Technical Report

Multi-Laboratory Validation Study for Quantitation of Total Saxitoxin (STX) by CAC-STX-1.0

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Technical questions concerning this method should be addressed to:

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This method was prepared under the contract with the State Water Resources Control Board, Division of Drinking Water 22-007-400-1

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1. Introduction:

1.1. Background

Saxitoxins (STX), also known as paralytic shellfish poisoning (PSP) toxins, are alkaloids produced by some marine dinoflagellates and a variety of cyanobacteria species. They were originally found in mollusks after poisonings of humans following consumption of seafood but have also been found in both marine and fresh water during the occurrence of some harmful algal blooms (HABs).

STX exposure in water primarily comes from consuming untreated surface water, either from water systems that don't sufficiently treat their surface water or through unintentional swallowing during recreational exposure in lakes and rivers.

1.2. Method Summary

The analytical method for this study was validated and the single laboratory validation results are summarized in the following section. Refinements were made to that method based on the comments and results of the participating three laboratories in the multilaboratory validation study (MLVS). Those updates are released as CAC-STX-1.1. This complete method is attached to this report as Appendix A.

The analytical method includes sample preparation and sample analysis procedures for source- and finished-surface water. The limited sample preparation includes a chlorine quenching agent for finished water and a buffer for sample stability. The method utilized enzyme-linked immunosorbent assay (ELISA) for the quantitative analysis of total saxitoxins in raw and finished surface-sourced drinking water.

1.3. Summary of the Single-Laboratory Study

The single-laboratory validation was performed by CDFA's Center for Analytical Chemistry Research and Development Unit (Sacramento, CA) (CAC-R&D), the laboratory originally contracted by State Water Resources Control Board, Division of Drinking Water (DDW) to develop a total saxitoxin analytical methodology, and validate its use in this MLVS.

A large variety of aqueous matrix samples, including surface water-sourced raw and finished drinking water, were spiked at five concentration levels from 0.050 ppb to 0.300 ppb and analyzed in the single-laboratory validation study; all of which had recoveries between 70-130%. The single-laboratory validation proposed a minimum reporting limit (MRL) of 0.03 ppb STX, after calculating an estimated lowest concentration minimum reporting limit (LCMRL) of 0.017 ppb STX. The LCMRL was calculated as an estimate, but was unable to be properly bracketed and confirmed due to it being below the lowest calibration point that was provided with the ELISA kits.

While automated plate washers and microplate pipetting workstations can simplify this method, the required plate reader has become commonplace in many full-service environmental laboratories. This means that this method should be accessible and possible to be implemented in a typical mid-sized full-service environmental laboratory.

2. Study Management, Objectives, Design, and Implementation:

While this MLVS is not designed to support an alternate test procedure (ATP) application, the number of matrices and statistical analyses of the data reflect, as much as possible, what would be required for an ATP for national use.

2.1. Study Management

Three laboratories (two commercial contract laboratories and one municipal water district laboratory) volunteered to participate in the study, under the authorization and stewardship from DDW. The three laboratories are listed in Table 2-1. All three laboratories contributed to the analysis of the aqueous matrices in this report (surface water, raw and finished). For the purposes of this study, the laboratories were randomly assigned numbers, which were used to maintain the anonymity of the results, these numbers do not correspond to their order of appearance in Table 2-1.

The CAC Quality Assurance unit (CAC-QA) managed the sample spiking, shipment, and received all data packages from the laboratories. Analytical standards and ELISA kits were provided to the participating laboratories by CAC. CAC-QA, with an established operational set-up and expertise to conduct Proficiency Testing (PT) schemes, prepared the PT samples shipped as part of this study, while CAC prepared the aqueous sample matrices.

CAC served as the method consultant to the MLVS and was available to clarify any questions or concerns.

Table 2-1. Participating Laboratories

Laboratory	**Location**	**Role**
Participating MLVS Labs		
Bend Genetics, LLC	Sacramento, CA	MLVS participant
L.A. Department of Water and Power	Pasadena, CA	MLVS participant
Weck Laboratories	Industry, CA	MLVS participant
Ancillary Labs		
CDFA Center for Analytical Chemistry – Research & Development	Sacramento, CA	Single laboratory validation, preparation of matrix spike standards
CDFA Center for Analytical Chemistry – Quality Assurance	Sacramento, CA	Preparation of PT standards, evaluation of data

2.2. Study Objective and Design

The focus of the MLVS is to generate the necessary data to document the precision and accuracy and overall performance of the analytical method for quantitation of STX in aqueous matrices. The primary objectives of this MLVS are to:

- Obtain data from aqueous matrices that are representative of the method's intended use.
- Obtain data from laboratories that are representative of those likely to use the method, but that were not directly involved in its development.
- Obtain feedback from laboratory users on the specifics of the method SOP.
- Use study data to evaluate the performance of the method.
- Develop QC acceptance criteria – average recovery and relative standard deviation – that will reflect method performance capabilities in real-world situations.

A brief description of the key points of this study design include:

- At least three laboratories, at least one of which is a municipal water laboratory.
- Two aqueous matrix samples from source and finished surface water.
- Initial calibration of STX by each laboratory.
- Initial Demonstration of Capability (IDOC) by each laboratory.
- Analyses of PT sample and matrix spike samples from each aqueous matrix.

This MLVS was conducted in two phases. The IDOC, which includes the initial calibration and verification of MRL level, and the method evaluation in the chosen aqueous matrices.

2.3. Matrix and Sample Selection

Two surface water samples (one source and one treated) were used for this phase of the MLVS. These were chosen to be representative of the expected real-world matrices analyzed by this method. These samples were collected from the Sacramento River Water Treatment Plant.

The MLVS was designed so that for each sample the following would be analyzed: an 'unspiked' (native) sample, two replicates spiked at low concentration, and two replicates spiked at a mid-level concentration.

2.4. Selection of Spiking Levels and Aqueous Media

The sample matrices were collected by DDW. The chosen sample matrices were raw and finished water from the West Sacramento River Water Treatment Plant. The saxitoxin spiking levels were chosen by CAC to be at the proposed MRL for the low-spike, and just above the center of the calibration range for the mid-spike. CAC-QA separately selected the PT spike level so that it would be blind to everyone participating, including all other CAC units.

2.5. Preparation of Study Samples

Due to the short hold time of the saxitoxin samples, the study samples were prepared by the participating laboratories. They were provided with a guidance document detailing the necessary preparations (included as Appendix B). Each laboratory was provided with working standards for both the matrix samples and the PT sample. The working standards were prepared by CAC-R&D for the matrix samples and CAC-QA for the PT sample.

The laboratories were instructed to fortify 1 mL samples using the working standards provided by CAC. The standards were frozen to -80°C, packed in insulated shipping containers (such as ThermoSafe® or similar) to ensure standard integrity during transport, and shipped to the laboratories.

2.6. Storage Stability Study

During method development, a storage stability study was conducted to determine the hold time of saxitoxin samples in finished surface-sourced drinking water. The matrix samples were spiked in triplicate for this study, and varying concentrations of chlorine quenching agent (ascorbic acid) were also tested to see the effect on saxitoxin stability. This data and the chosen concentration of chlorine quenching agent can be found in the method SOP in Appendix A, and the hold time was determined to be six days. Past day six, the recoveries begin to drop below the QC acceptance criteria.

