brownsville, Kitsap	8/4/2025	Summer
119-1	blue mussel	Penn Cove, Island	8/3/2025	Summer
120-1	California mussel	Bay Center Dock, Pacific	7/2/2025	Summer
121-1	blue mussel	Discovery Bay, Jefferson	8/4/2025	Summer
122-1	blue mussel	Kingston Marina, Kitsap	7/28/2025	Summer
124-1	blue mussel	Penn Cove, Island	7/24/2025	Summer
126-1	blue mussel	South Lopez, San Juan	8/9/2024	Summer
127-1	blue mussel	President Channel, San Juan	8/9/2024	Summer
128-1	blue mussel	President Channel, San Juan	8/23/2024	Summer
129-1	blue mussel	Kingston, Kitsap	9/12/2023	Summer
130-1	California mussel	East Straits, Clallam	8/21/2024	Summer
131-1	blue mussel	Sequim Bay, Clallam	9/18/2024	Summer
132-1	blue mussel	Port Susan, Island	8/22/2024	Summer
133-1	blue mussel	East Bainbridge, Kitsap	6/18/2024	Spring
134-1	blue mussel	West Fidalgo Island, Skagit	7/25/2024	Summer
135-1	California mussel	Oregon	6/25/2024	Summer
136-1	blue mussel	Port Townsend, Jefferson	8/28/2024	Summer
138-1	blue mussel	Sequim Bay, Clallam	9/8/2023	Summer
139-1	blue mussel	Lopez Sound, San Juan	8/23/2024	Summer

140-1	blue mussel	West Sound, San Juan	8/23/2024	Summer
141-1	blue mussel	Kilisut Harbor, Jefferson	7/26/2024	Summer
142-1	blue mussel	Kilisut Harbor, Jefferson	8/28/2024	Summer
143-1	blue mussel	Hood Canal #3, Jefferson	8/27/2024	Summer
144-1	blue mussel	Penn Cove, Island	8/19/2024	Summer
145-1	blue mussel	Birch Bay, Whatcom	8/7/2024	Summer
146-1	blue mussel	Saratoga Passage, Island	8/29/2024	Summer
147-1	blue mussel	Eagle Harbor, Kitsap	7/14/2025	Summer
148-1	blue mussel	Sequim Bay, Clallam	9/7/2022	Summer
149-1	blue mussel	Kingston, Kitsap	8/19/2024	Summer
150-1	blue mussel	Sequim Bay, Clallam	10/25/2022	Fall
151-1	blue mussel	Sequim Bay, Clallam	8/27/2024	Summer
152-1	California mussel	Clallam Coast, Clallam	10/1/2019	Fall
153-1	blue mussel	Sequim Bay, Clallam	10/18/2022	Fall
154-1	blue mussel	Port Susan, Island	8/1/2024	Summer
155-1	blue mussel	Saratoga Passage, Island	8/5/2024	Summer
156-1	blue mussel	Sequim Bay, Clallam	9/11/2023	Summer
157-1	blue mussel	Saratoga Passage, Island	8/14/2024	Summer
158-1	blue mussel	Liberty Bay, Kitsap	9/30/2019	Fall
159-1	blue mussel	Bellingham Bay, Whatcom	6/19/2024	Spring
160-1	blue mussel	Port Ludlow, Jefferson	8/30/2021	Summer
161-1	blue mussel	Sequim Bay, Clallam	10/25/2022	Fall
162-1	blue mussel	Sequim Bay, Clallam	8/23/2021	Summer
163-1	blue mussel	Discovery Bay, Jefferson	7/30/2024	Summer
164-1	blue mussel	Penn Cove, Island	7/17/2025	Summer
165-1	blue mussel	Saratoga Passage, Island	7/23/2024	Summer
166-1	blue mussel	Lopez Sound, San Juan	7/23/2024	Summer
167-1	blue mussel	Sequim Bay, Clallam	9/15/2022	Summer
168-1	blue mussel	Kingston, Kitsap	9/18/2023	Summer
169-1	blue mussel	San Juan Channel, San Juan	8/22/2024	Summer
170-1	blue mussel	Kilisut Harbor, Jefferson	8/19/2024	Summer
171-1	blue mussel	West Fidalgo Island, Skagit	9/3/2024	Summer
172-1	blue mussel	Kingston, Kitsap	9/13/2021	Summer
173-1	blue mussel	Kingston, Kitsap	8/12/2024	Summer
176-1	blue mussel	Sequim Bay, Clallam	10/1/2019	Fall
177-1	blue mussel	Liberty Bay, Kitsap	8/14/2023	Summer
178-1	blue mussel	Sequim Bay, Clallam	10/2/2019	Fall
179-1	blue mussel	Bellingham Bay, Whatcom	8/29/2024	Summer
180-1	California mussel	Grays Harbor Coast, Grays Harbor	8/3/2023	Summer
181-1	blue mussel	Discovery Bay, Jefferson	10/4/2021	Fall
182-1	blue mussel	Port Ludlow, Jefferson	6/5/2023	Spring
183-1	blue mussel	Sequim Bay, Clallam	9/23/2024	Fall
184-1	blue mussel	Port Ludlow, Jefferson	8/16/2021	Summer

185-1	California mussel	West Straits, Clallam	10/6/2021	Fall
186-1	blue mussel	East Sound, San Juan	8/9/2022	Summer
187-1	blue mussel	East Straits, Clallam	9/11/2024	Summer
188-1	blue mussel	East Straits, Clallam	8/17/2021	Summer
189-1	blue mussel	Sequim Bay, Clallam	11/1/2022	Fall
190-1	blue mussel	Drayton Harbor, Whatcom	9/7/2021	Summer
191-1	blue mussel	Sequim Bay, Clallam	9/6/2022	Summer
192-1	blue mussel	Bellingham Bay, Whatcom	7/28/2022	Summer

IX. #1 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Comparing Methods

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:
Sample type _____

Date	Sample/Station #	Conc. Recognized method	Conc. Alternative Method
	1		
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9		
	10		
	n		

n is the last sample in the comparison

For shellfish samples, repeat for each tissue type of interest

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method __RBA with **Rat** Brain Membrane Preparation = 0.55_____

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method _____RBA with **Porcine** Brain Membrane Preparation = 0.44_____

Variance of the data generated from the officially recognized analytical method __RBA with **Rat** Brain Membrane Preparation = 0.23_____

Variance of the data generated from the proposed alternative analytical method __RBA with **Porcine** Brain Membrane Preparation = 0.22_____

