ISSC Method Application Format for Biotoxin Methods Matrix Extension

The purpose of laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that methods under consideration for adoption by the NSSP are fit for their intended use in the Program. This document provides a detailed outline of the types of information and data the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Committee (LC) requests from submitters for extension of current NSSP methods to cover additional matrixes (i.e., molluscan shellfish species). These recommendations are intended for methods which have already undergone a single laboratory validation (SLV) and are being considered for use with a new matrix. Included are the method performance criteria that should be examined for inclusion in the validation package, along with LC recommendations for each criterion. Data generated for the more robust performance criteria may be used to satisfy multiple criteria, if applicable.

Method Overview
Method Title: Application of Receptor Binding Assay to Multispecies Monitoring for Paralytic Shellfish Poison
Method Submitter(s) and Contact Information: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield & Kelly Cusick, NOAA Charleston Lab Sitka Tribe of Alaska Environmental Research Laboratory
Intended or Target Use: (approved, approved limited use, or emergency use) The receptor binding assay (RBA) is intended for use as an NSSP Approved Method for the quantitative assessment of paralytic shellfish poison (PSP) in multiple species. The application of this project is specifically intended for use in regulatory laboratory settings. The RBA serves as an alternative to the mouse bioassay (MBA) in these applications, offering a measure of integrated toxicity with high throughput and the elimination of live animal testing.
Rationale for this Method in the NSSP: The overall goal of this project is to fully validate the Receptor Binding Assay for use in testing paralytic shellfish poison in multiple shellfish species. This project will take on the necessary work to make the RBA assay a viable regulatory method for PSP monitoring. It will address NOAA's Preventative, Control, and Mitigations of Harmful Algal Blooms (PCMHAB) program's goal of translating a method into a product that is widely available. By putting the method through conditions that simulate regulatory screening we will attempt to demonstrate that the assay is equivalent to the mouse bioassay. To date, only mussels have been fully approved for RBA testing in the NSSP, yet a number of matrices can be analyzed using limited use criteria. A significant effort is needed to expand the validations for the limited-use approved and other non-validated shellfish species using the RBA method for its utility to be realized by

PSP testing programs.

Method Principle/Basis:(receptor binding assay, immunoassay, LC-MS, etc.)
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 rat 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.

Target Matrix/Matrices:
(list shellfish species by common and scientific names)
The target matrices include Pacific geoduck clam (*Panopea generosa*) gutball, whole Pacific oysters (*Crassostrea gigas*), northern razor clam (*Siliqua patula*) meat only*,* and whole hard clams, comprising three species: butter clams (*Saxidomus gigantea*), Pacific littleneck clams (*Protothaca staminea*), and Japanese littleneck clams (also known as Manila clams; *Venerupis philippinarum).*

Target Toxin(s):
The target toxin class 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.

Existing Certification(s) of the Method:
(AOAC, etc.)
The RBA has successfully undergone AOAC single laboratory validation (Van Dolah et al. 2009 – Appendix II) and a full collaborative study (Van Dolah et al. 2012 – Appendix III). The RBA is now considered an AOAC Official Method of Analysis (OMA 2011.27 – Appendix IV) and was adopted in the FDA NSSP in the 2015 edition for analysis of mussels as an alternative to the mouse bioassay and as a limited use method for screening and precautionary closure. The current proposal provides data to seek consideration for the RBA to be a NSSP Approved Method for these additional matrices: geoduck gutball, whole oyster, whole clams, and razor clam meat.

Equipment Required:
(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

For rat brain isolation:
(x) Teflon/glass homogenizer (Motorized tapered Teflon pestle and glass tune (15 ml)
(y) Motorized tissue homogenizer (Polytron or small handheld blender)
(z) High-speed centrifuge and fixed angle rotor (20,000 xg rcf)
(aa) Centrifuge tubes (12-15 ml, rated for 20,000 xg)
(bb) plastic cryovials (2 ml)
(cc) Graduated beaker (300 or 500 ml)
(dd) Pipets (5-10 ml, disposable)
(ee) Forceps
(ff) Ice bucket and ice
(gg) top loading balance

Reagents Required:
(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])

For rat brain isolation:
(j) 20 rat brains (male, 6-week-old Sprague-Dawley; available through Hilltop Lab Animals, Inc., Scottdale, PA; www.hilltoplabs.com [or equivalent])
(k) MOPS, pH 7.4 (Sigma; Cat. No. M3183-500G [or equivalent])
(l) Choline chloride (100 mM; Sigma; Cat. No. C7527-500G [or equivalent])
(m) Phenyl methylsulfonyl fluoride (PMSF; Sigma: Cat. No. P7626)
(n) Isopropanol
(o) Micro bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL)

Proprietary Aspects:
(Provide any aspects of the method that are proprietary or trade secret.)
None. All reagents can be prepared or purchased.

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 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 3H-STX users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of radioisotopes. Please see Appendix C for further safety requirements.

Method Cost:
(Provide an estimate of cost per analysis, including start-up costs for specialized equipment, personnel, etc.)
The estimated cost per 96-well plate assay is ~$240. 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 ~$35.50. 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.

Sample Throughput:
(Provide a description of how many samples can be analyzed by this method in a given time frame; please specify under what conditions this throughput can be achieved.)
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.

DEFINITIONS

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

appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.

15. **Recovery –** The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – The ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – The ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** The range of analyte or measurand concentration over which the method is applied.

REFERENCES:

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

Background:

Proposal 13-114 was approved at the 2015 ISSC meeting for the analysis of paralytic shellfish toxins (PSTs) in mussels using the receptor binding assay (RBA) and was granted a limited-use designation for application in clams and scallops.

Successful implementation of the RBA method by the Sitka Tribe of Alaska and by countries supported by the International Atomic Energy Agency (IAEA) has generated continued interest in the adoption of RBA within laboratories operating under the National Shellfish Sanitation Program (NSSP). However, broader adoption has just begun, as the method is currently approved as an MBA replacement for mussel samples. The four target matrices under evaluation include Pacific geoduck clam (*Panopea generosa*) gutball, whole Pacific oysters (*Crassostrea gigas*), Northern razor clam (*Siliqua patula*) meat only, and whole hard clams, comprising three species: butter clams (*Saxidomus gigantea*), Pacific littleneck clams (*Protothaca staminea*), and Japanese littleneck (also known as Manila) clams (*Venerupis philippinarum).*

Organizations Involved:

- NOAA National Ocean Service**,** Charleston, SC (NOAA)
- Washington State Department of Health (WADoH)
- Sitka Tribe of Alaska Environmental Research Laboratory (STAERL)
- Alaska Department of Environmental Conservation (AKDEC)

A total of 1,572 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). However, sourcing suitable ‘blank’ material proved challenging. 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. PSP-free samples are rare, which made the execution of this study difficult and may present challenges for obtaining suitable ‘blank’ materials in future PSP method evaluations. 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 15 homogenates with 100 sub-aliquots needed for spikes at 1/4x, 1/2x 1x, 2x, and 4x the regulatory limit (80 ug STX/100 g of shellfish tissue) for each 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)

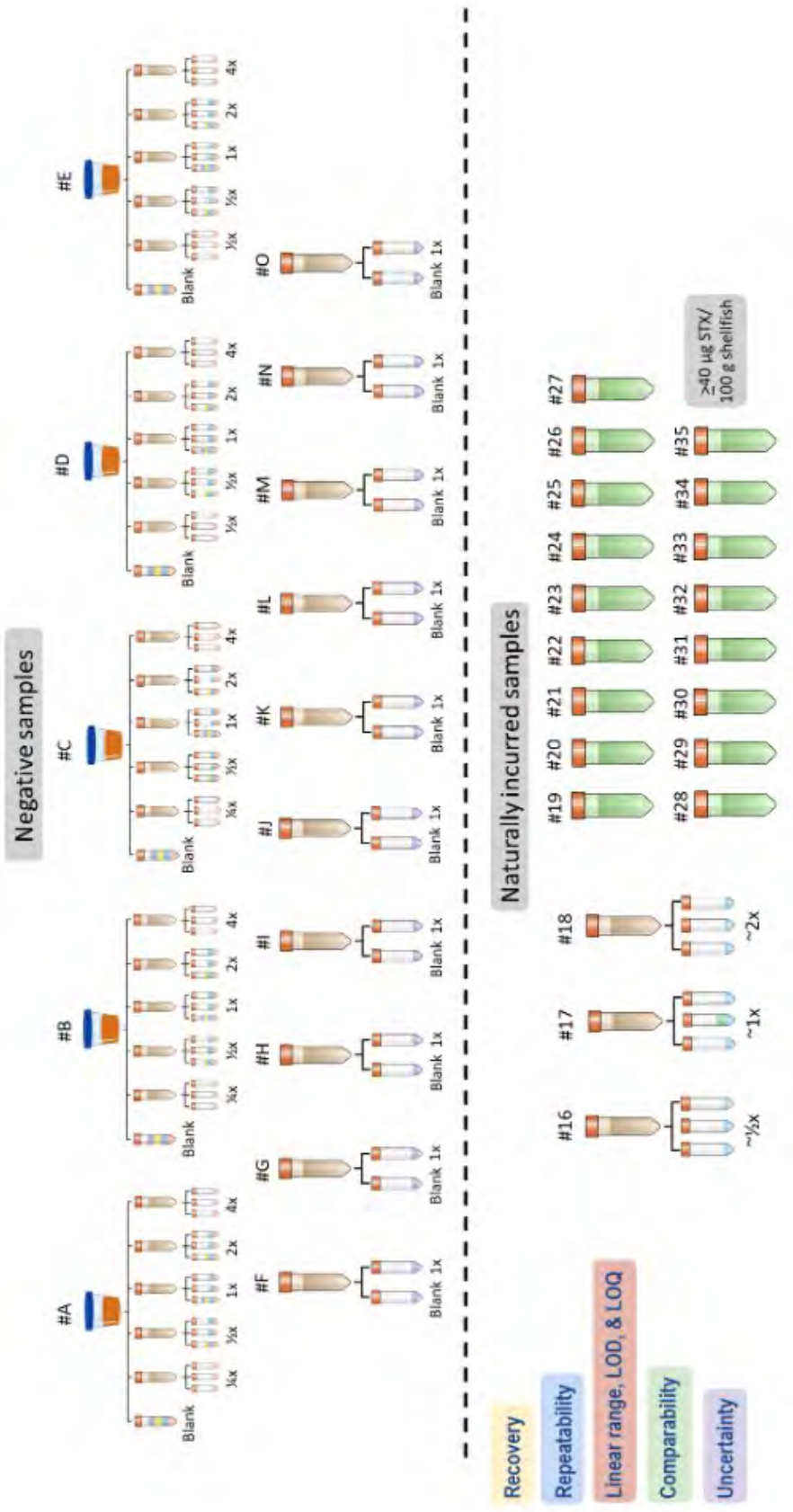

Negative samples
Naturally incurred samples
Recovery
Repeatability
Linear range, LOD, & LOQ
Comparability
Uncertainty
≥40 µg STX/ 100 g shellfish

Figure 1. Matrix Extension study design showing allocation of 126 samples per shellfish matrix type to meet five validation data criteria: recovery (yellow), repeatability (blue), linear range, LOD and LOQ (red), comparability (green), and uncertainty (purple).

In total, 952 PSP samples were below the MBA's limit of detection from both Washington state and Alaska (STAERL uses RBA for screening), and 598 Washington state samples were re-screened using a modified extraction procedure (0.5 g sample + 0.5 mL 0.1 N HCl). A total of 159 Pacific geoduck clam gutball MBA negative samples were re-screened using a modified RBA to identify 'blank' samples, and of these were selected for inclusion in the study. Of the 44 whole Pacific oyster samples, 35 were re-screened and used in the study. Similarly, 199 hard clam samples were re-screened, with 34 included in the study. Among 103 northern razor clam meat samples re-screened, 20 were used for further analysis (Figure 2).

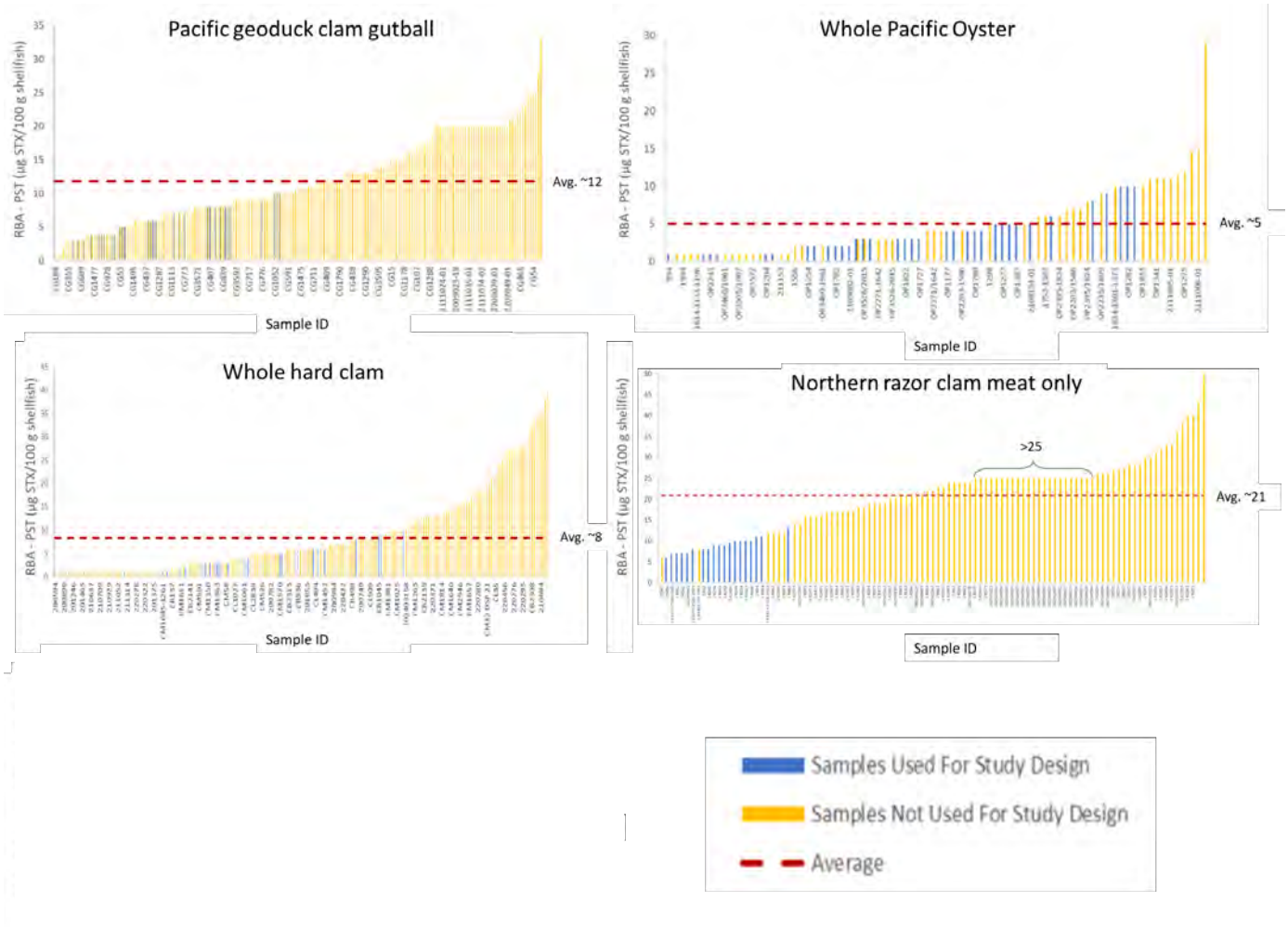


Figure 2. Samples from five shellfish matrices that tested negative for toxins using the MBA were re-screened with a modified RBA to identify "blank" samples for spiking in homogenates for the Matrix Extension study. Average PST values (indicated by the red dashed line) for Pacific geoduck clam gutball, whole Pacific oyster, northern razor clam meat, and whole hard clams are 12, 5, 21, and 8 µg STX/100 g shellfish tissue, respectively.

Samples were collected along the U.S. West Coast, specifically from Washington and Alaska, across various harvest days, locations, and calendar seasons. The 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 meet the required tissue weight for each homogenate, multiple individual samples were combined prior to spiking. This approach ensured sufficient sample mass while maintaining temporal and seasonal consistency across collections.

Table 1. Summary of shellfish tissue samples (Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam, and razor clam) collected across Washington and Alaska sites between 2020 and 2025, categorized by group, location, and season.

Group Letter	Shellfish Matrix	Location	Collection Date	Season
A	geoduck	Kingston, Kitsap - WA	3/2024	Winter
B	geoduck	Sequim Bay, Clallam - WA	5-6/2024	Spring
C	geoduck	Agate Passage, Kitsap - WA	7/2024	Summer
D	geoduck	South King County, King - WA	5-6/2024	Spring
E	geoduck	Admiralty Inlet, King - WA	4/2024	Spring
F	geoduck	East Bainbridge, Kitsap - WA	6-7/2022	Summer
G	geoduck	Port Madison, Kitsap - WA	7/11/2022	Summer
H	geoduck	Sequim Bay, Clallam - WA	6/7/2022	Spring
I	geoduck	Agate Passage, Kitsap - WA	5-6/2024	Spring
J	geoduck	Agate Passage, Kitsap - WA	3/4/2024	Winter
K	geoduck	Georgia Straits South, Whatcom - WA	4-5/2024	Spring
L	geoduck	Sequim Bay, Clallam - WA	3/4/2024	Winter
M	geoduck	Hale Passage, Pierce WA	6/21/2024	Summer
N	geoduck	Port Madison, Kitsap - WA	4/2024	Spring
O	geoduck	Sequim Bay, Clallam - WA	6/25/2024	Summer
A	oyster	Grays Harbor Coast, Grays Harbor - WA	7/2024	Summer
B	oyster	Willapa Bay, Pacific - WA	7/2024	Summer
C	oyster	Sequim Bay, Clallam - WA	7/2024	Summer
D	oyster	Willapa Bay, Pacific - WA	6/2024	Spring
E	oyster	Grays Harbor Coast, Grays Harbor - WA	6/2024	Spring
F	oyster	Canoe Lagoon, Blashke Islands - AK	10/30/2021	Fall
G	oyster	Peterson Bay, Kachemak Bay - AK	8/30/2021	Summer
H	oyster	Betton Island East, Ketchikan - AK	8/30/2021	Summer
I	oyster	Samish Bay, Skagit - WA	6/14/2022	Spring
J	oyster	East Passage, Kitsap - WA	6/4/2024	Spring
K	oyster	Shikat Bay, Sea Otter Sound East - AK	8/4/2021	Summer
L	oyster	Dabob Bay, Jefferson - WA	10/3/2022	Fall
M	oyster	Sequim Bay, Clallam - WA	6/6/2024	Spring
N	oyster	Port Madison, Kitsap - WA	7/9/2024	Summer
O	oyster	Grays Harbor Coast, Grays Harbor - WA	09-10/2022	Fall
A	butter clam	Fidalgo Bay, Skagit - WA	5/2024	Spring
B	manila clam	Haro Strait, San Juan - WA	07-08/2024	Summer
C	manila clam	Willapa Bay, Pacific - WA	06/07/2024	Summer
D	manila clam	Willapa Bay, Pacific - WA	05-06/2024	Spring
E	manila clam	Padilla Bay, Skagit - WA	06-07/2024	Summer
F	butter clam	Lone Tree Point, Skagit - WA	1/18/2023	Winter
G	littleneck clam	Sequim Bay, Clallam - WA	6/25/2024	Summer

H	butter clam	Skagit Bay, Skagit - WA	5/7/2024	Spring
I	manila clam	Sequim Bay, Clallam - WA	5/9/2024	Spring
J	butter clam	North King County, King - WA	5/24/2024	Spring
K	littleneck clam	Starrigavan North, Sitka - AK	6/24/2020	Summer
L	littleneck clam	Puget Cove, Yakutat - AK	12/15/2020	Fall
M	littleneck clam	False Island Boat Launch, Craig - AK	10/15/2021	Fall
N	manila clam	Drayton Harbor, Whatcom - WA	8/11/2022	Summer
O	littleneck clam	Tongass Beach, Ketchikan - AK	9/9/2020	Summer
A	razor clam	Pacific County Coast, Pacific - WA	1-2/2024	Winter
B	razor clam	Grays Harbor Coast, Grays Harbor - WA	2/13/2024	Winter
C	razor clam	Grays Harbor Coast, Grays Harbor - WA	4/16/2024	Spring
D	razor clam	Grays Harbor Coast, Grays Harbor - WA	5/2/2024	Spring
E	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/19/2024	Winter
F	razor clam	Pacific County Coast, Pacific - WA	9/25/2023	Fall
G	razor clam	Grays Harbor Coast, Grays Harbor - WA	1/30/2024	Winter
H	razor clam	Grays Harbor Coast, Grays Harbor - WA	1/2/2024	Winter
I	razor clam	Grays Harbor Coast, Grays Harbor - WA	2/27/2024	Winter
J	razor clam	Grays Harbor Coast, Grays Harbor - WA	4/2/2024	Spring
K	razor clam	Willapa Bay, Pacific - WA	4/14/2024	Spring
L	razor clam	Pacific County Coast, Pacific - WA	1/2/2024	Winter
M	razor clam	Grays Harbor Coast, Grays Harbor - WA	11/30/2023	Fall
N	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/12/2024	Winter
O	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/21/2024	Spring

Validation Data

1. **Recovery:** Recovery is the fraction or percentage of an analyte recovered following sample analysis. To determine method accuracy/trueness/recovery, the concentration of the target analyte as measured by the analytical method under study is compared to a true value or accepted reference concentration. Consider using certified reference materials (if available).

Suggested procedure**:** Use shellfish free of target analyte(s); analyze intended blank matrix tissue for background interferents. For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take four aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at half the action level. Spike a second aliquot with the target analyte(s) at the action level. Spike the third aliquot with the target analyte(s) at twice the action level. Do not spike the fourth aliquot; this is the sample blank. Process each aliquot to determine the concentration for the target analyte(s). Repeat this process with a minimum of five samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots. Additional samples may be required to examine the effects of seasonal and/or geographical differences in shellfish matrix components or analyte profiles on the method performance.

Data:
Working range/Range of interest: 40 µg saxitoxin diHCl equiv/100g shellfish tissue to 160 µg saxitoxin diHCl equiv /100g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oysters, whole hard clams (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.
5. Perform a one-way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		

Total 29

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary:

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant?

  - Pacific geoduck clam gutball: Not significant (F: 0.33 ≤ Fcrit: 3.2)
  - Whole Pacific oyster: Not significant (F: 1.84 < Fcrit: 3.2)
  - Whole hard clam: Not significant (F: 0.69 < Fcrit: 3.2)
  - Razor clam meat: Not significant (F: 0.43 < Fcrit: 3.2)

- Is the one-way analysis of variance to determine the consistency of recovery of the method under study significant?

  - Pacific geoduck clam gutball: Not significant (p = 0.72)
  - Whole Pacific oyster: Not significant (p = 0.17)
  - Whole hard clam: Not significant (p = 0.50)
  - Razor clam meat: Not significant (p = 0.65)

- At what concentrations is the one-way analysis of variance significant?
    - Pacific geoduck clam gutball: N/A
    - Whole Pacific oyster: N/A
    - Whole hard clam: N/A
    - Razor clam meat: N/A

- What is the overall percent recovery of the method under study?
    - Pacific geoduck clam gutball: 102.7%
    - Whole Pacific oyster: 103.6%
    - Whole hard clam: 102.1%
    - Razor clam meat: 95.00%

The data for each species were log-transformed to follow the ISSC's protocol for data handling prior to statistical analysis. A one-way ANOVA, conducted as described above, produced F-test results for each matrix type that were not statistically significant at the 95% confidence level for all species. These findings indicate that the method's recovery, as implemented by the laboratory, is consistent across the concentration range relevant to the method's intended application

Table 1.1. One-way ANOVA results for each shellfish matrix type.

Pacific geoduck clam gutball

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.003	2	0.002	0.33	0.72	3.22
Error	0.19	42	0.005			
Total	0.20	44				

Whole Pacific oyster

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.03	2	0.014	1.85	0.170	3.22
Error	0.33	42	0.008			
Total	0.358	44				

Whole hard clam

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.01	2	0.003	0.69	0.50	3.22
Error	0.21	42	0.005			
Total	0.21	44				

Razor clam meat

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.01	2	0.00	0.43	0.65	3.22
Error	0.29	42	0.01			
Total	0.29	44				

The method's overall percent recovery was 102.7% in Pacific geoduck clam gutball, 103.6% in whole Pacific oyster, 102.1% in whole hard clam, 95.0% in razor clam meat.

Full Data:

Sample ID	Shellfish Matrix	Spike Level	Spike Concentration (ug/100g)	RBA (ug/100g)	Blank Value	RBA - Blank	Percent Recovery (%)
CGa-16	geoduck	Blank	0	7	7	0	0.000
CGa-06	geoduck	1/2x	40	52	7	45	112.5
CGa-20	geoduck	1/2x	40	66	7	59	147.5
CGa-21	geoduck	1/2x	40	45	7	38	95.00
CGa-07	geoduck	1x	80	92	7	85	106.3
CGa-08	geoduck	1x	80	99.5	7	92.5	115.6
CGa-09	geoduck	1x	80	94	7	87	108.8
CGa-10	geoduck	2x	160	135	7	128	80.00
CGa-11	geoduck	2x	160	178	7	171	106.9
CGa-12	geoduck	2x	160	163	7	156	97.50
CGb-00	geoduck	Blank	0	6	6	0	0.000
CGb-20	geoduck	1/2x	40	52	6	46	115.0
CGb-21	geoduck	1/2x	40	39	6	33	82.50
CGb-05	geoduck	1/2x	40	46	6	40	100.0
CGb-07	geoduck	1x	80	86	6	80	100.0
CGb-08	geoduck	1x	80	85	6	79	98.75
CGb-09	geoduck	1x	80	77	6	71	88.75
CGb-10	geoduck	2x	160	192	6	186	116.3
CGb-11	geoduck	2x	160	138	6	132	82.50
CGb-12	geoduck	2x	160	167	6	161	100.6
CGc-16	geoduck	Blank	0	10	10	0	0.000
CGc-04	geoduck	1/2x	40	55	10	45	112.5
CGc-05	geoduck	1/2x	40	50	10	40	100.0
CGc-06	geoduck	1/2x	40	40	10	30	75.00
CGc-07	geoduck	1x	80	110	10	100	125.0
CGc-08	geoduck	1x	80	115	10	105	131.3
CGc-09	geoduck	1x	80	84	10	74	92.50
CGc-10	geoduck	2x	160	230	10	220	137.5
CGc-11	geoduck	2x	160	135	10	125	78.13
CGc-12	geoduck	2x	160	167	10	157	98.13
CGd-16	geoduck	Blank	0	11	11	0	0.000
CGd-20	geoduck	1/2x	40	49	11	38	95.00
CGd-21	geoduck	1/2x	40	49	11	38	95.00
CGd-22	geoduck	1/2x	40	46	11	35	87.50
CGd-07	geoduck	1x	80	103	11	92	115.0
CGd-08	geoduck	1x	80	84	11	73	91.25
CGd-09	geoduck	1x	80	80	11	69	86.25
CGd-10	geoduck	2x	160	199	11	188	117.5

CGd-11	geoduck	2x	160	197	11	186	116.3
CGd-12	geoduck	2x	160	157	11	146	91.25
CGe-00	geoduck	Blank	0	6	6	0	0.000
CGe-04	geoduck	1/2x	40	47	6	41	102.5
CGe-05	geoduck	1/2x	40	44	6	38	95.00
CGe-06	geoduck	1/2x	40	46	6	40	100.0
CGe-07	geoduck	1x	80	88	6	82	102.5
CGe-08	geoduck	1x	80	72	6	66	82.50
CGe-09	geoduck	1x	80	115	6	109	136.3
CGe-10	geoduck	2x	160	156	6	150	93.75
CGe-11	geoduck	2x	160	175	6	169	105.6
CGe-12	geoduck	2x	160	172	6	166	103.8
OPa-00	oyster	Blank	0	0	0	0	0.000
OPa-04	oyster	1/2x	40	47	0	47	117.5
OPa-21	oyster	1/2x	40	35	0	35	87.50
OPa-06	oyster	1/2x	40	46	0	46	115.0
OPa-07	oyster	1x	80	87	0	87	108.8
OPa-08	oyster	1x	80	102	0	102	127.5
OPa-09	oyster	1x	80	85	0	85	106.3
OPa-10	oyster	2x	160	186	0	186	116.3
OPa-11	oyster	2x	160	119	0	119	74.38
OPa-12	oyster	2x	160	159	0	159	99.38
OPb-00	oyster	Blank	0	6	6	0	0.000
OPb-04	oyster	1/2x	40	32	6	26	65.00
OPb-05	oyster	1/2x	40	41	6	35	87.50
OPb-06	oyster	1/2x	40	42	6	36	90.00
OPb-07	oyster	1x	80	101	6	95	118.8
OPb-08	oyster	1x	80	74	6	68	85.00
OPb-09	oyster	1x	80	85	6	79	98.75
OPb-10	oyster	2x	160	168	6	162	101.3
OPb-11	oyster	2x	160	142	6	136	85.00
OPb-12	oyster	2x	160	126	6	120	75.00
OPc-00	oyster	Blank	0	0	0	0	0.000
OPc-20	oyster	1/2x	40	28	0	28	70.00
OPc-21	oyster	1/2x	40	40	0	40	100.0
OPc-06	oyster	1/2x	40	42	0	42	105.0
OPc-07	oyster	1x	80	80	0	80	100.0
OPc-08	oyster	1x	80	75	0	75	93.75
OPc-09	oyster	1x	80	91	0	91	113.8
OPc-10	oyster	2x	160	128	0	128	80.00
OPc-11	oyster	2x	160	192	0	192	120.0
OPc-12	oyster	2x	160	91	0	91	56.88

OPd-16	oyster	Blank	0	5	5	0	0.000
OPd-04	oyster	1/2x	40	46	5	41	102.5
OPd-05	oyster	1/2x	40	45	5	40	100.0
OPd-22	oyster	1/2x	40	44	5	39	97.50
OPd-07	oyster	1x	80	79	5	74	92.50
OPd-08	oyster	1x	80	115	5	110	137.5
OPd-09	oyster	1x	80	100	5	95	118.8
OPd-10	oyster	2x	160	168	5	163	101.9
OPd-11	oyster	2x	160	167	5	162	101.3
OPd-12	oyster	2x	160	181	5	176	110.0
OPe-16	oyster	Blank	0	5	5	0	0.000
OPe-20	oyster	1/2x	40	48	5	43	107.5
OPe-21	oyster	1/2x	40	57	5	52	130.0
OPe-04	oyster	1/2x	40	66	5	61	152.5
OPe-07	oyster	1x	80	115	5	110	137.5
OPe-08	oyster	1x	80	116	5	111	138.8
OPe-09	oyster	1x	80	77	5	72	90.00
OPe-10	oyster	2x	160	202	5	197	123.1
OPe-11	oyster	2x	160	210	5	205	128.1
OPe-12	oyster	2x	160	153	5	148	92.50
CBa-00	butter clam	Blank	0	9	9	0	0.000
CBa-04	butter clam	1/2x	40	42	9	33	82.50
CBa-05	butter clam	1/2x	40	40	9	31	77.50
CBa-06	butter clam	1/2x	40	39	9	30	75.00
CBa-07	butter clam	1x	80	77	9	68	85.00
CBa-08	butter clam	1x	80	71	9	62	77.50
CBa-09	butter clam	1x	80	97	9	88	110.0
CBa-10	butter clam	2x	160	175	9	166	103.8
CBa-11	butter clam	2x	160	151	9	142	88.75
CBa-12	butter clam	2x	160	177	9	168	105.0
CMb-28	manila clam	Blank	0	0	0	0	0.000
CMb-04	manila clam	1/2x	40	42	0	42	105.0
CMb-05	manila clam	1/2x	40	45	0	45	112.5
CMb-06	manila clam	1/2x	40	47	0	47	117.5
CMb-07	manila clam	1x	80	87	0	87	108.8
CMb-08	manila clam	1x	80	85	0	85	106.3
CMb-09	manila clam	1x	80	70	0	70	87.50
CMb-10	manila clam	2x	160	159	0	159	99.40
CMb-11	manila clam	2x	160	153	0	153	95.60
CMb-12	manila clam	2x	160	132	0	132	82.50
CMc-00	manila clam	Blank	0	0	0	0	0.000
CMc-04	manila clam	1/2x	40	42	0	42	105.0