3. Data Management, Data Validation, and Data Rules for Statistical Analysis:

3.1. Programmatic Overview

The data management process involved documented and approved instructions, meetings, consultation and communication when required, and review of laboratory packages. A “kickoff” meeting over Teams was scheduled to explain the expectations for the MLVS to the participating laboratories. Laboratories were provided with the method prior to the meeting in preparation and given the opportunity to clarify any questions or concerns. They were also encouraged to ask questions via email or to schedule a one-on-one meeting throughout the MLVS as necessary. A written document containing the MLVS guidelines and passing QC criteria was also provided to the laboratories along with a data reporting template Excel workbook, to ensure uniform reporting.

3.2. Data Management

All result reporting forms, and raw data were submitted to CAC-QA via email. The labs were provided with an Excel workbook template to report their analysis batch data and results in, as well as a sample handling sheet to note how samples were prepared. The Excel template had password protected cells for any formulas to prevent accidental corruption or unintended changes. The laboratories also provided both their raw absorbance data as well as a plate map to identify the locations of each sample on the 96-well plate.

3.3. Data Validation

All data packages were reviewed by CAC-QA for completeness, compliance with the MLVS guidelines, and performance according to the QC metrics, see QC acceptance criteria in the SOP in Appendix A.

4. Calibration and Quantification:

Aqueous samples were analyzed by ELISA. As this method calls for the use of a commercial ELISA kit manufactured for use in analysis of water samples, the participating laboratories used the supplies provided in those kits for the calibration of the method. Each laboratory was advised to follow the manufacturer's instructions for the absorbance calibration. While manufacturer specifications may vary, the kits used in this study comprised of five calibration standards (in addition to a zero standard) that ranged from 0.02 ppb to 0.4 ppb in concentration.

4.1. Quantification

The preferred calculation for quantification using these ELISA kits is a Four Parameter Logistic (4PL) regression. This regression method is detailed in the method SOP in Appendix A.

4.2. Calibration Verification

A control standard was included in each of the ELISA kits. These standards were approximately at the mid-level (IC_{50} for 4PL) of the calibration set provided in the kits. The laboratories ran this control in each batch. The kit-provided controls were required to recover within ±30% of their true value. No sample results were eliminated from the study due to CV failures.

4.3. Instrument Sensitivity Check

Each laboratory created Low-CV spikes at the method MRL, from the standards provided in the ELISA kits. A Low-CV was analyzed in each batch. Low-CVs were required to recover within ±50% of their true value. No sample results were eliminated from the study due to Low-CV failures.

5. Initial Demonstration of Capabilities:

In addition to performing the initial calibration, laboratories submitted an (IDOC. The IDOC included a precision and accuracy analysis, system background check, and MRL confirmation.

5.1. Demonstration of Precision and Accuracy (P&A) Results

Laboratories were required to spike seven replicate Laboratory Fortified Blanks (LFBs) at 0.070 ppb STX. These LFBs were prepared and analyzed in the same manner as study samples, per the method. A percent relative standard deviation (%RSD) of less than 15% and a mean percent recovery of ±30% of the true value were the required criteria for this demonstration. All laboratories met these criteria.

5.2. Acceptable System Background

Five Laboratory Reagent Blanks (LRB) were run in the same batch as the LFBs for the P&A. The laboratories were required to have a background level of less than one-third of their MRL. All laboratories satisfied this requirement.

5.3. Minimum Reporting Limit Verification Analyses

The Minimum Reporting Limit for STX using this method was set by the method development (MD) laboratory at 0.030 ppb. The participating laboratories were required to confirm their MRLs using the MD level as an initial reference point. Seven LFBs were to be spiked at the proposed MRL for confirmation using the formulas in the method.

Three laboratories confirmed 0.030 ppb as the MRL, while the remaining lab confirmed 0.015 ppb as their MRL. It should be noted for future use of the method that the confirmed MRL should not be below the lowest standard provided in the ELISA kits. Any finding below the lowest standard level will be considered a ND in real samples.

5.4. Quality Control Sample (QCS)

A QCS prepared from a standard from a different source to that of the calibration standards was also required as part of the IDOC. The QCS was not required to be analyzed in the same batch as the P&A LFBs, and this requirement was satisfied by the PT samples sent to the laboratories (see section 6.3). Each laboratory analyzed a PT sample prepared from a standard sourced from a different vendor than the ELISA kit they were supplied with.

Table 5-1. IDOC Results

Laboratory	Precision (%RSD)	Accuracy (Mean %)	System Background (Mean ppb)	MRL (ppb)	UPIR	LPIR
1[#]	5.5%	79.0%	0.003	0.015	137.9%	71.6%
2	4.5%	108.4%	0.006	0.030	141.3%	82.5%
3	6.0%	100.6%	0.001	0.030	124.7%	77.2%

[#]during the MRL verification, the laboratory did not run the MRL and the LRB together as was required, originally resulting in LPIR below 50%. Upon being directed to follow the guidelines, the lab repeated the MRL study with an acceptable metric of PIRs, however, the MRL was studied below the lowest available standard, 0.02 ppb, which is to be considered ND in real sample situations.

6. Water Matrix Results:

Each participating laboratory was sent two 10 mL water matrix samples from the West Sacramento River Water Treatment Plant. One sample was raw surface water, the other was finished drinking water from the same treatment plant. The laboratories were provided with instructions on how to spike the required samples for this phase of the MLVS, as the short hold time for STX (six days) left limited feasibility for shipping pre-spiked samples.

6.1. STX Concentrations in Unspiked Matrices

Due to the laboratories being shipping bulk water matrix instead of prepared samples, each lab was required to analyze unspiked samples separately before preparing and running the matrix spikes. All laboratories reported native concentrations of STX well below one-third of their MRLs. These levels are included in Table 6-1.

6.2. Matrix Spike Results

The spike recovery data from all laboratories is also shown in Table 6-1. The raw and finished water matrices were spiked in duplicate at the proposed MRL-level (0.030 ppb) and at a mid-level (0.100 ppb). The results from all laboratories at all levels were acceptable with recoveries ranging from 73.3%-126.7% recovery.

6.3. PT Results

Each laboratory was provided with a PT working standard, prepared from a standard sourced from either Gold Standard Diagnostics or Creative Diagnostics. Each laboratory was sent the working standard prepared from the vendor that did not manufacture the ELISA kit they were supplied with, and instructions on how to prepare the PT sample for analysis. PT sample ensemble with matrices were packaged in an insulated shipping box (ThermoSafe® or similar) with ice packs ensuring standard integrity. All PT samples were shipped together to ensure process integrity and uniformity. Participating labs were allotted a two-week period to perform the analyses and submit results to the CAC - QA. Table 6-2 denotes the results. Since laboratory 2 did not initially report satisfactory results, CAC-EA laboratory, an independent unit of the CAC, was reached out as an additional laboratory participant and the final PT results from all laboratories are provided below in Table 6-2.

