Importance of Brevetoxin Determination

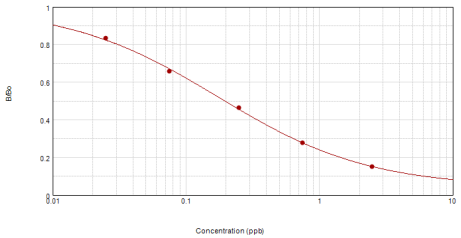
Neurotoxic shellfish poisoning (NSP) is caused by polyether toxins known as Brevetoxins. Brevetoxins (PbTxs) are produced by the dinoflagellate *Karenia brevis,* which causes harmful algal blooms (HABs) know as red tides. The Brevetoxins are toxic to fish, marine mammals, birds and humans, but not to shellfish. Contamination of shellfish with Brevetoxin has been associated with the presence of harmful algal blooms in various parts of the world.

Mortality events attributed to HABs have been documented for fish, manatee, dolphins, and seabirds. In man, NSP causes dose-dependent symptoms of diarrhea, nausea, and vomiting, chills, sweats, reversal of temperature, hypotension, arrhythmias, numbness, tingling, bronchoconstriction, paralysis, seizures, and coma.

The Brevetoxin ELISA allows the determination of up to 42 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in less than 2 hours.

Performance Data

Test sensitivity: The limit of detection for Brevetoxin is approximately 0.031 ng/mL in seawater (accounting for the dilution factor of 1.25 from the ABRAXIS® Seawater Pretreatment Solution) and 11.25 ng/g in shellfish (accounting for the extraction/dilution factor of 450). The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 0.2425 ng/mL in seawater and 87.3 ng/g in shellfish. Determinations closer to the middle of the calibration range of the test yield the most accurate results.



For demonstration purposes only. Not for use in sample interpretation.

Test reproducibility: The standard curve R^2 must be ≥ 0.98. The absorbance Coefficient of variation (CVs) for standards should be ≤10% and for the samples should be ≤15%. Standard 0 absorbance value should be between 0.8 - 3.000.

Selectivity: This ELISA recognizes Brevetoxin and other NSP toxins to varying degrees:

Cross-reactivities:

PbTx-3	100%
Desoxy PbTx-2	133%
PbTx-5	127%
PbTx-2	102%
PbTx-9	83%
PbTx-6	13%
PbTx-1	5%

No cross-reactivity was shown with any of the following common PSP shellfish toxins: saxitoxin, neosaxitoxin, dc-STX, gonyautoxins-1/4, gonyautoxins-2/3, B-2; B-1; C-1/2 and domoic acid.

Samples: Sea water and shellfish samples (after recommended dilution) were tested for matrix effects in the ELISA. No matrix effects were determined.

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ABRAXIS® Brevetoxin (NSP) ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Brevetoxin (NSP) in Water and Contaminated Samples

Product No. 520034

1. General Description

The ABRAXIS® Brevetoxin ELISA is an immunoassay for the quantitative and sensitive detection of Brevetoxin. Brevetoxin is one of the toxins associated with neurotoxic shellfish poisoning (NSP). This test is suitable for the quantitative and/or qualitative detection of Brevetoxin in water samples as well as shellfish samples. Seawater samples require addition of a pre-treatment solution and shellfish samples require an extraction (see Preparation of Samples, Section C) prior to analysis. If necessary, positive samples can be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Brevetoxin (PbTx-3). In addition, the substrate (color) solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The ABRAXIS® Brevetoxin ELISA should to be stored in the refrigerator (2-8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Brevetoxin by specific antibodies. Brevetoxin, when present in a sample, and a Brevetoxin enzyme-conjugate compete for the binding sites of sheep anti-Brevetoxin antibodies that have been immobilized in the wells of a microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Brevetoxin present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the ABRAXIS® Brevetoxin ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects cannot be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be: Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, exposure to direct or indirect sunlight during the substrate reaction, or extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The ABRAXIS® Brevetoxin ELISA kit provides screening results. As with any analytical technique (GC/MS, HPLC, etc.) positive samples requiring some action should be confirmed by an alternative method.

6. Working Instructions

A. Materials Provided

1. Microtiter plate coated with sheep anti-Brevetoxin
2. Standards PbTx-3 (6): 0, 0.025, 0.075, 0.25, 0.75, 2.5 ng/mL, 1.5 mL each
3. Lyophilized Brevetoxin-HRP Conjugate, 3 vials
4. Conjugate Diluent, 12 mL
5. Empty amber HDPE bottle for combining reconstituted Enzyme Conjugate (if necessary)
6. Sample Diluent (1X), 2 X 30 mL, used to dilute samples
7. ABRAXIS® Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section D)
8. Substrate (Color) Solution (TMB), 12 mL
9. Stop Solution, 12 mL
10. Sea Water Pretreatment Solution, 25 mL

B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200, and 200-1000 µL)
2. Multi-channel pipette (10-250 µL) or stepper pipette with plastic tips (10-250 µL), or electronic repeating pipette with disposable plastic tips (capable of delivering 50-250 µL)
3. Microtiter plate washer (optional)
4. Deionized or distilled water
5. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section D)
6. Microtiter plate reader (wave length 450 nm)
7. Paper towels or equivalent absorbent material
8. Timer
9. Materials and reagents for Sample Preparation (see Section C)