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____1.02_____

Is there a significant difference between the analytical methods Y/N → **paired t-test results: t = 3.74, p = 0.00028, df = 119**

Following the data handling procedure and utilizing log-transformed data, the initial paired t-test confirmed a significant difference between porcine and rat brain membrane preparations ($t(119) = 3.74$, $p<0.001$). This result is primarily an artifact of high statistical power from the large sample size (n=120) rather than a meaningful biological discrepancy. In null hypothesis significance testing (NHST), a paired t-test evaluates whether the mean difference between two methods is zero. Consequently, as sample size increases, the standard error decreases, resulting in large a t-value and a correspondingly small p-value; therefore, biologically irrelevant differences will likely reject the null hypothesis. Because a t-test evaluates difference rather than functional equivalence, relying on it alone risks an inappropriate rejection of a method substitution due to the test's over-sensitivity to irrelevant differences. To overcome this limitation, three complementary analyses such as linear regression, Bland-Altman agreement, and Two One-Sided Test (TOST), were performed to assess relative affinity, bias magnitude, and true analytical equivalence against predefined regulatory thresholds.

Linear regression analysis was conducted to evaluate how PST binding values vary between the two tissue types (porcine versus rat). The model demonstrated a near-perfect linear relationship ($R^2 = 0.99$), confirming that PSTs binding tightly to rat voltage-gated sodium channels exhibit the same relative affinity towards porcine sodium channels (Figure 3). However, a high correlation can mask proportional bias, so a Bland-Altman agreement analysis was performed. The analysis revealed a minor mean systematic bias of +0.024 log units (~ +5.7%), indicating that the assay using rat brain membrane preparation results in values on average 0.024 log units higher than the assay using porcine brain membrane preparation (Figure 4). In routine RBA for PST detection, inter-assay variability of ±10-20% is standard practice; thus, a systematic shift of 5.7% has a minimal effect on analytical accuracy and regulatory decision-making.

A TOST was performed to assess biological equivalence between assays using porcine versus rat brain membrane preparations. This test determines whether the mean difference between methods falls within a predefined, biologically acceptable interval. An equivalence margin of ± 0.097 log units (approximately 20% difference) was predefined for the analysis. The observed mean difference (+0.024 log units) and its 90% confidence interval fell within the upper and lower equivalence bounds, demonstrating a statistically significant equivalence ($t(119) = -11.0$, $p <0.01$; Figure 5). While the porcine brain membrane preparation yielded slightly lower average results than the rat brain membrane preparation (+5.7%), however, this minor variation represents a predictable baseline offset rather than a biological divergence. Because the relative compound affinities remained nearly identical ($R^2 = 0.99$) and absolute discrepancies fell within established equivalence bounds, porcine brain membrane preparation is a validated, functionally equivalent, scalable, and ethically favorable alternative to rat brain tissue for routine RBA to detect PSTs in mussels.

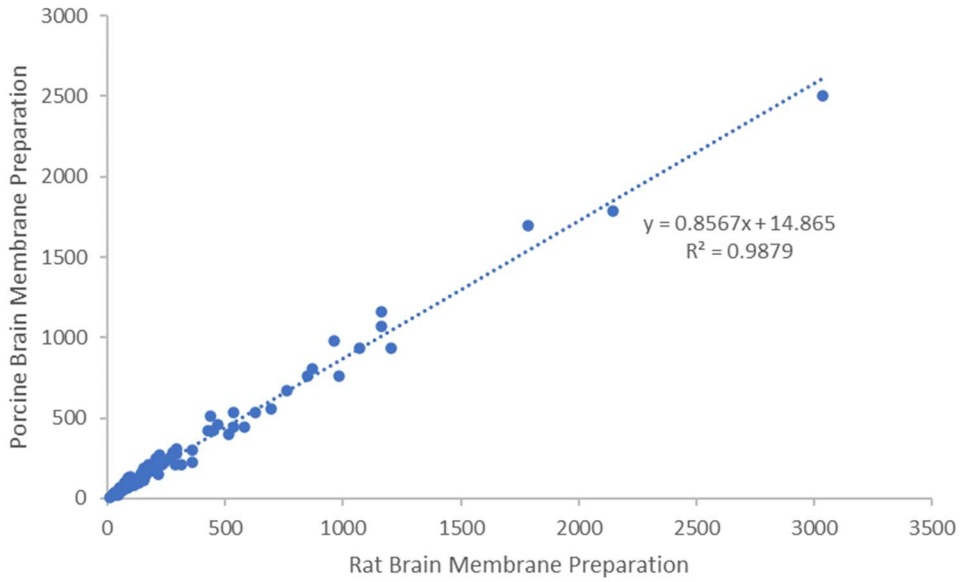


Figure 3. Scatterplot of comparing saxitoxin dihydrochloride equivalents (µg STX diHCl equiv./100 g shellfish) in naturally incurred mussel samples measured across seasons, harvest areas, and dates between the officially recognized method (rat brain membrane preparation) and the proposed alternative analytical method (porcine brain membrane preparation). The linear regression line (y = 0.86x + 14.87) and correlation coefficient (R^2 = 0.99) are shown.

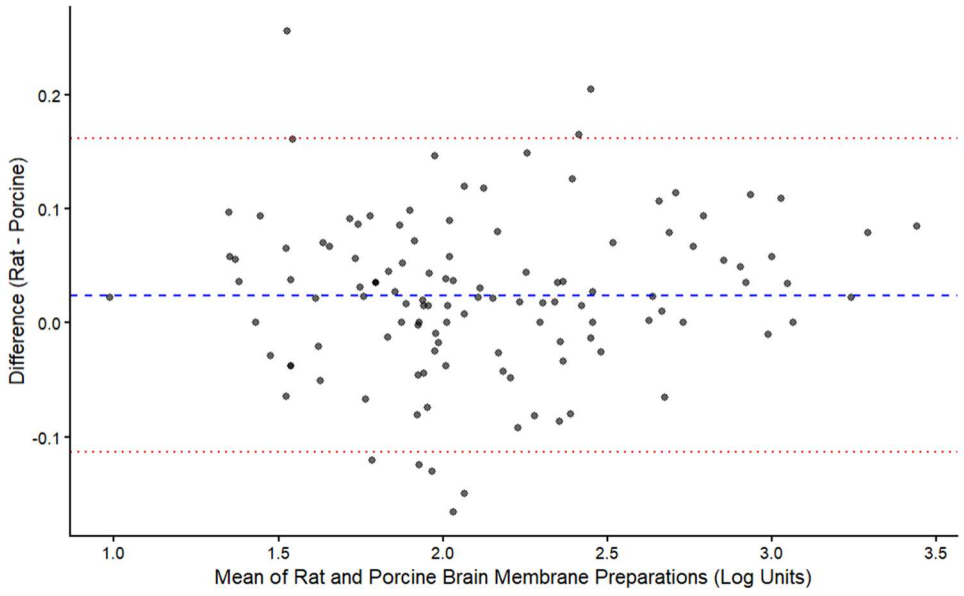


Figure 4. Bland-Altman plot assessing agreement between rat and porcine brain membrane preparations for Paralytic Shellfish Toxins (PSTs). Differences between paired log-transformed measurements (Rat - Porcine) are plotted against their paired mean values. The blue dashed line represents the mean systematic bias (+0.024 log units) and the red dashed lines represent the 95% limits of agreement (-0.113 to +0.161 log units).