Table 6-1. Unspiked and Spiked Matrix Results

Laboratory	Raw (%Recovery)					Finished (%Recovery)				
	Unspiked (ppb)	MRL Rep. 1	MRL Rep. 2	Mid-Level Rep. 1	Mid-Level Rep. 2	Unspiked (ppb)	MRL Rep. 1	MRL Rep. 2	Mid-Level Rep. 1	Mid-Level Rep. 2
1	0.010	80.0%	100.0%	83.0%	84.0%	0.006	113.3%	110.0%	95.0%	90.0%
2	ND	93.3%	90.0%	81.0%	84.0%	ND	83.3%	73.3%	82.0%	79.0%
3	0.010	116.7%	113.3%	124.0%	119.0%	0.002	123.3%	126.7%	111.0%	110.0%

Table 6-2. PT Results

Laboratory	Reagent Water PT			Tap Water PT			
	Result (ppb)	Target (ppb)	% Recovery	Result (ppb)	Target (ppb)	% Recovery	Mean % Rec.
1	0.064	0.074	87.1	0.081	0.074	110.2	98.7
2*	0.051	0.074	69.4	0.051	0.074	69.4	69.4
3	0.073	0.074	99.5	0.075	0.074	102.5	101.0

*laboratory analyzed PT samples with multiple dilutions and the results could not be computed in the initial run, the results presented are from their reanalysis.

7. Summary:

7.1. Preparatory Batch QC

Per the CAC-STX-1.0 method, an analysis batch consists of the calibration standards, field samples, a method blank, low-range calibration verification standard, laboratory fortified blank, and a laboratory fortified sample matrix and laboratory fortified sample matrix duplicate pair. This method is run on 96-well ELISA plates, and while the entire plate may be used in one batch, an analysis batch cannot consist of more than a single ELISA plate.

7.1.1. Method Blank

Method blanks, referred to as Laboratory Reagent Blanks (LRB) in this method, are included in the method to evaluate the potential for background contamination to be introduced during sample preparation in the laboratory. The saxitoxin concentration in the LRB must be less than one-third the MRL. This requirement was met by all participating laboratories.

7.1.2. Ongoing Precision and Accuracy Analyses

Ongoing Precision and Accuracy samples, represented in this method by the batch LFB requirements, are included to evaluate the efficiency of the sample preparation processes over time. A LFB prepared in the same manner as the study samples is included in every preparation batch.

This method also calls for the inclusion of Laboratory Fortified Sample Matrix (LFSM) and Laboratory Fortified Sample Matrix Duplicate (LFSMD). LFSM and LFSMD are field samples, spiked with a known amount of saxitoxin. These are included to check for matrix effects on the samples in each batch.

7.1.3. Low-Level Ongoing Precision and Accuracy Analyses

Low-Level Ongoing Precision and Accuracy samples, Low-CV samples in the method, are included in each analysis batch to evaluate method performance at the at the MRL.

7.2. Matrix Spike Analyses

Ongoing QC criteria of ±40% (Appendix A, Section 9.2.4) was applied to evaluate the results, and all participating labs performed the analyses matrix spikes satisfactorily, presented in Table 6-1.

The matrix results from the fourth laboratory (CAC-EA Lab, which was not a part of the initial MLV design) were not included in the final results as it was unable to be identified if the non-passing QC results were due to the laboratory staff performing the analyses or the potential degradation of the water matrices used, as the water matrices had been stored for approximately 2.5 months prior to CAC-EA preparing and analyzing the matrix samples.

7.3. PT Result Analyses

Ongoing QC criteria of ±40% (Appendix A, Section 9.2.4) was applied to evaluate the results, and all participating labs performed the analyses of the PT samples satisfactorily, presented in Table 6-2. The overall average recovery is 89.7% with an RSD of 19.3 % across the three participating labs.

PT results from the fourth laboratory (CAC-EA Lab, which was not a part of the initial MLV design) could not be used as the storage criteria for the PT samples were not met since the EA lab could not accommodate the analysis of the PT samples in a timely manner.

8. Conclusions:

The objectives of this MLVS were achieved: validation of the CAC-STX-1.0 method and the determination that the method can be implemented at a typical mid-sized full-service environmental laboratory. Overall, the data from the MLVS demonstrates that CAC-STX-1.0, as written, is robust and suitable for laboratories with similar instruments of different manufacturers and models. Points of additional clarity have been added to the SOP as version CAC-STX-1.1. Specifically, it has been noted that there must be a calibration curve run in each batch, and that any detection below the lowest calibration level is considered to be non-detect in real samples. The single-laboratory LCMRL results were also added to the SOP.

The results of the participating labs in this study have met the requirements stated in the method for:

- Calibration verification and sensitivity check
- Initial Precision and Accuracy
- Confirmation of MRL
- Batch QC samples

The suitability of the method to detect and quantify saxitoxin in surface-sourced drinking water (both raw and finished) was successfully demonstrated through the analysis of spiked samples of those real-world matrix types. Method blank results demonstrated no bias from background contamination during sample preparation. The Initial and Ongoing Recoveries and the Low-CV recoveries demonstrated that the QC acceptance criteria in the method were satisfactory for inclusion in the finalized method. ELISA kits from Gold Standard Diagnostics Horsham, LLC and Creative Diagnostics were used during this MLVS. It is noted that while kits from both vendors performed satisfactorily, Gold Standard Diagnostics was much more responsive and able to provide replacement kits on short notice. This could be an important consideration when determining a supplier to use, especially if unplanned or emergency sampling events are within the scope of the study this method is being used for.

9. References:

9.1. Ohio EPA Total (Extracellular and Intracellular) Saxitoxin by ELISA Analytical Methodology, Ohio EPA DES 702.0, Version 2.0, November 2016.

9.2. Maciel, Robert J. Standard Curve Fitting in Immunodiagnostics: a Primer. *Journal of Clinical Immunoassay*. 1985, Vol. 8, 98–106.

9.3. Sasaki, Diane and Mitchell, Robert A. How to Obtain Reproducible Quantitative ELISA Results. Oxford Biomedical Research website. https://www.oxfordbiomed.com/sites/default/files/2017-02/How%20to%20Obtain%20Reproducible%20Quantitative%20ELISA%20results.pdf (accessed February 2023).

9.4. ABRAXIS Saxitoxin (PSP) ELISA Microtiter Plate, 2022. https://www.goldstandarddiagnostics.us/media/15661/ug-21-081-rev-03-abraxis-saxitoxin-elisa_52255b.pdf (accessed 2023 January 10)

9.5. Eurofins Abraxis Excel Solver, 2020. https://www.goldstandarddiagnostics.us/media/9257/18urofins_abraxis_excel_solver-website.xlsm(accessed 2023 January 25)

9.6. U.S. EPA. August 2016. Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay.

Appendix A: Standard Operating Procedure for CAC-STX-1.1

[Changes from CAC-STX-1.0 to CAC-STX-1.1 have been underlined.]

CAC-STX-1.0
Quantitation of Total (Extracellular and Intracellular) Saxitoxin by Enzyme-linked Immunosorbent Assay (ELISA)

Version 1.1
June 13, 2025

Technical questions concerning this method should be addressed to:

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CAC Approvals for use:

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Date

This method was prepared under the contract to the State Water Resources Control Board, Division of Drinking Water 22-007-400-1.