CMc-05	manila clam	1/2x	40	50	0	50	125.0
CMc-06	manila clam	1/2x	40	47	0	47	117.5
CMc-07	manila clam	1x	80	113	0	113	141.3
CMc-08	manila clam	1x	80	72	0	72	90.00
CMc-09	manila clam	1x	80	92	0	92	115.0
CMc-10	manila clam	2x	160	189	0	189	118.1
CMc-11	manila clam	2x	160	146	0	146	91.30
CMc-12	manila clam	2x	160	197	0	197	123.1
CMd-00	manila clam	Blank	0	6	6	0	0.000
CMd-04	manila clam	1/2x	40	48	6	42	105.0
CMd-05	manila clam	1/2x	40	38	6	32	80.00
CMd-06	manila clam	1/2x	40	37	6	31	77.50
CMd-07	manila clam	1x	80	99	6	93	116.3
CMd-08	manila clam	1x	80	74	6	68	85.00
CMd-09	manila clam	1x	80	108	6	102	127.5
CMd-10	manila clam	2x	160	196	6	190	118.8
CMd-11	manila clam	2x	160	152	6	146	91.30
CMd-12	manila clam	2x	160	188	6	182	113.8
CMe-00	manila clam	Blank	0	4	4	0	0.000
CMe-04	manila clam	1/2x	40	45	4	41	102.5
CMe-05	manila clam	1/2x	40	48	4	44	110.0
CMe-06	manila clam	1/2x	40	37	4	33	82.50
CMe-07	manila clam	1x	80	91	4	87	108.8
CMe-08	manila clam	1x	80	94	4	90	112.5
CMe-09	manila clam	1x	80	76	4	72	90.00
CMe-10	manila clam	2x	160	197	4	193	120.6
CMe-11	manila clam	2x	160	180	4	176	110.0
CMe-12	manila clam	2x	160	156	4	152	95.00
CRa-00	razor clam	Blank	0	10	10	0	0.000
CRa-04	razor clam	1/2x	40	46	10	36	90.00
CRa-05	razor clam	1/2x	40	48	10	38	95.00
CRa-06	razor clam	1/2x	40	47	10	37	92.50
CRa-07	razor clam	1x	80	64	10	54	67.50
CRa-08	razor clam	1x	80	78	10	68	85.00
CRa-09	razor clam	1x	80	99	10	89	111.3
CRa-10	razor clam	2x	160	140	10	130	81.25
CRa-11	razor clam	2x	160	139	10	129	80.63
CRa-12	razor clam	2x	160	119	10	109	68.13
CRb-00	razor clam	Blank	0	10	10	0	0.000
CRb-04	razor clam	1/2x	40	66	10	56	140.0
CRb-05	razor clam	1/2x	40	39	10	29	72.50
CRb-20	razor clam	1/2x	40	53	10	43	107.5

CRb-07	razor clam	1x	80	69	10	59	73.75
CRb-08	razor clam	1x	80	104	10	94	117.5
CRb-09	razor clam	1x	80	81	10	71	88.75
CRb-10	razor clam	2x	160	144	10	134	83.75
CRb-11	razor clam	2x	160	165	10	155	96.88
CRb-12	razor clam	2x	160	212	10	202	126.3
CRc-00	razor clam	Blank	0	14	14	0	0.000
CRc-04	razor clam	1/2x	40	54	14	40	100.0
CRc-05	razor clam	1/2x	40	53	14	39	97.50
CRc-06	razor clam	1/2x	40	56	14	42	105.0
CRc-07	razor clam	1x	80	72	14	58	72.50
CRc-08	razor clam	1x	80	109	14	95	118.8
CRc-09	razor clam	1x	80	115	14	101	126.3
CRc-10	razor clam	2x	160	139	14	125	78.13
CRc-11	razor clam	2x	160	160	14	146	91.25
CRc-12	razor clam	2x	160	183	14	169	105.6
CRd-00	razor clam	Blank	0	11	11	0	0.000
CRd-04	razor clam	1/2x	40	50	11	39	97.50
CRd-05	razor clam	1/2x	40	45	11	34	85.00
CRd-06	razor clam	1/2x	40	37	11	26	65.00
CRd-07	razor clam	1x	80	95	11	84	105.0
CRd-08	razor clam	1x	80	84	11	73	91.25
CRd-09	razor clam	1x	80	105	11	94	117.5
CRd-10	razor clam	2x	160	117	11	106	66.25
CRd-11	razor clam	2x	160	169	11	158	98.75
CRd-12	razor clam	2x	160	168	11	157	98.13
CRe-00	razor clam	Blank	0	15	15	0	0.000
CRe-04	razor clam	1/2x	40	50	15	35	87.50
CRe-05	razor clam	1/2x	40	61	15	46	115.0
CRe-06	razor clam	1/2x	40	57	15	42	105.0
CRe-07	razor clam	1x	80	101	15	86	107.5
CRe-08	razor clam	1x	80	76	15	61	76.25
CRe-09	razor clam	1x	80	87	15	72	90.00
CRe-10	razor clam	2x	160	201	15	186	116.3
CRe-11	razor clam	2x	160	152	15	137	85.63
CRe-12	razor clam	2x	160	165	15	150	93.75

2. **Repeatability:** Repeatability is the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Suggested procedure**:** Use shellfish free of target analyte(s). For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take four aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at half the action level. Spike a second aliquot with the target analyte(s) at the action level. Spike the third aliquot with the target analyte(s) at twice the action level. Do not spike the fourth aliquot; this is the sample blank. For each aliquot, excluding the sample blank, prepare three sub-aliquots for analysis. Process each sub-aliquot, including the sample blank, to determine the method concentration of the target analyte(s). Repeat this process for each shellfish type of interest with a minimum of five samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

When available, shellfish with naturally incurred target analyte(s) should be included. Use a minimum of 10-12 animals per sample and prepare as a homogenate. For each shellfish type of interest, use three samples at a range of concentrations bracketing the action level (below, at or near, and above). For each sample homogenate prepare a minimum of three aliquots for analysis. Process each aliquot to determine the method concentration of the target analyte(s).

Data Handling

Response:
A doubly-nested ANOVA was selected as the most robust model to isolate within-run repeatability (technical replicates) from the variance introduced by the hierarchical spike levels and individual tissue homogenates.

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Precision
To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations.

If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA, variance component, Total.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Data:

Working range/Range of interest: 40 µg saxitoxin diHCl equiv/100g of shellfish tissue to 160 µg saxitoxin diHCl equiv /100g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

All data were log-transformed for the statistical analyses.

Nested ANOVA results for Pacific geoduck clam gutball:

	Degrees of freedom	Sum of Squares	Mean Squared Error (MSE)	F value	p-value
Homogenate	4	0.008	0.002	0.439	0.779
Homogenate:SpikeLevels	5	2.768	0.554	116.3	$< 2 \times 10^{-16}$
Residuals	35	0.167	0.005		

Following the data handling procedure and utilizing log-transformed data, the initial F-test confirmed significant variance components for Pacific geoduck clam gutball. To accurately model this variance, a doubly nested ANOVA was employed to match the hierarchical nature of the experimental design. In this model, the three technical replicates are nested within each spike level, which are in turn nested within the five individual homogenates. This doubly nested approach ensures that the analysis accounts for internal correlation within samples and provides a valid test of the effects of concentration across the species.

For all boxplots shown for each species below, the solid line inside the box represents the median, and the box edges (top and bottom lines of the box) represent the first and third quartiles. The whiskers are the lines that extend from the box and represent the maximum and minimum values within 1.5 times the interquartile range.

Doubly-nested ANOVA model - results:

- Pacific geoduck clam gutball:
  - F value: 0.0075
  - P-value: 0.9999

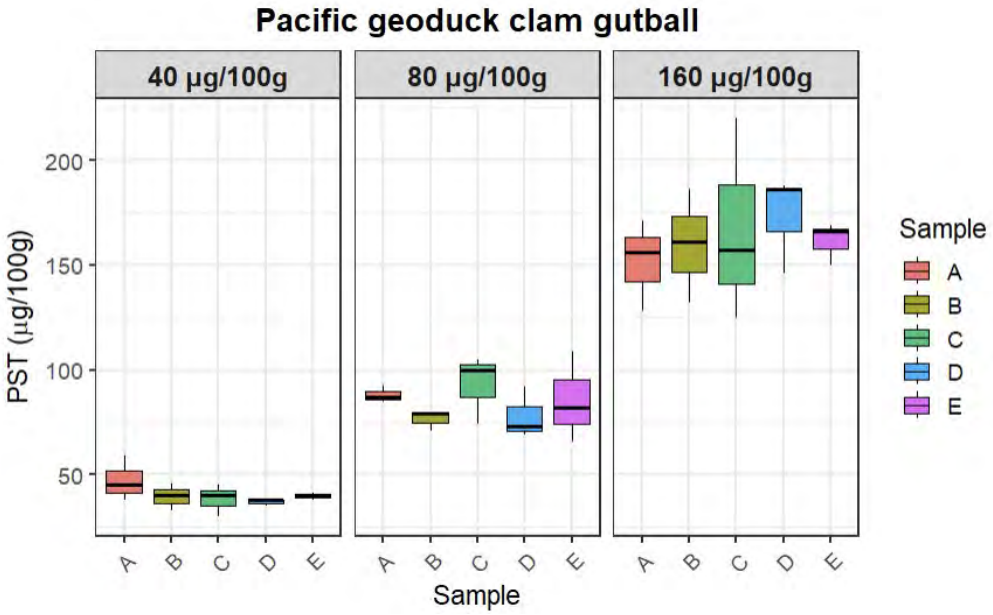


Figure 2.1. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five Pacific geoduck clam gutball samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Whole Pacific oyster:
  - F value: 0.1033
  - P-value: 0.9788

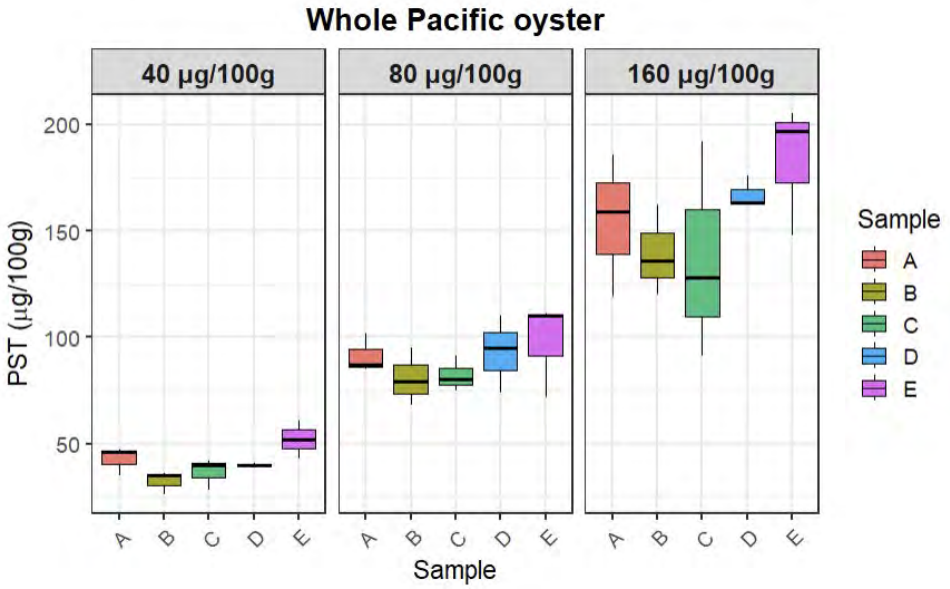


Figure 2.2. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five whole Pacific oyster samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Whole hard clam:
  - F value: 0.0425
  - P-value: 0.996

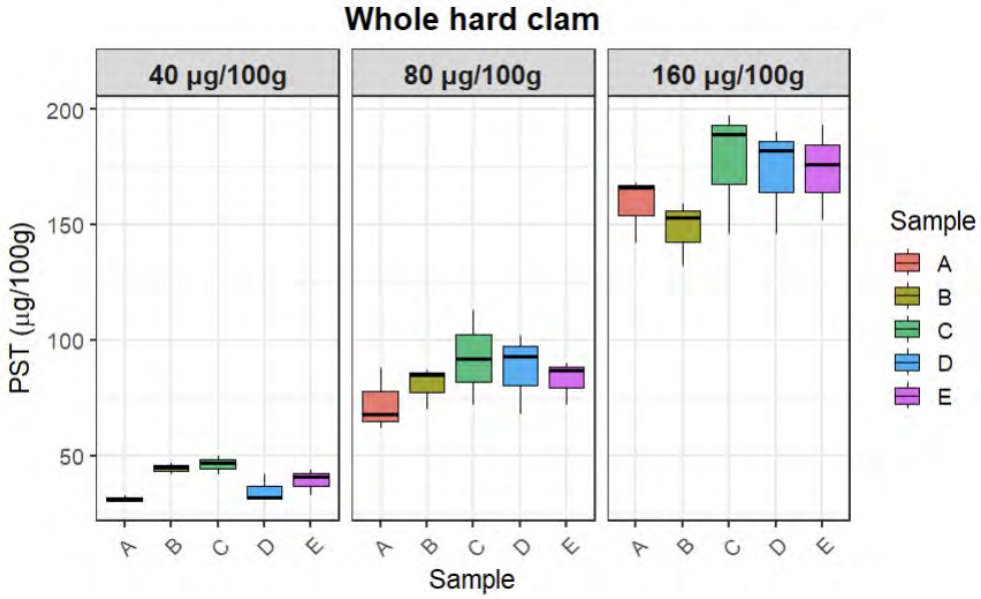


Figure 2.3. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five whole hard clam samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Razor clam meat:
  - F value: 0.0284
  - P-value: 0.9982

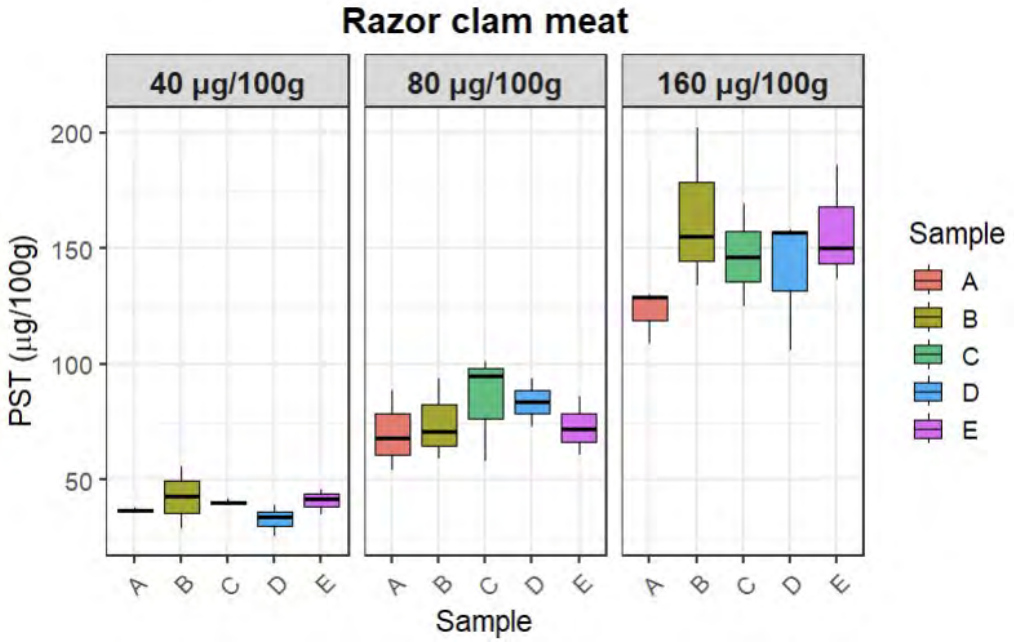


Figure 2.5. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five razor clam meat samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

• Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant?

- Pacific geoduck clam gutball: No
- Whole Pacific oysters: No
- Whole hard clams: No
- Razor clam meat: No

The resulting small F-value for the variance components indicates a high degree of consistency across the different spike levels. Rather than reflecting a lack of sensitivity, this tiny variance ratio demonstrates that the method's performance is stable and reproducible across the tested concentrations. This confirms that the subdivision of the five primary samples into nested spike levels and replicates did not introduce significant experimental error, highlighting the robustness of the data handling procedure for all shellfish species.

• If the variability of the method as implemented by the laboratory is consistent over the range in

concentrations important to its intended applications, what is the coefficient of variation? **See below.**

Table 2.1. Summary of RBA concentration results for Pacific geoduck clam gutball, whole Pacific oyster, and whole hard clam across three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Shown are the number of replicates (n), average measured concentration (µg saxitoxin diHCl equiv /100 g shellfish tissue), standard deviation, and percent coefficient of variation (%CV).

Pacific geoduck clam gutball

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	40.4	6.7	16.7
Medium (80 µg/100 g)	15	84.3	13.2	15.7
High (160 µg/100 g)	15	162.7	25.7	15.8

Whole Pacific oyster

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	40.7	8.8	21.6
Medium (80 µg/100 g)	15	88.9	14.5	16.4
High (160 µg/100 g)	15	156.3	32.7	20.9

Whole hard clam

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	39.3	6.9	17.6
Medium (80 µg/100 g)	15	83.3	14.3	17.1
High (160 µg/100 g)	15	166.1	20.9	12.6

Razor clam meat

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	38.8	7.1	18.2
Medium (80 µg/100 g)	15	77.3	15.5	20.1
High (160 µg/100 g)	15	146.2	26.5	18.1

Percent coefficient of variation (%CV) was calculated separately for each spike level to accurately assess precision and repeatability. Overall, the variability of the method is consistent over the range of concentrations for all shellfish species.

Full Data: The full data used for Repeatability can be found under the Recovery section.

Repeatability for Naturally Incurring Samples:

In this section, each sample was subdivided into three 5-gram tissue aliquots, each assigned to a specific concentration range: low (<60 µg saxitoxin diHCl equiv /100 g shellfish tissue), medium (60-120 µg saxitoxin diHCl equiv /100 g shellfish tissue), and high (>120 µg saxitoxin diHCl equiv /100 g shellfish tissue). Because these levels are unique to each sample and do not overlap across the experimental groups, a collective statistical analysis was not performed. This lack of shared reference levels precludes a direct comparison of main effects, as any observed variance could be attributed to either the group identity or the specific concentration range. Consequently, these two factors are statistically confounded and cannot be decoupled for a unified analysis.

Note: The naturally incurring northern razor clam meat was analyzed using post-column oxidation (PCOX) method rather than the traditional mouse bioassay.

Pacific geoduck clam gutball:

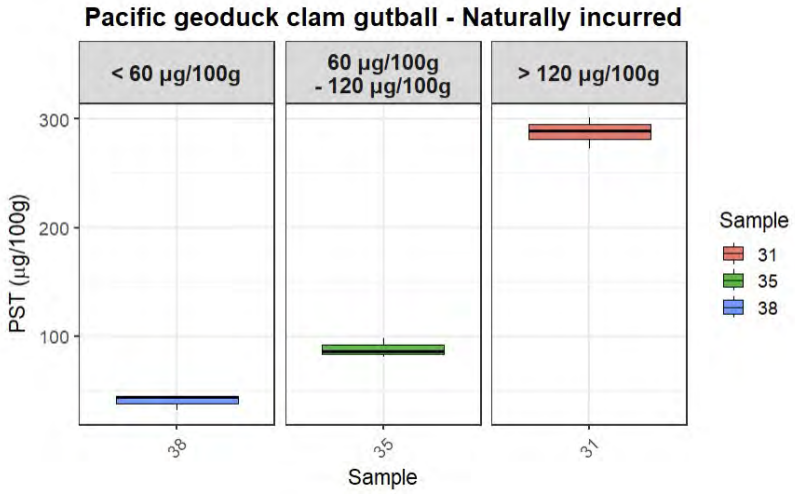


Figure 2.3. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred Pacific geoduck clam gutball samples, each sample representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue, 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Whole Pacific oyster:

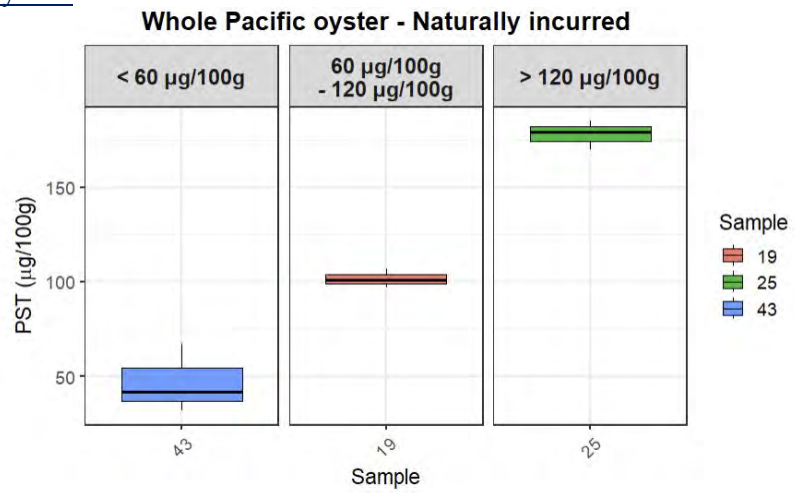


Figure 2.4. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred whole Pacific oyster samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue, 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Whole hard clam:

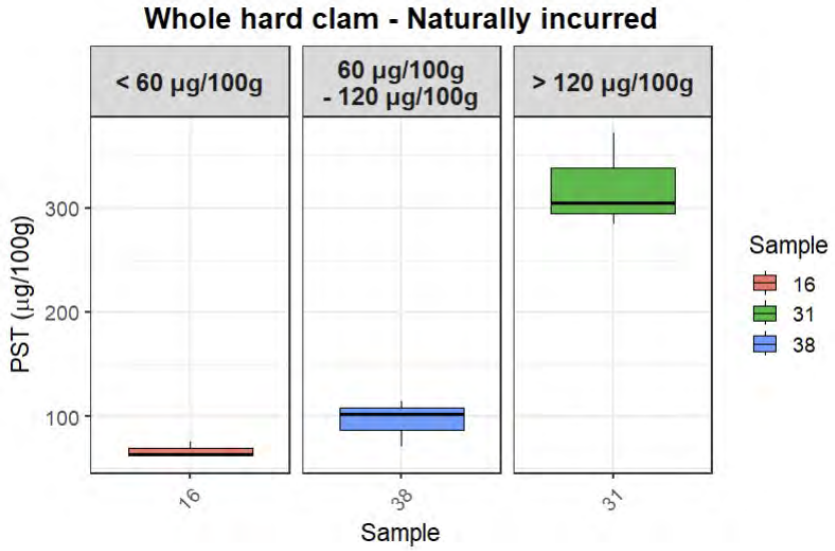


Figure 2.5. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred whole hard clam samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Northern razor clam meat:

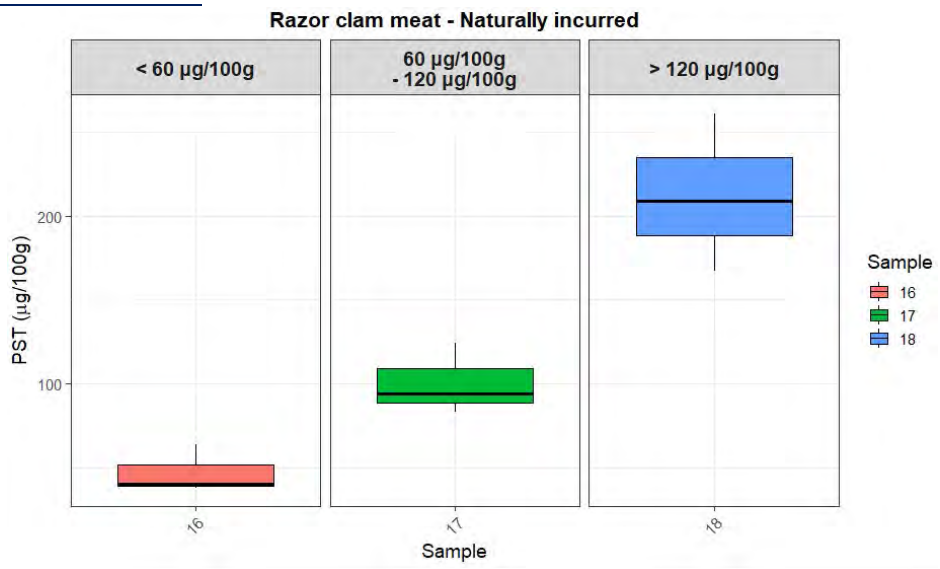


Figure 2.6. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred northern razor clam meat samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g; 60 to120 µg saxitoxin diHCl equiv /100 g; and > 160 µg saxitoxin diHCl equiv /100 g). Each sample was analyzed in triplicate.

• If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**

Table 2.2. Summary of PST concentration results for naturally incurred shellfish samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv/100 g shellfish tissue; 60 to 120 µg saxitoxin diHCl equiv /100 g shellfish tissue; and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Shown are the number of replicates (n), MBA concentration, and average measured concentration (µg saxitoxin diHCl equiv /100 g shellfish tissue), standard deviation, and percent coefficient of variation (%CV) for RBA PST values.

Pacific geoduck clam gutball

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (<60 ug/100 g)	3	50.00	40.33	7.234	17.94
Medium (60 - 120 ug/100 g)	3	87.00	88.33	8.737	9.891
High (>120 ug/100 g)	3	254.0	287.7	14.05	4.883

Whole Pacific oyster

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	40.00	47.00	18.03	38.86
Medium (80 µg/100 g)	3	87.00	101.7	5.033	4.951
High (160 µg/100 g)	3	235.0	178.0	7.550	4.241

Whole hard clam

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	40.00	66.67	8.145	12.22
Medium (80 µg/100 g)	3	95.00	95.67	22.19	23.19
High (160 µg/100 g)	3	236.00	320.33	45.96	14.35

Razor clam meat

	n	*PCOX (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	37.00	47.33	14.47	30.57
Medium (80 µg/100 g)	3	99.00	100.3	21.22	21.15
High (160 µg/100 g)	3	184.00	212.3	47.09	22.18

Full Data:

Pacific geoduck clam gutball

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	38-1	50	32
Low (< 60 µg/100g)	38-2	50	44
Low (< 60 µg/100g)	38-3	50	45
Medium (60-120 µg/100g)	35-1	87	81
Medium (60-120 µg/100g)	35-2	87	98
Medium (60-120 µg/100g)	35-3	87	86
High (> 120 µg/100g)	31-1	254	289
High (> 120 µg/100g)	31-2	254	301
High (> 120 µg/100g)	31-3	254	273

Whole Pacific oyster

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	43-1	40	32
Low (< 60 µg/100g)	43-2	40	67
Low (< 60 µg/100g)	43-3	40	42
Medium (60-120 µg/100g)	19-3	87	101
Medium (60-120 µg/100g)	19-4	87	97
Medium (60-120 µg/100g)	19-5	87	107
High (> 120 µg/100g)	25-1	235	179
High (> 120 µg/100g)	25-2	235	170
High (> 120 µg/100g)	25-3	235	185

Whole hard clam

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	16-1	40	63
Low (< 60 µg/100g)	16-2	40	61
Low (< 60 µg/100g)	16-3	40	76
Medium (60-120 µg/100g)	38-1	95	102
Medium (60-120 µg/100g)	38-2	95	71
Medium (60-120 µg/100g)	38-3	95	114
High (> 120 µg/100g)	31-1	236	284
High (> 120 µg/100g)	31-2	236	305
High (> 120 µg/100g)	31-3	236	372

Razor clam meat

Action Level	*Sample ID*	*PCOX (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	16-1	37	38
Low (< 60 µg/100g)	16-2	37	64
Low (< 60 µg/100g)	16-3	37	40
Medium (60-120 µg/100g)	17-1	99	94
Medium (60-120 µg/100g)	17-2	99	124
Medium (60-120 µg/100g)	17-3	99	83
High (> 120 µg/100g)	18-1	184	167
High (> 120 µg/100g)	18-2	184	261
High (> 120 µg/100g)	18-3	184	209

3. Linear Range, Limit of Detection, and Limit of Quantification: Linear range is the range within the working range where the results are proportional to the concentration of the analyte present in the sample. The limit of detection is the minimum concentration at which the analyte can be identified. Limit of detection is matrix and analyte dependent. The limit of quantification is the minimum concentration of the analyte that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Suggested procedure**:** Use samples free of target analyte(s); analyze intended blank matrix tissue for background interferents. For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. For each sample take a minimum of six aliquots of the homogenate appropriately sized for the work and spike five of the six aliquots with five different concentrations of the target analyte(s), spanning beyond the desired working range and including levels half, at, and twice the action level. Do not spike the sixth aliquot; this is the sample blank. Process each aliquot, including the sample blank to determine concentration for the target analyte(s). For each aliquot, excluding the sample blank, sub-aliquot for three replicate analyses. Repeat this process for each shellfish type of interest with a minimum of five samples collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots. Use the same spike levels for each of the samples analyzed.

Data:
Working range/Range of interest: 20 to 320 µg saxitoxin diHCl equiv /100 g shellfish tissue
Range in spiking levels used: 20, 40, 80, 160, & 320 µg saxitoxin diHCl equiv /100 g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oysters, whole hard clams (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Linear Range
To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to

it.

6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the

analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response

7. Multiply the value obtained in step 5 by 0.95 and 1.05.
8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects

either of the lines obtained above.

10. The linear range of the method as implemented by the laboratory is comprised of the range in

concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organism of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of “blank + 3σ” and “blank + 10σ” generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly, the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary

Data was produced according to the outlined procedure (ten samples spiked at five concentration levels, each analyzed in triplicate along with a blank) for every matrix type evaluated. However, the resulting data could not be processed using the methods specified in the data handling section of

this SOP. The majority of this data was not utilized for determining linearity or LOQ/LOD, it is included at the end of this data summary for reference.

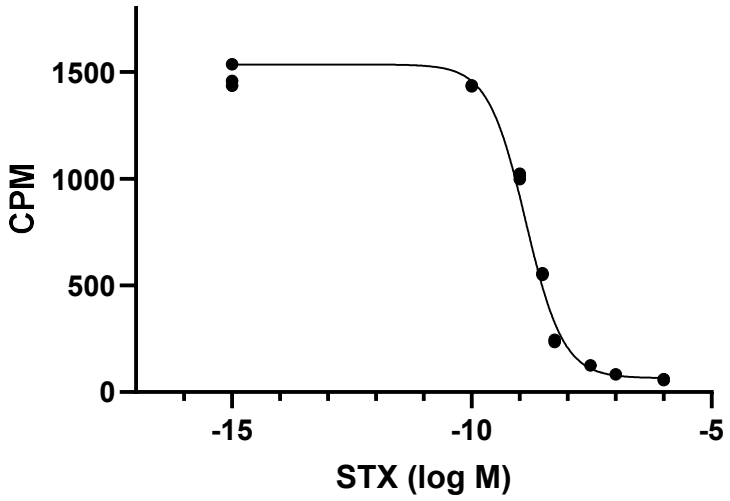
STX Standard Curve: RM 8642a FDA Saxitoxin Dihydrochloride Solution reference standard (100 µg/mL or 268.8 µM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6 x 10^{-6}, 6 x 10^{-7}, 1.8 x 10^{-7}, 6 x 10^{-8}, 1.8 x 10^{-8}, 6 x 10^{-9}, 6 x 10^{-10}, 6 x10^{-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 3H-STX binding in the absence of unlabeled STX.