Figure 5. A Two One-Sided Test (TOST) equivalence plot for rat vs. porcine brain membrane preparations in the RBA for PSTs. The black circle represents the mean difference (+0.024 log units), and the horizontal line represents the 90% confidence interval (+0.011 to +0.037 log units). Vertical dashed lines denote the predefined biological equivalence bounds ± 0.10 log units, corresponding to approximately ± 20% variance.

Table 2. PST values reported for each sample using two methods for RBA: rat brain membrane preparation and porcine brain membrane preparation (µg STX diHCl equiv./100 g shellfish).

Sample ID	Rat Brain Membrane Preparation	Porcine Brain Membrane Preparation
71-1	61	50
72-1	74	69.5
73-1	469	458
74-1	447	424
75-1	72	65
76-1	133	101
77-1	82.5	98
78-1	438	509
79-1	112	103
80-1	65	60
81-1	268	259
82-1	277	286
83-1	33	36
84-1	581	447
85-1	960	983
86-1	152	170
87-1	65	60
88-1	188	170
89-1	117	115
90-1	1206	938
91-1	81.5	67
92-1	143	152
93-1	223	214
94-1	92	89
95-1	105	101.5
96-1	76	91.5

97-1	89	86
98-1	223	241
99-1	31	25
100-1	357	304
101-1	80	108
102-1	197	197
103-1	98	107
104-1	223	268
105-1	161	134
106-1	29	31
107-1	36	31
108-1	54	63
109-1	65	60
110-1	80	89
111-1	58	51
112-1	152	188
113-1	95	99
114-1	89	71
115-1	286	214
116-1	232	214
117-1	107	98
118-1	357	223
119-1	871	804
120-1	10	9.5
121-1	514	402
122-1	205	197
124-1	286	286
126-1	83	92
127-1	58	47
128-1	112	98
129-1	3037	2501
130-1	1072	938
131-1	42	29
132-1	116	94.5
133-1	73.5	98
134-1	27	27
135-1	1161	1072
136-1	84.5	84.5
138-1	983	759
139-1	98	138.5
140-1	80	71
141-1	36	33
142-1	58	54
143-1	42	40

144-1	214	152
145-1	49	42
146-1	45	25
147-1	89	75.5
62-1	89	130.5
67-1	152	116
21-1	131.5	125
148-1	79	76
149-1	536	447
150-1	313	214
151-1	536	536
152-1	146	139
153-1	103	103
154-1	41	43
155-1	424	422
156-1	759	670
157-1	625	536
158-1	84	84.5
159-1	175	168
160-1	92	97.5
161-1	145	160
162-1	25	23
163-1	59	56
164-1	112	80
165-1	75	75
166-1	242	223
167-1	95.5	86.5
168-1	2144	1787
169-1	173	209
170-1	293	311
171-1	53	70
172-1	692	558
173-1	849	759
176-1	1787	1697
177-1	47	40
178-1	1161	1161
179-1	40	45
180-1	25	20
181-1	89	85
182-1	31	36
183-1	24	21
184-1	25	22
185-1	223	232
186-1	134	125

187-1	94	96
188-1	67	69
189-1	295	277
190-1	33	36
191-1	205	250
192-1	67	54

IX. #2 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Ruggedness

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of the new or modified method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals. For each sample take two aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine new method/modified method concentration for the target analyte/measurand/ organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike sample aliquots. Both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days if possible.

Data for demonstrating the ruggedness of the new or modified method:

Sample type _____

Sample	Conc. "Batch or lot 1" Media and/or Reagents	Conc. "Batch or lot 2" Media and/or Reagents
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method
In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 greater than 2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the statistical analysis based on the following considerations.

- If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the statistical analysis.
- If the distributions of the data from batch/lot1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the statistical analysis.
- If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the statistical analysis.
- If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both; but, the data lacks homogeneity of variance, use Welch's t-test to statistically analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

Value for the test of symmetry of the distribution of batch/lot 1 __-0.021_____

Value for the test of symmetry of the distribution of batch/lot 2 ____-0.039_____

Variance of batch/lot 1 data ___0.054_____

Variance of batch/lot 2 data ____0.055_____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 ___1.03____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples Y/N → **paired t-test results: t = -0.254, df = 9, p = 0.805**

To evaluate assay ruggedness under SOP #2 criteria, two distinct multi-well microplates were analyzed as Batch 1 and Batch 2 to test the inter-plate variability and consistency of saxitoxin dihydrochloride (µg STX diHCl equiv./100 g shellfish) detection in spiked mussel samples (n = 10). Assay values across three target spike concentrations (40, 80, and 160 (µg STX diHCl equiv./100 g shellfish) demonstrated strong inter-batch agreement, exhibiting a linear regression relationship of $y = 0.94x + 7.33$ and a correlation coefficient of $R^2 = 0.87$ between Batch 1 and Batch 2 (Figure 6, Table 3). This close alignment between distinct assay plates confirms that the receptor binding assay maintains reliable operational performance and robust analytical consistency despite normal inter-plate execution variables.