Quantitation of Total (Extracellular and Intracellular) Saxitoxin by Enzyme-linked Immunosorbent Assay (ELISA)

1. Scope:

This method is used for the quantitation of saxitoxin in drinking water using enzyme-linked immunosorbent assay (ELISA). Saxitoxin is one of the toxins associated with paralytic shellfish poisoning (PSP). This test is validated for use on drinking water samples pre- and post-treatment at water treatment plants.

2. Principle:

This test is a direct competitive ELISA based on the recognition of saxitoxin by specific antibodies. Saxitoxin, when present in a sample, competes for binding with a saxitoxin-enzyme conjugate to antibodies bound to an ELISA well-plate. With the addition of a substrate solution, a colorimetric signal is produced. The concentration of saxitoxin is inversely proportional to the intensity of the color produced. The color reaction is halted, and the color is evaluated using an ELISA-plate photometer. The concentrations of the samples are determined by a Four-parameter logistic analysis using the standard curve run with each batch. The single laboratory validation confirmed a reporting limit of 0.03 ppb.

3. Definitions:

3.1. ANALYSIS BATCH

3.1.1. The microtiter plate holds 12 plate strips of 8 wells each. Each batch sequence must include the calibration standards, laboratory reagent blank, low-range calibration verification standard, laboratory fortified blank, field samples, and laboratory fortified sample matrix and laboratory fortified sample matrix duplicate. A single analysis batch cannot consist of more than one microtiter plate, each plate must be its own batch.

3.1.2. WELL REPLICATES – this method requires that each component of the analysis batch be run in at least duplicates. Each well replicate will have both an absorbance from the plate reader and a concentration calculated from that absorbance. The well replicate absorbances will be used to calculate the %CV that must meet the QC requirements of the method. The well replicate calculated concentrations will be averaged, with this mean being used for reporting, all

method calculations, and evaluating QC acceptance criteria.

3.2. CALIBRATION STANDARDS – solutions of saxitoxin provided in the ELISA kit or prepared in the laboratory that are appropriate for the measurement range of the ELISA kit.

3.3. CALIBRATION CURVE

3.3.1. The calibration points are calculated using a four-parameter logistic function (section 3.3.2), relating concentration (x-axis) to the measured absorbance in the wells (y-axis). There is an inverse relationship between concentration and response. The zero-calibration standard gives the highest absorbance, and the highest calibration standard gives the lowest absorbance. Note also that the slope, or sensitivity, of the ELISA response is greatest in the middle of the curve and tends toward zero slope at extreme low and high concentrations. For a more detailed explanation of the four-parameter calibration model, see Maciel (1985) and Sasaki (web resource).

3.3.2. Four-Parameter Logistic Equation

$$y = \frac{(a-d)}{1+\left(\frac{x}{c}\right)^{b}} + d$$

y=absorbance
x=concentration
a=absorbance at the bottom plateau
b=slope related term at the inflection point
c=concentration at the inflection point
d=absorbance at the top plateau

3.4. LABORATORY FORTIFIED BLANK (LFB) - An aliquot of reagent water fortified with a known quantity of saxitoxin. The LFB is prepared to match the analytical procedure for field samples. The LFB is used during the IDC to verify method performance for precision and accuracy. The LFB is also a required QC element with each Analysis Batch. The results of the LFB verify method performance in the absence of sample matrix.

3.5. LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) - An aliquot of a field sample fortified with a known quantity of saxitoxin. The

purpose of the LFSM is to determine the bias contribution of the sample matrix to the analytical results.

3.6. LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – A second aliquot of the same field sample used to prepare the LFSM, fortified and analyzed in the same Analysis Batch as the LFSM. The LFSMD is used to verify method precision in sample matrices.

3.7. LABORATORY REAGENT BLANK (LRB) - An aliquot of reagent water prepared to match the sample processing procedures. The LRB is used to check if saxitoxin or other interferents are introduced from sample containers, processing equipment, or the reagents of the assay.

3.8. LOWEST CONCENTRATION MINIMUM REPORTING LEVEL (LCMRL) - The single-laboratory LCMRL is the lowest spiking concentration such that the probability of spike recovery in the 50 to 150% range is at least 99%.

3.9. LOW-RANGE CALIBRATION VERIFICATION (Low-CV) - The Low-CV is a calibration standard with a concentration equal to, or less than, the MRL. The purpose of the Low-CV is to confirm the accuracy of the calibration at concentrations near the MRL.

3.10. MINIMUM REPORTING LEVEL (MRL) - The minimum concentration that can be reported by a laboratory as a quantified value for total saxitoxins in a sample following analysis. This concentration must meet the criteria defined in Section 9.1.3 and must be no lower than the concentration of the lowest calibration standard.

3.11. PRIMARY DILUTION STANDARD (PDS) - a solution of saxitoxin in 1X Diluent Buffer. The PDS solutions are used to fortify the QC samples.

3.12. QUALITY CONTROL SAMPLE (QCS) - A solution containing saxitoxin at a known concentration that is obtained from a source different from the source of calibration standards. The purpose of the QCS is to verify the accuracy of the primary calibration standards.

3.13. REAGENT WATER - Purified water that does not contain any measurable quantity of saxitoxins or interfering compounds at or above half the MRL.

3.14. STOCK STANDARD SOLUTION - a concentrated standard in 1X Diluent Buffer that is purchased from a commercial source with a certificate of analysis.

4. Interferences:

There were no matrix interferences noticed during method development, however, the documentation from each ELISA kit should be reviewed for any manufacturer noted interferences or potential avenues of contamination.

5. Safety:

5.1. Read the Safety Data Sheet for all materials before use.
5.2. All general laboratory safety rules for sample preparation and analysis shall be followed.
5.3. Saxitoxin is highly toxic if swallowed: Causes irritation of mouth, pharynx, gullet, and gastrointestinal tract.
5.4. The substrate solution contains tetramethylbenzidine.
5.5. The stop solution contains diluted sulfuric acid.
5.6. All solvents should be handled with care in a ventilated area.

6. Equipment and Supplies:

6.1. Microtiter plate washer [optional], BioTek 50TS or equivalent
6.2. Microtiter plate reader (450nm), BioTek 800TS or equivalent
6.3. Microtiter plate shaker [optional]
6.4. Micropipette with disposable tips (10 – 100 µL)
6.5. Multi-channel, stepper, or electronic repeating pipette with disposable tips

6.5.1. Note: Multi-channel pipette is strongly recommended if following this protocol without a plate washer.

6.6. Vortex vibrating mixer.
6.7. Glass vials (1.5mL) with PTFE lined caps.

6.8. ABRAXIS Saxitoxins (PSP), ELISA kit (PN 52255B) or equivalent

6.8.1. Microtiter plate and plate strips

6.8.2. Calibration Standards and Control

6.8.3. Enzyme Conjugate solution

6.8.4. Antibody solution

6.8.5. Substrate (color) solution

6.8.6. Stop solution

6.8.7. Sample Diluent buffer concentrate 10X

6.8.8. Wash buffer concentrate 5X

7. Reagents and Standards:

7.1. Chlorine quenching agent

7.1.1. L-ascorbic acid, 99% CAS# 50-81-7

or

7.1.2. Sodium thiosulfate, anh. 99% CAS# 7772-98-7

7.2. Saxitoxin dichloride (20 µg/mL) CAS# 35554-08-06

7.2.1. Saxitoxin standard for Stock Standard Solution is purchased from Gold Standard Diagnostics (or other certified source) at a concentration of 20 µg/mL in 1X diluent buffer.