For a given sample, if none of the sample dilutions falls within the linear range of 0.2 to 0.7 (i.e., the concentration is too high, b/bo < 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/bo > 0.7) at sample dilution 1:10, the sample must be reported as below the LOQ.

1) Linear Range of Assay

To evaluate the linear range of the assay, 8-point STX standard curves (ranging from 0 to 1000 nM) from 80 RBA plates run in this validation were generated using the sigmoidal dose-response with variable slope, using a four-parameter logistic to perform curve fitting in Prism 10 (GraphPad Software). Based from the AOAC Official Method 2011.27, sample quantification should be done only on dilutions that on the linear part of the curve [b/bo = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and Bo is the maximum CPM)].

(a)



(b)

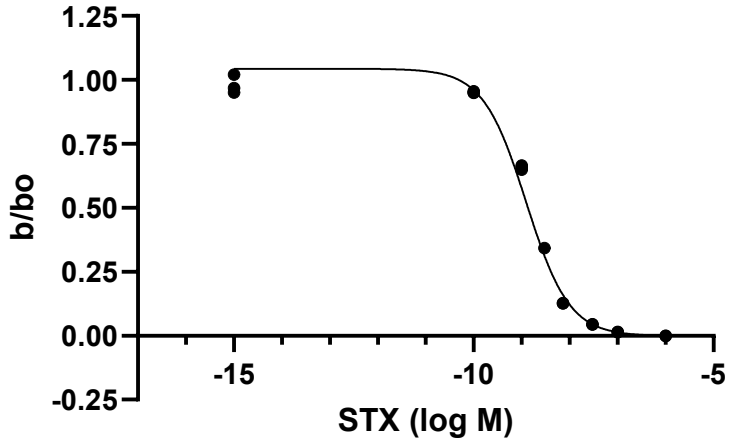
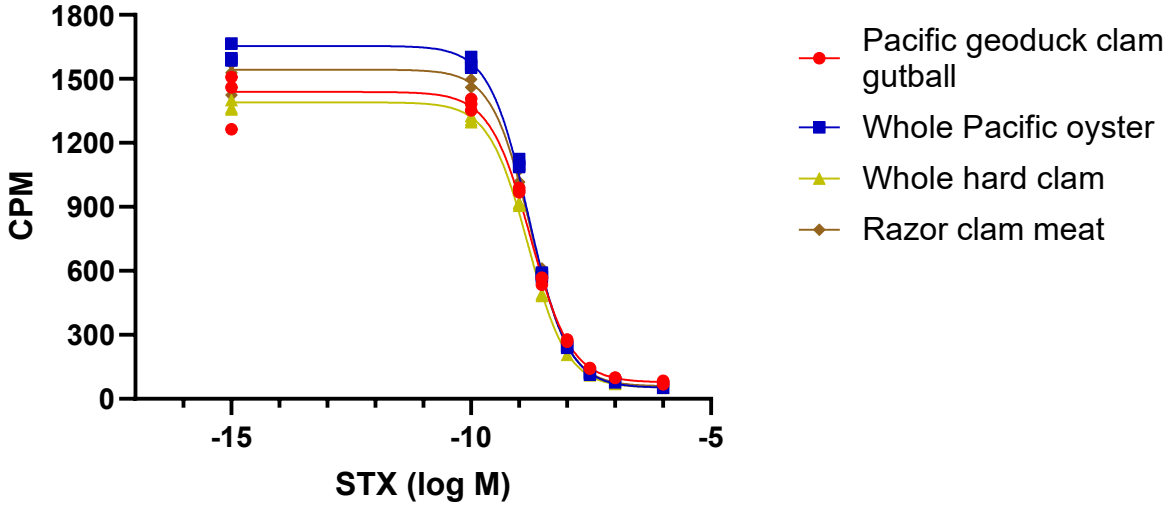


Figure 3.1. Average calibration curves derived from 80 independent assays performed by a single laboratory during the study period. The average IC_{50} was 1.65 ± 0.09 nM, with an average slope of 1.06 ± 0.024. Dots represent the average of each replicate. (a) Binding activity is measured in counts per minute (CPM). (b) Binding activity is measured in b/bo, where b represents the amount of radiolabeled ligand currently bound to the receptors and bo represents the total number of available receptor sites in the sample.

(a)



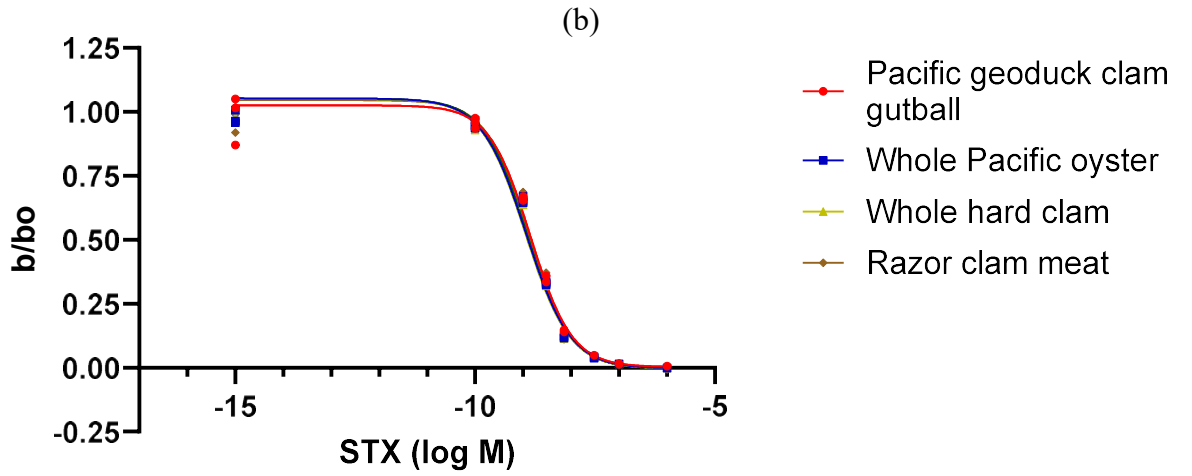


Figure 3.2. Competitive binding inhibition of saxitoxin (STX) in various shellfish tissues. Curves represent the displace of radiolabeled ligand by unlabeled STX (log M) across five shellfish matrices: Pacific geoduck clam gutball (n = 20; red), whole Pacific oyster (n = 19; blue), whole hard clam (n = 19; yellow), and razor clam meat (n = 19; brown). (a) Binding activity is measured in counts per minute (CPM). (b) Binding activity is measured in b/bo, where b represents the amount of radiolabeled ligand currently bound to the receptors and bo represents the total number of available receptor sites in the sample.

Table 3.1. Average and standard deviation of IC_{50} and slope values for each shellfish matrix type.

	Average IC_{50} (nM)	*Standard deviation IC_{50} (nM)*	*Average slope*	*Standard deviation slope*
All matrix types	1.65	0.20	1.068	0.123
Pacific geoduck clam gutball	1.74	0.15	1.034	0.089
Whole Pacific oyster	1.59	0.21	1.065	0.071
Whole hard clam	1.55	0.19	1.078	0.075
Razor clam meat	1.73	0.26	1.095	0.258

Linearity within the range defined by the bend points was evaluated using serial dilutions of shellfish samples spiked with STX at five concentrations (20, 40, 80, 160, and 320 µg STX diHCl equiv./100 g shellfish tissue). For each matrix type, five samples were prepared, and three replicate extractions were performed and analyzed per sample. According to the method protocol, the extraction procedure produces a twofold dilution (5 g of shellfish extracted to a final volume of 5 mL). A standardized dilution series (1:10, 1:50, and 1:200) was applied to each sample, ensuring that at least one dilution would fall within the linear region of the competition curve for shellfish containing approximately 5–1500 µg STX diHCl equiv./100g shellfish tissue. Each extract was subsequently diluted six times in MOPS/choline chloride assay buffer (pH 7.4) on the assay plate, yielding a total of five dilution levels per sample.

The expected and mean measured values are listed in Table 3.2. Figure 3.3 displayed the linear regression with the slope and r^2 value for each shellfish matrix.

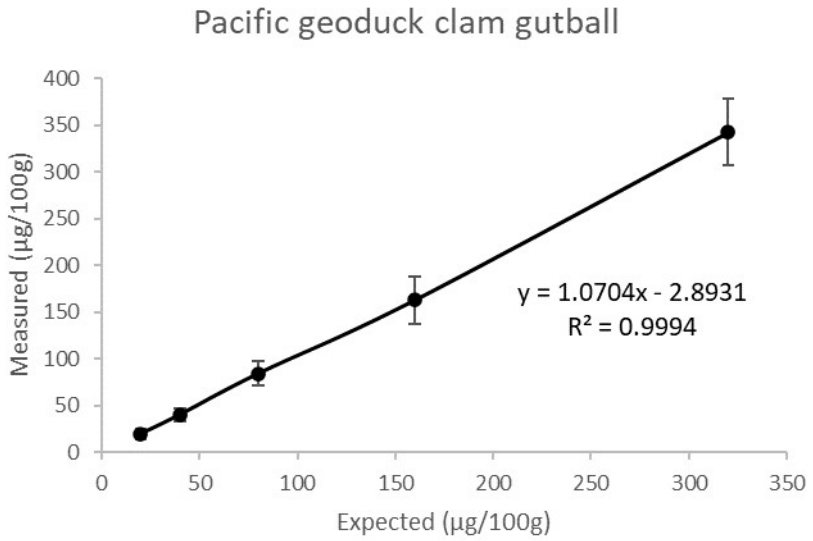
Table 3.2. Expected concentration and average measured concentrations measured by RBA (µg saxitoxin diHCl equiv /100 g shellfish tissue). Shellfish were spiked to five different concentrations with STX, and three replicates each of five samples per matrix were analyzed (n=20).

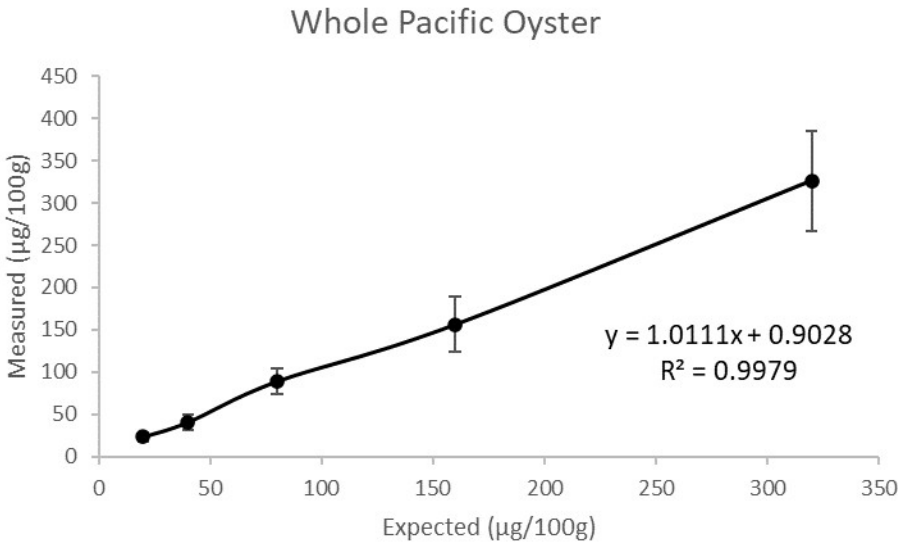
	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Pacific geoduck clam gutball	20	19.8	5.3	26.9
	40	40.4	6.7	16.7
	80	84.3	13.2	15.7
	160	162.7	25.7	15.8
	320	341.9	35.6	10.4

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Whole Pacific oyster	20	23.5	5.7	24.4
	40	40.7	8.8	21.6
	80	88.9	14.5	16.4
	160	156.3	32.7	20.9
	320	326.3	58.9	18.0

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Whole hard clam	20	20.1	4.2	21.0
	40	39.3	6.9	17.6
	80	83.3	14.3	17.1
	160	166.1	20.9	12.6
	320	316.1	39.1	12.4

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Razor clam meat	20	18.9	6.8	35.8
	40	38.8	7.1	18.2
	80	77.3	15.5	20.1
	160	146.2	26.5	18.1
	320	309.5	59.1	19.1


Pacific geoduck clam gutball
Measured (µg/100g)
Expected (µg/100g)
y = 1.0704x - 2.8931
R² = 0.9994
0
50
100
150
200
250
300
350
400


Whole Pacific Oyster
Measured (µg/100g)
Expected (µg/100g)
y = 1.0111x + 0.9028
R² = 0.9979
0
50
100
150
200
250
300
350
400
450

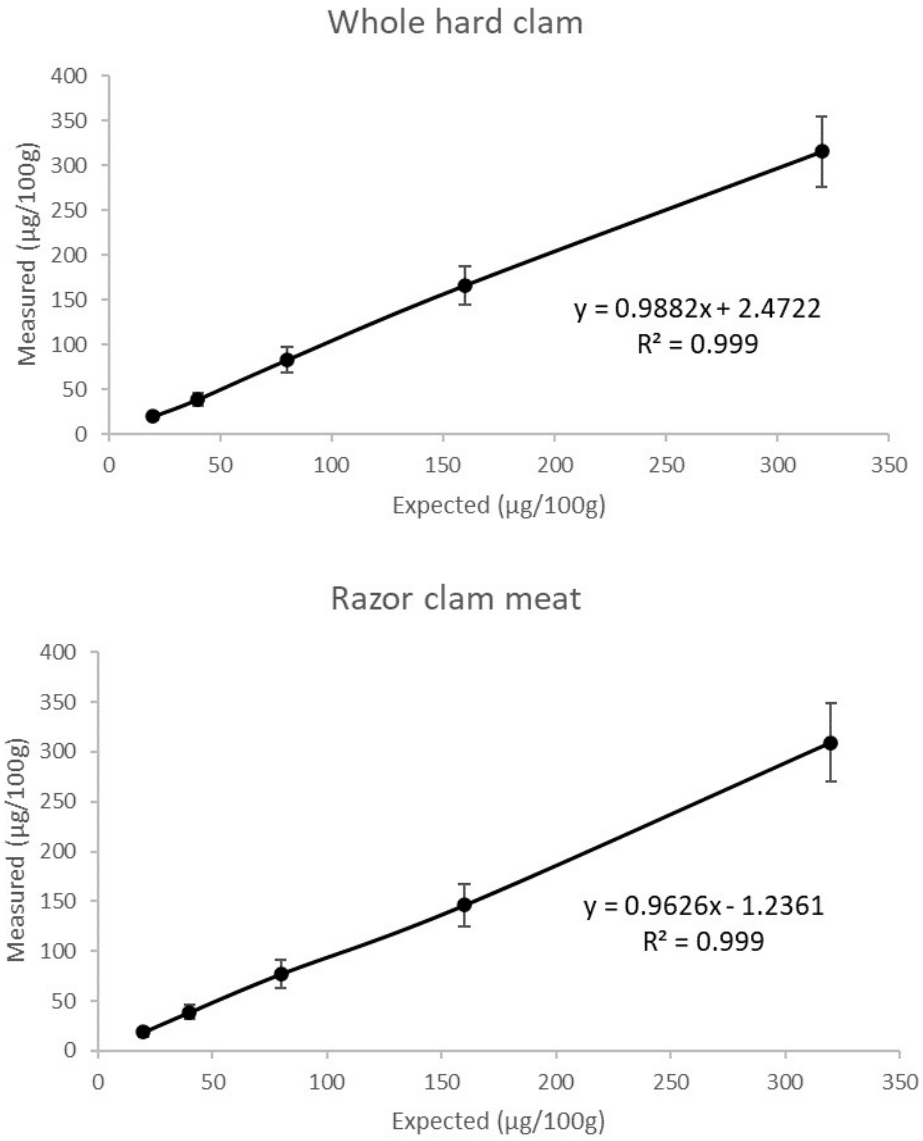


Figure 3.3. Expected concentrations vs. average concentrations measured by RBA (µg STX diHCl equiv./100g shellfish tissue). Five spike levels: 20, 40, 80, 160, and 320 µg STX diHCl equiv./100g shellfish tissue were used for five samples with three replicates per spike level. Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat were spiked with RM 8642a FDA Saxitoxin Dihydrochloride Solution and analyzed (n=60). Error bars represent standard deviation.

Calibration standard (QC check): A reference standard containing 1.8 x 10^{-8} M STX standard (3.0 x 10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquoted 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. 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.

In addition to evaluating the linear range, a calibration standard (QC check) was included to assess assay accuracy and consistency. At the WADoH laboratory, two QC checks were performed at the beginning and end of each assay plate. Across all shellfish matrices, a total of 160 QC checks were conducted, yielding an average concentration of 3.17 ± 0.48 nM.

2. Limit of Detection and Limit of Quantitation

The limits of detection (LOD) and quantitation (LOQ) were determined based on the average LOD values obtained from assay plates for each shellfish matrix. These values were derived from the IC_{80} data using the equations below, assuming a 1:10 sample dilution.

(nM STX eq) x (10 (sample dilution)) x (210 µL total volume/35 µL sample) = mM STX eq in extract

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

µg STX diHCl equiv./mL x (mL extract/g shellfish) x (100 g/g)
= µg STX diHCl equiv./100g shellfish tissue

Table 3.3. The average limit of detection (LOD) and limit of quantitation (LOQ) for each shellfish matrix in µg STX /100g shellfish tissue

.

	LOD	*LOQ*
All matrix types	2.01	3.31
Pacific geoduck clam gutball	2.04	3.42
Whole Pacific oyster	1.91	3.13
Whole hard clam	1.89	3.12
Razor clam meat	2.19	3.57

Full Data:

Sample ID	Shellfish Matrix	Spike Level	Spike (µg/100g)	RBA (µg/100g)	Blank value (µg/100g)	RBA-Blank (µg/100g)	Dilution
CGa-16	geoduck	Blank	0	7	7	0	10
CGa-01	geoduck	1/4x	20	32	7	25	50
CGa-03	geoduck	1/4x	20	33	7	26	50
CGa-18	geoduck	1/4x	20	23.5	7	16.5	Avg. of 10 & 50
CGa-06	geoduck	1/2x	40	52	7	45	50
CGa-20	geoduck	1/2x	40	66	7	59	50
CGa-21	geoduck	1/2x	40	45	7	38	50
CGa-07	geoduck	1x	80	92	7	85	50
CGa-08	geoduck	1x	80	99.5	7	92.5	Avg. of 10 & 50
CGa-09	geoduck	1x	80	94	7	87	50
CGa-10	geoduck	2x	160	135	7	128	200
CGa-11	geoduck	2x	160	178	7	171	200
CGa-12	geoduck	2x	160	163	7	156	200
CGa-13	geoduck	4x	320	316	7	309	200
CGa-14	geoduck	4x	320	361	7	354	200
CGa-15	geoduck	4x	320	316	7	309	200
CGb-00	geoduck	Blank	0	6	6	0	10
CGb-02	geoduck	1/4x	20	29.5	6	23.5	Avg. of 10 & 50
CGb-03	geoduck	1/4x	20	23.5	6	17.5	Avg. of 10 & 50
CGb-17	geoduck	1/4x	20	26	6	20	50
CGb-20	geoduck	1/2x	40	52	6	46	50
CGb-21	geoduck	1/2x	40	39	6	33	50
CGb-05	geoduck	1/2x	40	46	6	40	50
CGb-07	geoduck	1x	80	86	6	80	50
CGb-08	geoduck	1x	80	85	6	79	50
CGb-09	geoduck	1x	80	77	6	71	50
CGb-10	geoduck	2x	160	192	6	186	200
CGb-11	geoduck	2x	160	138	6	132	50
CGb-12	geoduck	2x	160	167	6	161	200
CGb-13	geoduck	4x	320	304	6	298	200
CGb-14	geoduck	4x	320	309	6	303	200
CGb-15	geoduck	4x	320	380	6	374	200
CGc-16	geoduck	Blank	0	10	10	0	10
CGc-19	geoduck	1/4x	20	29.5	10	19.5	Avg. of 10 & 50
CGc-01	geoduck	1/4x	20	24	10	14	10
CGc-02	geoduck	1/4x	20	21	10	11	50
CGc-04	geoduck	1/2x	40	55	10	45	50
CGc-05	geoduck	1/2x	40	50	10	40	50

CGc-06	geoduck	1/2x	40	40	10	30	50
CGc-07	geoduck	1x	80	110	10	100	50
CGc-08	geoduck	1x	80	115	10	105	50
CGc-09	geoduck	1x	80	84	10	74	50
CGc-10	geoduck	2x	160	230	10	220	200
CGc-11	geoduck	2x	160	135	10	125	200
CGc-12	geoduck	2x	160	167	10	157	200
CGc-13	geoduck	4x	320	343	10	333	200
CGc-14	geoduck	4x	320	354	10	344	200
CGc-15	geoduck	4x	320	442	10	432	200
CGd-16	geoduck	Blank	0	11	11	0	10
CGd-17	geoduck	1/4x	20	24	11	13	10
CGd-18	geoduck	1/4x	20	26	11	15	50
CGd-19	geoduck	1/4x	20	33	11	22	50
CGd-20	geoduck	1/2x	40	49	11	38	50
CGd-21	geoduck	1/2x	40	49	11	38	50
CGd-22	geoduck	1/2x	40	46	11	35	50
CGd-07	geoduck	1x	80	103	11	92	50
CGd-08	geoduck	1x	80	84	11	73	50
CGd-09	geoduck	1x	80	80	11	69	50
CGd-10	geoduck	2x	160	199	11	188	200
CGd-11	geoduck	2x	160	197	11	186	200
CGd-12	geoduck	2x	160	157	11	146	200
CGd-13	geoduck	4x	320	357	11	346	200
CGd-14	geoduck	4x	320	381	11	370	200
CGd-15	geoduck	4x	320	334	11	323	200
CGe-00	geoduck	Blank	0	6	6	0	10
CGe-01	geoduck	1/4x	20	25.5	6	19.5	Avg. of 10 & 50
CGe-02	geoduck	1/4x	20	33	6	27	50
CGe-03	geoduck	1/4x	20	34	6	28	50
CGe-04	geoduck	1/2x	40	47	6	41	50
CGe-05	geoduck	1/2x	40	44	6	38	50
CGe-06	geoduck	1/2x	40	46	6	40	50
CGe-07	geoduck	1x	80	88	6	82	50
CGe-08	geoduck	1x	80	72	6	66	50
CGe-09	geoduck	1x	80	115	6	109	50
CGe-10	geoduck	2x	160	156	6	150	200
CGe-11	geoduck	2x	160	175	6	169	200
CGe-12	geoduck	2x	160	172	6	166	200
CGe-13	geoduck	4x	320	323	6	317	200
CGe-14	geoduck	4x	320	379	6	373	200
CGe-15	geoduck	4x	320	350	6	344	200

OPa-00	oyster	Blank	0	0	0	0	10
OPa-32	oyster	1/4x	20	24	0	24	Avg. of 10 & 50
OPa-33	oyster	1/4x	20	23.5	0	23.5	Avg. of 10 & 50
OPa-34	oyster	1/4x	20	24.5	0	24.5	Avg. of 10 & 50
OPa-04	oyster	1/2x	40	47	0	47	50
OPa-21	oyster	1/2x	40	35	0	35	50
OPa-06	oyster	1/2x	40	46	0	46	50
OPa-07	oyster	1x	80	87	0	87	50
OPa-08	oyster	1x	80	102	0	102	50
OPa-09	oyster	1x	80	85	0	85	50
OPa-10	oyster	2x	160	186	0	186	200
OPa-11	oyster	2x	160	119	0	119	200
OPa-12	oyster	2x	160	159	0	159	200
OPa-13	oyster	4x	320	230	0	230	200
OPa-14	oyster	4x	320	319	0	319	200
OPa-15	oyster	4x	320	303	0	303	200
OPb-00	oyster	Blank	0	6	6	0	10
OPb-01	oyster	1/4x	20	29	6	23	50
OPb-02	oyster	1/4x	20	27	6	21	50
OPb-03	oyster	1/4x	20	48	6	42	50
OPb-04	oyster	1/2x	40	32	6	26	50
OPb-05	oyster	1/2x	40	41	6	35	50
OPb-06	oyster	1/2x	40	42	6	36	50
OPb-07	oyster	1x	80	101	6	95	50
OPb-08	oyster	1x	80	74	6	68	50
OPb-09	oyster	1x	80	85	6	79	50
OPb-10	oyster	2x	160	168	6	162	200
OPb-11	oyster	2x	160	142	6	136	200
OPb-12	oyster	2x	160	126	6	120	200
OPb-13	oyster	4x	320	357	6	351	200
OPb-14	oyster	4x	320	269	6	263	200
OPb-15	oyster	4x	320	394	6	388	200
OPc-00	oyster	Blank	0	0	0	0	10
OPc-01	oyster	1/4x	20	23.5	0	23.5	Avg. of 10 & 50
OPc-18	oyster	1/4x	20	24	0	24	Avg. of 10 & 50
OPc-19	oyster	1/4x	20	20.5	0	20.5	Avg. of 10 & 50
OPc-20	oyster	1/2x	40	28	0	28	50
OPc-21	oyster	1/2x	40	40	0	40	50
OPc-06	oyster	1/2x	40	42	0	42	50
OPc-07	oyster	1x	80	80	0	80	50
OPc-08	oyster	1x	80	75	0	75	50
OPc-09	oyster	1x	80	91	0	91	50

OPc-10	oyster	2x	160	128	0	128	200
OPc-11	oyster	2x	160	192	0	192	200
OPc-12	oyster	2x	160	91	0	91	200
OPc-13	oyster	4x	320	268	0	268	200
OPc-14	oyster	4x	320	324	0	324	200
OPc-15	oyster	4x	320	295	0	295	200
OPd-16	oyster	Blank	0	5	5	0	10
OPd-01	oyster	1/4x	20	30	5	25	Avg. of 10 & 50
OPd-02	oyster	1/4x	20	24	5	19	50
OPd-19	oyster	1/4x	20	28	5	23	50
OPd-04	oyster	1/2x	40	46	5	41	50
OPd-05	oyster	1/2x	40	45	5	40	50
OPd-22	oyster	1/2x	40	44	5	39	50
OPd-07	oyster	1x	80	79	5	74	50
OPd-08	oyster	1x	80	115	5	110	50
OPd-09	oyster	1x	80	100	5	95	50
OPd-10	oyster	2x	160	168	5	163	200
OPd-11	oyster	2x	160	167	5	162	200
OPd-12	oyster	2x	160	181	5	176	200
OPd-13	oyster	4x	320	400	5	395	200
OPd-14	oyster	4x	320	311	5	306	200
OPd-15	oyster	4x	320	375	5	370	200
OPe-16	oyster	Blank	0	5	5	0	10
OPe-01	oyster	1/4x	20	22	5	17	Avg. of 10 & 50
OPe-18	oyster	1/4x	20	24	5	19	Avg. of 10 & 50
OPe-19	oyster	1/4x	20	25.5	5	20.5	Avg. of 10 & 50
OPe-20	oyster	1/2x	40	48	5	43	50
OPe-21	oyster	1/2x	40	57	5	52	50
OPe-0	oyster	1/2x	40	66	5	61	50
OPe-07	oyster	1x	80	115	5	110	50
OPe-08	oyster	1x	80	116	5	111	50
OPe-09	oyster	1x	80	77	5	72	50
OPe-10	oyster	2x	160	202	5	197	200
OPe-11	oyster	2x	160	210	5	205	200
OPe-12	oyster	2x	160	153	5	148	200
OPe-13	oyster	4x	320	374	5	369	200
OPe-14	oyster	4x	320	449	5	444	200
OPe-15	oyster	4x	320	275	5	270	200
CBa-00	butter clam	Blank	0	9	9	0	10
CBa-01	butter clam	1/4x	20	26	9	17	50
CBa-02	butter clam	1/4x	20	23	9	14	50
CBa-03	butter clam	1/4x	20	20	9	11	50

CBa-04	butter clam	1/2x	40	42	9	33	50
CBa-05	butter clam	1/2x	40	40	9	31	50
CBa-06	butter clam	1/2x	40	39	9	30	50
CBa-07	butter clam	1x	80	77	9	68	50
CBa-08	butter clam	1x	80	71	9	62	50
CBa-09	butter clam	1x	80	97	9	88	50
CBa-10	butter clam	2x	160	175	9	166	200
CBa-11	butter clam	2x	160	151	9	142	200
CBa-12	butter clam	2x	160	177	9	168	200
CBa-13	butter clam	4x	320	330	9	321	200
CBa-14	butter clam	4x	320	265	9	256	200
CBa-15	butter clam	4x	320	307	9	298	200
CMb-28	manila clam	Blank	0	0	0	0	10
CMb-01	manila clam	1/4x	20	23	0	23	10
CMb-02	manila clam	1/4x	20	17	0	17	50
CMb-03	manila clam	1/4x	20	17.5	0	17.5	Avg. of 10 & 50
CMb-04	manila clam	1/2x	40	42	0	42	50
CMb-05	manila clam	1/2x	40	45	0	45	50
CMb-06	manila clam	1/2x	40	47	0	47	50
CMb-07	manila clam	1x	80	87	0	87	50
CMb-08	manila clam	1x	80	85	0	85	50
CMb-09	manila clam	1x	80	70	0	70	50
CMb-10	manila clam	2x	160	159	0	159	200
CMb-11	manila clam	2x	160	153	0	153	200
CMb-12	manila clam	2x	160	132	0	132	200
CMb-13	manila clam	4x	320	280	0	280	200
CMb-14	manila clam	4x	320	310	0	310	200
CMb-15	manila clam	4x	320	258	0	258	200
CMc-00	manila clam	Blank	0	0	0	0	10
CMc-01	manila clam	1/4x	20	23	0	23	Avg. of 10 & 50
CMc-02	manila clam	1/4x	20	23	0	23	50
CMc-03	manila clam	1/4x	20	21	0	21	50
CMc-04	manila clam	1/2x	40	42	0	42	50
CMc-05	manila clam	1/2x	40	50	0	50	50
CMc-06	manila clam	1/2x	40	47	0	47	50
CMc-07	manila clam	1x	80	113	0	113	50
CMc-08	manila clam	1x	80	72	0	72	50
CMc-09	manila clam	1x	80	92	0	92	50
CMc-10	manila clam	2x	160	189	0	189	200
CMc-11	manila clam	2x	160	146	0	146	200
CMc-12	manila clam	2x	160	197	0	197	200
CMc-13	manila clam	4x	320	342	0	342	200

