Importance of Cyclic Imines Determination

Cyclic Imines are a family of neurotoxins produced by some species of dinoflagellates. In humans and other animals, the skeletal neuromuscular junction constitutes a primary target for Cyclic Imine toxins such as spirolides and pinnatoxins. The neuromuscular junction is specialized for the rapid transmission of neuronal information from the pre-synaptic nerve terminal to the post-synaptic muscle fiber. This transmission is mediated by the synchronous release of the neurotransmitter acetylcholine (ACh), which activates nicotinic acetylcholine receptors (nAChRs) in the muscle endplate, triggering a series of events that lead to muscle contraction. Most ACh molecules are hydrolyzed by acetocholinesterases, which are highly concentrated at the neuromuscular junction. Cyclic Imines function as antagonists of nAChRs, blocking the activity of ACh and rendering the muscle unable to contract. Symptoms caused by Cyclic Imine toxins include diarrhea, paralysis, convulsions, and eventual death by asphyxiation.

Humans and other animals may be exposed to Cyclic Imines through ingestion of contaminated shellfish or during recreational activities in which water is swallowed. No known human fatalities have been conclusively linked to Cyclic Imines, however it has been suggested to be the causative agent in several outbreaks of food poisoning and shows a rapid rate of mortality in mouse bioassay. There are currently no regulatory limits for Cyclic Imine toxins.

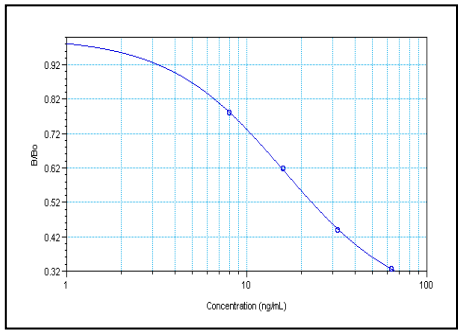
The Abraxis Cyclic Imines Receptor-Binding Assay can be performed in approximately 3.5 hours. Only a few milliliters of sample are required.

Performance Data

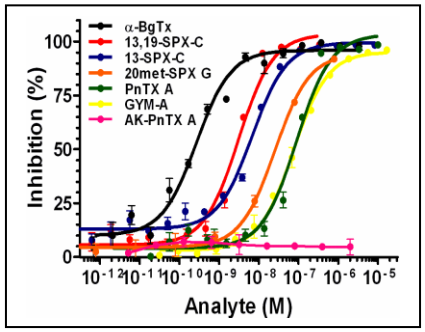
Test sensitivity: The limit of detection for 13-desmethyl spirolide C is 8 ng/mL. The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 25 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results.

The assay range is 80 ng/mL to 640 ng/mL in seawater (salinity range: 30-40 ppt) or bivalve tissue (for 13-desmethyl spirolide C; for other Cyclic Imine toxins (pinnatoxins, etc.), please see the appropriate technical bulletin).

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; for samples: <20% (based on absorbance).



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



Cross-Reactivity

References: *"*Procédé de fabrication d'un support d'analyse et utilisation pour la détection des toxines,"* French priority patent application No. 1150586, January 25, 2011 and International patent application No. FR2012050157, January 24, 2012. Licensed exclusively by Abraxis.

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R092316

Cyclic Imines Receptor-Binding Assay* (Microtiter Plate)

Receptor-Binding Assay for the Determination of Cyclic Imines in Marine Water and Bivalve Tissue

Product No. 520061

1. General Description

The Abraxis Cyclic Imines Receptor-Binding Assay is a patented* receptor-binding assay for the detection of Cyclic Imine toxins (spirolides, pinnatoxins, etc.) in marine water and bivalve samples. Sample preparation is required prior to analysis (see section C for marine water dilution and section E for bivalve sample extraction/preparation). This test is suitable for the quantitative and/or qualitative detection of Cyclic Imine toxins. Positive samples requiring regulatory action should be confirmed by HPLC/MS, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of 13-desmethyl spirolide C. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains dilute sulfuric acid. Avoid contact of these solutions with skin and mucous membranes. If these reagents come in contact with skin, wash thoroughly with water.