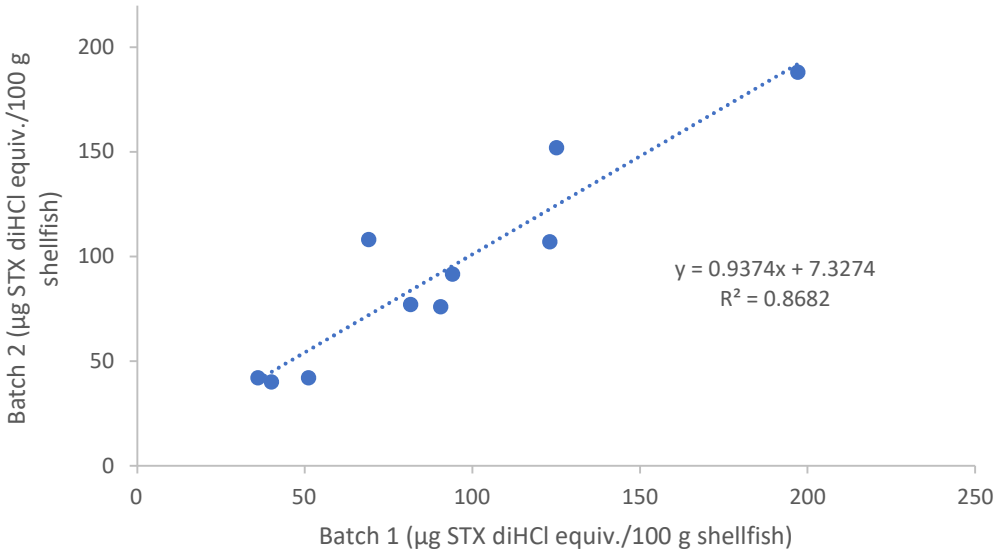


Figure 6. Scatterplot of comparing saxitoxin dihydrochloride (µg STX diHCl equiv./100 g shellfish) in spiked mussel samples measured across two batches (Batch 1 vs. Batch 2), showing the linear regression line ($y = 0.94x + 7.33$) and correlation coefficient ($R^2 = 0.87$).

Table 3. Comparison of target spike concentrations (µg STX diHCl equiv./ 100 g shellfish) and receptor binding assay values in spiked mussel samples across two assay batches (Batch 1 vs. Batch 2).

Sample	Expected Concentration (µg STX diHCl equiv./ 100 g shellfish)	Batch 1 (µg STX diHCl equiv./ 100 g shellfish)	Batch 2 (µg STX diHCl equiv./ 100 g shellfish)
1	40	40	40
2	40	51	42
3	40	36	42
4	80	90.5	76
5	80	81.5	77
6	80	69	108

7	80	94	91.5
8	160	197	188
9	160	123	107
10	160	125	152

IX. #3 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Specificity

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank, the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:
Name of suspected interfering organism/compound/toxin #1 **domoic acid**

Sample type **blue mussel**

Sample blank concentration for the target analyte/measurand/organism of interest **NIST Saxitoxin Dihydrochloride (RM 8642a)**

	Conc. of aliquot spiked with target analyte/measurand/organism of interest interest	Conc. of aliquot spiked with target analyte/measurand/organism of of interest in the presence of suspected interfering organism/compound/toxin
Replicate 1		
2		
3		
4		
5		

Repeat for each suspected interfering organism/compound/toxin tested.

Data handling for demonstrating specificity of the new or modified method
The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SIavg) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Interfering organism/compound/toxin #1- domoic acid $SI_{avg} = 1.01$

SI different from 1 $p = 0.70$

Specificity was evaluated to determine whether the co-occurrence of domoic acid (DA), a non-target marine toxin, interferes with the quantitation of saxitoxin dihydrochloride (STX diHCl) in the RBA using porcine brain membrane preparation. Across five experimental replications comparing STX diHCl alone versus STX diHCl in combination with DA, the mean Specificity Index SI_{avg} was 1.01, showing no statistically significant deviation from the ideal value of 1.00 ($p > 0.05$, Table 4). Further validation in blue mussel samples spiked at 40 µg STX diHCl equiv./100 g shellfish demonstrated consistent toxin recovery regardless of matrix co-fortification with DA (0.02 µg DA equiv./g shellfish). Specifically, samples fortified with STX diHCl alone an average RBA concentration of 38.4 µg STX diHCl equiv./100 g shellfish, while samples co-fortified with both STX diHCl and DA yielded an equivalent average of 36.2 µg STX diHCl equiv./100 g shellfish (Table 5). The blank homogenate (MBk-00) produced zero detectable signal, confirming no baseline matrix interference. Together, these data demonstrate

high method specificity, proving that DA does not cross-react or cause analytical interference during PST quantitation.

Table 4. Specificity index (SI) values derived from log-transformed toxicity concentrations of saxitoxin dihydrochloride (STX diHCl) alone and in combination with domoic acid (DA) across five experimental replications.

Replication	log (STX diHCl)	log (STX diHCl & DA)	Specificity Index (SI)
1	1.60	1.60	1.00
2	1.68	1.59	1.06
3	1.62	1.46	1.11
4	1.45	1.60	0.90
5	1.53	1.52	1.01
Average	1.58	1.56	1.01

Table 5. Blue mussel homogenate samples collected from Case Inlet, Mason County, Washington, in May–June 2023 (spring season), and spiked with saxitoxin (STX diHCl) and/or domoic acid (DA) standards. Samples MBk-01 through MBk-05 were fortified with NIST Saxitoxin Dihydrochloride (RM 8642a) at 40 µg STX diHCl equiv./100 g shellfish, while samples MBk-06 through MBk-10 were fortified with both Biosense Laboratories AS domoic acid standards (#A31300401-06) at 0.02 µg DA equiv./g shellfish and NIST Saxitoxin Dihydrochloride (RM 8462a) at 40 µg STX diHCl equiv./100 g shellfish. MBk-00 was not spiked with either toxin and served as a blank to correct for any matrix effects. The table reports the final concentration of ug STX diHCl equiv/100 g shellfish using RBA.

Sample ID	STX diHCl Spike (µg STX diHCl equiv./ 100 g shellfish)	DA Spike (µg DA equiv./ g shellfish)	RBA (µg STX diHCl equiv./ 100 g shellfish)
MBk-00	0	0	0
MBk-01	40	0	40
MBk-02	40	0	48
MBk-03	40	0	42
MBk-04	40	0	28
MBk-05	40	0	34
MBk-06	40	0.02	40
MBk-07	40	0.02	39
MBk-08	40	0.02	29
MBk-09	40	0.02	40
MBk-10	40	0.02	33