CMc-14	manila clam	4x	320	342	0	342	200
CMc-15	manila clam	4x	320	372	0	372	200
CMd-00	manila clam	Blank	0	6	6	0	10
CMd-01	manila clam	1/4x	20	33	6	27	50
CMd-02	manila clam	1/4x	20	29.5	6	23.5	Avg. of 10 & 50
CMd-03	manila clam	1/4x	20	25	6	19	10
CMd-04	manila clam	1/2x	40	48	6	42	50
CMd-05	manila clam	1/2x	40	38	6	32	50
CMd-06	manila clam	1/2x	40	37	6	31	50
CMd-07	manila clam	1x	80	99	6	93	50
CMd-08	manila clam	1x	80	74	6	68	50
CMd-09	manila clam	1x	80	108	6	102	50
CMd-10	manila clam	2x	160	196	6	190	200
CMd-11	manila clam	2x	160	152	6	146	200
CMd-12	manila clam	2x	160	188	6	182	200
CMd-13	manila clam	4x	320	329	6	323	200
CMd-14	manila clam	4x	320	310	6	304	200
CMd-15	manila clam	4x	320	408	6	402	200
CMe-00	manila clam	Blank	0	4	4	0	10
CMe-01	manila clam	1/4x	20	25	4	21	50
CMe-02	manila clam	1/4x	20	25	4	21	50
CMe-03	manila clam	1/4x	20	28	4	24	10
CMe-04	manila clam	1/2x	40	45	4	41	50
CMe-05	manila clam	1/2x	40	48	4	44	50
CMe-06	manila clam	1/2x	40	37	4	33	50
CMe-07	manila clam	1x	80	91	4	87	50
CMe-08	manila clam	1x	80	94	4	90	50
CMe-09	manila clam	1x	80	76	4	72	50
CMe-10	manila clam	2x	160	197	4	193	200
CMe-11	manila clam	2x	160	180	4	176	200
CMe-12	manila clam	2x	160	156	4	152	200
CMe-13	manila clam	4x	320	329	4	325	200
CMe-14	manila clam	4x	320	321	4	317	200
CMe-15	manila clam	4x	320	296	4	292	200
CRa-00	razor clam	Blank	0	10	10	0	10
CRa-01	razor clam	1/4x	20	25	10	15	Avg. 10 & 50
CRa-02	razor clam	1/4x	20	43	10	33	50
CRa-03	razor clam	1/4x	20	30	10	20	10
CRa-04	razor clam	1/2x	40	46	10	36	50
CRa-05	razor clam	1/2x	40	48	10	38	50
CRa-06	razor clam	1/2x	40	47	10	37	50
CRa-07	razor clam	1x	80	64	10	54	50

CRa-08	razor clam	1x	80	78	10	68	50
CRa-09	razor clam	1x	80	99	10	89	50
CRa-10	razor clam	2x	160	140	10	130	50
CRa-11	razor clam	2x	160	139	10	129	200
CRa-12	razor clam	2x	160	119	10	109	200
CRa-13	razor clam	4x	320	310	10	300	200
CRa-14	razor clam	4x	320	281	10	271	200
CRa-15	razor clam	4x	320	285	10	275	200
CRb-00	razor clam	Blank	0	10	10	0	10
CRb-01	razor clam	1/4x	20	27	10	17	50
CRb-02	razor clam	1/4x	20	38	10	28	50
CRb-03	razor clam	1/4x	20	35	10	25	50
CRb-04	razor clam	1/2x	40	66	10	56	50
CRb-05	razor clam	1/2x	40	39	10	29	50
CRb-20	razor clam	1/2x	40	53	10	43	50
CRb-07	razor clam	1x	80	69	10	59	50
CRb-08	razor clam	1x	80	104	10	94	50
CRb-09	razor clam	1x	80	81	10	71	50
CRb-10	razor clam	2x	160	144	10	134	200
CRb-11	razor clam	2x	160	165	10	155	200
CRb-12	razor clam	2x	160	212	10	202	200
CRb-13	razor clam	4x	320	317	10	307	200
CRb-14	razor clam	4x	320	360	10	350	200
CRb-15	razor clam	4x	320	261	10	251	200
CRc-00	razor clam	Blank	0	14	14	0	10
CRc-01	razor clam	1/4x	20	28	14	14	50
CRc-02	razor clam	1/4x	20	32	14	18	50
CRc-03	razor clam	1/4x	20	32	14	18	50
CRc-04	razor clam	1/2x	40	54	14	40	50
CRc-05	razor clam	1/2x	40	53	14	39	50
CRc-06	razor clam	1/2x	40	56	14	42	50
CRc-07	razor clam	1x	80	72	14	58	50
CRc-08	razor clam	1x	80	109	14	95	50
CRc-09	razor clam	1x	80	115	14	101	50
CRc-10	razor clam	2x	160	139	14	125	200
CRc-11	razor clam	2x	160	160	14	146	200
CRc-12	razor clam	2x	160	183	14	169	200
CRc-13	razor clam	4x	320	352	14	338	200
CRc-14	razor clam	4x	320	297	14	283	200
CRc-15	razor clam	4x	320	235	14	221	200
CRd-00	razor clam	Blank	0	11	11	0	10
CRd-01	razor clam	1/4x	20	37	11	26	50

CRd-02	razor clam	1/4x	20	21	11	10	Avg. 10 & 50
CRd-03	razor clam	1/4x	20	30.5	11	19.5	Avg. 10 & 50
CRd-04	razor clam	1/2x	40	50	11	39	50
CRd-05	razor clam	1/2x	40	45	11	34	50
CRd-06	razor clam	1/2x	40	37	11	26	50
CRd-07	razor clam	1x	80	95	11	84	50
CRd-08	razor clam	1x	80	84	11	73	50
CRd-09	razor clam	1x	80	105	11	94	50
CRd-10	razor clam	2x	160	117	11	106	50
CRd-11	razor clam	2x	160	169	11	158	200
CRd-12	razor clam	2x	160	168	11	157	200
CRd-13	razor clam	4x	320	268	11	257	200
CRd-14	razor clam	4x	320	452	11	441	200
CRd-15	razor clam	4x	320	407	11	396	200
CRe-00	razor clam	Blank	0	15	15	0	10
CRe-01	razor clam	1/4x	20	34	15	19	50
CRe-02	razor clam	1/4x	20	27	15	12	50
CRe-03	razor clam	1/4x	20	24	15	9	Avg. 10 & 50
CRe-04	razor clam	1/2x	40	50	15	35	50
CRe-05	razor clam	1/2x	40	61	15	46	50
CRe-06	razor clam	1/2x	40	57	15	42	50
CRe-07	razor clam	1x	80	101	15	86	50
CRe-08	razor clam	1x	80	76	15	61	50
CRe-09	razor clam	1x	80	87	15	72	50
CRe-10	razor clam	2x	160	201	15	186	200
CRe-11	razor clam	2x	160	152	15	137	200
CRe-12	razor clam	2x	160	165	15	150	200
CRe-13	razor clam	4x	320	286	15	271	200
CRe-14	razor clam	4x	320	376	15	361	200
CRe-15	razor clam	4x	320	335	15	320	200

4. **Measurement uncertainty:** Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including overall precision of the complete method, the method and laboratory bias and matrix effects.

Suggested procedure: Use shellfish free of the target analyte(s). For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take two aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at the action level. Do not spike the second aliquot as this is the sample blank. Process each aliquot to determine the concentration for the target analyte(s). Repeat this process with a minimum of 15 samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

Data:

Working range/Range of interest: 80 µg saxitoxin diHCl equiv/100 g shellfish tissue
Range in spiking levels used: 80 µg saxitoxin diHCl equiv/100 g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary

All data were log-transformed to measure uncertainty.

Calculate measurement uncertainty:

- Pacific geoduck clam gutball: 6.83×10^{-5} – 0.047
- Whole Pacific oyster: 0.017 – 0.068
- Whole hard clam: -2.90×10^{-4} – 0.056
- Razor clam meat: -0.074 – -0.006

Full Data:

Group	Sample ID	Shellfish Matrix	Spike (µg/100g)	Blank value (µg/100g)	RBA-Blank (µg/100g)
A	CGa-07	geoduck	80	7	85
A	CGa-08	geoduck	80	7	92.5
A	CGa-09	geoduck	80	7	87
B	CGb-07	geoduck	80	6	80
B	CGb-08	geoduck	80	6	79
B	CGb-09	geoduck	80	6	71
C	CGc-07	geoduck	80	10	100
C	CGc-08	geoduck	80	10	105
C	CGc-09	geoduck	80	10	74
D	CGd-07	geoduck	80	11	92
D	CGd-08	geoduck	80	11	73
D	CGd-09	geoduck	80	11	69
E	CGe-07	geoduck	80	6	82
E	CGe-08	geoduck	80	6	66
E	CGe-09	geoduck	80	6	109
F	CGf-01	geoduck	80	5	84
G	CGg-02	geoduck	80	5	99
H	CGh-01	geoduck	80	5	86
I	CGi-01	geoduck	80	11	78
J	CGj-01	geoduck	80	8	77
K	CGk-01	geoduck	80	0	88
L	CGL-01	geoduck	80	8	81
M	CGm-01	geoduck	80	4	86
N	CGn-01	geoduck	80	9	85
O	CGo-01	geoduck	80	4	100
A	OPa-07	oyster	80	0	87
A	OPa-08	oyster	80	0	102
A	OPa-09	oyster	80	0	85
B	OPb-07	oyster	80	6	95
B	OPb-08	oyster	80	6	68
B	OPb-09	oyster	80	6	79
C	OPc-07	oyster	80	0	80
C	OPc-08	oyster	80	0	75
C	OPc-09	oyster	80	0	91
D	OPd-07	oyster	80	5	74
D	OPd-08	oyster	80	5	110
D	OPd-09	oyster	80	5	95
E	OPe-07	oyster	80	5	110

E	OPe-08	oyster	80	5	111
E	OPe-09	oyster	80	5	72
F	OPf-01	oyster	80	0	100
G	OPg-01	oyster	80	0	101
H	OPh-01	oyster	80	0	87
I	OPi-01	oyster	80	0	82
J	OPj-01	oyster	80	0	105
K	OPk-01	oyster	80	7	74
L	OPL-01	oyster	80	0	88
M	OPm-03	oyster	80	0	83
N	OPn-01	oyster	80	0	89
O	OPo-01	oyster	80	0	85
A	CBa-07	butter clam	80	9	68
A	CBa-08	butter clam	80	9	62
A	CBa-09	butter clam	80	9	88
B	CMb-07	manila clam	80	0	87
B	CMb-08	manila clam	80	0	85
B	CMb-09	manila clam	80	0	70
C	CMc-07	manila clam	80	0	113
C	CMc-08	manila clam	80	0	72
C	CMc-09	manila clam	80	0	92
D	CMd-07	manila clam	80	6	93
D	CMd-08	manila clam	80	6	68
D	CMd-09	manila clam	80	6	102
E	CMe-07	manila clam	80	4	87
E	CMe-08	manila clam	80	4	90
E	CMe-09	manila clam	80	4	72
F	CBf-01	butter clam	80	0	78
G	CLg-01	littleneck clam	80	9.5	103.5
H	CBh-01	butter clam	80	7	87
I	CMi-03	manila clam	80	11	106
J	CBj-01	butter clam	80	9	85
K	CLk-01	littleneck clam	80	0	101
L	CLL-01	littleneck clam	80	0	84
M	CLm-01	littleneck clam	80	4	76
N	CMn-01	manila clam	80	3	100
O	CLo-01	littleneck clam	80	4	88
A	CRa-07	razor clam	80	10	54
A	CRa-08	razor clam	80	10	68
A	CRa-09	razor clam	80	10	89
B	CRb-07	razor clam	80	10	59
B	CRb-08	razor clam	80	10	94

B	CRb-09	razor clam	80	10	71
C	CRc-07	razor clam	80	14	58
C	CRc-08	razor clam	80	14	95
C	CRc-09	razor clam	80	14	101
D	CRd-07	razor clam	80	11	84
D	CRd-08	razor clam	80	11	73
D	CRd-09	razor clam	80	11	94
E	CRe-07	razor clam	80	15	86
E	CRe-08	razor clam	80	15	61
E	CRe-09	razor clam	80	15	72
F	CRf-01	razor clam	80	18	67
G	CRg-01	razor clam	80	10	74
H	CRh-01	razor clam	80	7	88
I	CRi-01	razor clam	80	9	80
J	CRj-01	razor clam	80	11	54
K	CRk-01	razor clam	80	10	72
L	CRL-01	razor clam	80	17	63
M	CRm-01	razor clam	80	8	58
N	CRn-01	razor clam	80	9	77
O	CRo-01	razor clam	80	8	62

5. **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 Handling:

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 – **Mouse Bioassay for all shellfish species except for northern razor clam meat, which was analyzed by post-column oxidation method.**

- Pacific geoduck clam gutball: 0.09
- Whole Pacific oyster: 0.99
- Whole hard clam: -0.04
- Razor clam meat: -0.30

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method – **Receptor Binding Assay**

- Pacific geoduck clam gutball: -0.01
- Whole Pacific oyster: 0.23
- Whole hard clam: -0.19
- Razor clam meat: -0.15

Variance of the data generated from the officially recognized analytical method– **Mouse Bioassay for all shellfish species except for northern razor clam meat, which was analyzed by post-column oxidation method.**

- Pacific geoduck clam gutball: 0.128
- Whole Pacific oyster: 0.110
- Whole hard clam: 0.136
- Razor clam meat: 0.050

Variance of the data generated from the proposed alternative analytical method– **Receptor Binding Assay**

- Pacific geoduck clam gutball: 0.161
- Whole Pacific oyster: 0.085

- Whole hard clam: 0.105
- Razor clam meat: 0.049

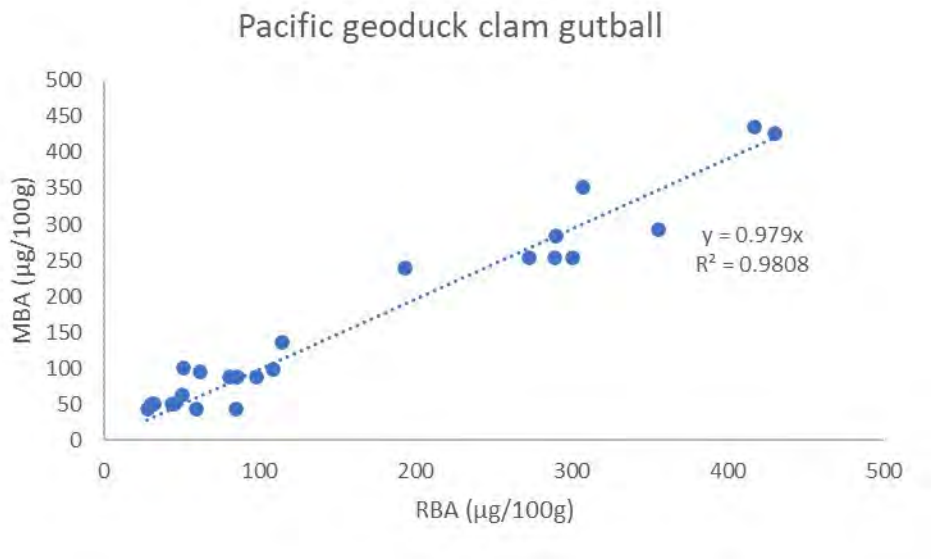
Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods

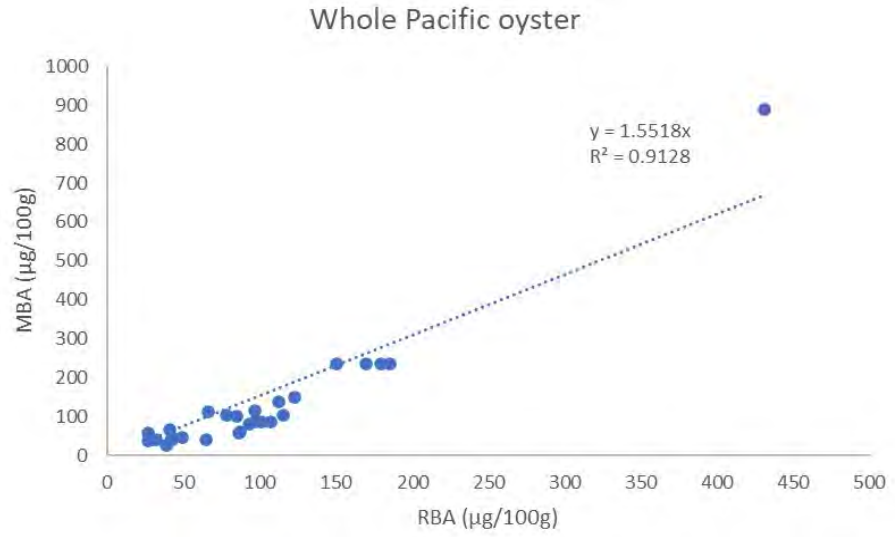
- Pacific geoduck clam gutball: 1.26
- Whole Pacific oyster: 1.29
- Whole hard clam: 1.29
- Razor clam meat: 1.02

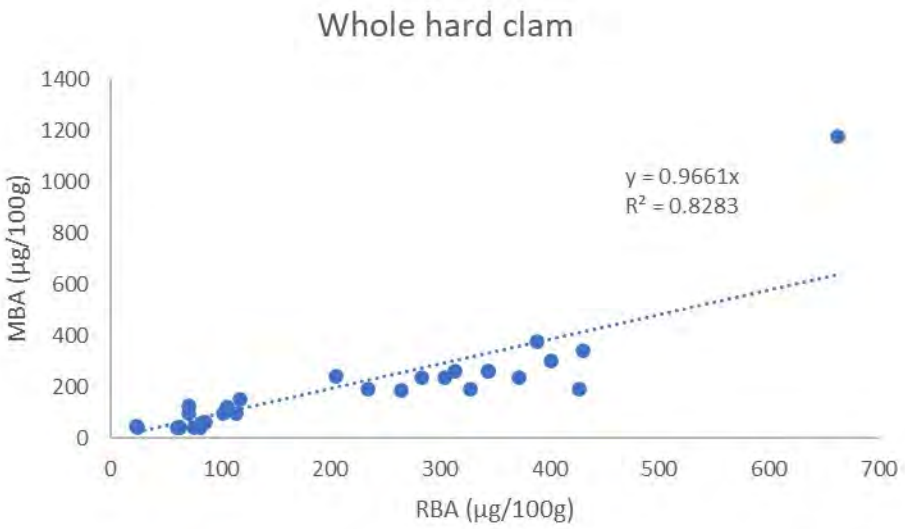
Is there a significant difference between the analytical methods Y/N

- Pacific geoduck clam gutball: No (p = 0.300)
- Whole Pacific oyster: No (p = 0.053)
- Whole hard clam: No (p = 0.0725)
- Razor clam meat: No (p = 0.086)

A total of 111 samples were tested using a previously established method with MBA for all shellfish matrix type except for razor clam meat, which used PCOX, and RBA was used for all shellfish matrix type: Pacific geoduck clam gutball (n=24), whole Pacific oyster (n=27), whole hard clam (n=29), and razor clam meat (n = 31).




Whole Pacific oyster
y = 1.5518x
R² = 0.9128
MBA (µg/100g)
RBA (µg/100g)
0
100
200
300
400
500
600
700
800
900
1000
0
50
100
150
200
250
300
350
400
450
500


Whole hard clam
y = 0.9661x
R² = 0.8283
MBA (µg/100g)
RBA (µg/100g)
0
200
400
600
800
1000
1200
1400
0
100
200
300
400
500
600
700

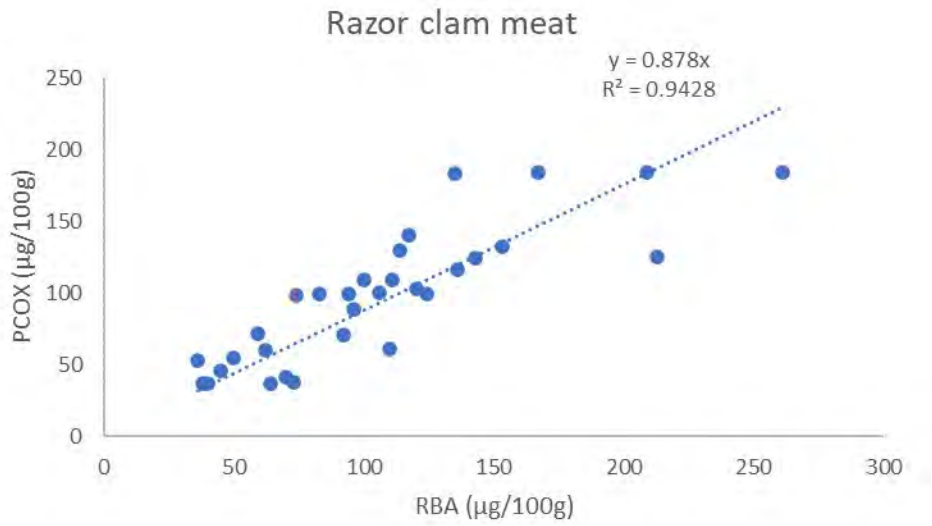


Figure 5.1. Linear regression of MBA versus RBA results in µg STX/100 g shellfish tissue for each shellfish matrix type except for razor clam meat, which was analyzed by PCOX and RBA.

Table 5.1. Average MBA and RBA concentrations for all shellfish matrix type except for razor clam meat (PCOX and RBA) in µg STX/100 g shellfish tissue, and average percent recovery (%) for each matrix type.

Matrix Type	*Avg. MBA Concentration*	*Avg. RBA Concentration*	*Average % Recovery*
Pacific Geoduck clam gutball	165.6	164.4	99.26
Whole Pacific oyster	127.4	98.38	77.22
Whole hard clam	191.3	239.0	124.9
Razor clam meat	97.26	106.3	109.3

Full Data:

Sample ID	Shellfish Matrix	Harvest Area	Harvest Date	Season	RBA (ug/100g)	MBA	% error	% recovery
CG31-1	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	289	254	13.78	113.8
CG31-2	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	301	254	18.50	118.5
CG31-3	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	273	254	7.480	107.5
CG32-1	geoduck	Agate Passage, Kitsap - WA	10/31/2023	Fall	290	283	2.473	102.5
CG33-1	geoduck	Beckett Beach, Kitsap - WA	10/29/2021	Fall	51	100	-49.00	51.00
CG34-1	geoduck	Dungeness West, Clallam - WA	9/25/2023	Fall	430	427	0.7030	100.7
CG35-1	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	81	87	-6.897	93.10
CG35-2	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	98	87	12.64	112.6
CG35-3	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	86	87	-1.149	98.85
CG36-1	geoduck	Agate Pass, Kitsap - WA	10/8/2023	Fall	193	239	-19.25	80.75
CG37-1	geoduck	Manzanita Tract, Kitsap - WA	10/1/2023	Fall	417	435	-4.14	95.86
CG38-1	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	32	50	-36.00	64.00
CG38-2	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	44	50	-12.00	88.00
CG38-3	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	45	50	-10.00	90.00
CG39-1	geoduck	Beckett Beach, Jefferson - WA	10/22/2021	Fall	114	136	-16.18	83.82
CG40-1	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	59	43	37.21	137.2
CG40-2	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	85	43	97.67	197.7
CG40-3	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	28	43	-34.88	65.12
CG41-1	geoduck	Beckett Beach, Jefferson - WA	9/25/2021	Fall	62	94	-34.04	65.96
CG42-1	geoduck	Beckett Beach, Jefferson - WA	11/19/2021	Fall	109	98	11.22	111.2
CG43-1	geoduck	Beckett Beach, Jefferson - WA	10/8/2021	Fall	307	352	-12.78	87.22
CG44-1	geoduck	Beckett Beach, Jefferson - WA	10/15/2021	Fall	356	293	21.50	121.5
CG45-1	geoduck	Beckett Beach, Jefferson - WA	11/12/2021	Fall	31	50	-38.00	62.00

CG46-1	geoduck	Beckett Beach, Jefferson - WA	6/22/2023	Summer	50	63	-20.64	79.37
OP19-3	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	101	87	16.09	116.1
OP19-4	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	97	87	11.49	111.5
OP19-5	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	107	87	22.99	123.0
OP20-1	oyster	Bay Center (Willapa Bay), Pacific - WA	5/29/2025	Spring	123	150	-18.00	82.00
OP21-1	oyster	Bruceport (Willapa Bay), Pacific - WA	5/30/2024	Spring	85	101	-15.84	84.16
OP22-1	oyster	Stony Point (Willapa Bay), Pacific - WA	5/30/2024	Spring	112	138	-18.84	81.16
OP23-1	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/30/2024	Spring	86	58	48.28	148.3
OP24-1	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	93	82	13.42	113.4
OP25-1	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	179	235	-23.83	76.17
OP25-2	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	170	235	-27.66	72.34
OP25-3	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	185	235	-21.28	78.72
OP26-1	oyster	Port Madison, Kitsap - WA	9/1/2023	Summer	150	237	-36.71	63.29
OP27-1	oyster	Kilisut Harbor, Jefferson - WA	9/15/2022	Summer	115	104	10.58	110.6
OP28-1	oyster	Drayton Harbor, Whatcom - WA	6/29/2022	Summer	42	47	-10.64	89.36
OP36-1	oyster	Agate Pass, Kitsap - WA	9/10/2023	Summer	431	888	-51.46	48.54
OP37-1	oyster	Agate Pass, Kitsap - WA	9/24/2023	Fall	78	104	-25.00	75.00
OP38-1	oyster	Samish Bay, Skagit - WA	9/28/2021	Fall	87	62	40.32	140.3
OP39-1	oyster	Samish Bay - West, Skagit - WA	9/28/2021	Fall	29	41	-29.27	70.73
OP40-1	oyster	Samish Bay - West, Skagit - WA	9/22/2021	Fall	41	68	-39.71	60.29

OP41-1	oyster	Samish Bay - West, Skagit - WA	9/27/2021	Fall	49	46	6.520	106.5
OP42-1	oyster	Samish Bay, Skagit - WA	9/12/2023	Summer	26.5	60	-55.83	44.17
OP43-1	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	32	40	-20.00	80.00
OP43-2	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	65	40	62.50	162.5
OP43-3	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	42	40	5.000	105.0
OP44-1	oyster	Bay Center Area, Pacific - WA	6/5/2024	Spring	97	115	-15.65	84.35
OP45-1	oyster	Stony Point, Pacific - WA	5/26/2024	Spring	66	114	-42.11	57.90
OP46-1	oyster	Chuckanut Bay, Whatcom - WA	5/28/2023	Spring	27	39	-30.77	69.23
CB16-1	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	63	40	57.50	157.5
CB16-2	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	61	40	52.50	152.5
CB16-3	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	76	40	90.00	190.0
CB18-1	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	328	192	70.83	170.8
CB18-2	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	427	192	122.40	222.4
CB18-3	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	235	192	22.40	122.4
CB28-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	5/16/2022	Spring	84	60	40.00	140.0
CB29-1	butter clam	Camano State Park, Island - WA	5/3/2022	Spring	401	300	33.67	133.7
CB30-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	5/4/2022	Spring	431	344	25.29	125.3
CB31-1	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	284	236	20.34	120.3
CB31-2	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	305	236	29.24	129.2
CB31-3	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	372	236	57.63	157.6
CB32-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	5/17/2022	Spring	389	379	2.639	102.6
CB33-1	butter clam	Grays Marsh Beach, Clallam - WA	2/4/2024	Winter	314	260	20.77	120.8
CB34-1	butter clam	Grays Marsh Beach, Clallam -	2/13/2023	Winter	86	63	36.51	136.5

		WA						
CB35-1	butter clam	Camano State Park, Island - WA	2/13/2023	Winter	265	187	41.71	141.7
CB36-1	butter clam	Camano State Park, Island - WA	3/21/2022	Spring	344	261	31.80	131.8
CB37-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/17/2024	Winter	205	240	-14.58	85.42
CB38-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	102	95	7.368	107.4
CB38-2	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	71	95	-25.26	74.74
CB38-3	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	114	95	20.00	120.0
CB39-1	butter clam	Fort Flagler, Jefferson - WA	1/3/2022	Winter	81	43	88.37	188.4
CM40-1	manila clam	South End/Blyn, Clallam - WA	9/7/2023	Summer	663	1175	-43.57	56.43
CM41-1	manila clam	Penn Cove, Island - WA	8/19/2021	Summer	118	152	-22.37	77.63
CB42-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	7/13/2022	Summer	344	264	30.30	130.3
CM43-1	manila clam	Samish Bay, Whatcom - WA	9/22/2021	Fall	23.5	47	-50.00	50.00
CM46-1	manila clam	Useless Bay, Island - WA	9/20/2021	Summer	24	40	-40.00	60.00
CM47-1	manila clam	Useless Bay, Island - WA	9/8/2021	Summer	106	123	-13.82	86.18
CM48-1	manila clam	Useless Bay, Island - WA	9/1/2021	Summer	71	126	-43.65	56.35

Sample ID	Shellfish Matrix	Harvest Area	Harvest Date	Season	RBA	PCOX	% error	% recovery
CR16-1	razor clam	Pacific, Pacific County Coast - WA	2/13/2024	Winter	167	184	-9.24	90.76
CR16-2	razor clam	Pacific County Coast, Pacific - WA	2/13/2024	Winter	261	184	41.85	141.8
CR16-3	razor clam	Pacific County Coast, Pacific - WA	2/13/2024	Winter	209	184	13.59	113.6
CR17-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	94	99	-5.051	94.95
CR17-2	razor clam	Grays Harbor, Grays Harbor -	9/5/2023	Summer	124	99	25.25	125.3

		WA						
CR17-3	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	83	99	-16.16	83.84
CR18-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	38	37	2.703	102.7
CR18-2	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	64	37	72.97	173.0
CR18-3	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	40	37	8.108	108.1
CR19-1	razor clam	Grays Harbor, Grays Harbor - WA	10/9/2023	Fall	213	125	70.40	170.4
CR20-1	razor clam	Pacific, Pacific County Coast - WA	10/23/2023	Fall	135	183	-26.23	73.77
CR21-1	razor clam	Willapa Spits, Pacific County Coast - WA	5/21/2025	Spring	153	132	15.91	115.9
CR22-1	razor clam	Grays Harbor, Grays Harbor - WA	11/6/2023	Fall	117	140	-16.43	83.57
CR23-1	razor clam	Pacific, Pacific County Coast - WA	9/25/2023	Fall	143	124	15.32	115.3
CR24-1	razor clam	Grays Harbor, Grays Harbor - WA	11/20/2023	Fall	114	130	-12.31	87.69
CR25-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	136	116	17.24	117.2
CR26-1	razor clam	Pacific, Pacific County Coast - WA	4/30/2024	Spring	120	103	16.50	116.5
CR27-1	razor clam	Mocrocks, Grays Harbor - WA	6/3/2025	Spring	100	109	-8.257	91.74
CR28-1	razor clam	Grays Harbor, Grays Harbor - WA	2/27/2024	Winter	111	109	1.834	101.8
CR29-1	razor clam	Grays Harbor, Grays Harbor - WA	10/23/2023	Fall	106	100	6.000	106.0
CR30-1	razor clam	Pacific, Pacific County Coast - WA	2/27/2024	Winter	74	98	-24.49	75.51
CR31-1	razor clam	Grays Harbor, Grays Harbor -	2/7/2024	Winter	110	61	80.33	180.3

		WA						
CR32-1	razor clam	Grays Harbor, Grays Harbor - WA	1/17/2024	Winter	96	89	7.865	107.9
CR33-1	razor clam	Grays Harbor, Grays Harbor - WA	12/1/2023	Fall	92	71	29.57	129.6
CR34-1	razor clam	Mocrocks, Grays Harbor - WA	5/21/2025	Spring	59	72	-18.06	81.94
CR35-1	razor clam	Quinault Res, Grays Harbor - WA	5/20/2025	Spring	62	60	3.333	103.3
CR36-1	razor clam	Grays Harbor, Grays Harbor - WA	2/27/2024	Winter	73	38	92.10	192.1
CR37-1	razor clam	Grays Harbor, Grays Harbor - WA	11/6/2023	Fall	50	55	-9.091	90.91
CR38-1	razor clam	Pacific, Pacific County Coast - WA	4/30/2024	Spring	70	41	70.73	170.7
CR39-1	razor clam	Copalis, Grays Harbor - WA	6/3/2025	Spring	36	53	-32.08	67.92
CR40-1	razor clam	Grays Harbor, Grays Harbor - WA	1/2/2024	Winter	45	46	-2.174	97.83

Additional Information
References (Provide references that are pertinent and supplemental to the validation data submitted; these may include peer-reviewed publications in which the method was validated and/or applied, validation packages submitted to other entities, etc. Do not provide references in lieu of data in the "Validation Data" section.) See References listed on the next page.
Standard Operating Procedure (SOP) (Provide a detailed procedure adequate for replication in additional laboratories.) See Appendix I.
Laboratory Evaluation Checklist (Provide any additions and/or modifications to the current method checklist for laboratory evaluation based on inclusion of the new matrix/ces.) See Appendix V.
Overview of Quality Systems (Provide an overview of the quality assurance/quality control systems utilized in the developer(s)/submitter(s) laboratory.) See Appendix VII.