3. Storage and Stability

The Cyclic Imines Receptor-Binding Assay kit should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

The test is a receptor-binding assay based on the affinity of Cyclic Imine toxins for nicotinic acetylcholine receptors (nAChRs). Cyclic Imine toxins, when present in a sample, will compete with the biotinylated alpha-bungarotoxin for the acetylcholine binding sites of nAChRs, from purified *Torpedo* electrocyte membranes, which were coated on the microtiter plate. After a washing step, a streptavidin-HRP solution is added to allow for the colorimetric detection of the biotinylated alpha-bungarotoxin bound on the receptor. After a second washing step, the substrate solution is added and a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of 13-desmethyl spirolide C present in the sample. The color reaction is stopped after a specified time and the color (yellow) is evaluated using a microplate photometer. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Cyclic Imines Receptor-Binding Assay, 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 environmental samples, test interferences caused by matrix effects cannot be completely excluded.

Marine water samples must be diluted prior to analysis to obtain accurate results (see section C).

Cyclic Imine toxins, such as pinnatoxin-a, will degrade when exposed to high pH conditions. Samples which have been preserved with reagents that raise the natural sample pH may produce inaccurate results (biased low). See Section C, Sample Collection and Handling, for appropriate sample handling information.

Mistakes in handling the test can also cause errors. Possible sources for such errors can include: a) inadequate storage conditions of the test kit, b) incorrect pipetting sequence or inaccurate volumes of the reagents, c) too long or too short incubation times, and d) inadequate temperatures during incubations (<35°C or >40°C during Standard/Sample, Biotinylated Alpha-Bungarotoxin, and Streptavidin incubations and <20°C or >25°C during Color incubation).

The Abraxis Cyclic Imines Receptor-Binding Assay kit provides screening results. As with any analytical technique (GC, HPLC, etc.), positive samples requiring regulatory action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Nicotinic Acetylcholine Receptor (nAChR) coated microtiter plate, 12 strips of 8 wells in a resealable pouch
2. 13-desmethyl spirolide C Standards (5): 0, 8, 16, 32, and 64 ng/mL (ppb), 1 mL each
3. Biotinylated Alpha-Bungarotoxin Solution, 6 mL
4. Streptavidin-HRP Conjugate Solution, 16 mL
5. Sample Diluent, 30 mL
6. Wash Solution (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
7. Color (Substrate) Solution (TMB), 16 mL
8. Stop Solution, 12 mL
9. Adhesive Film Plate Cover

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

1. Micro-pipettes with disposable plastic tips (20-200 µL)
2. Multi-channel pipette or stepper pipette (25-250 µL) with disposable plastic tips
3. Deionized or distilled water
4. Graduated cylinder

5. Container with 500 mL capacity (for 1X diluted Wash Solution, see Test Preparation, Section C)
6. Timer
7. Paper towels or equivalent absorbent material
8. Incubator capable of maintaining 37˚C
9. Microtiter plate reader (wave length 450 nm)

C. Sample Collection and Handling

Collect water samples in glass sample containers and store refrigerated for up to 5 days. Samples which must be held for more than 5 days should be stored frozen.

If total Cyclic Imine concentration (free and cell-bound) is required, an appropriate cell lysing procedure (freeze and thaw, etc.) must be performed prior to analysis. *Note: The use of sonication in cell lysing can negatively affect toxin concentrations, producing falsely low sample results.*

If large amounts of organic matter are present, samples may be filtered prior to analysis (PALL Life Sciences Acrodisc® 25 mm Syringe Filters (PALL part number 4612) are recommended). If determining total Cyclic Imines concentration, samples should be lysed prior to filtration to prevent the removal of cell-bound toxins, which would cause inaccurate results.