Standard Operating Procedure (SOP)
Appendix I

Method Modification: Step-by-Step Procedure

A. Sample Homogenate
    a. Each sample that was below the detection limit from the MBA (~40 µg STX/100g of shellfish) underwent mini saxitoxin extraction to be pre-screened by the RBA to detect true negative samples (<10µg/100g).
        i. Mini saxitoxin extraction was performed using the same protocol as Appendix A (Section A. Sample Extraction), by performing a 1:1 ratio of 0.5 g of tissue and 0.5 mL of 0.1 N HCl.
    b. Once the negative samples were identified, samples were combined to produce 30 sub-aliquots (~130 g of tissue) needed for spiking five homogenates for each shellfish matrix.
        1. We doubled the number of tissue samples for this study to maintain a backup supply, which safeguards against potential future issues such as lost, broken, or spilled samples.
        ii. To combine samples for one homogenate, it followed these criteria:
            1. The sample collection dates must be within a 30-day range and occur within the same season.
                a. Due to the limited availability of cockle tissue samples, collection dates must be within a 90-day window and fall within the same season.
            2. The samples must be harvested from the same water system.
        iii. Homogenates were distinct, reflecting differences based on the season of collection, harvest area, or year.
    c. The positive samples that have MBA data (>40 µg STX/100g of shellfish) were distinct, reflecting differences based on the season of collection, harvest area, or year.
    d. All homogenates were blended by a blender and/or a homogenizer to liquify the homogenate for spikes.
    e. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes for each spike level:
        i. 40 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
        ii. 80 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
        iii. 160 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
B. Spike Procedure
    a. Every homogenate was spiked with RM 8642a FDA Saxitoxin Dihydrochloride Solution (103 µg/g ± 4 µg/g)
    b. Prior to spiking the pre-weighed tissue, the laboratory technician gravimetrically verified the dispensed volume of water to ensure the spiking accuracy was within 3% of the designated volume.
    c. The volume of the RM 8642a FDA saxitoxin dihydrochloride solution was calculated based on the desired concentration and tissue weight available.
        i. 40 µg STX/100g of shellfish: 47 µL

ii. 80 µg STX/100g of shellfish: 93 µL
iii. 160 µg STX/100g of shellfish: 186 µL

d. Once spiked, each aliquoted homogenate was vortexed for approximately 2 minutes to ensure complete mixture of the spike and tissue.
e. Following the mixing of the sample, each sample was again aliquoted into 5 ± 0.3 grams, thereby achieving a minimum of three replicates for each spike level.
    i. Blank samples were not spiked and aliquoted into 5 ± 0.3 grams.
f. At least 16 samples for each homogenate (5) were frozen at -20°C (total of 80 aliquots).
    i. In addition,10 more homogenates from different seasons, harvest areas, and years were spiked to a concentration of 80 µg STX/100g of shellfish and aliquoted into 5 ± 0.3 grams (20 aliquots).
    ii. 20 naturally incurred samples were aliquoted into 5 ± 0.3 grams.
g. All samples, except for backups, were shipped to Washington State Department of Health for RBA using the protocol listed in Appendix A.

C. Spike Procedure for Interfering Toxin
a. Domoic acid and STX diHCl spike
    i. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes with the weight of 28.8 grams.
    ii. Using the domoic acid standard at 0.02 ppm, 7.2 mL of the domic acid standard was added to the tissue sample.
    iii. The addition of 7.2 mL of domoic acid standard resulted in a total sample mass/volume basis of approximately 36 grams and provided the target domoic acid concentration of 0.02 ug/g.
    iv. 140 uL of the STX diHCl standard was added to the sample.
    v. The sample was mixed thoroughly.
    vi. The final sample contained 0.02 ug/g domoic acid and 40 ug STX diHCl/100g shellfish.
b. STX diHCl Only Spike
    i. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes with the weight of 28.8 grams.
    ii. 7.2 mL of 10% acetonitrile in water was added to the sample in place of the domoic acid standard.
    iii. The sample was mixed thoroughly.
    iv. 140 uL of the STXdiHCl standard was added to the sample.
    v. The sample was mixed thoroughly to ensure uniform distribution of the spike.
    vi. The resulting sample contained a total of 36 grams and is targeted to contain 40 ug STX diHCl/100 g shellfish.
c. Following the mixing of the sample, each sample was again aliquoted into 5 ± 0.3 grams, thereby achieving a minimum of five replicates.
    i. Blank samples contained 7.2 mL of 10% acetonitrile in water and aliquoted into 5 ± 0.3 grams.

Appendix A: RBA Step-by-Step Procedure

A. Sample Extraction
   a. The extraction detailed below represents a small scale MBA procedure. The actual MBA extraction could be used instead of the small scale version described here.
   b. Accurately weigh 5.0 g of tissue homogenate into a tared, labeled 15 ml conical tube.
   c. Add 5.0 ml of 0.1 M HCl, vortex, and check pH.
      i. If necessary, adjust pH to 3.0-4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing.
   d. Place the tube in a beaker of boiling water on hot plate (or in a water bath) for 5 min with the caps loosened.
   e. Remove and cool to room temperature.
   f. Check pH and, if necessary, adjust cooled mixture to 3.0-4.0 as described above.
   g. Transfer entire contents to a labeled, graduated centrifuge tube and dilute volumetrically to 10 ml.
   h. Gently stir contents to homogeneity and then allow to settle until a portion of supernatant is translucent and can be decanted free of solids.
   i. Pour 5-7 ml of the translucent supernatant into a labeled centrifuge tube.
   j. Centrifuge at 3000 x g for 10 min.
   k. Retain clarified supernatant and transfer to a clean, labeled centrifuge tube.
   l. Store extracts at -20 °C until tested in RBA.

B. Preparation of Stock Solutions and Standards
   a. Assay buffer: 100 mM MOPS/100 mM choline chloride, pH 7.4
      i. Weigh 20.9 g MOPS and 13.96 g choline chloride and add to 900 ml distilled or milli-Q water.
      ii. Adjust pH to 7.4 with NaOH while stirring.
      iii. Bring to a final volume of 1 L with distilled or milli-Q water.
      iv. Store at 4 °C.
   b. Radioligand solution: 3H-STX
      i. Calculate the concentration of 3H-STX stock provided by the supplier. Suppliers generally provide specific activity in Ci/mmol (~10-30 Ci/mmol) and activity in mCi/ml (~0.05-0.1 mCi/ml), from which the molar concentration can be calculated.
      ii. Prepare 4 ml of a 15 nM working stock of 3H-STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate.
      iii. Measure total counts of each working stock prior to running an assay. Add 36 µl of working stock 3H-STX in buffer to a liquid scintillation counter vial with 4 ml scintillant and count on a traditional liquid scintillation counter to confirm correct dilution. The CPM should be consistent and within 15% of expected value.