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.

Appendix I: Standard Operating Procedure (SOP)

Matrix Extension: 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.
            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. 20 µg STX/100g of shellfish: 33 ± 0.3 grams of tissue
        ii. 40 µg STX/100g of shellfish: 33 ± 0.3 grams of tissue
        iii. 80 µg STX/100g of shellfish: 17 ± 0.3 grams of tissue
        iv. 160 µg STX/100g of shellfish: 17 ± 0.3 grams of tissue
        v. 320 µg STX/100g of shellfish: 17 ± 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. 20 µg STX/100g of shellfish: 64 µL
        ii. 40 µg STX/100g of shellfish: 128 µL
        iii. 80 µg STX/100g of shellfish: 132 µL

iv. 160 µg STX/100g of shellfish: 264 µL
v. 320 µg STX/100g of shellfish: 528 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.

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

FRANCES M. VAN DOLAH, TOD A. LEIGHFIELD, and GREGORY J. DOUCETTE
NOAA National Ocean Service, Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412
LAURIE BEAN
Maine Department of Natural Resources, McKown Point, W. Boothbay Harbor, ME 04575
BARBARA NIEDZWIADEK and DOROTHEA F.K. RAWN
Health Canada, Food Research Division, Sir Frederick Banting Research Centre, Ottawa, ON, Canada K1A 0L2

A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 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.

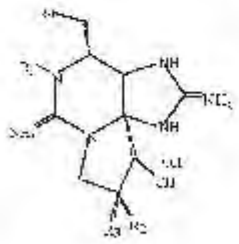
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fmn.vandolah@noaa.gov

Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 μg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/μmole
Carbamate	STX	H	H	H	$CONH_2$	2182
	Neo STX	OH	H	H	$CONH_2$	2285
	GTX1	OH	OSO_3^-	H	$CONH_2$	2768
	GTX2	H	OSO_3^-	H	$CONH_2$	732
	GTX3	H	H	OSO_3^-	$CONH_2$	1624
	GTX4	OH	H	OSO_3^-	$CONH_2$	1932
N-sulfocarbamoyl	GTX5 (B1)	H	H	H	$CONHSO_3^-$	180
	GTX6 (B2)	OH	H	H	$CONHSO_3^-$	-
	C1	H	OSO_3^-	H	$CONHSO_3^-$	16
	C2	H	H	OSO_3^-	$CONHSO_3^-$	277
	C3	OH	OSO_3^-	H	$CONHSO_3^-$	33
	C4	OH	H	OSO_3^-	$CONHSO_3^-$	43
Decarbamoyl	dcSTX	H	H	H	H	1274
	dcNeoSTX	OH	H	H	H	
	dcGTX1	OH	OSO_3^-	H	H	-
	dcGTX2	H	OSO_3^-	H	H	16.7
	dcGTX3	H	H	OSO_3^-	H	18.2
	dcGTX4	OH	H	OSO_3^-	H	
Decarbamoyl-N-sulfocarbamoyl	dcGTX5	H	H	H	H	-
	dcGTX6	H	H	OSO_3^-	H	
	dcGTX7	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.

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

U = unknown sample

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

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(a) *Microplate scintillation counter.*—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(b) *Microplate filtration manifold.*—Millipore (Bedford, MA).

(c) *Hot plate.*—Fisher Scientific (Suwannee, GA).

(d) *Countertop centrifuge.*—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(e) *Microtiter filter plates (96 well) with 1.0 μm pore size type FB glass fiber filter/0.65 μm pore size 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

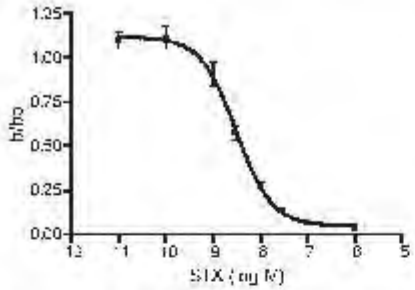


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

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	6.6	16.7	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	86	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

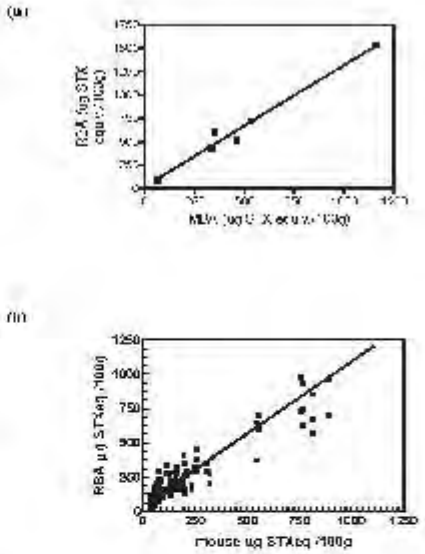


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days ($r^2 = 0.98$, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with 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 equivalents/100 g shellfish using the following formulas:

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

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

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

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.

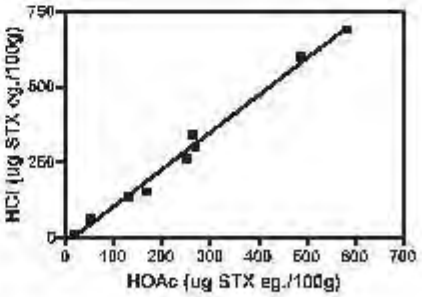


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

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	42.8	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.

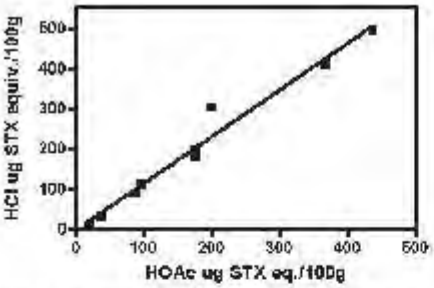


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

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.

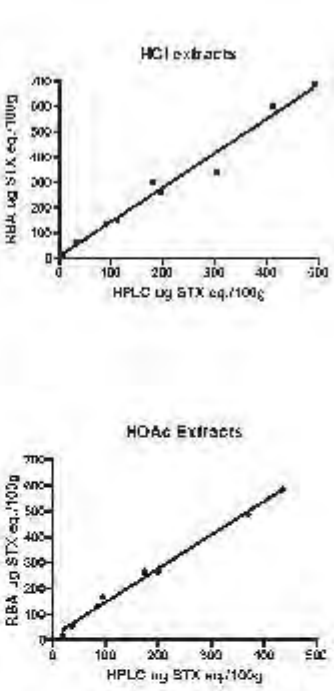


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

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.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**
(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2006) *J. AOAC Int.* **88**, 1714–1732
(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**
(4) Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009) *J. AOAC Int.* **92**, 190–207
(5) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425
(6) Hall, S., Strichartz, G., Moczydlowshi, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65
(7) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636
(8) Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196
(9) Powell, C.L., & Doucette, G.J. (1999) *Nat. Toxins* **7**, 393–400
(10) Suarez-Isla, B.A., & Valez, P. (2000) in *Seafood and Freshwater Toxins*, L.M. Botana (Ed.), Marcel Dekker Inc., New York, NY, pp 187–202
(11) Llewellyn, L.E., Doyle, J., Jellett, J., Barrett, R., Alison, C., Bentz, C., & Quilliam, M. (2001) *Food Addit. Contam.* **18**, 970–980
(12) Ruberu, S.R., Liu, Y.-G., Wong, C.T., & Perera, S.K. (2003) *J. AOAC Int.* **86**, 1–9
(13) Usup, G., Leaw, C.-P., Cheah, M.-Y., Ahmad, A., & Ng, B.-K. (2004) *Toxicon* **44**, 37–43
(14) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667
(15) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

Appendix III: Van Dolah et al., 2012

FOOD CHEMICAL CONTAMINANTS

Appendix III

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

FRANCES M. VAN DOLAH, SPENCER E. FIRE, TOD A. LEIGHFIELD, CHRISTINA M. MIKULSKI, and GREGORY J. DOUCETTE
Center for Coastal Environmental Health and Biomolecular Research, NOAA Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412

Collaborators: Å. Andersson; L. Bean; D. Couture; S. DeGrasse; A. DeLeon; V. Dell'Ovo; L. Flewelling; P. Holland; G. Langlois; R. Lewis; M. Masuda; P. McNabb; C. Mikulski; B. Niedzwiadek; B. Porntepkasemsan; D. Rawn; E. Sombrito; K. Srisuksawad; B. Suarez; S. Subsinserm; A. Tubaro

A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chilliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

Submitted for publication December 3, 2011.
The recommendation was approved by the Method Committee on Paralytic Shellfish Toxins as First Action. *See* "Methods News," (2011) *Inside Laboratory Management*, January/February issue.
This publication does not constitute an endorsement of any commercial product or intend to be an opinion beyond scientific or other results obtained by the National Oceanic and Atmospheric Administration (NOAA). No reference shall be made to NOAA, or this publication furnished by NOAA, to any advertising or sales promotion which would indicate or imply that NOAA recommends or endorses any proprietary product mentioned herein, or which has as its purpose an interest to cause the advertised product to be used or purchased because of this publication.
Corresponding author's e-mail: Fran.vandolah@noaa.gov
DOI: 10.5740/jaoacint.CS2011_27

Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Plactopecten 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 (*Plactopecten 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 (*Plactopecten 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 I). 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. chilliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus giganteus*) from U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method **959.08**), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**); however, final concentrations in μg/kg and μg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well CVs for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_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 MΩ).

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

Assay	Sample No.	ID	Lab 1	2	3	4	5	6	7	8	9	All labs Mean	S_R	RSD_R, %	HorRat	Labs 1–8 Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	820	410	690	1070	630	680	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV08	1100	1340	1320	1440	1260	1720	2080	2130	890	1478	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	180	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	880	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[a]	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	346	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	490	340	840	490	240	800	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[a]	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[a]	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	1490	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	689	23.3	1.7
	20	MLV11	430	350	490	280	550	620	1148[a]	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	480	620	
7	530	880	3090	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	670	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*	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.

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:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC_{50}) \cdot \text{Hill slope}}}$$

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	1526	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_0 of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_0 represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_0 of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

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

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

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCL equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1380**	**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**	**834**	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**	**1060**

[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)*

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^{-6}	3×10^{-6}	3×10^{-6}	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^{-6}	10^{-6}	10^{-6}	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^{-6}	3×10^{-6}	3×10^{-6}	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^{-6}	10^{-6}	10^{-6}	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			

* 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%.

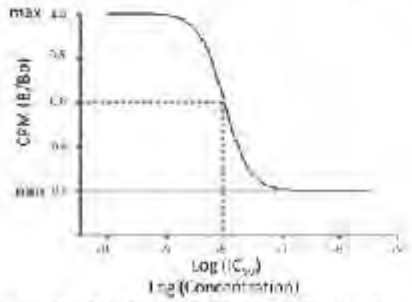


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

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 C3,4–1.3 (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	864.3
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	1880.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.8	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	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	884.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHO 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	1060	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	690	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_d	RSD_R,%	Recovery,%
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

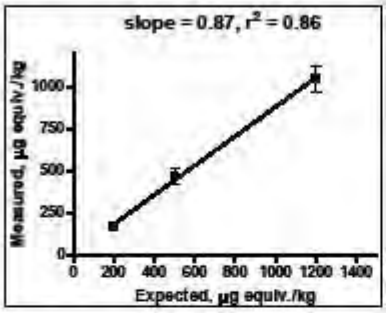


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

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

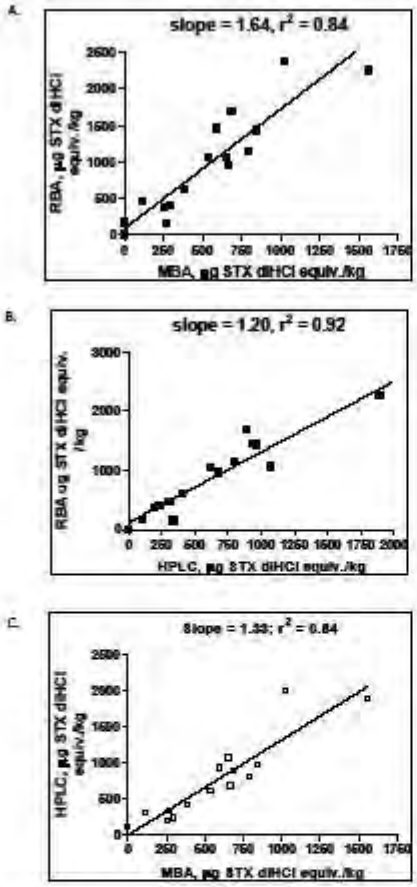


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

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.

Acknowledgments

We would like to thank Laurie Bean (Maine Department of Marine Resources), Stacey DeGrasse (U.S. Food and Drug Administration), Patrick Holland (Cawthron Institute), Gregg Langlois (California Department of Health Services), Bob Lona (Washington State Department of Health), and Benjamin Suarez (University of Chile) for providing the shellfish material used in the study. We are grateful to Barbara Niedzwiadek and Dorothea Rawn (Health Canada) for HPLC analyses and to Laurie Bean and Darcie Couture (Maine Department of Marine Resources), Benjamin Saurez, and Supanoi Subsinserm (Thailand Department of Fisheries) for performing mouse bioassays. We are indebted to the following collaborators for their dedication and perseverance in carrying out the RBAs for this study:

Leanne Flewelling, Florida Wildlife and Marine Research Institute, St. Petersburg, FL

Stacey DeGrasse, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD

Patrick Holland and Paul McNabb, Cawthron Institute, Nelson, New Zealand

Gregg Langlois and Melisa Masuda, California Department of Public Health, Richmond, CA

Richard Lewis and Åsa Andersson, University of Queensland, Queensland, Australia

Christina Mikulski, NOAA Marine Biotoxins Program, Charleston, SC

Elvira Sombrito and Aileen DeLeon (Philippine Nuclear Research Institute, Manila, the Philippines

Kanitha Srisuksawad and Boonsom Porntepkasemsan, Thailand Nuclear Research Institute, Bangkok, Thailand

Aurelia Tubaro and Valeria Dell'Ovo, Dipartimento dei Materiali e Risorse Naturali, Trieste, Italy

This project was funded by the International Atomic Energy Agency Interregional Project INT7017 9001 and NOAA Marine Biotoxins Program Project 02E0017. FDA saxitoxin diHCl used for tritiation by IICH and STX diHCl reference standard (NIST RM 8642) were provided by Sherwood Hall, FDA Office of Seafoods.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) LaDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(3) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(5) *Official Methods of Analysis* (2011) AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.02**

(6) Jellett, J.F., Robart, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425. http://dx.doi.org/10.1016/S0041-0101(02)00153-8

(7) Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(8) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667. http://dx.doi.org/10.1021/tx050277i

(9) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636. http://dx.doi.org/10.1016/S0041-0101(96)00189-4

(10) Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., & Rawn, D.F.K. (2009) *J. AOAC Int.* **92**, 1705–1713

(11) McFarren, E.F. (1959) *J. AOAC Int.* **42**, 263–271

(12) EU Reference Laboratory for Marine Biotoxins (2010) *Report*

on the EURIAB 2010 proficiency testing on saxitoxin group (PSP) toxins determination, Vigo, Spain

(13) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*, http://www.eurachem.org/index.php/publications/guides/mv

(14) Kleivdal, H., Kristiansen, S.-I., Nilson, M.A., Goksoyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(a) *Teflon/glass homogenizer.*—Motorized tapered Teflon pestle and glass tube, 15 mL.

(b) *Motorized tissue homogenizer.*—Polytron or small hand-held blender.

(c) *High-speed centrifuge and fixed angle rotor.*—Capable of 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.

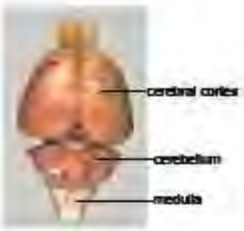


Figure 1. Rat brain.

(*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

© 2012 AOAC INTERNATIONAL

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv/kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

Assay	Sample No.	Sample ID	Lab 1	2	3	4	5	6	7	8	9	All labs Mean	S_R	RSD_R, %	HorRat	Labs 1–8 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	860	1478	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	90	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	256	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	280	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	1460	2420[a]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1820	1580	1110	1700	3180	1400	2780	820	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	2680	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	670	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	149	34.4	1.9
	14	MLV13	400	1240[a]	580	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[a]	90	385	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	1290	1481	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	2680	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	480	280	550	620	1149[a]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg RSD_R														33.2				28.7	
Avg HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

© 2012 AOAC INTERNATIONAL

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	560	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	860	2090	1240	1750	1150	1460	1830	230*	1149*	
8	660	940	2130	870	1210	2150	820	1120	500	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		336		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^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk [*Appendix 1; J. AOAC Int.* (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

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

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

© 2012 AOAC INTERNATIONAL

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

Laboratory	ID	Day 1	Day 2	Mean	s,	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*	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:

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

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure 2011.27); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(a) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

© 2012 AOAC INTERNATIONAL

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{75}, 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	2068	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[b]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	−1.0	1.6	2.7	619	0.70	None			
	3	−0.9	2.1	3.2[a]	693	0.82	None			
8	1	−1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	−1.1	1.4	1.5[b]	1095	0.78	None			
	3	−1.1	2.4	2.3	886	1.04	None			
9	1	−1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	−1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	−1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

© 2012 AOAC INTERNATIONAL

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	540	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**	585	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**	**1580**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	**690**	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

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

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

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

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

(b) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(c) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(d) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

© 2012 AOAC INTERNATIONAL

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

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

[a] REF = Reference; QC = quality control check; U = unknown sample. [Note: The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

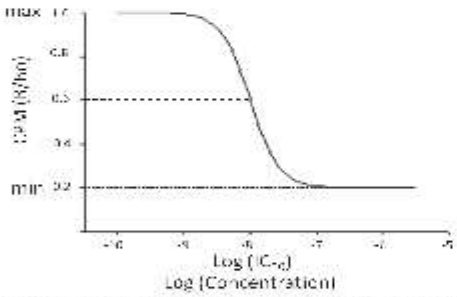


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}

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)

© 2012 AOAC INTERNATIONAL

Appendix V: Shellfish Laboratory Evaluation Checklist

<table>
<tr><td colspan="3">PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV</td></tr>
<tr><td colspan="3">SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)</td></tr>
<tr><td colspan="3">LABORATORY:</td></tr>
<tr><td colspan="3">ADDRESS:</td></tr>
<tr><td colspan="2">TELEPHONE:</td><td>FAX:</td></tr>
<tr><td colspan="3">EMAIL:</td></tr>
<tr><td>DATE OF EVALUATION:</td><td>DATE OF REPORT:</td><td>LAST EVALUATION:</td></tr>
<tr><td colspan="2">LABORATORY REPRESENTED BY:</td><td>TITLE:</td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2">LABORATORY EVALUATION OFFICER:</td><td>SHELLFISH SPECIALIST:</td></tr>
<tr><td colspan="2">OTHER OFFICIALS PRESENT:</td><td>TITLE:</td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="3">Items which do not conform are noted by: Conformity is noted by a “√”
C- Critical K - Key O - Other N/A- Not Applicable</td></tr>
</table>

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan: (check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			b. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
			c. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			d. Internal performance assessment.
			e. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**2.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		2.2.2 In state/county laboratories, the analyst(s) meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**2.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		2.2.4 In commercial/private laboratories, the analyst(s) must have at least a high school diploma and at least three months of experience in laboratory sciences.

C	**6**		**2.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**2.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**
		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.

K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
K	2		1.4.12 Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.
K	1		1.4.13 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**
K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.

O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
			1.5 Reference Solution Reagent Storage, Preparation and Security
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of ^{3}[H]STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** a. **100 mM MOPS/L.** b. **100 mM choline chloride/L.** c. **pH adjustment to 7.4 with NaOH.** d. **Refrigerated storage at 4 °C.** e. **Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**

K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.
	1.6 Rat Brain Membrane Tissue Preparation and Storage		
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use**.
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5 The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue.
K	10		1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.

C	10		1.6.8 Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months
	1.7 Rat Brain Membrane Tissue Protein Receptor Determination		
C	**10**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**
C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA			
	2.1 Collection and Transportation of Samples		
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	6, 13		2.1.5 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
	2.2 Preparation of Samples for Analysis – Homogenization		
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.

C	**5, 6**		**2.2.9 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender with all freeze thaw liquid for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender with all freeze-thaw liquid for homogenization.**
K	6, 13		2.2.10 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
	2.3 Preparation of Samples for Analysis – Extraction		
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.
C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**
C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**
C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted, if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3,000 x g for 10

			minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4°C in a sealed container for up to 24 hours, or frozen at -20°C.
	2.4 Sample Assay		
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
K	10		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension**
K	10		2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**

K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	10		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC50 is in acceptable range (2.0 nM +/- 30%). If the IC50 is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
	2.5 Calculation of Sample Toxicity		
C	**10**		**2.5.1 When more than one dilution falls within B/Bo of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX eq) x (sample dilution) x [(210 µL total volume/35 µL sample] = mM STX eq in extract** **(nM STX diHCl eq in extract) x (1L/1000 mL) x (372 ng/nmol) x (1 µg/1000 ng) = µg STX diHCl eq/mL** **µg STX diHCl equiv./mL x (mL extract/g shellfish) x (1000 g/kg) = µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl eq/100 g) of sample is actionable.**

C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

REFERENCES

1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.

2. American Public Health Association (APHA). 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.

3. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.

4. Appendix C: Radiation Safety Requirements, ISSC Proposal 13-114 Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination.

5. American Public Health Association (APHA). 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, Fourth Edition. APHA, Washington, D.C.

6. Good Laboratory Practice.

7. Fisher J. 1985. Measurement of pH. *American Laboratory* 16:54-60.

8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.

9. Consult pH electrode product literature.

10. Association of Official Analytical Chemists (AOAC). 2016. Official Method 2011.27 Paralytic Shellfish Toxins (PSTs) in Shellfish Receptor Binding Assay.

11. Consult instrument manufacturer instructions.

12. Technical Data Sheet, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63146.

13. Wilt, d. s. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U. S. Food and Drug Administration, Washington, D.C.

14. U. S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. NSSP *Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington D.C. and Columbia, S.C.

15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results	____
Total # of Critical (C) Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical (C), Key (K), and Other (O) Nonconformities	_____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a.
   b.
   c.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a.
   b.
   c.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a.
   b
   c.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

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

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

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

C. Performing the Assay
a. Plate setup: When possible use a multichannel pipet to minimize effort and increase consistency.
i. Run standards, samples, and QC check in triplicate.
ii. For quantitation, multiple dilutions per extract should be analyzed in order to obtain a value that falls within the dynamic range of the assay. A minimum sample extract dilution of 1:10 is recommended to minimize potential matrix effects.
iii. Use of a standard plate layout (Figure 1) is recommended. This will improve ease of analysis and can help maximize the number of samples/standards that can be analyzed per plate.
b. Addition of samples/standards: Add in the following order to each well
i. 35 µl assay buffer
ii. 35 µl STX standard/QC check/sample extract
iii. 35 µl 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. $y = \min + (\max-\min)/1+10^{(x-\log IC_{50})\text{Hill slope}}$
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: Rat Brain Membrane Preparations

B. Equipment/Supplies
   a. Teflon/glass homogenizer: Tapered Teflon pestle and glass tube, 15 ml
   b. Motorized tissue homogenizer: Polytron or small hand-held blender
   c. High-speed centrifuge and fixed angle rotor: capable of 20,000 x g
   d. Centrifuge tubes: 12-15 ml, rated for >20,000 x g
   e. Plastic cryovials: 2 ml
   f. Glass beaker: 300-500 ml
   g. Pipets: disposable 5 and 10 ml
   h. Forceps.

C. Reagents
   a. 20 rat brains: male, 6-week old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA) or equivalent
   b. MOPS: pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G)
   c. Choline chloride: 100 mM (Sigma; Cat. No. C7527-500G)
   d. Phenyl methylsulfonyl fluoride (PMSF): (Sigma; Cat. No. P7626)
   e. Isopropanol.

D. Procedure
   a. Prepare 1 L of 100 mM MOPS, pH 7.4, containing 100 mM choline chloride (as described in Appendix A) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol: dissolve 0.174 g PMSF in 10 ml isopropanol to make 100 mM stock. Aliquot stock and store at -20 °C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh in the day of use.
   b. Remove the medulla and cerebellum from each brain using forceps and discard. Place cerebral cortex in a small amount of ice-cold buffer and place on ice.
   c. Place one cerebral cortex in 12.5 ml MOPS/choline Cl/PMSF, pH 7.4, in glass/Teflon homogenizer. Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes and ensure there are no visible chinks remaining in the homogenate. Keep tube in ice at all times. Pour homogenized tissue into 250 ml beaker on ice and repeat procedure with remaining cortices.
   d. Transfer pooled homogenate tissue to centrifuge tubes, balance the tubes (pairwise: using ice-cold buffer to balance), and centrifuge at 20,000 x g for 15 min at 4 °C.
   e. Aspirate the supernatant and resuspend pellets in ice-cold MOPS/choline Cl/PMSF, using an adequate amount to fully resuspend the pellet (5-10 ml per brain).
   f. Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume up to 200 ml (keep on ice).
   g. Keeping the beaker on ice, polytron (or homogenize with small handheld blender) at 70% full speed for 20 s to obtain a homogeneous solution.
   h. Aliquot 2 ml per tube into cryovials. It is critical to keep the preparation well mixed while dispensing. Keep cryotubes on ice.
   i. Freeze and store at -80 □C. This preparation is stable for at least 6 months.

E. Protein Assay

a. Determine the protein concentration of the membrane preparation using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) or equivalent. The above protocol should yield ~6-8 mg protein/ml of rat membrane preparation.
b. Determine the membrane dilution needed for the assay. The protein concentration in the daily working stock should be 1 mg/ml (which yields a diluted concentration of 0.5 mg/ml in-assay concentration). Based on the protein concentration determined using the protein assay, dilute rat membrane preparation with buffer to 1 mg/ml. It is this diluted membrane preparation that is used in the assay.
c. Protein concentrations must be determined and new dilutions calculated accordingly for each new batch of membranes prepared.

Appendix C: Radiation Safety Requirements

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

Approved and current. Effective starting 4/14/2025. 69790.12 (version 8.2) Quality Management Plan

Public Health Laboratories

Quality Management Plan



Washington State Department of Health
Public Health Laboratories

Uncontrolled copy printed by Jeffery Lahti on 9/1/2026 11:20 AM (PDT). Page 1 of 42

For more information or additional copies contact:

Washington State Public Health Laboratories
1610 NE 150th Street
Shoreline, WA 98155-9701

Telephone: (206) 418-5400
Facsimile: (206) 418-5445
www.doh.wa.gov/public-health-healthcare-providers/public-health-laboratories
69790.12 *Quality Management Plan*

Washington State Department of Health

All rights reserved. Permission to use or distribute all or parts of this document as part of the development of a quality assurance program is hereby granted provided that the user attributes original authorship to the Department of Health. All other requests for permission to use and / or distribute all or part of this document may be addressed to the Public Health Laboratories. Permission to use figures or text contained in this document which is credited to other sources must be obtained from those authors or publishers.

Although every effort has been made to provide valid information in this manual the Public Health Laboratories makes no assurance as to the accuracy of data and statements in this document. The user assumes all responsibility for verification and validation of the information provided.

1 INTRODUCTION 6

2 MISSION OF THE PUBLIC HEALTH LABORATORIES 6

3 STATED GOALS AND OBJECTIVES OF QUALITY ASSURANCE PROGRAM 6

4 QUALITY MANAGEMENT RESPONSIBILITIES 7

4.1 Clinical and Technical Consultant 7
4.2 Delegation of Duties 8
4.3 Quality Assurance Monthly Meeting 8
4.4 QA Officer 8
4.5 Office Directors and Supervisors 8
4.6 Public Health Laboratories Staff 8
4.7 Laboratory Safety 9

5 DOCUMENT CONTROL 9

5.1 Document Control Process 9
5.2 Quality Management Program Review 9
5.3 Procedures, Forms & Records 10
5.4 Management and correction of laboratory records 10
5.5 Document Retention 10
5.6 Electronic Signature 10

6 DESCRIPTION OF SERVICES 10

6.1 Laboratory Director 10
6.2 Office of Operations 10
6.3 Office of Public Health Microbiology 10
6.4 Office of Environmental Laboratory Sciences 11
6.5 Office of Newborn Screening 11

7 IMPORTANT ASPECTS OF SERVICE 11

8 LICENSURE, ACCREDITATION, AND CERTIFICATION 11

8.1 Compliance Policies 12
8.2 Medicare/Medical Test Site Licensure Program 12
8.3 College of American Pathologists 12
8.4 Department of Ecology, Washington State 12
8.5 Food and Drug Administration 12
8.6 Federal Emergency Management Administration 13
8.7 Select Agent Inspections 13
8.8 Department of Transportation 13
8.9 Washington Radiation Inspections 13
8.10 ANSI National Accreditation Board 13

9 PERSONNEL 13

9.1 Educational and Experience Requirements 13
9.2 Office Organization 14
9.3 Training and Competency 15
9.4 Performance Evaluation 15
9.5 Color Deficiency 15

10 SAMPLE TEST MANAGEMENT 16

10.1 Unique Identification of Specimens and Samples 16
10.2 Limited Exceptions in Specimen and Sample Labeling 16

10.3 Retaining Samples 16
10.4 Specimens Sent to Reference Laboratories 16
10.5 Selection of Reference Laboratories 17
10.6 Identification of specimens, specimen types, samples, and aliquots 18
10.7 Accessioning Specimens and Samples 18
10.8 Review of Specimen and Sample Acceptance 19
10.9 Verbal Test Orders 19
10.10 Specimen and Sample containers and analytical interference 19
10.11 Analyst Identification 19
10.12 Reporting Results 19
10.13 Amended Reports 20
10.14 Retrieval of Information 20
10.15 Availability of Test Methods to Clients 20
10.16 Change in Analytical Methodology 20
10.17 Reagent Labeling, Storage & Expiration 21
10.18 Unknown Submitter 21
10.19 Loss of Critical Documents 21
10.20 Testing Control Specimens 21
10.21 Specimen Handing for Molecular Procedures 21

11 ANALYTICAL PROCEDURES 21

11.1 Intermittent Testing 21

12 QUALITY CONTROL 22

12.1 Proficiency Testing 22
12.2 Monitoring of Laboratory Environmental Conditions and Equipment 25
12.3 Calibration Check of Thermometers 26
12.4 Pipettes and Liquid Handlers 26
12.5 Weights & Balances 27
12.6 Autoclaves and Sterilizers 27
12.7 Timers 28
12.8 Reagent Water Quality 28
12.9 Glassware Washing 28
12.10 Environmental Maintenance and Equipment Preventative Maintenance 28
12.11 Monitoring of Controls 29
12.12 Monitoring Analytic Performance 29
12.13 Detection and Correction of Significant Clerical and Analytic Errors 29
12.14 Control Charting 30
12.15 Reagent Quality Control Testing 30
12.16 Calibration, Analytical Measurement Range, etc. 30
12.17 Telepathology 31
12.18 Patient and Environmental Safety Goals 31
12.19 Reporting Quality Concerns 31

13 QUALITY INDICATORS 32

13.1 Turnaround Time 32
13.2 Specimen Acceptability 32
13.3 Proficiency Testing Performances 32
13.4 Employee Quality Communication 32

14 INFORMATION SYSTEMS 33

14.1 Security 33
14.2 Computer System Operations 33
14.3 Office of Newborn Screening 33

14.4 Office of Public Health Microbiology 34
14.5 Office of Environmental Laboratory Sciences 34
14.6 QA Office 35

15 EXTERNAL COMMUNICATIONS 35

15.1 Client Complaints 35
15.2 Communication Breakdowns 35
15.3 Critical Results and Timely Communication 35
15.4 Securing Healthcare Information 36
15.5 Reporting of Adverse Reactions 36
15.6 Notifications from Vendors 37

16 QUALITY ASSURANCE REVIEW 37

16.1 Effectiveness of the Quality Management Plan 37
16.2 Directorship Review 38
16.3 Laboratory Reports and Forms Review 38
16.4 Root Cause Analysis 38
16.5 Material Transfer Agreements 38

17 MEMBERSHIP ORGANIZATIONS 38

18 DEBRIEFING PROCESS FOR REVIEW OF RESPONSE TO PUBLIC HEALTH THREATS OR DISEASE OUTBREAKS 39

19 METHOD EVALUATION PARAMETERS 39

20 Listing of Procedures at the Public Health Laboratories 40

21 Measurement of Client Satisfaction of the Laboratory Service 40

22 References 40

23 Appendix 41

1 INTRODUCTION

The Washington State Department of Health, Public Health Laboratories (PHL) is committed to the continuous improvement of quality management and assurance. A quality management program must systematically evaluate the quality and appropriateness of laboratory services. The program must be designed to identify and resolve important problems in patient care and identify opportunities to improve patient care. Quality Assurance (QA) is defined as planned or systematic actions necessary to provide adequate confidence that a product or service will satisfy defined needs.