Marine water samples must be diluted at a ratio of 1:10 prior to analysis (for example, 0.1 mL of sample into 0.9 mL of sample diluent) to remove matrix interference and obtain accurate results.

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and samples are necessary. A multi-channel pipette or a stepping pipette is recommended for adding the Biotinylated Alpha-Bungarotoxin, Streptavidin-HRP Conjugate, substrate (color), and stop solutions in order to equalize the incubation periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Allow the microtiter plate, reagents, and samples to reach room temperature before beginning the test.
2. The microtiter plate consists of 12 strips of 8 wells. The recommended run size is 6 strips or less, as drift may occur. Use of more than 6 strips in an analytical run may cause inaccurate results. Analysis in **triplicate** is highly recommended.
3. Remove the number of microtiter plate strips required from the foil bag. The remaining strips must be stored in the foil bag with desiccant and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
4. The standard solutions, assay buffer, Biotinylated Alpha-Bungarotoxin, Streptavidin-HRP, substrate (color), and stop solutions are ready to use and do not require any further dilutions.
5. Dilute the wash buffer concentrate at a ratio of 1:5 with deionized or distilled water. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
6. The adhesive film plate cover should be used to cover the wells during the incubation periods. Wells should be sealed closed to prevent evaporative loss. The cover can be used for multiple runs; do not discard the backing – replace on the adhesive portion of the cover after each use.

E. Bivalve Sample Extraction/Preparation

(for the extraction of 13-desmethyl spirolide C from bivalve tissue; for other Cyclic Imine toxins (pinnatoxins, etc.), please see the appropriate technical bulletin).

Additional Materials Required

Methanol; Acetonitrile; 0.45 µm PES membrane syringe filters with prefilters (Environmental Express part number SF145E are recommended) with 10 mL disposable syringes; Centrifuge capable of 3000 rpm; 15 mL conical tubes; Glass vials with Teflon-lined caps (4 mL and 20 mL); Vortex mixer with 15 mL conical tube adaptor or overhead tube rotator, Strata-X 33µ Polymeric Reversed Phase SPE Columns, 3 mL with 30 mg sorbent bed (Phenomenex part number 8B-S100-TBJ); Vacuum manifold; Preconditioning Solvent (1:2:12 methanol/acetonitrile/deionized water, prepare by adding 10 mL methanol and 20 mL acetonitrile to 120 mL deionized water); 60% methanol solution (prepare by adding 60 mL of methanol to 40 mL of deionized water); RapidVap nitrogen evaporation system; Blender or food processor; Disposable spatula.

