Importance of Anatoxin-a Determination

Anatoxin-a is an alkaloid neurotoxin produced by some species of cyanobacteria (blue-green algae). It is one of the most toxic of the cyanobacterial toxins. In humans and other animals, the skeletal neuromuscular junction constitutes a primary target for Anatoxin-a (Anatoxin-a can also cross the blood-brain barrier). 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 acetylcholinesterases, which are highly concentrated at the neuromuscular junction. Anatoxin-a functions as an agonist of nAChRs, like ACh, but is about 20 times more potent. Unlike ACh, it is not degraded by acetylcholinesterases and produces sustained depolarization of the muscle endplate, causing over stimulation of the muscles, leading to muscle fatigue and ultimately paralysis. Symptoms begin within 5 minutes of ingestion of Anatoxin-a and progress rapidly, resulting in cyanosis, convulsions, cardiac arrhythmia, and respiratory paralysis, which ultimately results in death due to suffocation.
Humans and other animals may be exposed to Anatoxin-a through ingestion of contaminated water, through drinking or during recreational activities in which water is swallowed. Due to the potential for serious harm and even death, many countries are expanding monitoring programs to include Anatoxin-a and are establishing regulations regarding the amount of Anatoxin-a in drinking and recreational waters. New Zealand is among those taking regulatory action, establishing a 6.0 µg/L provisional maximum acceptable value (MAV) for Anatoxin-a.
The ABRAXIS® Anatoxin-a ELISA can be performed in less than 90 minutes. Only a few milliliters of sample are required.

Performance Data

Test sensitivity: The detection limit, based on Anatoxin-a, (90% B/B_0) is approximately 0.1 ppb (µg/L). The middle of the test (50% B/B_0) is approximately 1.38 ppb (ng/mL). Determinations closer to the middle of the calibration curve give the most accurate results.

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 and Control should be ≤15%. The Control should be within its acceptable range and Standard 0 absorbance value should be between 0.8 - 3.000.

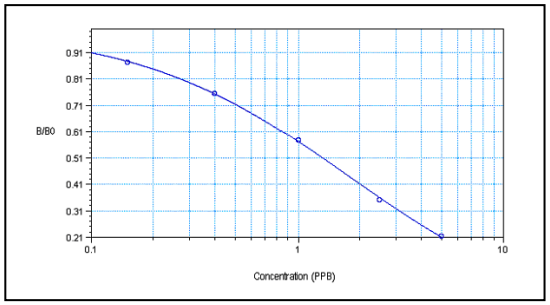
Recoveries:

Level (ppb)	% Recovery
0.25	103.3
0.50	98.0
1.50	104.4
3.00	103.1

Specificity: Cross-reactivity of the ABRAXIS® Anatoxin-a Plate Kit for various congeners:

(+)Anatoxin-a	100.0%
Homoanatoxin-a	124.8%
(-)Anatoxin-a	0.3%
Synthetic Dihydroanatoxin-a cis	1%

Standard Curve:



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

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*The monoclonal antibody and enzyme conjugate included in the ABRAXIS® Anatoxin-a ELISA have been licensed (Patent # 11,054,415) from the Spanish National Research Council (CSIC) and the University of Valencia (UVEG).

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GOLD STANDARD DIAGNOSTICS

ABRAXIS® Anatoxin-a ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Anatoxin-a* in Water and Contaminated Samples

Product No. 520060

1. General Description

The ABRAXIS® Anatoxin-a ELISA Plate Kit is an immunoassay for the quantitative and sensitive screening of Anatoxin-a. This test is suitable for the quantitative and/or qualitative screening of Anatoxin-a in drinking water, recreational water, or seawater samples (please refer to Sample Collection and Handling, section C); Please see the Gold Standard Diagnostics website for the Anatoxin-a and Saxitoxin in Benthic Mats technical bulletin for the sample preparation procedure for benthic mat samples. Samples requiring regulatory action should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Anatoxin-a. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted 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 ABRAXIS® Anatoxin-a ELISA Kit should 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. Consult state, local, and federal regulations for proper disposal of all reagents.

