PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST
MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
	:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "√"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
K	3, 6		1.1.1 Written Plan adequately covers all the following: (check √ those that apply) a. Organization of the laboratory. b. Staff training requirements. c. Standard operating procedures. d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established. e. Laboratory safety. f. Internal performance assessment. g. External performance assessment.
C	3		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	State's Human Resources Department		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
			1.3 Work Area
O	3, 6		1.3.1 Adequate for workload and storage.
O	6		1.3.2 Clean and well lighted.
O	6		1.3.3 Adequate temperature control.
O	6		1.3.4 All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment
O	4		1.4.1 The pH meter has a standard accuracy of 0.1 unit.
K	4		1.4.2 pH paper in the appropriate range (i.e. 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	3		1.4.3 The pH meter is calibrated daily when in use. Results are recorded, and records are maintained.
K	6		1.4.4 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	6		1.4.5 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions (pH 7 and 10) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.
K	3, 7		1.4.6 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	2, 4		1.4.7 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	6		1.4.8 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded, and records are maintained.
K	1		1.4.9 Refrigerator temperature is maintained between 0 and 4 ºC.
K	6		1.4.10 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	11		1.4.11 Freezer temperature is maintained at -10 ºC or below.

K	6		1.4.12 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**9**		**1.4.13 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.14 All glassware is clean.
C	**11**		**1.4.15 Absorbance Microplate reader equipped with filter for measurement at 450 nm is used.**
O	2		1.4.16 Absorbance Microplate reader performance is evaluated at least annually using manufacturer instructions or a check standard microplate at the appropriate wavelength (450) to assess alignment, accuracy, reproducibility, and linearity. Method used: _____
K	2		1.4.17 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded, and records are maintained.
O	11		1.4.18 A centrifuge capable of holding 15 mL or 50 mL polypropylene tubes is used.
			1.5 Reagents and Reference Solution Preparation and Storage
C	**11**		**1.5.1 All solvents and reagents used are ACS grade materials or better.**
O	6		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded, and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	6		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**11**		**1.5.4 Brevetoxin-3 (BTX-3 or PbTx-3) provided with the MARBIONC ELISA kit is used as the reference standard.**
C	**11**		**1.5.5 Stock standard solution is made by diluting brevetoxin-3 reference standard to 1 µg/ml in 100% methanol in a volumetric flask.**
C	**11**		**1.5.6 Working standard solution (100 ng/ml) is made by diluting 1 ml of stock solution to 10 ml in a volumetric flask using 100% methanol.**
K	11		1.5.7 Extraction solvent (80% methanol) is made by adding 800 ml of methanol to a 1 L graduated cylinder and bringing the total volume to 1 L with water.
K	11		1.5.8 Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are used within 1 week of preparation. pH of prepared media is determined to ensure it is consistent with manufacturers requirements. Results are recorded, and records are maintained.
K	11		1.5.9 Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are stored in refrigerator for no longer than 1 week and brought to room temperature before use.
K	11		1.5.10 Gelatin stock solution is prepared by dissolving 5 g gelatin in 100 ml water and stirring the solution over gentle heat on a stir plate until clear. Gelatin stock solution is aliquoted into smaller volumes (e.g. 15 ml centrifuge tubes) and refrigerated.
K	11		1.5.11 Blocking buffer is prepared by dissolving 1 pouch in 200 ml water. Blocking buffer solution is aliquoted into 50-ml centrifuge tubes and refrigerated.
K	11		1.5.12 PGT (PBS, 0.05% Tween, 0.5% gelatin) is made fresh daily by measuring 5 ml liquified gelatin stock solution into a 50-ml centrifuge tube and filling to 50 ml with PBS-Tween.
C	**11**		**1.5.13 Stock and working standard solutions are stored -10 ºC or below.**
C	**5**		**1.5.14 All standards used are within expiration date (or 1 year if not provided).**
			1.6 Collection and Transportation of Samples
O	4, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	4, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.

C	**4, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	2, 10		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ASSAY OF SHELLFISH FOR NSP TOXINS			
			2.1 Preparation of Sample
C	**4**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	4		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	4		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	4		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	4		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**4**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	4		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	4		2.1.8 Pieces of shell and drainage are discarded.
C	**2, 4**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
			2.2 Sample Extraction
K	4		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**11**		**2.2.2 One (1) gram of homogenized sample is weighed into a 15 ml or 50 ml polypropylene centrifuge tube and subsequently extracted.**
C	**11**		**2.2.3 The sample homogenate is extracted by adding 9 ml extraction solvent (80% aqueous methanol) and vortexing at highest speed for 2 minutes.**
C	**11**		**2.2.4 The homogenate/methanol mixture is centrifuged at a minimum of 3.000*xg* for 10 minutes.**