A detailed monitoring and evaluation process enables the Laboratories to effectively use their resources to manage the quality of the service they provide. This process involves the ongoing examination of services provided, identification of deficiencies in the services, and improvement, as necessary, of the quality of the services. Appropriate monitoring and evaluation activities are ongoing and integrated with other managerial and planning activities throughout the organization.

The monitoring and evaluation process assists in identifying patterns of service that may not be evident when only a case-by-case review is performed. The process may also identify situations in which a case review is likely to be most useful in identifying correctable deficiencies in service and opportunities to improve service. Although this process will not identify every case of substandard service, monitoring, and evaluation help the organization distinguish situations on which its attention could be most productively focused.

2 MISSION OF THE PUBLIC HEALTH LABORATORIES

PHL's vision is to be a world-class laboratory in providing diagnostic and analytical measurements to support the Washington State Department of Health's programs and Local Health Jurisdictions to improve and protect the health of citizens of Washington State.

PHL's mission is to put the best diagnostic and analytical tests to accurately and rapidly provide timely results to our partners by:

- Continually interacting and supporting the public health community.
- Soliciting and encouraging all interested parties to communicate with us.
- Building, maintaining, and sharing technical expertise in the latest laboratory technology available to the public health community.
- Providing a wide range of diagnostic and analytical functions for the assessment and surveillance of infectious/communicable, heritable/genetic, and chronic diseases as well as environmental contamination.
- Improving the QA and analytical performance of clinical, surveillance, and environmental laboratories through training and consultation as well as providing scientific and managerial leadership in developing public health policy.

Our Quality Management Plan (QMP) is designed to support our vision, mission, and values as well as help us provide the highest level of analytical support to our many partners throughout the state of Washington as well as the country.

3 STATED GOALS AND OBJECTIVES OF QUALITY ASSURANCE PROGRAM

PHL Quality Assurance program has an obligation to the customers that the PHL serves. QA includes all actions planned and implemented to provide confident test results and exemplary customer service. It is those planned or systematic actions necessary to provide adequate confidence that a product or service

will satisfy defined needs. This includes control of all processes and procedures that occur within the Laboratories, from the time the sample/specimen is received, through the analytical testing, to the reporting and archiving of data. QA also extends beyond the physical confines of the Laboratories, from the time a test is requested, through the collection process, to the medical or scientific use and interpretation of the test results.

The QA Program was established to facilitate achieving the highest standards of quality customer service. The program has been developed to provide a:

- Continuous program monitoring and evaluation of QA activities
- Working structure for identification and resolution of QA concerns
- Means for integrating and monitoring information between support and scientific areas
- Mechanism for integrating the QA within PHL management and operation
- Framework such that the standards established by the Laboratories will meet or exceed the standards and requirements for certification of all accrediting agencies

4 QUALITY MANAGEMENT RESPONSIBILITIES

The PHL Deputy Director has the overall responsibility for the PHL QA Program. The QA team is the organizational structure that supports the Deputy Director in carrying out this responsibility. The Deputy Director has designated the QA Officer and the QA team to be responsible for the review of the PHL QA program. The Office Directors and Supervisors are responsible for the implementation and feedback for the QA program. The QA Officer reports directly to the Deputy Director. Currently, the Deputy Director also serves as CLIA Director.

4.1 Clinical and Technical Consultant

The primary role of Clinical Consultant for communicable disease-related issues at the PHL is filled by Dr. Scott Lindquist MD, MPH Washington State Epidemiologist for Communicable Diseases. Dr. Lindquist and the staff of the Office of Communicable Disease Epidemiology are physically located within the PHL building and are available for consultation during Laboratory operating hours and on-call during weekends and holidays.

In addition to Dr. Lindquist, Epidemiologists within the Office of Communicable Disease Epidemiology are available for client and laboratory consultation relating to laboratory testing for communicable diseases, foodborne illness, STI, etc.

The Clinical Consultants are:

- Marcia Goldoft, MD, DOH Epidemiologist
- Scott Lindquist, MD State of Washington Epidemiologist
- Michael Bender, MD, PhD - Pediatric Hematologist - Fred Hutchinson Cancer Research Center
- Patricia Fechner, MD, Pediatric Endocrinologist – Seattle Children's Hospital
- The Cystic Fibrosis clinical consultant is Margaret Rosenfeld, MD, MPH, Pediatrics Pulmonologist at Seattle Children's Hospital and Regional Medical Center, Associate Professor, Pediatrics/Pulmonary, University of Washington, School of Medicine
- Sihoun Hahn, MD, PhD, Director, Biochemical Genetics Program, Professor, Department of Pediatrics, Division of Genetic Medicine, University of Washington School of Medicine, Seattle Children's Hospital
- Maria Teresa de la Morena MD, Immunologist at Seattle Children's Hospital
- Seth Perlman MD, Neurologist Seattle Children's Hospital

4.2 Delegation of Duties

The Office Directors in Microbiology, Newborn Screening, and Environmental Laboratory Sciences serve as **Technical Consultants** and **General Supervisors** along with the PHL Deputy Director for the laboratory's CAP accreditation. They are delegated appropriate duties by using the form:
69790.21 *Delegation Form Office Director*

The Office Directors delegate appropriate duties to the supervisors using **69790.22** *Delegation Form Supervisor*. The supervisor delegates appropriate duties to the Leads using **69790.125** *Delegation Form Lead*. Additional delegation forms are:
69790.18 *Delegation Form Deputy Director*
69790.19 *Delegation Form IT Applications*
69790.20 *Delegation Form IT Hardware*

These delegation form templates are kept in document control and approved by the Deputy Director prior to the signature workflow for approving the delegation of duties to an individual.

4.3 Quality Assurance Monthly Meeting

The PHL Deputy Director, upper management, and QA team meet monthly, all staff are invited to attend via Teams. This has enabled staff who were previously unable to attend because of time constraints, meeting room capacity, etc. to attend. The agenda is presented each month and the meeting minutes are available to all staff on the PHL QA SharePoint page. The meeting minutes are approved at the following month's QA meeting.

4.4 QA Officer

The QA Officer is the primary contact for all QA-related activities at PHL. This position coordinates both internal and external inspections as well as PHL's response to any deficiencies and recommendations noted. The duties of the QA Officer include but are not limited to chair of the QA team, presentation of the monthly QA meeting, and revision of the QMP as necessary with approval from the Deputy Director. The QA Officer participates in QA-related training, guides and reviews the performance of the biennial client survey, assists with new method validation/verification, and directs follow-up of nonconformance reports. The QA Officer is responsible for administrating MediaLab software, which is PHL's cloud-based document control platform.

4.5 Office Directors and Supervisors

Office Directors and Supervisors have the responsibility of reviewing the accuracy and authenticity of the data generated for laboratory reporting and test method validation/verification. They are responsible for ensuring the daily quality control activities have been performed accordingly, to ensure that the test results are accurate and reliable. Supervisors and lead workers have the primary responsibility of managing daily testing operations for their respective units.

4.6 Public Health Laboratories Staff

All staff are responsible for:

- Reporting to their leads or supervisors QA problems encountered while performing their duties and responsibilities.
- Assisting in the resolution of problems.
- Providing suggestions for ongoing monitoring and developing improvements in service.
- Providing pertinent information to their supervisors as it relates to QA.
- Signing the Proficiency testing (PT) attestation for samples analyzed.

- Completing a **69790.14** *PT Quality Improvement Assessment Sheet* when results are not graded as Passing or Acceptable.
- Completing a **69790.793** *PHL Customer Feedback Form* for client complaints and compliments (in MediaLab IQE).
- Reading and following the rules and guidance in the QMP and requesting variances from the QA Officer, if necessary.
- Completing the **69790.67** *New Test & Instrument Checklist* before for new procedures are placed online.
- Reporting laboratory quality issues or concerns. See **69790.945** *How to Record a New Nonconforming Event in MediaLab* for details on how to use the IQE module in MediaLab to report quality issues and concerns.
- Submitting feedback intended to improve QA systems at the PHL using the *Internal (Laboratory Staff) Customer Feedback Form* found on the PHL QA Welcome page at PHL QA Welcome! (sharepoint.com). Staff feedback may be submitted anonymously using this form.

4.7 Laboratory Safety

The Laboratory Safety Program is administered by the Safety Office with assistance from the Laboratory Safety Committee. The Safety Office helps to ensure a safe work environment for staff and clients. The Safety Officer provides training, information, technical assistance, policy development, and continual oversight that enables management to maintain a safe and effective workplace environment at the PHL. The Safety Officer helps to ensure that the activities of the laboratories are compliant with statutory requirements. The PHL promotes safety and quality as an institutional core value and ensures that analytical activities are conducted in compliance with applicable policies and regulations.

Please refer to the PHL manuals listed below for further information regarding safety practices.

• Safety & Health Manual	69790.38
• Biosafety Manual	69790.17
• Chemical Hygiene Plan	69790.32
• Respirator Protection Program	69790.37
• Emergency Response Plan	69790.34
• Radiation Safety Manual	69790.178
• Confined Space Program	69790.33
• Hearing Conservation Program	69790.36
• VHP Management Plan	69790.3876

5 DOCUMENT CONTROL

5.1 Document Control Process

The PHL uses an electronic cloud-based software system called MediaLab (www.medialab.com). All employees stationed at the PHL have been given access to MediaLab with varying permission levels of access. The document control process is described in **69790.1057** *Document Control*.

5.2 Quality Management Program Review

The Deputy Director is responsible for the annual Quality Management Program (QMP) review and approval, along with input from the QA Officer, QA team, Office Directors, and Supervisors. Upon a

change to the Deputy Director position, the new appointee will re-review and approve the QMP, over a reasonable period of time (typically 90 days after appointment to the Deputy Director position).

A paper copy of the QMP is located at the front office. The QA Officer is responsible for replacing any old copies when a new version of the QMP is issued.

5.3 Procedures, Forms & Records

Supervisors are responsible for adhering to the authorized document control process, and for ensuring that testing personnel have read the policies and procedures relevant to their job activities, as well as conducting biennial procedure reviews in the assigned section, along with the Office Director. All procedures and forms should adhere to regulatory guidelines governing that unit. Next generation sequencing (NGS) procedures should include the classification process and interpretation of sequence variants.

5.4 Management and correction of laboratory records

The detailed process is described in **69790.1057** *Document Control.*

5.5 Document Retention

The detailed process is described in **69790.1308** *Record and Specimen Retention Policy.*

5.6 Electronic Signature

Per Department of Health Policy number 17.008, electronic signatures may be used where feasible and especially in cases where the document workflow is already conducted electronically. Many methods of implementing an E-Signature process are accepted under this policy. At the PHL, the most common method is the Clickwrap method and the symbol indicating intent to sign is /s/.

6 DESCRIPTION OF SERVICES

The PHL consists of four technical offices:

- Office of Operations
- Office of Public Health Microbiology
- Office of Environmental Laboratory Sciences
- Office of Newborn Screening

6.1 Laboratory Director

The Laboratory Director provides operational and strategic direction and leadership. The office consists of the Deputy Director, administrative support staff (payroll, human resources, travel, etc.), and the QA Officer.

6.2 Office of Operations

The Office of Operations (OPS) provides auxiliary services such as glassware sterilization and preparation, mailroom activities, facilities management, and media preparation. OPS also includes the Safety Office and security. The Safety Office reports to the OPS Office Director.

6.3 Office of Public Health Microbiology

The Office of Public Health Microbiology (MICRO) supports and assists programs of the Department of Health, other state agencies, federal agencies, private and public laboratories, and local community health departments by providing analytical and diagnostic services on infectious diseases and communicable outbreak response.

6.4 Office of Environmental Laboratory Sciences

The Office of Environmental Laboratory Sciences (ELS) supports the Department of Health Programs, local health jurisdictions, and the general public in supplying qualitative and quantitative testing on the microbial, radiological, and chemical conditions in the environment.

6.5 Office of Newborn Screening

State law and regulation (Chapter 246-650 WAC) requires hospitals to submit a blood specimen from every newborn to the WA Newborn Screening Laboratory (NBS). Parents may opt out, based on religious tenets or practices by signing a refusal form. The NBS is required to perform appropriate screening tests to detect:

Amino Acids disorders: Phenylketonuria (PKU), Maple syrup urine disease (MSUD), Homocystinuria (HCY), Argininosuccinic academia (ASA), Citrullinemia (CIT), Tyrosinemia type 1 (TYR-1)

Fatty acid oxidation disorders: Medium chain acylCoA dehydrogenase (MCADD), Carnitine uptake defect (CUD), Long chain L-3-hydroxy acyl-CoA dehydrogenase (LCHAD) deficiency, Trifunctional protein (TFP) deficiency, very long chain acyl-CoA dehydrogenase (VLCAD) deficiency.

Organic acid disorders: 3-Hydroxy-3methylglutaric aciduria (HMG), Beta –ketothiolase deficiency (BKT), Glutaric acidemia type 1 (GA-1), Isovaleric acidemia (IVA), Methylmalonic acidemias (CblA, CblB & Mutase), Multiple carboxylase deficiency (MCD), Propionic acidemia (PROP) AND

Other disorders: Congenital hypothyroidism (CH), Congenital adrenal hyperplasia (CAH), Hemoglobinopathies, Galactosemia, Biotinidase deficiency, Cystic fibrosis (CF), Severe combined immunodeficiency (SCID), X-linked adrenoleukodystrophy (X-ALD), Pompe disease & Mucopolysaccharidosis type I (MPS-I), spinal muscular atrophy (SMA) and other heritable disorders as determined by the State Board of Health.

7 IMPORTANT ASPECTS OF SERVICE

The services provided by the PHL support the initiatives to develop healthy children and protect Washingtonians from environmental hazards and contamination, infectious, communicable, genetic, and chronic diseases.

8 LICENSURE, ACCREDITATION, AND CERTIFICATION

Accreditation or certification is the recognition for meeting certain predetermined standards conferred upon a facility by a nationally recognized agency. Standards of the accrediting agency assure quality for performance in both administration and analytical testing. The basic principle of accreditation is that the laboratory complies with the accrediting agency's standards continuously.

The laboratories are licensed, accredited, certified, or inspected by the following agencies:

- WA Medical Test Site Licensure Program, CLIA
- College of American Pathologists, CAP
- State of California Clinical and Environmental Testing
- Washington State Department of Ecology, WA DOE
- United States Food and Drug Administration, FDA
- Federal Emergency Management Administration, FEMA
- State of Washington Office of Radiation Protection
- Center for Disease Control Select Agent Program, SA
- Department of Transportation, DOT

- ANSI National Accreditation Board, ANAB ISO/IEC 17025

These agencies periodically inspect the laboratories they certify. Inspections determine the degree to which a laboratory is following the agency's standards.

Any deficiency revealed during an inspection is addressed and corrected by the appropriate laboratory section within an agreed-upon timeframe with the inspecting agency Follow-up report(s) for corrected deficiencies are sent to the certifying agency with a copy maintained on the P: drive or in the IQE module of MediaLab.

Successful accreditation is dependent on satisfactory performance on Proficiency Tests. Unsuccessful performance on Proficiency Tests may result in the loss of accreditation status and cessation of the testing.

8.1 Compliance Policies

The PHL shall comply with applicable laws and regulations such as the Washington State Labor & Industries, OSHA worker safety regulations, and Clinical Laboratory Improvement Amendments (CLIA). WA PHL develops, implements, and maintains policies, processes, and procedures throughout the organization that are designed to meet relevant requirements.

8.2 Medicare/Medical Test Site Licensure Program

Clinical laboratories in Washington State are required to be licensed under the WA Medical Test Site Licensure Program. Laboratory inspections are performed by inspectors from the Department of Health Office of Laboratory Quality Assurance or by CLIA-deemed accrediting agencies.

The requirements and standards are described in the federal register, dated Friday, February 28, 1992, in Part II Department of Health and Human Services, titled Clinical Laboratory Improvements Amendment, 1988 (CLIA88). A copy is maintained in the QA Officer's office.

The PHL sections performing clinical testing are licensed by the WA Medical Testing Site Licensure Program and biennially inspected by the College of American Pathologists (CAP). Internal audits are performed during the year when no external inspection is scheduled. A detailed audit procedure can be found in **69790.957** *CAP Internal Inspection*.

The summary reports from the internal inspection are prepared by the QA Officer and submitted to the external inspectors upon request.

8.3 College of American Pathologists

CAP is a well-recognized clinical laboratory-certifying agency and CLIA-deemed entity. The PHL has maintained accreditation through CAP since the 1970's. Further details on CAP accreditation can be found in **69790.958** *CAP Accreditation*.

8.4 Department of Ecology, Washington State

The WA DOE Laboratory Accreditation Section accredits laboratories performing testing of drinking water throughout the state. The PHL is accredited by the DOE for performing lead testing in drinking water, i.e., school drinking water testing. The DOE also regulates hazardous waste management.

8.5 Food and Drug Administration

Under provisions of the National Shellfish Sanitation Program, State Laboratory evaluations are the responsibility of the FDA to ensure the uniform application of standard procedures and methods in split samples, the examination of shellfish growing waters, quality control of market shellfish, and the monitoring of depuration systems and paralytic seafood poisoning. An additional purpose of laboratory

evaluations is to determine the adequacy of facilities, equipment, and personnel to perform the level of analytical testing necessary to meet the State's Shellfish Program requirements.

A satisfactory evaluation indicates that the agency recognizes that the laboratory 1) complies with recommended procedures and 2) has the capabilities to produce quality analytical results in support of the State's Shellfish Control Program.

The FDA recommends on-site evaluations at least every three years or more frequently if necessary. Inspections follow guidelines set by the Food and Drug Administration and typically include a review of laboratory apparatus, materials, media preparation, bacteriological procedures, and laboratory techniques. The analysts must also demonstrate competence in performing appropriate tests as described in the fourth edition of the *Recommended Procedures for the Examination of Sea Water and Shellfish, APHA Official Methods of Analysis of the Association of Official Analytical Chemists*, Section 46.117-46.119, Thirteenth Edition, 1980, AOAC, and *Interim Guides for the Depuration of the Northern Quahaug, Mercenaria*, Marine Health Sciences Laboratory, 1986.

The FDA may also inspect laboratories that do reference testing on blood supply products.

8.6 Federal Emergency Management Administration

The FEMA evaluates the PHL Environmental Radiation laboratory, as a part of the State of Washington Radiation Emergency Response Team. Every twelve years through observation of the laboratory performance during a simulated drill, FEMA evaluates the laboratory preparedness for the radiation emergency response by their ability to follow the Laboratory Standard Operating Procedures (*Radiation Emergency Response Manual,* a hard copy).

8.7 Select Agent Inspections

As part of the registration process for the CDC Select Agent Program, the PHL is subject to unannounced inspections, at any time, by inspectors from the Select Agent Program. There are no routinely scheduled inspections, annual or otherwise.

8.8 Department of Transportation

As part of the requirement to ship diagnostic and infectious substances, the PHL can be inspected at any time to ensure that they are compliant with shipping regulations.

8.9 Washington Radiation Inspections

The State of Washington Office of Radiation Program issues a license every two years to the PHL to handle radioactive materials. Prior to the renewal of the licensures the PHL will be inspected by the Office of Radiation Protection.

8.10 ANSI National Accreditation Board

As of Nov 2019, the PHL Radiation Laboratory is accredited by ANAB ISO/IEC 17025.

9 PERSONNEL

9.1 Educational and Experience Requirements

Personnel education and experience qualifications are a major aspect of QA. It is the policy of the PHL to comply with all applicable state and local laws and regulations regarding personnel policies. Sufficient education and/or job experience is required for each position at the PHL, including those of clinical consultants whether employed by DOH or under contract. For detailed job requirements see the appropriate Washington State Department of Personnel website:

http://www.dop.wa.gov/CompClass/JobClassesSalaries/Pages/ClassifiedJobListing.aspx (accessed June 2024).

Certain positions have specific educational requirements the lab must follow to maintain accreditation and license to perform testing. The Center for Medicare and Medicaid Services (CMS) requires specific credentials for clinical testing personnel. Those requirements can be found in **69790.5052** *CAP Personnel Requirements*.

Required qualifications for a position are documented in the position posting and job description. At the time of recruitment and hiring, Human Resources collects and records proof of position qualifications which may include but are not limited to diplomas, transcripts, board certifications, licenses, and work history.

Some units within the PHL have responsibilities that occur outside of regular business hours (after hours, weekends, and holidays) when Supervisor and Lead staffing is minimal. To ensure testing is completed in a satisfactory manner only qualified employees who have completed training and competency requirements for their job duties are permitted to work outside of regular business hours.

9.2 Office Organization

The PHL is an office within the division of Disease Control and Health Statistics in the State of Washington Department of Health. The Laboratories are under the directorship of Laboratory Director, Dr. Vince Aoki, and consist of four offices: Public Health Microbiology, Environmental Laboratory Sciences, Newborn Screening, and Operations. The Office Directors currently serve roles as General Supervisor and Technical Supervisor as defined by the CLIA/CAP. Level 4 Microbiologists and Chemists act as subject matter experts (SME) in their specialty of testing, providing technical advice to Office Directors. Diagnostic testing is performed by Microbiologists, Chemists, and Laboratory Technicians.

Public Health Microbiology

The Office of Public Health Microbiology is divided into the following laboratory sections: Reference Bacteriology / Antibiotic Resistance laboratory, Sexually Transmitted Diseases/Tuberculosis laboratory, Molecular/AMD (Advanced Molecular Detection)/Virology laboratory, Enteric/Food/Parasitology laboratory, Bioterrorism/Biowatch laboratory and High Throughput (Covid)/ Central Accessioning laboratory. Each laboratory has a Microbiologist 4 as a Supervisor and a Microbiologist 3 as a Lead.

Environmental Laboratory Sciences

The Office of Environmental Laboratory Sciences is divided into the following laboratory sections: Shellfish Biotoxins, Marine Water Bacteriology, Chemical Incident Response, and Environmental and Radiation Chemistry. Each section has a Supervisor (Chemist 4), who reports directly to the Office Director of Environmental Laboratory Sciences.

Newborn Screening

The Office of Newborn Screening is divided into three sections: laboratory, operations, and follow-up. The follow-up section is supervised by the Follow-Up Supervisor (Epidemiologist 2), the operations section is supervised by the NBS Program Manager (MA5), the laboratory is supervised by two laboratory supervisors (Chemist 4) and the entire program is supported by a NBS epidemiologist (Epi2). A laboratory quality assurance coordinator (WMS1) provides quality oversight to the NBS laboratory operations. The laboratory is further divided into sections based on testing methodology. These are MS/MS (amino acid disorders, fatty-acid oxidation disorders, organic acid disorders & X-linked adrenoleukodystrophy), Autodelfia (CH, CAH & CF), isoelectric focusing for hemoglobinopathies (reflex

to HPLC and DNA testing), enzyme activity assays (biotinidase deficiency and galactosemia), DNA-based tests (SCID & SMA) and digital microfluidics (Pompe disease & MPS-I).

Operations

The Office of Operations is divided into three units: Facilities, Support Services, and Safety Office.

An organizational chart is available through the Microsoft Teams or Outlook contact cards and in Service Central under "My Org Chart".

9.3 Training and Competency

From a laboratory perspective, training is the documented process where an employee becomes proficient in performing an assay or protocol safely according to the parameters outlined. Successful completion of training is required prior to performing duties. Training documents are kept by Supervisors in each section in the Compass module of MediaLab

Ensuring that all analysts are competent to perform their analyses is basic to Quality Assurance. To be effective, the competency assessment must be performed throughout the year (twice for the first year of employment, then annually). Duties related to the training and competency evaluation are delegated to the Office Directors.

Detailed procedure can be found in **69790.965** *Personnel Training and Competency*.

9.4 Performance Evaluation

The State Department of Personnel requires that all state employees complete a performance evaluation annually. PHL employees are evaluated by their respective supervisor. Evaluations are maintained by the Department of Personnel in Olympia.

9.5 Color Deficiency

All technical personnel performing testing or other tasks that require color discrimination are to be evaluated for difficulty with visual color discrimination. Evaluation is not required for personnel that do not perform such functions. The evaluation may be limited to discrimination of those colored items pertinent to the job. The Ishihara Test for Color Deficiency is utilized, and a record of the testing is recorded on the Color Deficiency Form. Currently, the Administrative Assistant to the PHL Director (AA4) is responsible for color deficiency testing as a part of onboarding orientation. If the AA4 is unavailable, color deficiency testing will be administered by Office Administrative Assistance 3 (AA3). In circumstances where social distancing is necessary, an online version of the Ishihara test is used. The employee will copy their test results from the online module to a modified **69790.2077** *Ishihara's Online Test for Color-Deficiency* form. The original result form will be kept in the employee's medical file. The AA4 will provide a notification to the employee as well as their supervisor indicating a pass or failure. If the employee fails, they are not disqualified from performing the test, especially if this is a part of their essential duties. If an employee fails the test, The Reasonable Accommodations Manager should be contacted to engage in the reasonable accommodations process and further steps.

The online Ishihara Test for Color Deficiency can be found at the below link:

https://www.colorlitelens.com/ishihara-test.

10 SAMPLE TEST MANAGEMENT

It is the WA PHL policy that all testing is compliant with applicable state and local laws and regulations. Test requests shall be made from authorized personnel such as hospitals, health care clinics, and various government agencies. A signed Patient Consent Form for testing may be required to process specimens.

More specific testing information can be found in the individual procedures in the laboratory and on the laboratory test menu website.

Most samples and specimens are delivered to the PHL by U.S. mail, UPS, or by a courier service. Further details can be found in **69790.468** *Specimen Handling in Central Accessioning*.

The PHL will make an effort to provide necessary training to our customers in specimen and sample collection. Information for the collection and handling of laboratory specimens is available in the Public Health Laboratories Directory of Services as well as the PHL Lab Test Menu website. The QA Officer and unit Supervisors are responsible for maintaining updated information posted on the website.

https://doh.wa.gov/sites/default/files/legacy/Documents/Pubs//301-016-PHLDirectoryServices.pdf (accessed June 2022)

https://doh.wa.gov/public-health-healthcare-providers/public-health-laboratories/lab-test-menu (accessed June 2022)

10.1 Unique Identification of Specimens and Samples

All specimens and samples must be labeled at the time of collection to provide unique identification. For clinical specimens, two unique identifiers are to be used to label the primary specimen container.

10.2 Limited Exceptions in Specimen and Sample Labeling

If laboratory personnel identify a potential error on a specimen or sample label, the best practice is to reject this specimen/sample and recollect the specimen/sample. However, there may be circumstances when recollection is not possible or practical. Circumstances under which clerical correction of the information on a specimen/sample label is permitted are determined by a supervisor in a given specialty of testing. A record of all such corrections should be maintained on the original specimen/sample paperwork and laboratory log. The PHL promptly notifies the submitter and asks them to investigate the reason for the errors in specimen/sample labeling. If needed, recommendations can be made for the submitter to develop corrective/preventive action as appropriate, including an education of personnel who collect specimens/samples.

The PHL has created a **69790.46** *Specimen Label Deficiency Authorization Form* to address the issue when the PHL receives a specimen with missing or discrepant labeling. This form clearly states that the submitters shall take full responsibility for the identity of the patient from whom this specimen was collected. Laboratory results will not be reported out until this form has been completed and received by the PHL.

10.3 Retaining Samples

Laboratory samples are to be retained for a defined period as listed in **69790.1308** *Record and Specimen Retention Policy*.

10.4 Specimens Sent to Reference Laboratories

In several instances, the PHL must send specimens, nucleic acid extractions, aliquots, cultures, NGS testing processes (analytical wet bench, analytical bioinformatics, interpretation), samples, etc. to a reference laboratory for additional testing.

Shipment of specimens, samples, shipping documentation, and shipment receipt is predominately handled by Central Accessioning (CA) and in rare circumstances by the laboratory unit directly. The Office of Newborn Screening accessions their own samples in the N-wing. The CA Supervisor ensures the appropriate communication with recipient laboratories regarding technical details for the particular requirement on shipment, if applicable. The Deputy Director is responsible for monitoring the quality of test results received from reference laboratories. The detailed shipping procedure is described in

69790.467 *Central Accessioning Shipping* and the procedure for receipt and distribution of reports from reference laboratories is described in **69790.466** *Central Accessioning Reporting Send Out*.

NGS bioinformatics data files may be transferred to reference laboratories. The conditions and details when a data file can be transferred to a reference laboratory are detailed in the specific NGS method standard operating procedure (SOP). Procedures for file transfer are found in **69790.3152** *Bioinformatics – IT Infrastructure*. Data files are only transferred using secure and approved methods (eg, FASTQ file format and secure file transfer systems such as PulseNet FTP or BaseSpace Sequence Hub). Records of file transfer and receipt to reference laboratories must be kept.

Lab reports from all the referred testing laboratories are retained according to the DOH retention policy, **69790.1308** *Record and Specimen Retention Policy*.

The essential elements of referred test results are reported by the WA PHL as received from the referral laboratory, without alterations that could affect clinical interpretation. Transcribed results from the referral laboratory report, the test result(s), interpretation, and information directly related to the interpretation are copied as reported by the referral laboratory.

10.5 Selection of Reference Laboratories

The selection of reference laboratories is under the authority of the CLIA director and is designated on a case-by-case basis. Any diagnostics testing sent out shall be pre-authorized by the CLIA director and the CLIA director shall evaluate the authenticity of the recipient reference laboratory.

The PHL is fully responsible for the selection and transport of the specimen to reference laboratories. PHL will properly follow all requisition, collection and handling specifications of the reference laboratories, and state and federal transportation regulations.

The selection and evaluation of reference laboratories must include the following criteria:

- The reference laboratory must meet appropriate accreditation requirements for the area of testing they are performing. For instance, clinical laboratories must meet CLIA-88 testing requirements and the laboratory must be a CLIA-certified laboratory or a laboratory meeting equivalent requirements as determined by the Center for Medicaid Services. Environmental laboratories must meet the Environmental Protection Agency, State of Washington Department of Ecology, Food and Drug Administration, Office of Radiation Protection, or ISO 17025 certification requirements.
- The PHL is responsible for verifying that the reference laboratory has the appropriate certification.
- Laboratory quality of performance must be accounted for. This can be done by previous experience with the laboratory, other users of the laboratory, contacting the accreditation agency for the laboratory, requesting documentation of proficiency performance from the reference laboratory, or past accreditation inspections.
- Turnaround times must meet the testing needs of the PHL and their clients.

PHL is responsible for closely controlling pre-analytic variables to maintain specimen integrity. Also, it may be necessary to collect specific patient information required by the testing laboratory.