7.2.2. The 20 µg/mL standard is serially diluted to 100 ng/mL and 10 ng/mL using 1X diluent buffer, the 10 ng/mL standard is used for the PDS.

7.3. Calibration Standards

7.3.1. The calibration standards used in each batch come in the ELISA kit.

7.3.2. Calibration standards obtained from the ELISA Kits must be discarded within 2 weeks of unsealing.

7.4. Parafilm or PTFE sealing tape.

7.5. DPD (N,N-diethyl-p-phenylenediamine) chlorine test

8. Sample Collection, Preservation and Storage:

8.1. All water samples must be preserved upon collection with the addition of 10X Concentrated Sample Diluent (included in ELISA kit) at the time of collection.

8.1.1. Samples should be collected in amber glass vials. Collect enough sample volume to meet the method requirements, including sufficient volume for QC samples.

8.1.2. Add 10X Concentrated Sample Diluent to samples in a 1:10 ratio (i.e. add 100 µL of 10X Diluent per 900 µL of sample). Mix thoroughly.

8.1.3. Note: Samples preserved with 10X Concentrated Sample Diluent must have their results multiplied by a dilution factor of 1.1.

8.2. Treated drinking water samples must, in addition to preservation with 10X Concentrated Sample Diluent, have the residual chlorine quenched with either sodium thiosulfate or ascorbic acid.

8.2.1. Prior to sample collection, add neat quenching agent to empty sample containers. Minimum quenching concentration is 0.1 mg/mL, but up to 1.0 mg/mL may

be used if necessary. This may be done before taking the empty sample containers to the collection point.

8.3. Pre-treatment drinking water samples do not require the addition of a chlorine quenching agent, however, quenching agent may be added as it does not affect analysis.

8.4. Collected samples must be cooled on ice to 0-4°C immediately, and kept at this temperature range, away from sunlight, until analysis.

8.5. SAMPLE SHIPMENT AND STORAGE – Samples must be chilled during shipment. Samples must be confirmed to be at or below 10 °C when they are received at the laboratory. In the laboratory, samples must be stored at or below 4 °C and protected from light.

8.5.1. For treated drinking water samples, analyze one sample using a common assay for total residual chlorine, such as the DPD-colorimetric technique. The total chlorine concentration should be below the detection limit of the assay. A duplicate sample may be collected for use in this assay.

8.6. SAMPLE HOLDING TIMES – Analyze samples as soon as possible. Samples that are collected and stored as described in Section 8 must be analyzed within 6 days of collection.

9. Quality Control:

9.1. INITIAL DEMONSTRATION OF CAPABILITY (IDC)

9.1.1. DEMONSTRATION OF PRECISION AND ACCURACY - Prepare seven replicate LFBs, fortified with saxitoxin at 0.07 ng/mL. LFBs must include the chlorine quenching agent and be processed in a single Analysis Batch. The Analysis Batch should also include the LRBs from section 9.1.2, as well as a Low-CV standard. The %RSD for the LFBs must be ≤15%. The mean recovery for the LFBs must be ≥70% and ≤130%.

9.1.2. DEMONSTRATION OF LOW SYSTEM BACKGROUND - Included in the Analysis Batch in section 9.1.1, prepare five LRBs. The LRBs must also contain the chlorine quenching agent. Distribute the

LRBs throughout the plate. The results for each LRB must be less than half the MRL.

9.1.3. MINIMUM REPORTING LIMIT (MRL) CONFIRMATION - The Minimum Reporting Limit (MRL) for saxitoxins by this method has been set at 0.03 ppb. EPA methods require that the MRL be confirmed by analyzing seven samples spiked at the proposed MRL. The Analysis Batch for the MRL confirmation must include two LRBs and a Low-CV. The results of these spikes must meet the following requirements for the Prediction Interval of Results (PIR).

Half Range=3.963*S, where S is the standard deviation and 3.963 is a constant for seven replicates.

Upper PIR Limit=$\frac{Mean+HR}{Spiked\ Conc.} x100\% \leq 150\%$

Lower PIR Limit=$\frac{Mean-HR}{Spiked\ Conc.} x100\% \geq 50\%$

9.1.4. QUALITY CONTROL SAMPLE (QCS) - Analyze a mid-level QCS prepared as in section 9.2.6, to confirm the accuracy of the calibration standards.

9.2. ANALYSIS BATCH QC REQUIREMENTS

9.2.1. WELL REPLICATES - All field and QC samples must be added to at least two wells. The coefficients of variation (CVs) for standards must be <10%, and CVs for samples must be <15%. If the standard CVs fail to meet the limits, the batch is invalid, and the samples must be included in a subsequent batch. If a field or QC sample fails to meet the limits, that sample is invalid.

9.2.2. LABORATORY REAGENT BLANK (LRB) - For each Analysis Batch, include one LRB. If quenching agents were used for the sample preservation as in section 8, the LRB must also include the chlorine quenching agent. Analyze the LRB in duplicate, placing one set of well replicates on opposite sides of the plate. The

total saxitoxin concentration must be less than half the MRL. If the concentration is greater than or equal to that level, any positive results from that Analysis Batch are invalid.

9.2.3. LOW CALIBRATION VERIFICATION (Low-CV) - One Low-CV must be analyzed in each Analysis Batch. The Low-CV is a calibration standard at or below the MRL. A calibration standard from the kit may be used. The concentration of the Low-CV must be ±50% of the true value, else the entire Analysis Batch is invalid.

9.2.4. LABORATORY FORTIFIED BLANK (LFB) - At least two LFBs, at identical concentrations, must be included in each Analysis Batch. If quenching agents were used for the sample preservation as in section 8, the LFBs must also include the chlorine quenching agent. Fortify near the center of the calibration curve. The percent recovery for each LFB must be ±40% of the true value, else the entire Analysis Batch is invalid.

9.2.5. LABORATORY FORTIFIED SAMPLE MATRIX / LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE - One set of LFSM and LFSMD is required with each Analysis Batch. Two sets are required if more than 20 field samples are present in the batch. If samples with and without quenching agents are in the same analytical batch, one set is required for each sample type. The native background concentration must be determined from a separate field sample. The source of the sets should be distributed among the various water sources for the laboratory over time.

9.2.5.1 Three separate aliquots of a field sample are required for each set, one to determine the native background concentration and one each for the LFSM and LFSMD. Homogenize the sample before separating into three

vials. Fortify the LFSM and LFSMD near the center of the calibration curve.

9.2.5.2 Calculate the mean percent recovery for each LFSM and LFSMD set:

$$\%R = \frac{(A-B)}{C} \times 100\%$$

A= mean measured concentration of set
B= measured native background
C= fortification concentration

9.2.5.3 The mean percent recovery for each set should be ±40% of the true value. If the percent recovery is outside this range, and the performance of the LFBs is in control for the same batch, the recovery may be matrix biased. Mark the result for the sample from which the LFSM was prepared as “suspect-matrix”.