C	**11**		**2.2.5 The supernatant is transferred to a clean, labeled graduated 15-ml centrifuge tube and brought to a final volume of 10 ml with extraction solvent.**
K	11		2.2.6 Crude extracts are sealed tightly in glass vials and stored at -10 ºC or below until analyzed.
			2.3 Analysis
C	**11**		**2.3.1 Only high-binding flat-bottom plates no older than 1 year are used (e.g. Nunc Maxisorp Immunoplates).**
C	**2, 11**		**2.3.2 A standard calibration curve of seven concentrations (0.078-5 ng PbTx-3/ml) is included on each plate. Results are recorded, and records are maintained.**
C	**11**		**2.3.3 When pipetting kit reagents that are pre-diluted in glycerol (A, C, and D):** **a. the pipet tip is not pre-rinsed,** **b. only the very tip of the pipet tip is inserted into the vial to withdraw the required amount,** **c. the tip is submerged into the buffer when dispensing and rinsed several times with buffer to ensure complete transfer**
K	2		2.3.4 Crude extracts are thoroughly mixed before withdrawing an aliquot for analysis.
C	**11**		**2.3.5 Crude extracts are diluted with PGT before analysis. The minimum dilution for shellfish extracts is 1:40 (25 ul + 975 ul PGT) (resulting in a sample dilution of 1:400).**
C	**11**		**2.3.6 For quantitative (actionable) results, serial dilutions (n=7) of each sample extract are assayed. Fewer dilutions are permissible for screening purposes only.**
C	**11**		**2.3.7 Assay Step 1: Reagent A is diluted by 300 (or as specified in kit instructions) in PBS, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS (no Tween for this wash step).**
C	**11**		**2.3.8 Assay Step 2: Each well is filled with 250 µl of blocking buffer. The plate is incubated on a plate shaker for 30 minutes. After 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.9 Assay Step 3: Serial dilutions (n=7) of each crude sample extract and a standard calibration curve of seven concentrations (0.078-5.0 ng PbTx-3/ml) are prepared in PGT.**
C	**11**		**2.3.10 Assay Step 4: 100 µl of each sample or standard dilution is loaded on to the microplate as well as two reference wells (containing PGT only) adjacent to each set of sample dilutions. Each dilution of standard or sample is added to duplicate wells. Plate layout identifying locations of samples and standards on the plate is documented.**
C	**11**		**2.3.11 Assay Step 5: Reagent C is diluted by 300 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate (which contains samples or standards), and the plate is incubated on a plate shaker for 90 minutes. After 90 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.12 Assay Step 6: Reagent D is diluted by 800 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, all wells are rinsed 3 times with 300 µl PBS-Tween, and one final time with 300 µl PBS only to ensure no Tween remains on the plate. TMB should be brought to room temperature in the dark.**
C	**11**		**2.3.13 Assay Step 7: Each well is filled with 100 µl of room temperature TMB (3,3'5,5'-Tetramethylbenzidine) and incubated until a blue color develops in the reference wells. The reaction is stopped by adding 100 µl of 0.5M sulfuric acid solution to each well, and the absorbance in the wells at 450 nm is measured in a microplate reader.**
K	11		2.3.14 Plates are covered with microplate sealing film during all incubation steps (Steps 1-6 above).

C	11		**2.3.15 Plates are protected from light by covering with aluminum foil during color development (Step 7 above).**
K	11		2.3.16 The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities (at 450 nm) of 1.0 +/- 30%.
			2.4 Quality Control
C	11		**2.4.1 Acceptance of assay (plate) results is dependent on meeting the following criteria:** **a. Absorbance of standard reference wells (Amax) must be ≥ 0.6.** **b. CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (30-70% inhibition) must be < 20%.**
C	11		**2.4.2 Acceptance of individual sample results is dependent on meeting the following criteria:** **a. CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (30-70% inhibition) must be < 20%.** **b. CV of calculated concentrations of different sample dilutions within the linear range of the assay (30-70% inhibition) must be < 20%.**
			2.5 Calculation of Sample Toxin Concentration
C	11		**2.5.1 Absorbance values are converted to % color inhibition:** **a. % inhibition = [1 - (Avg of duplicate A/Amax)] x 100% where Amax is the average absorbance of the reference wells oriented below the sample or standard dilutions**
C	11		**2.5.2 Using the 4-parameter logistic (4PL) curve in an appropriate software program, a curve is fit to the positive control with ng toxin/ml on the x-axis (log scale) and % inhibition on the y-axis (linear scale).**
C	11		**2.5.3 The concentrations for sample dilutions falling within 30%-70% inhibition are interpolated from the standard curve.**
C	11		**2.5.4 Sample toxin concentration is calculated by multiplying the interpolated concentration by the sample dilution factor and dividing by 1000 to obtain PbTx-3 eq. results in ppm.**
C	11		**2.5.5 If more than one dilution of a sample falls within 30%-70%, the mean of the two calculated concentrations is used.**
C	8		**2.5.6 A result of ≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters is considered negative and can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.** **A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method to support management actions.**
O	8		2.5.7 Laboratory reports to the Shellfish Management Authority detail sample date, location, species (matrix), date tested, analyst name, result of ELISA in ppm, and the actionable ELISA threshold for the species tested.

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
4. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
5. Consult reference standard product literature.

6. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
7. Consult pH electrode product literature.
8. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. *NSSP Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
9. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
10. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
11. MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish. (ISSC proposal 17-107, supporting documents Appendix A)

LABORATORY:			DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**
LABORATORY STATUS			

LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON (NSP or Brevetoxin) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the brevetoxin (NSP) ELISA Component

1. **Conforms Status: The NSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The NSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The NSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥ 4**.
   - b. or the total # of Key nonconformities is **≥ 6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform – Provisionally Conforms – Conforms

Acknowledgement by Laboratory Director/Supervisor:

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

Laboratory Signature:_____ Date:_____

LEO Signature:_____Date:_____