c. Unlabeled STX standard working solution: The STX diHCl standard (NIST RM 8642 STX diHCl) is provided at a concentration of 268.8 µM (100 µg/ml).
   i. A bulk standard curve can be made up in advance and stored at 4 °C for up to one month. The use of a bulk standard curve minimizes time needed for routine analyses and improves repeatability.
   ii. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µl in 50 ml) and use for the serial dilutions.
   iii. Serial dilutions should result in the following stock concentrations (M):
      1. 6 x 10-6 [100 µl 268.8 µM STX + 4.38 ml 0.003 M HCl]
      2. 6 x 10-7 [500 µl 6 x 10-6 M STX + 4.5 ml 0.003 M HCl]
      3. 1.8 x 10-7 [1.5 ml 6 x 10-7 M STX + 3.5 ml 0.003 M HCl]
      4. 6 x 10-8 [500 µl 6 x 10-7 M STX + 4.5 ml 0.003 M HCl]
      5. 1.8 x 10-8 [500 µl 1.8 x 10-7 M STX + 4.5 ml 0.003 M HCl]
      6. 6 x 10-9 [500 µl 6 x 10-8 M STX + 4.5 ml 0.003 M HCl]
      7. 6 x 10-10 [500 µl 6 x 10-9 M STX + 4.5 ml 0.003 M HCl]
      8. 5 ml 0.003 M HCl.

d. Interassay calibration standard (QC check): Reference standard STX (1.8 x 10-8 M STX) in 3 mM HCl. For long-term storage keep at -80 °C; for routine use (up to one month), store at 4 °C.

e. Porcine brain membrane preparation: Store bulk rat brain membrane preparations at -80 °C.
   i. Thaw an aliquot of porcine brain preparation on ice.
   ii. Dilute membrane preparation with cold (4 °C) 100 mM MOPS/100 mM choline chloride, pH 7.4 to yield a working stock with a protein concentration of 1.0 mg/ml.
   iii. Vortex vigorously to achieve a visibly homogeneous suspension.
   iv. Keep the diluted membrane preparation on ice.

C. Performing the Assay

a. Plate setup: When possible use a multichannel pipet to minimize effort and increase consistency.
   i. Run standards, samples, and QC check in triplicate.
   ii. For quantitation, multiple dilutions per extract should be analyzed in order to obtain a value that falls within the dynamic range of the assay. A minimum sample extract dilution of 1:10 is recommended to minimize potential matrix effects.
   iii. Use of a standard plate layout (Figure 1) is recommended. This will improve ease of analysis and can help maximize the number of samples/standards that can be analyzed per plate.

b. Addition of samples/standards: Add in the following order to each well
   i. 35 µl assay buffer
   ii. 35 µl STX standard/QC check/sample extract
   iii. 35 µl 3H-STX
   iv. 105 µl membrane preparation (ensure solution is homogeneous)
   v. Cover the plate and incubate at 4 °C for 1 h.

c. Assay filtration: Use the vacuum manifold attached to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process.

i. Set the vacuum pressure gauge on the pump or manifold to ~4-8" Hg (~135-270 millibar).
ii. Place the 96-well plate on the vacuum manifold.
iii. Fill any empty wells with 200 µl MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate.
iv. Turn on vacuum. Optimum vacuum will pull the wells dry in 2-5 s.
v. With vacuum pump running, quickly rinse each well twice with 200 µl ice cold MOPS/choline chloride buffer using a multichannel pipet. Maintain vacuum until liquid is removed.

d. Preparation of the assay for counting: Remove the plastic bottom from the plate and blot the plate bottom once on absorbent towel.
   i. For counting in microplate scintillation counter:
      1. Seal the bottom of a counting cassette with sealing tape.
      2. Place the microplate in the counting cassette.
      3. Add 50 µl scintillation cocktail per well using multichannel pipet.
      4. Seal the top of the plate with sealing tape.
      5. Incubate for 30 min at room temperature.
      6. Place the plate in the scintillation counter and count for 1 min per well.
   ii. For counting in traditional scintillation counter:
      1. Place the microplate in the MultiScreen punch system apparatus and place the disposable punch tips on top of the microplate.
      2. Punch the filters from the wells into scintillation vials and fill with 4 ml scintillation cocktail.
      3. Place caps on the vials and vortex.
      4. Allow vials to sit overnight in the dark.
      5. Count using a tritium window in a traditional scintillation counter.

D. Analysis of Data
   a. Curve fitting: Perform curve fitting using a four-parameter logistic fit (sigmoidal dose response curve with variable slope).
      i. $y = \text{min} + (\text{max-min})/1+10^{(\text{x-log } IC_{50})\text{Hill slope}}$
      ii. where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX (also known as B_0); min is the bottom plateau, equal to nonspecific binding in CPM in the presence of saturating nonradiolabeled STX; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is the total igand binding in CPM (B/B_0).
   b. Sample quantification: Sample quantification is only carried out on dilutions having a B/B_0 in the range of 0.2-0.7.
      i. Where B represents the bound 3H-STX in CPM in the sample and B_0 represents the max bound 3H-STX in the sample.

ii. Sample concentration is calculated in µg STX diHCl equivalents (eq)/kg shellfish as described below:

(nM STX eq) x (sample dilution) x [(210 µl total volume)/35 µl sample]
= nM STX eq in extract

(nM STX diHCl eq in extract) x (1 L/1000 ml) x (372 ng/nmol) X (1 µg/1000 ng)
= g STX diHCl eq/ml

µg STX diCHl eq/ml x (ml extract/g shellfish) x (1000g/kg)
= µg STX diHCl eq/kg

Figure 1. Example plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

Concentrations indicate those of the STX standard curve; REF = reference; QC = quality control; U = unknown sample (with dilutions indicated). The same standard curve made be used for additional plates run on the same day using the same reagents (i.e., 11 samples can be run on subsequent plates).

Appendix B: Radiation Safety Requirements

A. All users must follow all local, state, and federal laws and procedures regarding receipt, use and disposal of isotopes.
B. All users must be trained in and follow all in-house safety procedures for working with radiolabeled materials.
C. All isotopes and work stations where isotopes are used should be controlled access areas. Any one with access to the area must also receive radiation safety training.
D. Freezers where the isotopes are stored must be locked.
E. Personal protective equipment must include lab coats (designated specifically for use with radioactive materials), safety glasses, and gloves.
F. Radioactive materials will only be handled and manipulated in designated areas, which have been clearly identified and labeled accordingly.
G. Work with source radiation material must be conducted in a fume hood.
H. Radioactive materials will be stored and/or carried in secondary containment.
I. When possible, disposable supplies such as pipet tips, absorbent paper, and kim wipes will be used so that contaminated supplies can be readily disposed of as radioactive waste.
J. Wipe surveys will be conducted at the end of each experiment as well as monthly to ensure that there is no contamination in the laboratory.
K. The filter plates used in the assay will be designated as solid radioactive waste, while the washes from the filter plates (containing buffer and unbound 3H-STX) will be handled as liquid radioactive waste. There will be a dry active waste container to hold contaminated items such as the plates, gloves, absorbent paper and kim wipes. There will be a liquid waste jug to hold the contaminated liquid radioactive waste.
L. All wastes must be disposed of according to state and local laws.