9.2.5.4 Calculate the relative percent difference (RPD):

$$RPD = \frac{|LFSMD-LFSM|}{(LFSMD+LFSM)/2} \times 100\%$$

9.2.5.5 The RPD for each set should be ≤40%. If the RPD is outside this range, and the performance of the LFBs is in control for the same batch, the precision may be matrix biased. Mark the result for the sample from which the LFSMD was prepared as “suspect-matrix”.

9.2.6. QUALITY CONTROL SAMPLE (QCS) - A QCS must be analyzed during the IDC, and again with each new set of calibration standards. The saxitoxin used for the QCS must be procured from a source that is independent of the source of the ELISA kit. The concentration of the QCS should be near the center of the calibration curve. The percent recovery for the QCS must be ±30% of the true value.

10. Instrument calibration:

10.1. The calibration standard curve consists of six levels that come with the ELISA kit. Do not add or remove calibration levels. Laboratories may prepare their own calibration standards, but the number of standards and their concentrations must match those provided in the kit. Ensure the lowest level is below the reporting limit. Each calibration standard must be run at least in duplicate.

10.2. Calibration is calculated using a four-parameter logistic regression. The calibration curve is validated based on the %CV of the well replicates for each calibration level and the coefficient of determination. The %CV is calculated for each level's absorbance values, including the 'zero' standard. The %CV must be ≤10%, though a single pair is allowed to be higher but must still be ≤15%. The coefficient of determination (R^2) must be ≥ 0.990.

10.3. If the calibration fails validation based on either %CV or R^2, the entire analysis batch is invalid. Reanalyze the samples in a subsequent batch.

10.4. Each batch must include a calibration set. Analysis may not be done for samples using a calibration curve from a different batch.

11. Procedure:

11.1. Preparation of blank and fortified samples

11.1.1. LRB: mix 900 µL of reagent water with 100 µL of 10X diluent buffer into a 1.5mL vial.

11.1.2. LFB / LFSM / LFSMD: spike a concentration greater than or equal to the native background concentration, if known, of saxitoxin (or 0.100 ng/mL) into 900 µL of reagent water (or field sample / duplicate) and mix with 100 µL of 10X diluent buffer into a 1.5mL vial.

11.2. Test sample preparation.

11.2.1. Allow samples to come to room temperature.

11.2.2. Aliquot 1 mL of each sample into a 1.5 mL vial, return remaining samples to refrigerator for continued preservation.

11.2.3. If there is a concern of algal cells being present in ambient water samples, a cell lysis by freeze-thaw should be performed.

11.2.3.1 Perform three freeze-thaw cycles. The sample volume for lysis should be less than 25% of the vial capacity. Samples must be completely frozen and thawed during cycles. Thaw samples in a water bath at approximately 35°C and mix after each cycle.

11.3. All samples, standards, and reagents must come to room temperature.

11.4. It is required to process samples in at least duplicates.

11.5. Note: When using commercially available kits, follow manufacturer instructions, which may differ from the procedure identified below.

11.5.1. Dilute the 5x Wash Buffer Concentrate 1:5. If diluting the whole bottle (100 mL), add 400 mL of reagent water.

11.5.2. For the number of samples in each analysis batch, load the microtiter plate with the required number of plate strips.

11.5.3. Aliquot 50 µL of each standard, sample, and QC into the plate wells. An example batch plate is illustrated below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std0	Std4	etc.									
B	Std0	Std4	etc.									
C	Std1	Std5										
D	Std1	Std5										
E	Std2	Sam1										
F	Std2	Sam1										
G	Std3	Sam2										
H	Std3	Sam2										

Std 0 – Std 5 are the standards.

Sam 1, Sam 2, etc. are either QC or samples.

11.5.4. Aliquot 50 µL of the enzyme conjugate solution to each well in sequence, using a multichannel, stepper, or electronic repeating pipette.

11.5.5. Aliquot 50 µL of the antibody solution to each well, as before. Cover the wells in parafilm or PTFE tape. Mix wells by moving the plate in a circular motion on the benchtop for 60 seconds. Ensure the contents do not spill. A microtiter plate shaker may also be used.

11.5.6. Incubate the plate at room temperature for 30 minutes.

11.5.7. This step may be done manually, or with a microtiter plate washer. Decant the plate. Wash each well 4 times using diluted wash buffer. Wash each well with 250 µL of 1X wash buffer each time. Remove excess wash buffer from wells when finished, if washing

plates manually, pat inverted plate on a dry stack of paper towels.

11.5.8. Aliquot 100 µL of the substrate (color) solution to each well as in 11.5.5, recovering and mixing the plate.

11.5.9. Incubate the plate at room temperature for 30 minutes, protected from direct sunlight.

11.5.10. Aliquot 100 µL of the stop solution to each well in the same sequence as 11.5.8.

11.6. Read the absorbance at 450nm using a microtiter plate reader within 15 minutes of the addition of the stop solution. Readings taken beyond 15 minutes from addition of the stop solution are inaccurate and must be re-analyzed.

12. Data Analysis and Calculations:

12.1. The preferred calculation of the acquired data is by a Four-Parameter Logistic regression. Calculate the concentration for each well. For each sample (field and QC), use the average of the replicate wells to report and evaluated against the acceptance criteria.

12.2. If a result exceeds the calibration curve, dilute the sample with reagent water. Select a dilution factor to result in the diluted concentration being near the inflection point of the 4PL curve. Run this diluted sample in a subsequent analysis batch.

12.3. <u>If a result is below the level of Standard 1 (the lowest non-blank standard), the result is considered to be non-detect.</u>

13. Method Performance:

13.1. EPA's Alternative Testing Procedure (ATP) protocol was followed, where possible, for method development and validation.

13.2. Method Detection Limits (MDL) refers to the lowest concentration of the analyte that a method can report with 99% confidence that the measured concentration is distinguishable from method blank results. To determine the MDL, seven drinking water samples were spiked at 0.15 ppb saxitoxin and processed through the entire method along with seven drinking water blanks. The standard

deviation derived from the spiked sample recoveries was used to calculate the MDL using the following equation:

$MDL_S = (t)*(S)$
Where t is the Student single tailed t-test value for the 99% confidence level with n-1 degrees of freedom and S denotes the standard deviation obtained from n replicate analyses. For the n=7 replicates used to determine the MDL, t=3.143.

The MDL from the blanks (MDL_B) was set to the highest numerical result for a blank, based on the EPA's procedure for having some, but not all, method blanks giving numerical results. The results for the standard deviations and MDL are in Table 1.

13.3. The LCMRL fortification levels and calculated result are shown in Table 2. Due to the calculated LCMRL being below the lowest calibration point associated with the ELISA kit, the LCMRL was unable to be properly bracketed for final confirmation. The estimated LCMRL is 0.017 ppb.

13.4. MRL passed the EPA confirmation criteria at 0.03 ppb. The results for this MRL confirmation are in Table 3.

13.5. Method Validation consisted of the analysis of background water collected before and after treatment for drinking water, from 2 separate treatment plants. These waters were spiked at five different levels (0.050, 0.075, 0.100, 0.200, and 0.300 ppb) and analyzed five separate data sets on separate days. Recoveries for these validation samples are shown in Table 4.