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Anatoxin-a by a monoclonal antibody. Anatoxin-a, when present in a sample, and an Anatoxin-a enzyme conjugate compete for the binding sites of mouse anti-Anatoxin-a antibodies in solution. The Anatoxin-a antibodies are then bound by a second antibody (anti-mouse) immobilized on the microtiter plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Anatoxin-a 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® Anatoxin-a ELISA, Possible Test Interference

Although many organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects cannot be completely excluded.
Immediately upon collection, freshwater samples must be preserved with the provided Sample Diluent (10X) Concentrate to prevent degradation of Anatoxin-a (please refer to Sample Collection and Handling, section C).
Anatoxin-a will degrade when exposed to natural or artificial light and/or high pH conditions. Samples that have been exposed to natural or artificial light and/or treated with reagents that raise the natural sample pH may produce results that are falsely low. Adjust sample pH to between pH 5 and pH 7 within 24 hours of collection. Protect samples from light.
Samples containing methanol must be diluted to a concentration < 2.5% methanol to avoid matrix effects.
Seawater samples up to 37 parts per thousand were tested and no matrix effects were detected. Average recovery of spiked seawater samples was 104%.
Anatoxin-a is an intracellular, as well as extracellular, toxin. Therefore, to measure total Anatoxin-a, cell lysing will be required. Once the sample is preserved, three freeze/thaw cycles are recommended for cell lysing.
No matrix effects have been observed with samples that have been treated with ascorbic acid at concentrations ≤ 1 mg/mL. ***Sodium thiosulfate should not be used to treat samples, as sodium thiosulfate will degrade Anatoxin-a, producing inaccurate (falsely low) results.***
Mistakes in handling the test can also cause errors. Possible sources for such errors include: 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 (lower than 10°C or higher than 30°C) during the test performance.
As with any analytical technique (GC/MS, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.

6. Working Instructions

A. Reagents and Materials Provided

1. Microtiter plate coated with a secondary antibody (anti-mouse), in a re-sealable aluminum pouch
2. Lyophilized Anatoxin-a-HRP Enzyme Conjugate, 3 vials
3. Conjugate Diluent, 12 mL
4. Lyophilized Anti-Anatoxin-a Antibody, 3 vials
5. Antibody Diluent, 12 mL
6 Empty clear and amber HDPE bottles for combining reconstituted Enzyme Conjugate and Antibody (if necessary)
7. (+)Anatoxin-a Standards (6): 0, 0.15, 0.40, 1.0, 2.5, 5.0 ppb, 1.5 mL each
8. Control at 0.75 ± 0.185 ppb, 1.5 mL
9. Sample Diluent (10X) Concentrate, 2 X 25 mL
10. ABRAXIS® Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section D)
11. Substrate (Color) Solution (TMB), 12 mL
12. Stop Solution, 12 mL (handle with care)

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 (10-300 µL), stepper pipette (10-300 µL), or electronic repeating pipette with disposable plastic tips (capable of delivering 50-300 µL)
3. Microtiter plate washer (optional)
4. Microtiter plate reader (wavelength 450 nm)
5. Deionized or distilled water
6. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section D)
7. Paper towels or equivalent absorbent material
8. Timer

C. Sample Collection and Handling

Collect water samples in amber glass sample containers. Drinking water samples should be treated with ascorbic acid (up to 1 mg/mL) immediately after collection to remove residual chlorine. *Do not use sodium thiosulfate. Sodium thiosulfate will degrade Anatoxin-a.*

Immediately upon collection, fresh water samples must be preserved using the Sample Diluent (10X) Concentrate (1 mL of 10X Sample Diluent Concentrate per 9 mL of water sample), to prevent degradation of Anatoxin-a. Samples must be adjusted to between pH 5 and pH 7 and protected from exposure to natural and artificial light, as exposure to light and/or high pH will cause degradation of Anatoxin-a. Sample pH must be adjusted within 24 hours of collection. Store samples refrigerated (up to 28 days). For storage periods greater than 28 days, samples should be stored frozen. Seawater samples do not need to be preserved but the same pH and storage conditions should be applied.