Extraction/Preparation

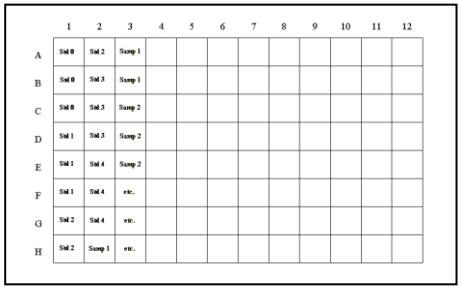
1. Remove and discard shells from bivalve samples.
2. Homogenize sample using a blender or food processor *(note: thoroughly clean the blender or food processor between samples to prevent cross-contamination of samples)*.
3. Weigh 1 g of homogenized sample into an appropriately labeled 15 mL conical tube.
4. Add 2 mL of methanol. Place in vortex adaptor and vortex for 10 minutes.
5. Centrifuge for 10 minutes at 3000 rpm. Transfer the supernatant to a clean, appropriately labeled 15 mL conical tube.
6. Repeat steps 4 and 5 for a second extraction of the sample, combining the supernatants.
7. Add methanol to the combined supernatants to a final volume of 5 mL. Vortex thoroughly.
8. Filter the methanol extract (using a PES membrane syringe filter) into an appropriately labeled 20 mL glass vial.
9. Transfer 1 mL of the filtered extract into a clean, appropriately labeled 15 mL conical tube. Add 2 mL of acetonitrile and vortex. Allow sample to incubate at room temperature for 20 minutes (a precipitate will form).
10. Centrifuge for 10 minutes at 3000 rpm. Transfer 3 mL of the supernatant into a clean, appropriately labeled 15 mL conical tube.
11. Add 12 mL deionized water. Vortex thoroughly.
12. Precondition the Strata-X column with 3 mL of methanol then 3 mL of Preconditioning Solvent Solution (see Additional Materials Required section for solution preparation). Load the 15 mL sample immediately after preconditioning; do not apply the vacuum during preconditioning or sample loading as **allowing the column to dry between preconditioning and sample loading** will adversely affect SPE performance and may cause inaccurate results.
13. Add 3 mL of 60% methanol (see Additional Materials Required section for solution preparation) to the column.
14. Add a second 3 mL aliquot of 60% methanol to the column. Apply the vacuum to dry the column.
15. Add 1 mL of methanol (100%) to the column. Apply vacuum to a flow rate of 1 mL/minute and collect the extract in a clean, appropriately labeled 4 mL vial. After the methanol is completely eluted, add a second 1 mL aliquot of methanol to the column then re-apply the vacuum at a flow rate of 1 mL/minute until methanol is completely eluted.
16. Evaporate the methanol extract under a gentle stream of nitrogen.
17. Reconstitute by adding 2 mL of Sample Diluent and vortex thoroughly.
18. Analyze as sample (Assay Procedure, step 1).

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The recommended run size is 6 strips or less, as drift may occur. Use of more than 6 strips in an analytical run may cause inaccurate results. Analysis in **triplicate** is highly recommended. 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 4: Standards
0; 8; 16; 32; 64 ppb

Samp1, Samp2, etc.: Samples



G. Assay Procedure

1. Add 100 µL of the **standard solutions and samples** into the appropriate wells of the test strips according to the working scheme given. Analysis in **triplicate** is recommended. Cover the wells with the adhesive film plate cover and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. Incubate the strips in an incubator at 37˚C for 2 hours.
2. Add 50 µL of **biotinylated alpha-bungarotoxin solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with the adhesive film plate cover and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. Incubate in an incubator at 37˚C for 30 minutes.
3. Remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips **three times** using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
4. Add 150 µL of **streptavidin-HRP conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with the adhesive film plate cover and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. Incubate in an incubator at 37˚C for 30 minutes.
5. Remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips **three times** using the 1X washing buffer solution. Use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
6. Add 150 µL of **substrate (color) solution** to the wells using a multi-channel pipette or a stepping pipette. Cover the wells with the adhesive film plate cover and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Incubate for 30 minutes at room temperature. Protect the strips from direct sunlight.
7. Add 100 µL of **stop solution** to the wells using a multi-channel pipette or a stepping pipette in the same sequence as for the substrate solution.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

H. Evaluation

The evaluation of the assay can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). Results can also be determined using a spreadsheet macro available from Abraxis upon request. For manual evaluation, calculate the mean absorbance value for each of the standards (B_i). Calculate the $\%B_i/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance (B_0). Construct a standard curve by plotting the $\%B_i/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Cyclic Imines (13-desmethyl spirolide C) concentration on the horizontal logarithmic (x) axis on graph paper. $\%B_i/B_0$ for samples will then yield levels in ppb of Cyclic Imines (13-desmethyl spirolide C equivalents) by interpolation using the standard curve; final sample results are then obtained by multiplying the concentration determined by the curve by a factor of 10. Samples showing lower concentrations of Cyclic Imines compared to Standard 1 (8 ng/mL) should be reported as containing < 80 ng/mL of Cyclic Imines. Samples showing a higher concentration than Standard 4 (64 ng/mL) should be reported as containing > 640 ng/mL. If a quantitative result is necessary, samples must be diluted further and re-analyzed to obtain accurate results.