13.5.1. Two pre-treatment data points were above the control limits during the method validation on day five, Fairbairn Plant 0.200 ppb and Sacramento River Water Plant 0.300 ppb. As these are both raw water samples and not treated drinking water, these points were left in the data set facilitate realistic and meaningful control limits with an ability to capture process deviations of significance.

13.6. STORAGE STABILITY STUDY - A storage stability study was completed. The storage stability study consisted of three replicates spiked at 0.1 ppb tested over a seven-day period. Glass bottles containing background groundwater were spiked and stored in the refrigerator, and 1 mL aliquots of each were removed to be analyzed on each day 0-7. A matrix blank and a matrix spike (0.1 ppb) were also on each analysis day and analyzed with the storage

stability samples. This storage stability study shows saxitoxin stability through day six. The results are shown in Table 5.

14. Pollution Prevention

14.1. For information about pollution prevention applicable to laboratory operations described in this method, consult: Less is Better, Guide to Minimizing Waste in Laboratories, a web-based resource available from the American Chemical Society at www.acs.org.

15. Waste Management

15.1. The Agency requires that laboratory waste management practices be consistent with all applicable rules and regulations, and that laboratories protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. In addition, compliance is required with any sewage discharge permits and regulations, particularly the hazardous waste identification rules and land disposal restrictions.

16. References:

16.1. Ohio EPA *Total (Extracellular and Intracellular) Saxitoxin by ELISA Analytical Methodology*, Ohio EPA DES 702.0, Version 2.0, November 2016.

16.2. Maciel, Robert J. Standard Curve Fitting in Immunodiagnostics: a Primer. *Journal of Clinical Immunoassay*. 1985, Vol. 8, 98–106.

16.3. Sasaki, Diane and Mitchell, Robert A. How to Obtain Reproducible Quantitative ELISA Results. Oxford Biomedical Research website. https://www.oxfordbiomed.com/sites/default/files/2017-02/How%20to%20Obtain%20Reproducible%20Quantitative%20ELISA%20results.pdf (accessed February 2023).

16.4. ABRAXIS Saxitoxin (PSP) ELISA Microtiter Plate, 2022. https://www.goldstandarddiagnostics.us/media/15661/ug-21-081-rev-03-abraxis-saxitoxin-elisa_52255b.pdf (accessed 2023 January 10)

16.5. Eurofins Abraxis Excel Solver, 2020. https://www.goldstandarddiagnostics.us/media/9257/17urofins_abraxis_excel_solver-website.xlsm(accessed 2023 January 25)

16.6. U.S. EPA. August 2016. Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay.

17. Tables, Figures, and Method Performance Data

Table 1. The Determination of Method Detection Limit (MDL) in Treated Drinking Water Spiked at 0.150 ppb.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	SD	MDL
Spike	0.152	0.153	0.152	0.154	0.152	0.150	0.142	0.004	0.013
Blank	0.006	ND	0.011	ND	0.005	ND	0.010		0.011

EPA MDL definitions set the MDL to be the higher value of the MDL_S and MDL_B. Therefore, the saxitoxin MDL=0.013ppb.

Table 2. Lowest Concentration Minimum Reporting Limit for Saxitoxin

Analyte	Fortification levels, ng/mL	LCMRL
STX	0.0, 0.020, 0.025, 0.030, 0.050, 0.075, 0.10, 0.20, 0.30	0.017*

*The LCMRL was calculated but unable to be properly bracketed and confirmed due to it being below the lowest calibration point that was provided with the kit.

Table 3. The Confirmation of Method Reporting Limit (MRL) in Treated Drinking Water Spiked at 0.03 ppb.

Sample	Conc. (ppb)
1	0.030
2	0.028
3	0.031
4	0.033
5	0.032
6	0.029
7	0.036
Mean	0.031
SD	0.002
Half Range	0.010
Upper Limit	137%
Lower Limit	71%

Lower Limit >50% and Upper Limit <150%
0.03 ppb PASSES as the MRL for saxitoxin.

Table 4. Method Validation in Pre- and Post-treated Drinking Water

		Spike Level Recovery				
Water Source	Day	0.050 ppb	0.075 ppb	0.100 ppb	0.200 ppb	0.300 ppb
Fairbairn Plant - Raw	1	79%	87%	91%	93%	96%
	2	99%	103%	101%	107%	103%
	3	91%	103%	97%	100%	95%
	4	94%	101%	108%	98%	89%
	5	98%	109%	102%	132%	90%
Fairbairn Plant - Treated	1	75%	90%	93%	99%	93%
	2	93%	104%	109%	110%	104%
	3	91%	109%	103%	104%	89%
	4	93%	99%	97%	98%	93%
	5	109%	110%	101%	97%	90%
Sacramento River Water Plant - Raw	1	85%	93%	97%	106%	107%
	2	106%	120%	112%	119%	119%
	3	91%	105%	106%	106%	97%
	4	93%	97%	102%	101%	97%
	5	104%	105%	104%	100%	127%
Sacramento River Water Plant - Treated	1	91%	96%	101%	105%	104%
	2	105%	114%	110%	114%	114%
	3	89%	99%	101%	96%	96%
	4	99%	105%	96%	93%	88%
	5	104%	106%	106%	102%	93%

Control Limits	
Mean	100.1%
SD	8.5%
RSD	8.5%
UCL	125.7%
LCL	74.5%

Table 5. Storage Stability Study in Treated Drinking Water
Spiked at 0.100 ppb.

Average Daily Recovery (n=3)						
Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	Day 8
100.1%	87.0%	82.8%	75.1%	79.6%	77.8%	72.3%

Table 6. Initial Demonstration of Capability (IDC) QC Requirements

Method Reference	**Requirement**	**Specification**	**Acceptance Criteria**
9.1.1	Demonstration of precision and accuracy	Analyze 7 replicate Laboratory Fortified Blanks (LFBs) at 0.07 ng/mL.	Percent relative standard deviation <15%. Mean percent recovery >70% and <130%.
9.1.2	Demonstration of acceptable system background	Analyze 5 Laboratory Reagent Blanks (LRBs) distributed throughout a plate.	STX concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.
9.1.3	MRL confirmation	Fortify and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR meet the recovery criteria.	Upper PIR ≤150% Lower PIR ≥50%
9.1.4	Quality Control Sample (QCS)	Prepare a QCS near the center of the calibration with STX from a source independent from the calibration standards.	Percent recovery >70% and <130% of the true value