PHL ensures access to reference laboratory test reports either via direct access to the reference laboratory portal or via keeping a hard copy of the original reports. Results are typically released directly to ordering providers from the reference laboratory, or PHL releases the original report from the reference laboratory without alterations. This is captured in document **69790.466** *Central Accessioning Reporting Send Out Results*.

10.6 Identification of specimens, specimen types, samples, and aliquots

A system must be in place to positively identify all patient specimens, specimen types, samples, and aliquots at all times. Each specimen and sample container must identify the patient uniquely. This may be text-based, numeric, bar-coded, etc. The form of this system is entirely at the discretion of each laboratory, so long as all primary collection containers and their aliquots have a unique label that can audit back to full the particulars of patient or sample identification, collection date, specimen type, etc. Practical considerations of container size may limit the extent of such details. Aliquots must not be returned to the original container for specimens to be used for molecular-based testing. For specimens used for non-molecular types of testing, the laboratory must consider contamination and the potential for specimen mix-up when defining this in its procedure. If previously aliquoted specimens are used for additional testing, the procedure must define when and how they can be used.

When a specimen aliquot is made, care must be taken to prevent cross-contamination of the aliquot containers. No aliquot is ever returned to the original container.

10.7 Accessioning Specimens and Samples

The date (and time, if appropriate) that the specimen or sample was received by the laboratory is to be recorded. The specimens and samples for the Offices of Microbiology and Environmental Laboratory Sciences are received and accessioned in the central accessioning facility, B103. Newborn Screening accessions their specimens in N-wing (N22) after having been received in the central receiving facility (B103). Detailed accessioning process is found in **69790.465** *Central Accessioning Data Entry* and NBS **69790.368** *Specimen Processing*.

Clinical laboratory specimens are to be analyzed only at the written or electronic request of an authorized person. An authorized person would be a physician licensed under chapter 18.71 RCW, Physicians; chapter 18.57 RCW, Osteopathy—Osteopathic medicine and surgery; or chapter 18.22 RCW, Podiatric medicine and surgery; advanced registered nurse practitioner, licensed under chapter 18.79 RCW, Nursing care; Midwife licensed under chapter 18.50 RCW, Midwifery; Physician assistant licensed under chapter 18.71A RCW, Physician assistants; Naturopath licensed under chapter 18.36A RCW, Naturopathy; or Dentist licensed under chapter 18.32 RCW, Dentistry. WAC 246-338-020, *Licensure — Types of medical test site licenses*, was used as a guide in determining who is an authorized person. If this authorization is lacking, the Public Health Laboratories will follow up and get the appropriate authorization.

Extracted nucleic acid specimens for clinical testing are only accepted from a CLIA-certified (or equivalent) laboratory and are clearly indicated on the test requisition form.

Starting January 1, 2024, to be in compliance with the Washington State Notifiable Conditions Rule Chapter 246-101 WAC, the PHL Microbiology Office will no longer accept paper-based test requisitions for clinical test submissions. Specimen submissions are to be completed through the electronic test ordering portal, Lab Web Portal (LWP), which will collect all required demographic data included in the Notifiable Conditions Rule. First-time users of the LWP can sign up on the PHL Lab Test Menu home page at Lab Test Menu | Washington State Department of Health. Paper-based requisitions will be accepted with preauthorization from the PHL on a limited basis if the submitter cannot access the LWP or due to some other barrier preventing the use of the LWP.

This change does not apply to Newborn Screening submissions. Environmental Laboratory Sciences submissions are currently being transitioned to LWP, once this transition is complete, paper-based requisitions will be phased out.

10.8 Review of Specimen and Sample Acceptance

An integral part of providing timely results is the receipt of samples/specimens by the laboratory that are properly collected, labeled, packaged, and shipped. Each laboratory unit monitors the unsatisfactory package/specimen to collect and do appropriate follow-up, such as re-training submitters regarding specimen shipment.

The condition of the specimen is recorded in the laboratory information management system (LIMS) system and a report is generated to the submitting physician or the follow-up team (NBS) that the specimen was unsuitable for testing. Starting January 1, 2024, Microbiology clinical specimens submitted with a paper requisition will be rejected unless preauthorized to do so (see section 10.7).

To improve this process, some laboratory units track the frequencies of the unsatisfactory package/ specimen to collect and analyze the data when unacceptable samples or specimens are received by the PHL. An example of such a log is **69790.501** *Specimen Rejection Log* in the Reference laboratory in the Microbiology office. The root cause analysis and/or statistical analysis to identify the potential trends will assist PHL in determining where to focus our quality process improvement efforts. In addition, such data analysis will provide each office with guidelines on which quality indicators to track yearly. The QA Officer is responsible for summarizing findings in the annual *Quality Management Plan Effectiveness Report*.

10.9 Verbal Test Orders

It is the PHL policy that personnel receiving verbal or phone orders for testing must read back the entire order to verify the accuracy of the transcription. All verbal order is required to submit a written test order, prior to release test results. Exceptions may be case-by-case approved by the CLIA director. If modifications of existing orders are verbally made by submitters, such as adding a test panel, such changes are noted in the comment section of a final report.

10.10 Specimen and Sample containers and analytical interference

Specimen and sample containers are to be assessed to ensure that they do not interfere with the assays performed. Exhaustive testing is not mandated for each laboratory, rather, this may be done through some combination of:

- direct testing by the laboratory
- review of the clinical literature
- evaluation of information from manufacturers.

Documentation of a summary of their analytical interference evaluation is to be kept either in the method validation report and/or in the testing procedure(s) for which the specimen containers are utilized.

10.11 Analyst Identification

The analyst, testing system and date of test at all stages of a test must be able to be identified. At PHL, this can be done by either initialing/dating paper worksheets, reports, etc. or tracked by the computer, depending on the assay.

10.12 Reporting Results

The laboratory communicates diseases of particular significance promptly and retains records of the communication. The method of reporting results may vary with each respective Office. Detailed procedures for reporting results are found in Office or Section specific SOPs and/or in individual test SOPs. All patient results are handled and reported in compliance with Health Insurance and Portability Accountability Act (HIPAA) requirements and the respective regulatory or certifying body requirements (eg, CLIA, ISO).

Result reports will include the method(s) used, analyte(s) tested for, test result(s), and when appropriate reference intervals, and the clinical interpretation. All report template content and format are reviewed by the Deputy Director prior to test implementation and any edits post-implementation. Preliminary results must be documented with the same level of detail as final reports.

Reports interpreting NGS sequence variants must comply with a standardized written algorithm. This algorithm is used to determine the clinical significance of any detected organisms and genes linked to resistance and pathogenicity. When sequencing identifies normal microbiota in a patient sample it is clearly differentiated from pathogenic organisms in the report. Each NGS SOP must address this policy in the reporting structure. Before releasing reports, any microbial phenotypes identified through traditional methods are to be checked against the phenotype identified through NGS.

Disclaimers for Reports and Amended Reports are kept in MediaLab for document control. They will follow the full approval process and be linked to the SOP to which they pertain to. Once approved, the supervisor will forward the updated disclaimer to the LIS manager to update the LIS report.

10.13 Amended Reports

All revised preliminary or final reports for previously reported incorrect or incomplete results are to be identified as amended, regardless of the format of the reports (paper, verbal, e-FAX etc.). In the case of multiple sequential corrections of a single test result, all corrections are to be referenced in sequential order on subsequent reports. Reports that display amended results must clearly indicate the new result is a change from a previously reported result. Submitters are immediately notified of the amendment of the previous results.

The amended and original data must be clearly identified as such, and the original data is to be readily accessible to the user for comparison, such as the original data must be present in the amended report (for paper reports) or linked electronically or logically to the amended information (in electronic reports). The precise format of corrected reports is at the discretion of the individual laboratory.

Events with amended reports are documented using MediaLab/IQE platform. QA Officer reviews and determines the necessary follow-ups. Detailed procedure for non-conformance reporting is found in **69790.945** *Non-Conformance Report* and **69790.946** *Managing Non-Conforming Work with Continuous Quality Improvement.*

10.14 Retrieval of Information

Copies or files of reported results are to be retained by the laboratory in a manner that permits prompt retrieval of the information. The length of time that reported data is retained in the laboratory may vary. Detailed procedure for the record retention is found in **69790.1308** *Record and Specimen Retention Policy.*

10.15 Availability of Test Methods to Clients

If a client request is made to obtain a laboratory's current test methods and/or performance specifications, the section Supervisor and/or Office Director shall address their needs.

10.16 Change in Analytical Methodology

If the laboratory changes its analytical methodology so that test results or their interpretations may be significantly different, the change is to be explained to clients. This can be accomplished in any of several different ways, depending on local circumstances. Some methods may include a change in the *Directory of Services*, directed mailings, laboratory newsletters, or part of the test report itself.

10.17 Reagent Labeling, Storage & Expiration

Reagents and solutions are to be labeled, stored according to manufacturer specifications, and used by the expiration date. Detailed procedure is found in **69790.1038** *Reagent Handling*.

10.18 Unknown Submitter

When specimens are received without the submitter's identification (name, address), specimen will be rejected for testing. The exception to this rule is newborn screening specimens are tested even if the submitter's identification is blank because many of the newborn screening conditions can be life-threatening during the first weeks of life. Newborn screening staff can usually identify a specimen's submitter by a barcode search.

10.19 Loss of Critical Documents

Upon determining laboratory documents critical to reproducing data are lost, immediately notify the incident to the section Supervisor, Office Director, and QA Officer. Type of laboratory document, root cause of the incident and any other information of importance to the investigation is documented. Non-conformance is reported via MediaLab IQE platform for QA review. The Office Director is responsible for notifying appropriate stakeholders including submitters.

10.20 Testing Control Specimens

Control specimens are tested in the same manner and by the same personnel who routinely perform testing. Each operator does not necessarily need to perform QC daily, so long as each instrument and/or test system has QC performed at required frequencies, and all operators participate in performing QC on a regular basis.

10.21 Specimen Handing for Molecular Procedures

Due to the high sensitivity and potential for contamination in molecular testing involving nucleic acid special laboratory hygiene and sample handling procedures shall be followed as detailed in **69790.1229** *Laboratory Contamination Prevention and Monitoring: Nucleic Acid Testing*.

11 ANALYTICAL PROCEDURES

Each office is required by their certifying agencies to have a procedure for all analytical work they perform. The procedures may cover a grouping of testing or be very specific in its emphasis. All PHL procedures must follow the PHL Procedure format as described in ***69790.107*** *Results Generating PHL Procedure Template* and **69790.70** *Non-results Generating PHL Procedure Template* .

11.1 Intermittent Testing

When a test is put back into production, the following requirements must be met:

- PT or alternative assessment performed within 30 days prior to restarting patient testing.
- Method performance specifications verified, as applicable, within 30 days prior to restarting patient testing.
- Competency assessed for analysts within 12 months prior to restarting patient testing.

This applies to tests that are taken out of production for a certain period of time (for example, seasonal testing for influenza). A test is considered to be taken out of production when (1) patient testing is not offered AND (2) PT or alternative assessment, as applicable, is suspended.

For tests for which PT is required by CAP, if a PT challenge is not offered during the 30-day period prior to restarting patient testing, the laboratory may perform an alternative assessment of the test. In such a case, the laboratory must participate in the next scheduled PT event.

12 QUALITY CONTROL

Quality control more narrowly describes the techniques and activities by which a department validates and documents proof of the quality of a specific test or service, most notably testing results. The quality control system is monitored using both statistical and non-statistical techniques. Quality control should be viewed as a system or Quality Control Program that includes many different procedures, such as checking reagent lot numbers, monitoring temperatures of refrigerators, freezers, and heating baths and measuring quality control samples of known values. Control of analytical testing may be broadly divided into internal and external types.

Results are validated and monitored in real-time using internal control systems that include information from stable control samples as well as from other samples/specimens. The main objective of internal quality control is to ensure the day-to-day consistency of measurements.

External quality control refers to a process of retrospectively comparing results from different laboratories through the use of an external source. The major purpose of external quality control is to assess accuracy by comparing results with other laboratories with similar and different methodologies. External Controls are useful to determine lot to lot variability of testing kits.

Documents related to the Total Quality Control Program (TQCP) are:

69790.1365 *TQCP Implementation Guidance*
69790.1356 *TQCP IQCP Flow Plan*
69790.1357 *TQCP Summary Sheet*
69790.1358 *TQCP Summary Example & Notes*
69790.1359 *Westgard Sigma Rules*
69790.1360 *Quality Control MTS Checklist*
69790.1373 *Sigma Quality Determination of Test Systems*

Individual Quality Control Programs allow the laboratory to customize the quality control practices of a test system. An IQCP consists of three steps critical to ensuring QC practices are compliant and reduce potential errors or problems. These three steps include a risk assessment, definition of the quality control plan (QCP) and ongoing monitoring and assessment of the QCP. For more information on developing and IQCP for a test system, review the documents listed below.

Documents related to the Individual Quality Control Program (IQCP) are:
69790.1060 *IQCP Implementation Guidance*
69790.1041 *IQCP Assay Summary Form*

12.1 Proficiency Testing

The objectives of proficiency testing are:

- Provide a measure of the quality of performance for individual laboratories
- Promote improvement of performance
- Allow potential customers and accreditation agencies to evaluate/compare laboratory performance
- Provide a training tool for employees that are being trained and evaluated in the procedure

PT or an alternative performance assessment (for analytes with no commercially available PT provider) will be performed on all regulated analytes performed at the PHL by those who routinely perform the testing. The alternative PT's will be handled in the same manner as with an external vendor, twice per

year (approximately every 6 months). Alternate PTs should always contain at least two to five test specimens per event.

Detailed procedure for PT process is in **69790.1330** *Proficiency Testing*.

Centers for Disease Control
CDC distributes samples to be tested for Blood Lead, Fluoride Analyses, Newborn Screening, Bioterrorism and Chemical Terrorism (Center of Toxicology, Quebec, Canada). The CDC is not a proficiency-testing agency *per se*. Although there is no accreditation or certification involved with the testing of analytes from CDC, the PHL participates in the survey. Summaries of the testing performed are sent to the CDC for review. The QA Officer will be informed when these PT samples have arrived as well as when the results are sent to the PHL.

College of American Pathologists
To perform successfully on a College of American Pathologists' testing survey, the laboratory must achieve at least an 80% success rate. Usually, five different analyses are required per testing event. Currently, most surveys are distributed 3 times per year.

Environmental Resource Associates (ERA)
ERA provides material for the quality control and PT needs of environmental chemical, radiochemical and pharmaceutical laboratories. ERA provides the products and support to help with accreditation or the verification of the quality of critical pharmaceutical analyses. ERA is the oldest and largest company in the industry with the most accreditations, staff, instrumentation, and facilities.
http://www.eraqc.com/ (accessed June 2022).

Food and Drug Administration
PT is performed annually for both the Food and Shellfish microbiology laboratories. The FDA Laboratory Quality Assurance Branch, 6502 south Archer Rd, Summit-Argo, IL 60501-1957, provides Food Split Sample Proficiency Test and the Shellfish Split Sample Proficiency Tests.

Inter-laboratory Comparison (Alternative PT)
Biannual alternative PTs are set up when there is no commercial proficiency test available. Usually, participants take turns in preparing and sending out the proficiency specimen to all participating laboratories. Acceptance criteria must be established based upon mutual agreement among participants and a summary of comparison results must be generated and sent to all participants.

Laboratory Proficiency (REML)
REML provides the PT for the water microbiology laboratory. Each method in the water microbiology laboratory will receive one set of samples with ten samples per set. The water microbiology laboratory can receive these samples all at one time or at staggered intervals. Acceptable test results are scored against the assigned values.

Laboratory Response Network (LRN)
LRN was established by the Department of Health and Human Services, CDC in accordance with Presidential Decision Directive 39, which outlined national anti-terrorism policies and assigned specific missions to federal departments and agencies. Through a collaborative effort involving LRN founding partners, the Federal Bureau of Investigation, and the Association of Public Health Laboratories, the LRN became operational in August 1999. Its objective was to ensure an effective laboratory response to bioterrorism by helping to improve the nation's public health laboratory infrastructure, which had limited ability to respond to bioterrorism. Today, the LRN is charged with the task of maintaining an integrated network of state and local public health, federal, military, and international laboratories that can respond to bioterrorism, chemical terrorism, and other public health emergencies.

https://emergency.cdc.gov/lrn/index.asp (accessed June 2022)

www.inspq.qc.ca/en (accessed June 2022)

- **LRN Select Agents**: the LRN sends proficiency events three to four times per year with five samples per event.

LRN Chemical Terrorism: PT specimen for cyanide in blood, organophosphate nerve agent metabolites (OPNA) in urine, and volatile organic compounds (VOCs) in blood, OPNA in serum, Abrine/Ricinine in Urine, Tetramine in Urine, Mercury in Urine, Toxic Element in Urine (Urine Metals), and Blood Metals are received three times a year on a pre-announced schedule from LRN contractors. Once a year the LRN-C will also send an unannounced PT from one of the methods to selected laboratories. Each proficiency set is comprised of ten samples per set. The Wadsworth Center in New York (CDC contractor) provides matrix-matched Priority Metals Quality Assessment Screen (PMQAS) PT specimen for multiple trace elements in urine and blood matrices, three times per year. Each proficiency set varies, at times all samples have analytes (typically metals-urine or blood matrix) spiked into the samples, but it is not uncommon to receive one or more (sometimes two) non spiked samples. It is typical to receive one or more non spiked samples for the rest of the LRN-C PTs, but it is usually two (not always) for those methods. Blood metal's PT samples are provided by WSLH (WI State Lab of Hygiene). Mixed Analyte Proficiency Evaluation Program (MAPEP, US Department of Energy).

The PHL Environmental Radiation Laboratory participates in MAPEP, conducted by the US Department of Energy Radiological and Environmental Measures Laboratory (RESL). The analytical performance is evaluated on the relative bias of the laboratory results vs. reference value for radiological and inorganic analytes.

Model Performance Evaluation Program (MPEP)
The CDC provides the MPEP program for PT testing for retroviral, AID related and *Mycobacterium tuberculosis* testing. Well characterized samples resembling patient specimens encountered in clinical testing are sent out as two surveys per year.

- **Retroviral and AIDS Related Testing**: HIV-1 antibody; HTLV-I/II antibody; HIV-1 RNA; HIV-1 p24 antigen; and T-lymphocyte immunotyping evaluations
- **Mycobacterium Testing**: five culture panels consisting of strains of *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria.

www.cdc.gov/tb/topic/laboratory/mpep/default.htm (accessed June 2022)

National Institutes of Standards (NIST)
Founded in 1901, NIST is a non-regulatory federal agency within the U.S. Commerce Department's Technology Administration. NIST's mission is to promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve our quality of life. The PHL Radiation Laboratory participates in the NIST Radiation Inter-comparison Program. This program prepares and distributes PT samples of different matrices, such as air, water, soil, urine, and for radiochemical analytes. This program was founded for developing laboratory capability to respond to radiation emergencies.

www.nist.gov/ (accessed June 2022)

WISCONSIN STATE LABORATORY OF HYGIENE (WSLH)
WSLH provides clinical, environmental, and industrial analytical services, specialized public health procedures, reference testing, training, technical assistance and consultation for private and public health agencies. To perform successfully on a WSLH proficiency survey, the laboratory must achieve at least an 80% success rate. Each event consists of six analytes, there are three events per year with the exception of blood metals PT which only receives three analytes per event.

www.slh.wisc.edu/ (accessed June 2022)

TEXAS DEPARTMENT OF STATE HEATLH SERVICES – MUTATIONAL ANALYSIS

NBS conducts PT testing in conjunction with the Texas Newborn Screening Program. Two times a year (May and November) the NBS sends the Texas State Laboratory previously tested blood spots to verify our results as followings: one specimen with the following GALT genotypes - Q188R, K285N, N314D, S135L or not detected; three blood spots each with Hb S, Hb C, or Hb E mutations. The paper results are reviewed and signed off by the CLIA Director. Documentation is in MediaLab IQE module (previously found in P:\EHSPHL\PHL\NEWBORN\COMMON\DNA\QC\PTs\Exchange Program).

12.2 Monitoring of Laboratory and Equipment Environmental Conditions

If the environmental conditions in a laboratory are not stable, the substances being used may physically change or equipment may malfunction resulting in unreliable test results. Adequate environmental monitoring is critical to ensuring quality results are produced by the laboratories.

Monitoring of environmental conditions required by the test method, test kit, or equipment manufacturer must occur, be documented, and a monthly review completed by the supervisor or their designee. Environmental conditions include but are not limited to temperature, humidity, and CO_2. Monitoring must be done with calibrated equipment suitable for the range required by the test method, reagents, specimens, or equipment used in the laboratory.

The PHL uses the SensoScientific monitoring system for temperature, humidity, and CO_2 monitoring. Because temperature is one of the most universally impactful conditions in the laboratory, local backup min/max thermometers are to be installed in equipment identified as critical by the laboratory sections. To learn more about the SensoScientific environmental monitoring system refer to the following documents.

- **69790.2235** *SensoScientific Cloud Monitoring System User Manual*
- **69790.2513** *PHL SensoScientific Equipment Monitoring Job Aid*
- **69790.3874** *Sensoscientific Probe Removal and Installation Job Aid*
- **69790.81** *Temperature Monitoring System*

Guidelines For Monitoring

Follow the guidelines below for laboratory spaces or equipment with specific environmental requirements for temperature, humidity, or CO_2.

- Establish a specific operating range appropriate to the test method, reagents, or specimens and the length of time those conditions may be out of range before a data alarm is triggered.
- Use SensoScientific for continuous monitoring and notifications when measurements go out of range.
- If the condition being monitored is outside the acceptable range, investigate the cause and take appropriate action to ensure test results remain viable. Use the SensoScientific Cloud application to document the root cause and corrective actions taken.
- Follow the manufacturer's instructions for operating thermometers, probes, and monitoring equipment.
- All non-certified thermometers used for clinical specimen storage must be checked against a NIST thermometer before use. All NIST thermometers used in the PHL must have a certificate of analysis, which requires them to be renewed yearly or biennially.
- Mercury thermometers should not be used unless there is a specific reason they need to be used. Mercury spill kits must be present wherever mercury thermometers are used.
- SensoScientific CO_2 probe measurements must be verified at a minimum annually with a calibrated instrument or method.

12.3 Calibration Check of Thermometers

Calibration checks of thermometers are done annually by calibrating the thermometer to a NIST traceable thermometer. This is to be done by sending the thermometer for calibration to a vendor who can provide a calibration certificate. If requested, manufacturers can provide a certificate of calibration when a thermometer is purchased. The laboratory is responsible for keeping a copy of the calibration report. Note that the *Statement of Accuracy* may not be sufficient if it does not list the calibration dates or expiration date of the calibration.

12.4 Pipettes and Liquid Handlers

Automatic pipettes are used in the laboratories to accurately transfer small liquid volumes. Glass pipettes are not highly accurate for volumes less than one milliliter, but automatic pipettes are both accurate and precise. It is up to each laboratory to determine the type of pipettes that meet their needs. The laboratory needs to take into consideration the level of accuracy and precision needed at the bench for performing their assays.

Pipettes are to be purchased with certified performance specifications (received with a calibration certificate) or be calibrated and certified before use. Pipette manufacturers do not always test every pipette that is manufactured. Instead, data from a sampling of each batch may be used to create a certificate for the entire lot. Request individual pipette certificates when placing the initial order. QA also strongly recommends users of pipettes complete an initial training and assessment followed by an annual assessment thereafter.

Pipettes are to be calibrated at least annually through a qualified external vendor. External calibrations are performed by sending pipettes to a third-party vendor or by having the vendor come into the facility to professionally perform the calibrations. When performing onsite calibration, the level of accuracy is not as high as when performed in the vendor's facilities. We primarily have used Rainin and Eppendorf for performing the calibrations.

Periodic checks are recommended between calibrations for pipettes used in critical testing. These checks may be performed by the laboratory. For volumes less than 100 µL, the Artel system is recommended.

Liquid handlers are to be calibrated at least annually. Details outlining the calibration can be found in **69790.1534** *Laboratory Equipment Preventative Maintenance Guidelines.*

Some documents available for your review are:
69790.1828 *10-tips-to-improve-your-pipetting-technique*
69790.2078 *Rainin Pipet-Lite Operating Instructions*
69790.120 *Eppendorf Pipette Standard Operating Procedure*
69790.119 *Pipetman Users Guide*
69790.1534 *Laboratory Equipment Preventative Maintenance Guidelines*
69790.1628 *Compass - End User Training & Competency Assessment*
69790.116 *Artel PCS User Guide*
69790.1826 *Liquid Handling Assessment Artel PCS System*
9790.1806 *Multichannel Verification system Procedure Guide*
69790.1831 *Multichannel Verification System, MVS*

12.5 Weights & Balances

Analytical balances must meet the level of accuracy needed for the laboratory work. Before purchasing a balance, the accuracy necessary for the laboratory work should be determined.

An analyst is to check the balance before each use or once per day, whichever is less, with at least two different reference weights. Reference weights are to be kept in a clean container and handled appropriately. Reference weights are to be checked against Class 1, Class S, or Class U weights (stored in Q1, QA Officer's office or purchased by each laboratory) referenced to NIST standards. A monthly check is required if the balance goes to three or four decimal points. If the balance goes to two decimal points or less, then the balance should be checked quarterly. All frequently used balances, i.e., used practically every day, are to be checked monthly regardless of the type.

To determine the range of reference weights used, consider the weights that are used routinely in the laboratory, the maximum weight used in the laboratory, and the maximum capacity of the balance or follow the manufacturer's recommendation.

Daily reference weight checks are to be kept in a bound laboratory notebook or a controlled sheet designed for that purpose. The date, analyst initials, and weight readings are to be legibly recorded. Use the same form to record required corrective action due to failing checks. The records are to be reviewed and initialed monthly by the responsible Supervisor. The QA Officer keeps the certification records for the class 1 weights and is responsible for sending the weights out for certification. The certification of the weights is good for five years.

12.6 Autoclaves and Sterilizers

Autoclaves use a combination of pressure, heat, and steam to sterilize media, glassware, and utensils. They are also used to destroy microorganisms on inoculated media prior to the media's disposal. WA PHL autoclaves are located in the BSL3, media preparation, and in the glassware washing area.

For each day of use, the temperature of the sterilizer is documented on a log sheet located near the autoclave. Temperatures and time cycles are monitored on charts which are dated and stored within each area. Any deviation of temperature from the validated conditions for the content type requires notification of the supervisor and/or the building maintenance supervisor. The autoclaves are on preventative maintenance schedules and all maintenance records are maintained by building maintenance.

The dry oven sterilizer is located in the glassware preparation room. The temperature is monitored by a thermometer placed in a sand container inside the oven. The thermometer is checked semi-annually for calibration using a certified thermometer. Each run is documented on a log sheet. The temperature is maintained at validated conditions for the specific content type. The run is also monitored for sterility by the use of a dated sterility indicator placed on the container to be sterilized.

All sterilizers are checked once a month, with Biological Sterility Indicators, to ensure they are functioning properly. The Sterility Indicators are to be placed in situations that are similar to regular usage, i.e., placed in mock waste containers, coffins, etc. Chemical indicators or non-biological indicators are used in conjunction with biological indicators. The advantage is that the chemical indicators or non-biological indicators can give immediate feedback that sterilization has been achieved.

Each autoclave should display in clear view the CERTIFICATE OF INSPECTION issued by the Department of Labor and Industries, Division of Building and Construction Safety Inspection Services. This is required

by RCW 70.79.320. Inspection by Washington state Labor and Industries (L&I) is required every two years.

Room	Department	Sterilizer	Indicator	Schedule
R20	Operations	Dry Heat – HAFO 1600	Paper	Monthly
R21	Operations - Media	Steam – Primus, A1	SterilAmp II	Monthly
R41	Operations - Glassware	Steam – Primus, P1	SterilAmp II	Monthly
R41	Operations - Glassware	Steam – Primus, P2	SterilAmp II	Monthly
R21	Operations - Media	Steam – Primus, A2	SterilAmp II	Monthly
C25	BSL3	Steam -	SterilAmp II	Monthly

12.7 Timers

Timers are to be checked when their function is critical to test performance. A stopwatch is located in the Facilities Office and can be used for this purpose. Documentation of the timer check should be noted on the instrument and/or in the Preventative Maintenance log for the instrument. Employees need to follow the manufacturer's product insert for checking timers. If an employee suspects that any timer is incorrect, the timer should be checked and/or replaced. The laboratory standard of practice for instruments with timing functions such as centrifuges, manual coagulation machines, anaerobic chambers, cell washers, radioactive particle counters, blood cell counters, or nucleic acid equipment, is to check yearly as a part of Preventative Maintenance.

12.8 Reagent Water Quality

All laboratory testing will utilize clinical laboratory reagent water (CLRW, previously type II) water unless otherwise specified by the individual procedure. Special reagent water (SRW, previously Type I water) is to be used for testing that requires a higher quality of reagent water. For example, the Molecular Laboratory also purchases reagent water that is RNase free.

The PHL monitors its CLRW by performing chemistry and microbiological checks. Checks are made monthly for free chlorine and Heterotrophic Plate Counts (HPC). Checks for silver, cadmium, chromium, copper, nickel, lead, and zinc are performed annually. Conductivity is monitored continually by an internal probe in the CLRW system and recorded monthly. Further details are found in **69790.1331** *Laboratory Reagent Water Quality Testing*

12.9 Glassware Washing

Glassware washing follows predetermined standards to ensure that the glassware is clean and free of contaminants/detergent before it can be used or recirculated. Appropriate documented procedures are in place for handling and cleaning glassware.

All glassware is washed with a detergent, with the final rinse in deionized water. Organic glassware is also rinsed with a solvent (EPA guidelines followed). The glassware is checked for effective detergent removal by following procedures **69790.28** *Glassware Procedure* and ***69790.211** pH Testing for Clean Glassware*. Sterilization of glassware is performed by the hot oven sterilizer or by autoclaving.

Records for detection of detergent residues resulting from improper rinsing are located in the Dish Room QA/QC Records binder located in R41. The detergent removal check is performed for each dishwasher load. Records include the test date, types of glassware tested, and test results.

12.10 Environmental Maintenance and Equipment Preventative Maintenance

Bench tops, cabinets, drawers, and sinks are to be clean and well-maintained. Preventative maintenance (PM) ensures that equipment continues to function at the level that is required for quality analysis. The majority of critical testing instruments at the PHL are under annual full-service contracts with the

instrument manufacturers that include both preventative maintenance either annually or biannually and repair services, on an as-needed basis for parts and labor. A handful of legacy systems without available service contracts due to the age of the instruments are maintained in-house with a defined maintenance schedule. All laboratory units should document the PM records including documentation from outside vendors (Spectrum, Precision Mechanical). Preventative maintenance records and records from vendors performing preventative maintenance are signed by the section supervisor.

Cleaning of the instrument and/or troubleshooting is performed either by the laboratory analysts, the laboratory maintenance department, or contracted maintenance services depending on the degree of difficulty. Records of cleaning are kept in the preventative maintenance logbook.

Biological Safety Cabinets and fume hoods are inspected annually. All personnel working in the laboratory using Biological Safety Cabinets and Fume hoods will be instructed in the use of the above by their immediate supervisor.

Instrument/equipment performance verification includes processes to verify that the instruments and equipment perform according to expectations for the intended use and within defined tolerance limits. The performance of all instruments and equipment is verified and documented prior to initial use, after major maintenance or service, and after relocation to ensure that they run according to expectations.

12.11 Monitoring of Controls

The monitoring of control is an important aspect of the analysis. If control results are not within acceptable range, the patient or sample results of the same run should not be reported. Each laboratory unit determines the frequency, concentration requirements (for quantitative testing), and the acceptable range for quality controls in a run. The results of controls must be reviewed by the laboratory unit Lead or Supervisor for acceptability before releasing result reports. Details are found in the standard operating procedure for the method.