Appendix C. Abbreviations and Acronyms

3H-STX	Tritiated saxitoxin
AOAC	Association of Analytical Communities
ARC	American Radiolabeled Chemicals
B	Bound CPM
B_0	Maximum bound CPM
CFSAN	Center for Food Safety & Applied Nutrition
CPM	Counts per minute
diHCl	Dihydrochloride
Eq	Equivalents
HCl	Hydrochloric acid
IC_{50}	Inhibitory concentration at which CPMs are at 50% max
LC-FD	Liquid chromatography with fluorescence detection
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Mouse bioassay
MOPS	3-Morpholinopropanesulfonic acid
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NSSP	National Shellfish Sanitation Program
OMA	Official method of analysis
PMSF	Phenyl methylsulfonyl fluoride
PCOX	Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX	Pre-column oxidation liquid chromatography with fluorescence detection
PSP	Paralytic shellfish poisoning
PSTs	Paralytic shellfish toxins
QA	Quality assurance
QC	Quality control
QS	Quality System
RBA	Receptor binding assay
RSD	Relative standard deviation
SLV	Single laboratory validation
STX	Saxitoxin

Appendix II: Van Dolah et al. 2009

FOOD CHEMICAL CONTAMINANTS

Appendix II

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

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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 ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 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.

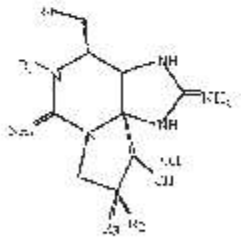
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Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently 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/µmole
Carbamate	STX	H	H	H	$OCONH_2$	2182
	Neo STX	OH	H	H	$OCONH_2$	2295
	GTX1	OH	OSO_3^-	H	$OCONH_2$	2766
	GTX2	H	OSO_3^-	H	$OCONH_2$	792
	GTX3	H	H	OSO_3^-	$OCONH_2$	1684
	GTX4	OH	H	OSO_3^-	$OCONH_2$	1938
Sulfocarbamoyl	GTX5 (B1)	H	H	H	$OCONHSO_3^-$	180
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$	-
	C1	H	OSO_3^-	H	$OCONHSO_3^-$	16
	C2	H	H	OSO_3^-	$OCONHSO_3^-$	225
	C3	OH	OSO_3^-	H	$OCONHSO_3^-$	33
	C4	OH	H	OSO_3^-	$OCONHSO_3^-$	143
Decarbamoyl	dcSTX	H	H	H	OH	1274
	dcNeoSTX	OH	H	H	OH	
	dcGTX1	OH	OSO_3^-	H	OH	-
	dcGTX2	H	OSO_3^-	H	OH	16.7
	dcGTX3	H	H	OSO_3^-	OH	18.2
	dcSTX4	OH	H	OSO_3^-	OH	
Deoxydecarbamoyl	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO_3^-	H	
	doGTX3	H	OSO_3^-	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.

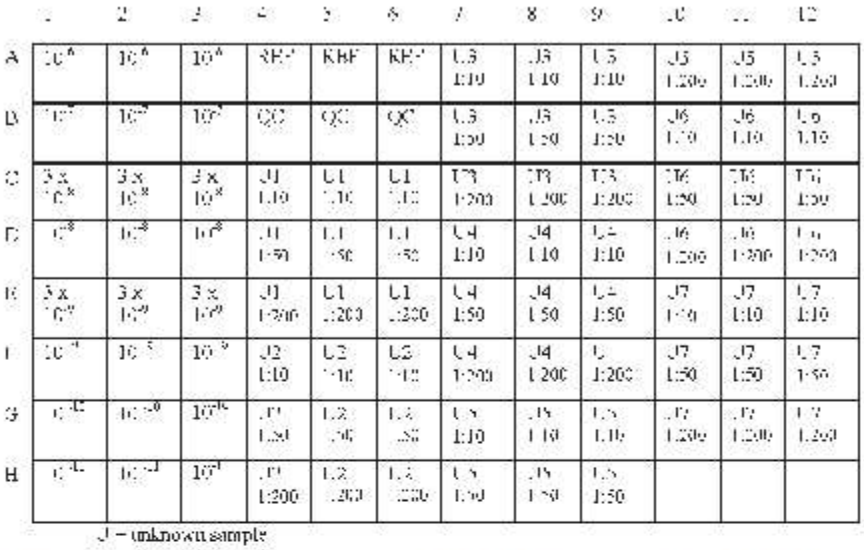


Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

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 Durapore 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

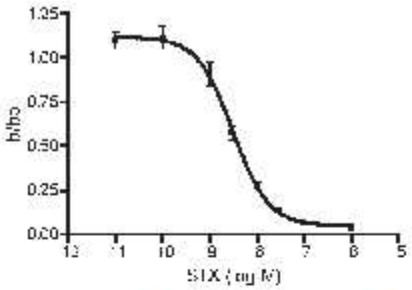


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

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.

(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 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

Table 2. Recovery of analyte from spiked samples (µg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD,	Recovery, %
0	<dl*			
40	47	8.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

* <dl = Less than LOQ (5 µg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; $n = 5$) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	65	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

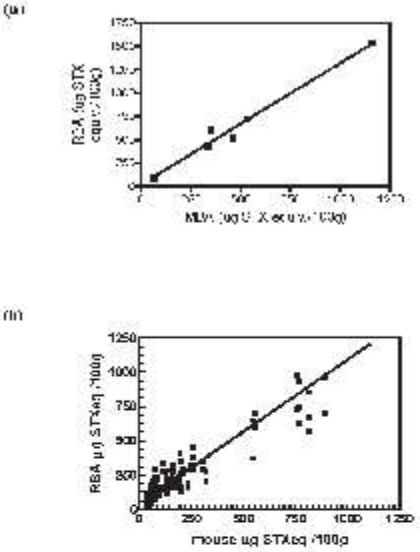


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days ($r^2 = 0.98$, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