C. Preparation of Samples

Shellfish (Mussels, Clams, Oysters)

1. Remove the shellfish from their shells, wash with deionized water, drain and blot dry. Homogenize using a Waring blender, Polytron or equivalent.
2. Weigh a 1.0 g portion of the homogenized shellfish into an appropriately labeled 40 mL glass vial.
3. Add 9.0 mL of methanol/deionized water (9:1 v/v) solution.
4. Cap vial and shake by hand vigorously for 2 minutes.
5. Centrifuge mixture for 10 minutes at 3000 x g. Collect and transfer the supernatant to an appropriately labeled glass vial.
6. Add 980 µL of Sample Diluent to an appropriately labeled 4 mL glass vial. Add 20 µL of the collected extract (from Step 5) to the Sample Diluent (equals a 1:50 dilution).
7. Analyze diluted extracts as samples (Assay Procedure, Section F, Step 1).

The Brevetoxin concentration contained in the samples is determined by multiplying the ELISA result by a factor of 450. Highly contaminated samples outside the range of the curve should be diluted further and re-analyzed. Additional ABRAXIS® Sample Diluent (PN 205226) can be purchased from Gold Standard Diagnostics.

Seawater

1. Collect 2 mL of sea water sample in a 4 mL glass vial.
2. To prevent loss of Brevetoxin to the glass surface, immediately add 0.5 mL of Sea Water Pretreatment Solution, mix by hand. (Alternatively, a larger seawater sample can be collected in a glass container and 0.5 mL of Sea Water Pretreatment Solution added for every 2 mL of sample.)
3. Analyze preserved sample as samples (Assay Procedure step 1)

The Brevetoxin concentration contained in the sea water sample is determined by multiplying the ELISA result by a factor of 1.25. Highly contaminated samples outside the range of the curve should be diluted in ABRAXIS® Sample Diluent (PN 205226), and re-analyzed. Additional ABRAXIS® Sample Diluent or ABRAXIS® Sea Water Pretreatment Solution (PN 205227) can be purchased from Gold Standard Diagnostics

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. The use of a multi-channel pipette, stepping pipette, or electronic repeating pipette is recommended for the addition of the antibody, enzyme conjugate, substrate, and stop solutions in order to equalize the incubation periods on the entire microtiter plate.

To avoid drift and obtain accurate results, the addition of the antibody, conjugate, color, and stop solutions should be performed in less than 2 minutes for each reagent. If additions to the entire microtiter plate cannot be completed in less than 2 minutes, run size should be decreased to the number of rows, which can be pipetted in less than 2 minutes. Please use only the reagents and standards from one kit lot in one test, as they have been adjusted in combination.

1. Allow the microtiter plate and the reagents to room temperature before use.
2. The enzyme conjugate must be reconstituted prior to use. Add 3 mL of the Conjugate Diluent to the Bevetoxin-HRP conjugate vial and vortex well. Let sit for at least 10 minutes and re-vortex prior to use. ***If more than one vial is required for testing, combine the reconstituted enzyme conjugate vials in the amber HDPE bottle a**nd mix thoroughly prior to use.* The reconstituted conjugate solution should be stored frozen.
3. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (2-8°C).
4. The standards, enzyme conjugate, substrate (color) and stop solutions are ready to use and do not require any further dilutions.
5. Dilute the ABRAXIS® Wash Buffer (5X) Concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
6. The stop solution should be handled with care as it contains diluted H_2SO_4.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 5: Standards
0; 0.025; 0.075; 0.25; 0.75,
2.5 (ng/mL) or ppb

Sam1, Sam2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Sam1										
F	Std 2	Sam1										
G	Std 3	Sam2										
H	Std 3	Sam2										

F. Assay Procedure

1. **Add 50 µL of standards, samples (water) or sample extracts (shellfish)** into the wells of the test strips according to the working scheme given. Analysis in at least duplicate is recommended.
2. **Add 50 µL of the reconstituted enzyme conjugate solution** to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the benchtop for about 30 seconds. Be careful not to spill contents. **Incubate for 60 minutes.**
3. After incubation, remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. **Wash the strips three times using the diluted wash buffer.** Use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
4. **Add 100 µL of substrate (color) solution** to the wells successively using a multi-channel, stepping, or electronic repeating pipette. **Incubate the strips for 30 minutes at room temperature.** Protect the strips from direct sunlight.
5. **Add 100 µL of stop solution** to the wells in the same sequence as for the substrate (color) solution using a multi-channel, stepping, or electronic repeating pipette.
6. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Brevetoxin concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Brevetoxin by interpolation using the standard curve.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing lower concentrations of Brevetoxin compared to standard 1 (0.025 ng/mL) are considered as negative. Samples showing a higher concentration than standard 5 (2.5 ng/mL) must be diluted further to obtain accurate results. Results must be multiplied by the appropriate dilution factor (see Sample Preparation, Section C).

As with any analytical technique (GC/MS, HPLC, etc.) samples requiring regulatory action should be confirmed by an alternative method.