12.12 Monitoring Analytic Performance

Performance monitoring, or quality control, is used both to detect shifts in measurement processes over time and to determine the acceptability of analysis for reporting. The parameters monitored and their associated control limits are specified in the standard operating procedure for the method, or instructions specific to the instruments and tools that are used in those methods. Typically, quality control material is used according to the manufacturer's instructions.

Please refer to the individual standard operating procedures and related guidance documents covering the method in question for a listing of quality control materials and acceptance criteria. As applicable, controls, metrics, and quality control parameters must be established during validation or verification of the method and analyte and defined in the standard operating procedure for the method or analyte.

When appropriate, statistics on test results are maintained and periodically reviewed to identify changes in test performance and detect systemic errors. See individual method or analyte standard operating procedures for methods of calculating statistics, recording statistical data, evaluation frequency, and corrective action if indicated.

12.13 Detection and Correction of Significant Clerical and Analytic Errors

It is important to detect laboratory reporting errors as quickly as possible in the testing process, monitor the causes, and reduce their impact by (1) timely intervention when errors are recognized and (2) determining the underlying reasons for reporting mistakes so that adjustments can be made to prevent recurrences. It is imperative that all laboratory staff actively watch for errors and investigate their causes when they are found. Knowledge obtained from these investigations may identify methods to improve reporting procedures so that errors do not recur.

Possible mechanisms to detect clerical errors are:

- Select random reports and review the work cards, worksheets, and final charted results for accuracy and completeness.
- Lead workers should compare results on worksheets and requisition forms when reviewing final reports.
- Review process for clerical errors regularly.
- Follow up on any quality control issues or problems.
- Make sure that satisfactory competency assessments have been completed for all personnel performing the work.
- Each unit is responsible for implementing specific steps to detect and correct possible sources of errors.

Details concerning the error correction procedure can be found in **69790.1332** *Error Correction.*

12.14 Control Charting

Quality Control Charts are normally presented graphically in the form of Levy-Jennings plots. Control limits may also be established by statistical analysis of a set of data (e.g., the mean and ± two and three standard deviations, from a set of a minimum of twenty data points). Control limits may also be based on regulatory requirements, criteria specified by a vendor, or the data quality objectives for which the method was developed. Details are further found in the individual standard operation procedure for each test.

12.15 Reagent Quality Control Testing

Reagent performance and adequacy per different lot must be verified before placing the material in service or concurrent with use. Various methods, such as direct analysis, use of reference materials, or parallel testing of old *versus* new reagents, are acceptable. The results of verification checks must be recorded.

12.16 Calibration, Analytical Measurement Range, etc.

These criteria are primarily for assays where a quantitative result is reported or where the manufacturer package insert stipulates that it be performed. Please note that this does not replace the standard method validation/verification requirements that the performance of all instruments and equipment are verified prior to initial use, after major maintenance or service, and after relocation to ensure that they run according to expectations.

Calibration

Calibration is the set of operations that establish, under specified conditions, the relationship between reagent system/instrument response and the corresponding concentration/activity values of an analyte. Calibration procedures are typically specified by a method manufacturer but may also be established by the laboratory.

Calibration Verification is the process of confirming the current calibration is still valid for a process. The frequency of verification is determined by the governing regulatory guidance and/or by the manufacturer's recommendation, whichever is more frequent. In addition, if the instrumentation undergoes a major repair or is modified or a change of critical reagents, then it is necessary to perform the calibration verification. If the calibration verification is unsuccessful, then remedial action is necessary. It is the individual laboratory unit's responsibility to establish the criteria for a successful calibration verification process.

Analytical Measurement Range (AMR)

AMR must be determined at a minimum of every six months or after a major change in the instrumentation system or a change in analytically critical reagents. The materials used for the establishment of the AMR must closely approximate the matrix characteristics of patient specimens used in the analysis. A minimum of three (low, midpoint, and high) values over a linear range is required. It is the individual laboratory unit's responsibility to establish the criteria for a successful AMR verification process.

AMR can be further expanded, either at the high or low end, when the specimen has been diluted, concentrated, or another pretreatment that can extend the AMR. The process for when to dilute or concentrate must be prescribed by a written protocol which includes the diluter or process for concentrating the specimen.

Instrument Comparability

The performance of each instrument is checked against each other at least twice a year for comparability of results when the laboratory uses more than one instrument/method to test for a given analyte. Quality control data may be used for this comparison for tests performed on the same instrument platform, with both control materials and reagents of the same manufacturer and lot number. Otherwise, the use of human samples, rather than stabilized commercial controls, is preferred to avoid potential matrix effects. The use of pooled patient samples is acceptable since there is no change in the matrix. Details procedure is found in **69790.1112** *Performance Specification & Instrument Comparison*.

12.17 Telepathology

The PHL does not provide telepathology service.

12.18 Patient and Environmental Safety Goals

The PHL's Laboratory Patient and Environmental Safety Goals are to improve:

- Specimen identification at collection, analysis, and result delivery.
- Verification and communication of life-threatening or life-altering testing as well as environmental threats to public safety.
- Identification, communication, and correction of errors.
- The coordination of the laboratory's patient and environmental safety role within the Public Health system (local health jurisdictions, nursing, administration, point of care (POC) personnel, and providers).
- Monitoring of quality indicators at the PHL.

The annual progress will be evaluated and documented in the *Effectiveness of the Quality Management Plan* report.

12.19 Reporting Quality Concerns

Laboratory personnel are always encouraged to report any quality concerns directly to their supervisor or Office Director. Personnel may also communicate with the relevant accreditation body directly if they have a concern that is not being addressed by laboratory management. Accreditation bodies hold such communications in strict confidence. Signs explaining this right are posted in the main corridor above the drinking fountain near C-wing and the main laboratory entrance to Newborn screening, outside N30. The PHL prohibits harassment or punitive action against an employee in response to a complaint or concern made to an accreditation body or other regulatory organization regarding laboratory quality or safety. The dedicated, confidential CAP telephone line for quality or safety concerns is 866-236-7212 (US, toll-free).

13 QUALITY INDICATORS

Every year, the PHL establishes quality indicators that each Office Director determines as meaningful to their office. The progress of selected quality indicators is presented during QA team meetings on a quarterly basis. For the quality indicators exhibiting persistent or substantial deterioration of performance, the respective Officer Director will present a plan for improvement. QA Officer is responsible for monitoring the progress of the proposed plan. By the end of the year, the QA Officer generates an annual report with compiled quality indicators, summaries outlining the observations, follow-up actions being taken to address any concerns, and the improvement plan, together with each Office Director. CLIA Director is responsible for the appropriate selection of quality indicators based upon SMART (Specific, Measurable, Attainable, Relevant, and Time-based) practice and responsible for the timely evaluation of the effectiveness and the improvement of quality of works through selected quality indicators. The CLIA director is the driving force of follow through on the entire cycle of quality indicator-based quality assurance practice across offices.

13.1 Turnaround Time

Turnaround time (TAT) is an important aspect of quality assurance. They provide the customer with the assurance that laboratory results will be reported in a timely manner. Since there is such a diverse amount of testing performed by the Public Health Laboratories, turnaround times are established by each Office. The listing of turnaround times for tests performed at the Washington Public Health Laboratories are listed in the Directory of Services as well on the Test Menu available on DOH's website:

http://www.doh.wa.gov/Portals/1/Documents/Pubs/301-016-PHLDirectoryServices.pdf (accessed June 2022).

Each office is responsible for monitoring and investigating TATs that indicate systemic or acute problems in laboratory operations. When the established turnaround times are expected to be exceeded by more than two working days, the laboratory will notify the submitter verbally, in writing, or both. This is done in conjunction with the supervisor, Office Director, clinical consultant, and DOH programs. Documentation of submitter notification is required, including the date, submitter institution, name of the person notified, and initial of the PHL staff member who performed the notification.

13.2 Specimen Acceptability

The acceptability of specimens received is determined by monitoring the "less than optimal" specimens received and the specimen rejection rate. This is usually done at the time of accessioning the specimen for the particular laboratory. Criteria for acceptability are located in the individual procedures and the PHL Lab Test Menu.

13.3 Proficiency Testing Performances

The evaluation of the performance on proficiency testing is crucial to determining the performance of the assays. Each Office is responsible for monitoring their performance on proficiency testing. Review for bias or trends will also be reviewed by the Supervisors and Office Directors.

13.4 Employee Quality Communication

Any employees can file quality issues with the QA Officer through IQE/MediaLab (PHL document control system). When received, the issue is reviewed, and follow-up is made until the issue is resolved. It is strictly prohibited to retaliate against the employee who reports quality issues.

14 INFORMATION SYSTEMS

The PHL Information Management Systems supports a wide range of laboratory functions at the PHL, from test ordering to result reporting. The LIMSs are managed by the agency IT division, Health Technology Solution (HTS).

14.1 Security

Physical Security
The computer equipment is located in an environmentally controlled room (the "computer room") with no exterior walls. The door to this room is secured with a key card lock that only authorized personnel can open. Access to the computer room is gained by passing through an outer room, which is occupied by Information Services staff during working hours and is locked during non-working hours.

System Security
LIMS users are assigned a unique login identification and password. Passwords are confidential. Users are required to change their password regularly; the standard is every 180 days. If a user chooses not to change their password, the system automatically denies access. Only authorized Information Services staff can allow access to the user's account. If a user tries to log in three times unsuccessfully, the system will automatically respond with an Intruder Lockout. This lockout status can only be removed by authorized Information Services staff upon the request of the user who has received this status. The File Server records all Intruder Lockout information and Information Services staff regularly monitor this server. Each PHL Office management has established the appropriate access privilege for each user when accessing information.

Data Storage and Security
All laboratory data is backed up. Local backup files are kept in a secure and environmentally controlled area. Remote backup is controlled by the agency's IT group periodically. Off-site information can be retrieved only by authorized staff.

14.2 Computer System Operations

The system is in operation 24 hours a day, seven days a week. The monthly maintenance schedule by the IT group is pre-determined to minimize workflow disruption. Unscheduled downtime (e.g., crashes, power outages) is rare. The majority of users access the computer system during the extended workday of 6:00 AM to 8:00 PM, Monday through Friday (excluding holidays). Exception users with 24/7 access require pre-approval by management.

The applications utilized by PHL staff have been pre-screened. Users are not allowed to install random software onto the system.

IT Support
During normal operation work hours (8:00 AM to 5:00 PM, M-F), the PHL Help Desk is your primary IT support. During non-normal work hours, please follow the steps as outlined in **69790.23** *IT 24 Hour Support*.

14.3 Office of Newborn Screening

Neometrics' Metabolic Screening Database System (MSDS) is currently utilized in NBS. This system consists of applications software for tracking and managing the processing of specimens through a variety of biochemical tests for newborn screening. Neometrics automates the daily workflow of testing staff and provides a mechanism for triggering follow-up activities through the Neometrics case management system (CMS).

MSDS is tightly integrated into the CMS. Presumed positives and any other specimens requiring follow-up are automatically merged from MSDS into CMS. Within CMS a case is created for each patient, and all of the patient's specimen data from MSDS are linked to the case.

The Laboratory portion of MSDS includes capabilities for demographic data capture, batch building, result entry, supervisory review, and mailer production. Instrument Merges enable the test results computed by the instruments to be automatically posted to the appropriate specimen records in the system. In addition, MSDS includes reporting and online look-up for all specimens processed through the system. Through MSDS, staff have access to complete specimen records. Additionally, MSDS provides call logs, notebook entries, and audit trails so staff can keep a record of all their activities.

The databases and related files are backed up daily and stored at an offsite location. Other files are backed up daily on PCs within the laboratory. Hard copies of the specimen forms and the birth rosters are professionally stored by Eastlake Management Inc.

14.4 Office of Public Health Microbiology

StarLIMS

The Microbiology information technology system (StarLIMS) collects demographics and test results for a large number of specimens in selected clinical areas. The system assists Microbiology staff in tracking and analyzing clinical specimens for various tests. Demographic information is entered into the LAN system by laboratory staff and CA staff. Test results are entered and verified by lead laboratory staff. Logbooks, physicians' reports, and summary information are generated and printed by laboratory staff. The entry of data and printing of reports can be done on any PC connected to the PHL intranet. All laboratory data stored on the LIMS is backed up daily and stored at an offsite location weekly.

All Microbiology PCs are Microsoft Windows-compatible and connected to the PHL intranet. Print capability within the labs has been standardized with HP-compliant printers. PCs running the Microbiology database applications are interchangeable.

The PHL Microbiology Office is in the transition of replacing its existing LIMS with a new system. The project duration is scheduled to be from 2020 through 2024.

NGS Analytical Bioinformatics Pipeline Infrastructure

Policy and procedures governing information technology (IT) infrastructure for the NGS analytical bioinformatics pipeline and its management are detailed in **69790.3152** *Bioinformatics - IT Infrastructure Manual*.

14.5 Office of Environmental Laboratory Sciences

Radiation

Applications are currently run on a variety of hardware, software, and operating systems, respective to the purchase date of, and the upgrades available for, the instruments. The logbook is run on the SQL Server in Olympia. Some counters are connected to the DECnet (instrumentation network), and other counters are connected directly to PCs. All staff are connected to the PHL intranet and have email access. Applications were developed in MS ACCESS and PASCAL. Databases are mostly ASCII files. Word processing, spreadsheets, and other PC-based applications are used by laboratory staff.

Environmental Chemistry

Instrument controllers are directly connected to laboratory equipment. Most of the instrument controllers are connected to the PHL Instrument Network, affording common printing and secure backup services. Other, older instrument controllers are stand-alone. Data from instruments and procedures not controlled via computer is collected in laboratory notebooks.

Staff use word processing, spreadsheets, and other PC-based applications on desk computers on the separate, DOH network. Information technology for ELS consists of the standard, approved software bundle from DIRM, with separate approval for specialized application software.

Shellfish
Database information is on a shared Microsoft Access platform maintained by the program in Olympia. Some instrumentation is directly connected to instrumentation but most of the laboratory activities use paper-based systems.

Chemical Incident Response
Applications are currently run on a variety of hardware, software, and operating systems. All staff computers are connected to the DOH network and have email access. Word processing, spreadsheets, and other PC-based applications are used by laboratory staff. Instrument applications are vendor specific. Individual stand-alone instrument controllers are connected to the PHL Instrument Network, where all data is backed up automatically. Reports are printed on dedicated printers not connected to the DOH network.

14.6 QA Office

QA Office
Administrative information technology consists of word processing, spreadsheets, email, project manager, and communication applications. All computers are IBM PC compatible and are connected to the PHL intranet. The print capability has been standardized with HP-compliant printers with shared access through the PHL intranet. Document control is performed using the online-based platform MediaLab, with secure access. Each user has differential access privileges.

15 EXTERNAL COMMUNICATIONS

15.1 Client Complaints

Client complaints are usually resolved by the affected office. If the complaint is not resolved by the office, the complaint and its resolution are transferred to the CLIA Director. All actions in resolving the complaint are documented in the IQE MediaLab module using **69790.793** *PHL Customer Feedback Form*.

15.2 Communication Breakdowns

Communication breakdowns, like complaints, are resolved by the affected office. If the breakdown is not resolved by the office, the problem and resolution is transferred to the QA Officer. All actions in resolving the breakdown are documented in the IQE MediaLab module using **69790.793** *PHL Customer Feedback Form*.

15.3 Critical Results and Timely Communication

Laboratory test results may require the immediate notification of a physician or other personnel when results fall within an established "alert" or "critical" range. Determination of alert or critical ranges is done in consultation with the Supervisor, Office Director, CLIA Director, Clinical Consultant, or DOH program. Each laboratory office is responsible for establishing a process for this immediate notification including how the notification is documented, this process is to be included in the test method SOP or reporting results SOP.

When any laboratory result, including critical values, is reported by telephone (including to DOH programs), the PHL staff member reporting the results must ask the receiver of the information to read back the values given to ensure that accurate communication of the results was made. This is to be documented by recording the date, to whom the result was made, laboratory specimen identifying information, verification of read-back, and initials of the employee giving the laboratory information.

- **Microbiology:** The notification procedure for critical results is documented in the test method SOP.
- **NBS:** NBS has a dedicated task group (follow-up staff) to immediately communicate with submitters regarding all abnormal results with severe clinical implications. Follow-up staff communicate those results to the clinician or providers following their protocol **69790.845** *Follow-up Protocol for Abnormal Laboratory Results*.
- **Chemical Incident Response (CIR):** CIR tests the specimens of victims of chemical exposure, all of which are reported according to CDC protocols. CIR results will go to the CDC Toxicologist/Washington Department of Health Toxicologist and/or Poison Control for review and action. Washington Environmental Biomonitoring Services, WEBS, reports all results to the Office of Non-Infectious Conditions Epidemiology of the Washington Department of Health. Critical results are reported back to the participants with recommendations according to the regulations set forth in the institutional review board (IRB) for the grant.

15.4 Securing Healthcare Information

The federal law known as HIPAA, the Health Insurance Portability and Accountability Act (HIPAA) of 1996, set standards for the protection of individually identifiable health information by covered entities, such as health care providers. All PHL employees must protect the confidentiality of healthcare information, also known as protected health information (PHI), under both federal and state law. Further details are found in **69790.1334** *Patient Healthcare Information Protection.*

15.5 Reporting of Adverse Reactions

When information reasonably suggests that any laboratory instrument, reagent, or other device has or may have caused or contributed to a patient death or serious patient injury, the FDA requires hospitals and outpatient diagnostic facilities, including independent laboratories, to report the event. If the event is death, the report must be made both to the FDA and the device manufacturer. If the event is serious patient injury, the report may be to the manufacturer only, unless the manufacturer is unknown, in which case the report must be submitted to the FDA. Individual medical device reports (MDR) must be submitted on FDA Form 3500A (or an electronic equivalent) as soon as practical but no later than 10 days from the time medical personnel become aware of the event.

Device malfunctions or problems that are reportable may relate to any aspect of a test, including hardware, labeling, reagents, or calibration; or to user error (since the latter may be related to faulty instrument instructions or design). An adverse patient event that may have resulted from inherent limitations in an analytic system (e.g., limitations of sensitivity, specificity, accuracy, precision, etc.) is not reportable.

The PHL follows FDA guidance on 1) the identification and evaluation of adverse patient events, 2) the timely submission of MDR (medical device reporting) reports, and 3) compliance with record-keeping requirements. Further details in FDA guidance are found at: https://www.fda.gov/medical-devices/postmarket-requirements-devices/mandatory-reporting-requirements-manufacturers-importers-and-device-user-facilities (accessed June 2022).

The PHL provides all laboratorians medical device safety reporting training through the Washington State Learning Center, course Medical Device Reporting Training (course code DOH_PHL_410).

The PHL will submit an annual report of device-related deaths and serious injuries to the FDA if any such event was reported during the previous year. This will be handled by the QA Officer. Annual reports will be submitted on form 3419 (or an electronic equivalent) by January 1 of each year. The PHL will keep records of MDR reports for two years in the QA Officer's Office.

Additional information is available on the FDA websites:

- https://www.fda.gov/medical-devices/medical-device-safety/medical-device-reporting-mdr-how-report-medical-device-problems
- https://www.fda.gov/medical-devices/medical-device-safety/medical-device-reporting-mdr-how-report-medical-device-problems#overview

15.6 Notifications from Vendors

Notifications may take the form of product recalls, market withdrawals, or software patches and upgrades. The PHL documents all notifications from vendors of defects or issues with supplies or software under the QA directory on the P: drive at:

\\Dohfltum01\phl\EHSPHL\PHL\QA\Vendor Notifications

Immediate action will be taken if the notifications from vendors have the potential to affect testing results or laboratory services.

16 QUALITY ASSURANCE REVIEW

16.1 Effectiveness of the Quality Management Plan

The PHL QMP is reviewed annually at the QA Meeting for its effectiveness. An annual report is compiled by the QA Officer and reviewed by PHL management including the CLIA Director. This review is to ensure that recurrent problems have been addressed and that new or redesigned activities have been evaluated. Follow-up activities taken as a result of the monitoring and evaluation of activities are documented as well. Additionally, an annual Management Review is performed for all ISO 17025-accredited laboratories. This procedure is described in document 69790.791 Management Review of the Quality System.

The annual QMP review report includes:

- Follow through on previous *Effectiveness Quality Management Plan* reports.
- Summary of reported nonconforming events (NCE)
- Quality indicator summary
- Client survey summary
- A review of the proficiency testing program
- A review of the PHL's Patient Safety Goals is to include:
  1. Improve patient and sample identification at specimen collection, analysis, and result delivery.
  2. Improve verification and communication of life-threatening or life-altering information regarding malignancies.
  3. Improve identification, communication, and correction of errors in a timely manner.
  4. Improve the coordination of the laboratory's patient safety role within healthcare organizations (nursing, administration, POC personnel, providers).
- Feedback from employees
- Lesson learned and future directions

16.2 Directorship Review

The CLIA Director will review the laboratory procedures every two years. The director will ensure that the collection of policies and technical protocols are complete, current, and have been thoroughly reviewed by a subject matter expert. Following the review, the CLIA Director will sign off on procedures and laboratory manuals that they are satisfactory. The CLIA Director may designate the Office Director of that section to review/sign off on the procedures where no major changes have occurred. If there is a change in the CLIA Director position, the laboratory procedures and manuals must undergo a review

within 90 days of the new director's appointment. Currently, controlled document review and sign-off are done electronically using the MediaLab platform.

16.3 Laboratory Reports and Forms Review

The Deputy Director or designee will review the templates and format of laboratory reports and forms at least every two years.

16.4 Root Cause Analysis

Tracing or investigating the underlying symptoms of a problem or event can be referred to as determining the root cause of the event. Determining the root cause of deficiencies is instrumental to developing effective corrective action to prevent recurrence. One model for the root cause analysis often used at the PHL is asking the 5-Why's.

All areas of the laboratory should be considered when investigating the root cause of a deficiency. KATTAR analysis can be useful in identifying the area of need so that the root cause can be addressed.

1. **Knowledge**: was the requirement to accomplish the task or item known?
2. **Assignment**: has the task or item been clearly assigned?
3. **Tools**: have procedures been developed and are they adequate?
4. **Training**: have individuals been trained and do they fully understand what they are to do?
5. **Accountability**: are procedures, training, and assignments in place?
6. **Resources**: have appropriate resources been applied to the problem?

Documentation of the root cause analysis is done by utilizing the IQE module in MediaLab following procedures **69790.945** *How to Record a New Nonconforming Event in MediaLab* and **69790.946** *Managing Non-Conforming Work with Continuous Quality Improvement*.

16.5 Material Transfer Agreements

Occasionally infectious agents need to be transferred to other institutions. Form **69790.86** *Material Transfer Agreement Pathologic* is used and communication is made to stipulate the conditions of use by the recipient. If the purpose of the transfer is other than quality control, appropriate approval such as research, surveillance, or IRB, approval is required. Copies of such requests are kept for two years in the section Supervisor's files.

17 MEMBERSHIP ORGANIZATIONS

The PHL maintains memberships of the following organizations:

- Clinical Laboratory Standards Institute (formerly NCCLS)
- American Society of Testing Materials (ASTM)
- Association of Official Analytical Collaboration AOAC International (formerly Association of Official Analytical Chemists)
- Association of Public Health Laboratories (APHL)

Clinical Laboratory Standards Institute

The PHL is an active member of the Clinical Laboratory Standards Institute. The Institute has published some 200 standards, guidelines, and committee reports. Document topics include safety, chemistry, microbiology, and toxicology. The membership includes a report of their guidelines and standards. Many of the guidelines, though directed at clinical laboratories, are appropriate to the laboratories as a whole. All guidelines, upon receipt, are routed to the laboratories that may have an interest. The guidelines are maintained in office R1.

American Society of Testing Materials

Organized in 1898, ASTM International is one of the largest voluntary standards development organizations in the world. ASTM Intl. is a not-for-profit organization that provides a forum for the development and publication of voluntary consensus standards for materials, products, systems, and services. More than 20,000 members representing producers, users, ultimate consumers, and representatives of government and academia develop documents that serve as a basis for manufacturing, procurement, and regulatory activities.

Association of Official Analytical Collaboration
AOAC INTERNATIONAL is a 120-year-old not-for-profit scientific association committed to worldwide confidence in analytical results. AOAC serves the communities of analytical sciences by providing the tools and processes necessary for community stakeholders to collaborate and, through consensus building, develop fit-for-purpose methods and services for assuring quality measurements. AOAC has a number of communities in various stages of development including food safety and security, agricultural materials, marine and freshwater toxins, dietary supplements, food allergens, and disinfectants.

Association of Public Health Laboratories
The APHL is a membership organization in the United States representing the laboratories that protect the health and safety of the public. In collaboration with members, APHL advances laboratory systems and practices and promotes policies that support healthy communities. APHL serves as a liaison between laboratories and federal and international agencies and ensures that the network of laboratories has current and consistent scientific information to be ready for outbreaks and other public health emergencies. Membership consists of local, territorial, county, and state public health laboratories; environmental, agricultural, and veterinary laboratories; and corporations and individuals with an interest in public health and laboratory science.

18 DEBRIEFING PROCESS FOR REVIEW OF RESPONSE TO PUBLIC HEALTH THREATS OR DISEASE OUTBREAKS

A debriefing process for review of response to public health threats or disease outbreaks has been established. The regular Office Director meetings will be used or if needed, additional meetings will be added to supplement the debriefing process. This includes the consideration of surveillance, staff roles, investigation procedures, and communications. When a public health threat or outbreak exists, the PHL Laboratory Director or the PHL Deputy Laboratory Director will coordinate with the laboratory management, Office of Epidemiology, Risk Management, and other appropriate offices for the response of the public health laboratory.

19 METHOD EVALUATION PARAMETERS

Many methods are developed by PHL personnel both for clinical and environmental analysis. Guidance on method development is offered by various organizations. The PHL has utilized a variety of resources in developing method evaluation parameters some of which include: the EPA, the National Committee for Clinical Laboratory Standards (NCCLS), FDA, AOAC, published literature, etc.

Method evaluation parameters include but are not limited to, precision (intra- and inter-assay), accuracy, sensitivity, specificity, reference range (normal range), reportable range, limit of detection, clinical cut-off, and/or linearity. Further details are found in **69790.959** *Method Validation Verification* and **69790.1112** *Performance Specification & Instrument Comparison.*

Some laboratory units may apply different method evaluation parameters, called data quality objectives (DQO). DQO must be established for bias and precision for each test. Analytical testing is performed on

instrumentation of differing capabilities and with different performance standards. Therefore, the details for each DQO are method-dependent. DQO must be realistic compared to method expectations.

Each laboratory must set accuracy and precision goals either by client-specific requests or by reference to applicable regulations or statutes. The point of contact of each laboratory shall solicit written documentation of the client's DQO for the laboratory work prior to accepting samples or specimens for analysis. Each of the laboratories must meet or exceed the client's DQO. If the client's DQO cannot be met by the laboratory, the point of contact of the laboratory should notify the client and consult on the client's options.

Method validation prior to the introduction of new analytic methods yields an estimation of the method bias and precision. Analysis of matrix-matched Quality Control samples with each batch provides an ongoing check of the method bias and precision.

20 LISTING OF PROCEDURES AT THE PUBLIC HEALTH LABORATORIES

As part of the PHL's CAP accreditation, PHL is required to document a listing of laboratory-developed tests (LDT). This listing is kept in the PHL's document control system, **69790.39** *LDT Test List*. The listing is reviewed at least every two years. For tests that have been retired over two years, the test will be deleted from the listing since it will be available in previous listings in document control.

21 MEASUREMENT OF CLIENT SATISFACTION OF THE LABORATORY SERVICE

The input from the clients of the PHL is essential for the continual improvement of the services that PHL provides. Since 2011, DOH has mandated that all sections of the DOH perform a survey of the clients they serve. This will also serve to meet the needs of the PHL accreditation requirement. Every two years, the PHL will survey clients from the Office of Microbiology, Environmental Laboratory Sciences and Operations. Newborn Screening does a quarterly client screening. The questions are designed by the Office Directors. Survey recipients are selected randomly. Survey documentation can be found at P:\EHSPHL\PHL\QA\Client Survey's

22 REFERENCES

Cited MediaLab Documents

69790.3152	Bioinformatics - IT Infrastructure
69790.958	CAP Accreditation
69790.957	CAP Internal Inspection
69790.465	Central Accessioning Data Entry
69790.466	Central Accessioning Reporting Send Out Results
69790.20	Delegation Form IT Hardware
69790.125	Delegation Form Lead
69790.21	Delegation Form Office Director
69790.22	Delegation Form Supervisor
69790.1057	Document Control
69790.1332	Error Correction
69790.845	Follow-up Protocol for Abnormal Laboratory Results
69790.28	Glassware Procedure
69790.945	How to Record a New Nonconformance Event in MediaLab
69790.2077	Ishihara's Online Test for Color-Deficiency

69790.23	IT 24 Hour Support
69790.1229	Laboratory Contamination Prevention and Monitoring: Nucleic Acid Testing
69790.1331	Laboratory Reagent Water Quality Testing
69790.39	LDT Test List
69790.1862	Liquid Handling Assessment Artel PCS System
69790.946	Managing Non-Conforming Work with Continuous Quality Improvement
69790.86	Material Transfer Agreement Pathologic
69790.959	Method Validation Verification
69790.67	New Test & Instrument Checklist
69790.70	Non-results Generating PHL Procedure Template
69790.1334	Patient Healthcare Information Protection
69790.1112	Performance Specification & Instrument Comparison
69790.965	Personnel Training and Competency
69790.211	pH Testing for Clean Glassware
69790.793	PHL Customer Feedback Form
69790.1330	Proficiency Testing
69790.14	PT Quality Improvement Assessment Sheet
69790.12	Quality Management Plan
69790.1038	Reagent Handling
69790.1308	Record and Specimen Retention Policy
69790.107	Results Generating PHL Procedure Template
69790.468	Specimen Handling in Central Accessioning
69790.46	Specimen Label Deficiency Authorization Form
69790.368	Specimen Processing
69790.501	Specimen Rejection Log
69790.81	Temperature Monitoring System

CLIA Brochures | CMS

23 APPENDIX

Appendix A Abbreviations and Definitions

Definitions – See **69790.1335** *Terms and Definitions*

Abbreviations

Alternative PT	Inter-laboratory comparison
AMR	Analytical measurement range
ANAB	ANSI National Accreditation Board
AOAC	Association of Official Analytical Collaboration International
APHL	Association of Public Health Laboratories
ASTM	American Society of Testing Materials
CAP	College of American Pathologists
CIR	Chemical incident response
CLIA	Clinical Laboratory Improvement Amendments
CLRW	Clinical laboratory reagent water, previously type II
CMS	Neometrics case management system
DOE	Department of Ecology
DOT	Department of Transportation
DQO	Data quality objectives

EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FEMA	Federal Emergency Management Administration
HIPAA	Health Insurance Portability and Accountability Act
HTS	Health Technology Solution, previously IT
IEC	International Electrotechnical Commission
IQCP	Individual Quality Control Program
IQE	Intelligent quotient engine (MediaLab)
IRB	Institutional Review Board
ISO	International Organization for Standardization
L&I	Labor and Industries, Washington State
LDT	Laboratory developed test
LIMS	Laboratory information management system
LRN	Laboratory response network
MAPEP	Mixed Analyte Proficiency Evaluation Program
MDR	Medical device reports
MPEP	Model Performance Evaluation Program
MSDS	Metabolic screening database system
NCCLS	National Committee for Clinical Laboratory Standards
NGS	Next generation sequencing
NIST	National Institutes of Standards
OSHA	Occupational Safety and Health Administration
PHI	Protected Health Information
PM	Preventative maintenance
POC	Point of care
PT	Proficiency testing
QMP	Quality Management Program
REML	Laboratory proficiency
RESL	Radiological and Environmental Measures Laboratory
SA	CDC Select Agent Program
SOP	Standard operating procedure
SRW	Special reagent water, previously type I
TAT	Turnaround time
TQCP	Total Quality Control Program
WSLH	Wisconsin State Laboratory of Hygiene
WAC	Washington Administrative Code