Assay Procedure

(a) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(b) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equialents/100 g shellfish using the following formulas:

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

$$(\text{nm equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, $b/b_o < 0.2$), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., $b/b_o > 0.7$) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD_r = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

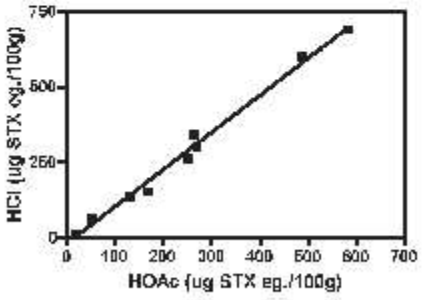


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.6	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Placopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

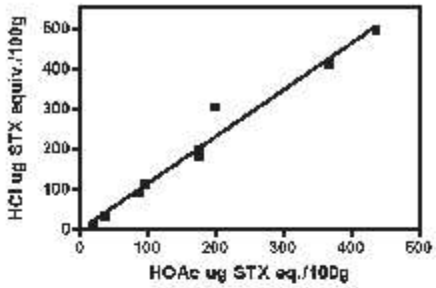


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

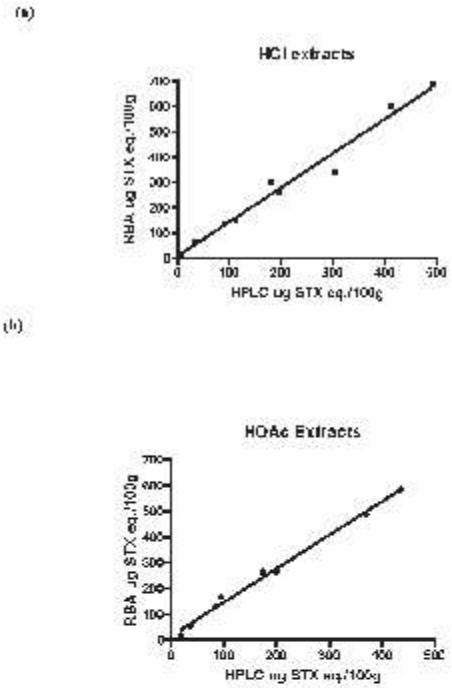


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

Acknowledgments

We thank Karen Mao and Steven Eaker for excellent technical support in the conduct of this study.

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Appendix III: Van Dolah et al., 2012

FOOD CHEMICAL CONTAMINANTS

Appendix III

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

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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.

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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 (*Placlopecten 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. chilensis*) from Chile	
10	MLV04	Scallop (*Placlopecten 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. chilensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Placlopecten 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*℠ 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 *CV*s for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_0), 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*	1149*	
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

* Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(c) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(d) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(a) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(c) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8" Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \mathrm{IC}_{50}) \times \text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_0; 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_0, 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**	**1150**	**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_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 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., $B/B_0 > 0.7$), 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 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).]

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%.

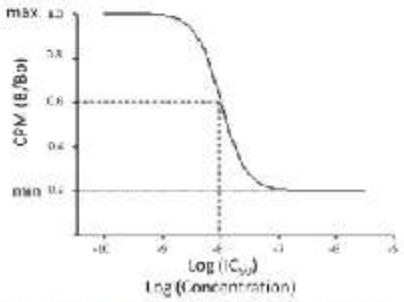


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

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, 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		508.4			2153.9	1070.9
MLV10	Butter clam	1783.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	238.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	684.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHCl equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	890	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method **2006.02**, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10×SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0 <0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table **2011.27D**. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3×SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10×SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_R	RSD_R, %	Recovery, %
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

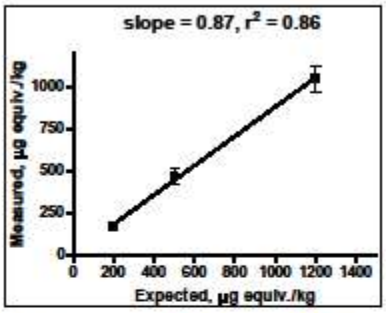


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table **2011.27E**). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8" Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_0 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_0 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

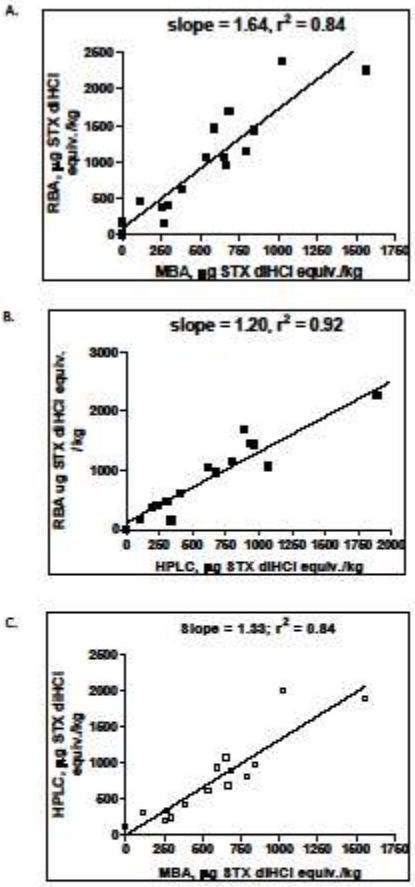


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

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.

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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 20000 × *g* (rcf).

(d) *Centrifuge tubes.*—12–15 mL rated for >20000 × *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.

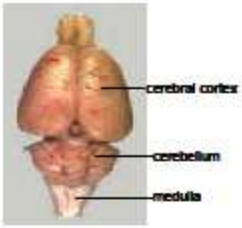


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20000 × 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.

Appendix IV: AOAC OM 2011.27

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

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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.

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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*	1149*	
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

* 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^{-8} 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_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

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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,	RSD, %
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*	1150*			
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

* 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:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)(HillSlope)}}$$

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

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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_{50}, 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.

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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

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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).]

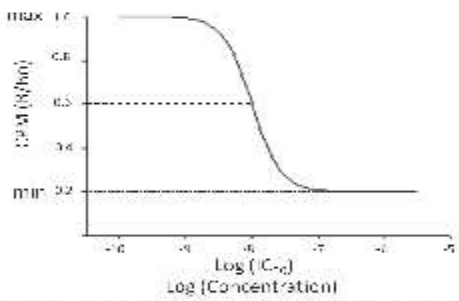


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}

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_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 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., $B/B_0 > 0.7$), 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.* **95**, 795(2012)

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