

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

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Technical questions concerning this method should be addressed to:

John Kelly, Ph.D.
john.kelly@cdfa.ca.gov
California Department of Food and Agriculture, Center for Analytical Chemistry (CAC)
3292 Meadowview Rd.
Sacramento, CA 95832

and

Mohammadreza Chehelamirani, Ph.D.
mohammadrez.chehelamirani@cdfa.ca.gov
California Department of Food and Agriculture, Center for Analytical Chemistry (CAC)
3292 Meadowview Rd.
Sacramento, CA 95832

CAC Approvals for use:

Teresa Bowers
CAC Environmental Program Manager I

Date

Sarvamangala Balachandra
CAC Quality Assurance Officer

Date

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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 μ L)
- 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 μ L of 10X Diluent per 900 μ L 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.

$$\text{Upper PIR Limit} = \frac{\text{Mean} + \text{HR}}{\text{Spiked Conc.}} \times 100\% \leq 150\%$$

$$\text{Lower PIR Limit} = \frac{\text{Mean} - \text{HR}}{\text{Spiked Conc.}} \times 100\% \geq 50\%$$

- 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 $\pm 50\%$ 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 $\pm 40\%$ 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 $\pm 40\%$ 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 $\leq 40\%$. 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 $\pm 30\%$ 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 $\leq 10\%$, though a single pair is allowed to be higher but must still be $\leq 15\%$. 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 μ L of the enzyme conjugate solution to each well in sequence, using a multichannel, stepper, or electronic repeating pipette.
- 11.5.5. Aliquot 50 μ L 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 μ L 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 μ L 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 μ L 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. If a result is below the level of Standard 1 (the lowest non-blank standard), the result is considered to be non-detect.

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 Immunodiagnosics: 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/9257/17eurofins_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

Water Source	Day	Spike Level Recovery				
		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