Drinking water samples treated with ascorbic acid (0.1 mg/mL) and sodium bisulfate (1 mg/mL) according to EPA Method 545 do not need to be preserved with Sample Diluent (10X) Concentrate. Samples must be adjusted to between pH 5 and pH 7. The same storage conditions (light, temperature, and duration) apply as described above.

Anatoxin-a is an intracellular, as well as extracellular, toxin. Therefore, to measure total Anatoxin-a, cell lysing will be required. Once the sample is preserved, three freeze/thaw cycles are recommended for cell lysing (the three freeze/thaw cycles will not degrade Anatoxin-a).

Preserved fresh water or seawater samples may be filtered following cell lysing and prior to analysis using any of the following syringe filters: Environmental Express 0.2 µm PES (PN SF020E), Pall Acrodisc® 0.2 µm PVDF (PN 4450), Supor® membrane syringe filters (PN 4612), or Environmental Express 1.2 µm Glass Fiber (PN SF012G). *Note: Fresh water samples must be preserved (and lysed) prior to filtration or Anatoxin-a may bind to the filter, removing it from the sample, and producing falsely low sample results.*

D. Test Preparation

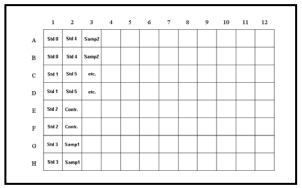
Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. A multi-channel, stepping, or electronic repeating pipette is recommended for adding the enzyme conjugate, antibody, substrate, and stop solutions in order to equalize the incubation periods across the entire microtiter plate. Please only use 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 use.
2 The enzyme conjugate and antibody must be reconstituted prior to use. Add 3 mL of the appropriate diluent to the appropriate 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 and the reconstituted antibody vials in the clear HDPE bottle prior to use.*** The solutions are stable for up to 2 weeks if stored at 2-8°C and up to 1 month if stored frozen.
3. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag with desiccant and zip-locked closed.
4. The standard solutions, substrate, 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. Dilute the Sample Diluent (10X) Concentrate at a ratio of 1:10 with deionized or distilled water (i.e. 1 mL of Sample Diluent (10X) Concentrate into 9 mL of deionized water) as needed for sample dilutions.
7. The stop solution must be handled with care as it contains diluted H_2SO_4.
8. After analysis, store the remaining kit components in the refrigerator (2-8°C).

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-Std5: Standards
Contr.: Control
Samp1, Samp2, etc: Samples



F. Assay Procedure

1. **Add 50 µL of the standard solutions, control, or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate 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.
3. **Add 50 µL of the reconstituted antibody 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 circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. **Incubate the strips for 60 minutes at room temperature.**
4. 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 four 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.
5. **Add 100 µL of substrate (color) 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 circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. **Incubate the strips for 20-30 minutes at room temperature.** Protect the strips from sunlight.
6. **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.
7. Read the absorbance at 450 nm using a microtiter plate 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 a 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 a vertical linear (y) axis versus the corresponding Anatoxin-a concentration on horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb of Anatoxin-a by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Gold Standard Diagnostics upon request.

Results for freshwater samples which have been preserved with Sample Diluent (10X) Concentrate as described in Sample Collection and Handling (section C) must be multiplied by a factor of 1.1 to account for the initial dilution.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Anatoxin-a than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Anatoxin-a (< 0.165 ppb for preserved water samples). Samples showing a higher concentration than standard 5 (5.0 ppb) should be reported as containing > 5.0 ppb of Anatoxin-a (> 5.5 ppb for preserved water samples) or must be diluted using 1X Sample Diluent to obtain accurate results. The concentration of the positive control provided should be 0.75 ± 0.185 ppb.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Anatoxin-a greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Anatoxin-a less than that standard.

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