Table 7. Analysis Batch QC Requirements

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
10.2	ELISA Calibration	Use kit-recommended levels and concentrations. Minimum two well replicates (Sect. 3.1.2) per standard.	%CV of absorbance <10%; <15% allowed for 1 pair. $r^2 > 0.990$.
9.2.1	Well replicates	Analyze field and QC samples in minimum two wells.	Sample invalid if %CV of absorbance values >15%
9.2.2	Laboratory Reagent Blank (LRB)	Analyze one LRB per Analysis Batch. Analyze in duplicate on opposite sides of the plate.	STX concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.
9.2.3	Low Calibration Verification (Low-CV)	Calibration standard at, or below, the MRL concentration. One per Analysis Batch.	Percent recovery >50% and <150% of the true value
9.2.4	Laboratory Fortified Blank (LFB)	Reagent water fortified near the center of calibration. Analyze 2 per Analysis Batch.	Percent recovery for each LFB >60% and <140% of the true value
9.2.5	Laboratory Fortified Sample Matrix (LFSM) and LFSM Duplicate	Fortify near the center of calibration. One set in Analysis Batches containing drinking water; two if 20 or more field samples. One set in Analysis Batches containing ambient water; two if 20 or more field samples.	Mean percent recovery of LFSM and LFSMD pair >60% and <140%. Relative percent difference (RPD) <40%. Qualify results for samples failing these limits as “suspect–matrix”.
9.2.6	Quality Control Sample (QCS)	Assay 1 QCS for each new lot of calibration standards. Prepare the QCS near the center of calibration with STX from a source independent of the calibration standards.	Percent recovery >70% and <130% of the true value

Appendix B: Guidelines for Interlaboratory Validation of Method CAC-STX-1.0

Guidelines for Interlaboratory Validation of Method CAC-STX-1.0

This set of guidelines is written for the purpose of guiding the labs who volunteered to participate in in the interlaboratory validation of method CAC-STX-1.0: Quantitation of Total (Extracellular and Intracellular) Saxitoxin by Enzyme-linked Immunosorbent Assay (ELISA).

1.1. Initial Demonstration of Capability (IDOC)

1.1.1. Labs will be provided with the SOP and sent ELISA kits via overnight shipping.

1.1.1.1. Verify the integrity of the kits upon receipt.

1.1.1.1.1. Inspect package for damage

1.1.1.1.2. Confirm all components are present.

1.1.1.1.3. Verify expiration dates.

1.1.1.2. Store kits and standards according to label conditions.

1.1.1.3. Follow the SOP to perform the Initial Demonstration of Capability (IDOC)

1.1.1.4. Note: all dilutions of standards need to be done with 1X diluent buffer (prepared from 10X diluent buffer provided in kits).

1.1.2. IDOC Data submission and competency verification. Submit IDOC data to ensure competency and address any procedural challenges before Water Matrix / PT sample distribution.

1.1.2.1. Table 1: QC requirements summary.

Method Reference	**Requirement**	**Specification**	**Acceptance Criteria**
9.1.1	Demonstration of precision and accuracy	Analyze 7 replicate Laboratory Fortified Blanks (LFBs) at 0.07 ng/mL.	Percent relative standard deviation (RSD) <15%. Mean percent recovery >70% and <130%.
9.1.2	Demonstration of acceptable system background	Analyze 5 Laboratory Reagent Blanks (LRBs) distributed throughout a plate.	STX concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB. STX < 0.5 x MRL

Method Reference	Requirement	Specification	Acceptance Criteria
9.1.3	MRL confirmation	Fortify and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR meet the recovery criteria.	Upper PIR ≤150% Lower PIR ≥50%
9.1.4	Quality Control Sample (QCS)	Prepare a QCS near the center of the calibration with STX from a source independent from the calibration standards.	Percent recovery >70% and <130% of the true value

1.1.2.2. The initial proposed MRL should be 0.03 ppb based on the method development results.

1.1.2.2.1. MRL confirmation spikes can be made by diluting the kit calibration standards in 1X diluent buffer.

1.1.2.2.2. *For example*: To achieve a 0.03 ppb spikes, dilute 75µL of Standard 5 (0.4ppb) to 1mL in 1X diluent buffer.

1.2. Method Evaluation in Surface-source Water

1.2.1. Labs will be sent 2 sample waters, one each from a treated surface-source drinking water and a raw surface-source water. 5 aliquots need to be taken from each water. These aliquots will be used to prepare a single unspiked sample, and duplicate low- and duplicate mid-spiked samples for each water source.

1.2.1.1. Verify the receipt of these samples as in 1.1.1.1 with the following addition:

1.2.1.1.1. Verify TempDot has not gone out of range during shipping.

1.2.2. A Sample Handling sheet will be provided in the Report Template workbook, please fill it out for sample traceability.

1.2.3. Working standards for spiking these samples will be prepared and provided by the CDFA/CAC QA unit.

1.2.3.1. Spike the sample aliquots provided according to Table 2

Sample	Volume Standard (µL)	Volume Sample (µL)
Unspiked x1	0	1000
Low-spike x2 (0.03 ppb)	30.0	970
Mid-spike x2 (0.10 ppb)	100	900

1.2.4. Immediately prepare 1mL samples by mixing 100 µL of 10X diluent buffer with 900 µL of the spiked samples. This will result in a dilution factor of 1.1.

1.2.5. Unspiked samples should be run in a separate batch to confirm absence of incurred saxitoxin prior to running spiked samples.

1.3. Proficiency Testing

1.3.1. After acceptance of IDOC data, each lab is sent one blind PT standard

1.3.1.1. Lab is required to spike the PT standard into reagent AND local tap water as follows:

1.3.1.1.1. Aliquot 100 µL of PT working standard and dilute to 1 mL in reagent/tap water.

1.3.1.1.2. Immediately prepare each 1mL sample by mixing 100 µL of 10X diluent buffer with 900 µL of the spiked reagent/tap water. Tap water samples should also include 0.1mg/mL of ascorbic acid to quench residual chlorine.

1.3.1.1.2.1. Ascorbic acid should be added as a neat compound, to not affect the final volume of the samples.

1.3.2. Submit all data to CDFA/CAC QA unit.

1.3.2.1. CDFA QA Supervisor, Sarva Balachandra, sarvamangala.gunjur@cdfa.ca.gov

1.3.2.2. Use provided Report Template workbook to report results for IDOC, water samples, and PT

1.3.2.2.1. Additional Batch Data Worksheets may be duplicated from the template for each batch.

1.3.2.3. In addition to the resulting data, please also submit:

1.3.2.3.1. Raw absorbance data

1.3.2.3.2. Microtiter plate legend

Appendix C: Data Reporting Template for MLVS of Method CAC-STX-1.0

Sample Handling Sheet

Method # : CAC-STX-1.0
Analysis:
Matrix:

CDFA SampleID	LAB SampleID	prepared by/date:	Matrix ID	sample volume (mL)	spk soln. ID	Volume spiked (mL)	Final Volume (mL)		comments

QC Samples

QC SampleID	LAB SampleID	prepared by/date:	Matrix ID	sample volume (mL)	spk soln. ID	Volume spiked (mL)	Final Volume (mL)		comments
LRB			Reagent		n/a		1.00		
LFB			Reagent						

Reagent _____ _____ _____
Water _____ _____ Pipette ID _____

Approved By: Mohammad Chehelamirani Rev. 6 12/18/2024

Batch: _____
Kit Vendor: _____
Kit Lot#: _____

Please also attach raw absorbance data and plate map legend

Date Analyzed	SampleID	Spiked Conc. (ppb)	Calculated Conc. (ppb)	Recovery	%CV
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	
				N/A	

4PL Parameters	Value
A	
B	
C	
D	
R^2	