Importance of Anabaenopeptins Determination

Most of the world's population relies on surface freshwaters as its primary source for drinking water. The drinking water industry is constantly challenged with surface water contaminants that must be removed to protect human health. Toxic cyanobacterial blooms are an emerging issue worldwide due to increased source water nutrient pollution caused by eutrophication.

Cyanobacterial harmful algal blooms occur in freshwater lakes, ponds, rivers, and reservoirs, and in brackish waters throughout the world. The wide variety of cyanotoxins and their congeners can lead to frequent exposure of humans through consumption of meat, fish, seafood, blue-green algal products and water, accidental ingestion of contaminated water and cyanobacterial scum during recreational activities, and inhalation of cyanobacterial aerosols. Cyanotoxins can also occur in the drinking water supply. In order to monitor human exposure, sensitive analytical methods such as enzyme linked immunosorbent assay (ELISA) are often used.

Anabaenopeptins (APs) are cyclic peptides comprised of a ring of five amino acid residues connected to an exocyclic residue through an ureido linkage. Anabaenopeptins were first isolated from the cyanobacteria Anabaena flos-aquae. To date at least 96 anabaenopeptins have been reported, the various congeners are structurally related. Planktothrix, Nodularia, Microcystis, Lyngbya, and Schizothrix, have also been reported as producers of anabaenopeptins. APs have been shown to be inhibitors of protein phosphatases and proteases.

Performance Data

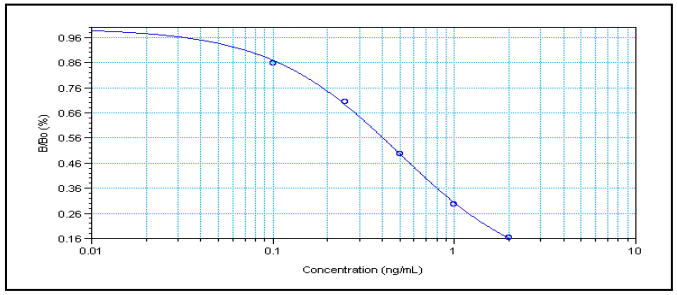
Test sensitivity: The Anabaenopeptins ELISA has an estimated detection limit (90% B/B_o) of 0.08 ppb (ng/mL). The middle of the test (50% B/B_o) is approximately 0.5 ppb. Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; for samples: <15%.

Selectivity: Cross-reactivity of the Abraxis Anabaenopeptins Plate Kit for various congeners:

Anabaenopeptin A	100.0%
Anabaenopeptin B	100.0%
Anabaenopeptin F	166.9%
Anabaenopeptin 872	53.9%
Ferintoic Acid A	133.1%

This ELISA does not recognize Cylindrospermopsin, Microcystin-LR, and Nodularins up to 1000 ppb.



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

Samples: Sample correlation between LC/MS, PPA, and ELISA methods showed a good correlation.

References

(1) Elisabeth Entfellner et al.,Evolution of Anabaenopeptin Peptide Structural Variability in the Cyanobacterium Planktothrix. *Frontiers in Microbiology*, Feb 2017, Vol. 8, Article 219.

(2) Herman Schreuder et al., Isolation, Co-Crystallization and Structure-Based Characterization of Anabaenopeptins as Highly Potent Inhibitors of Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFIa). *Sci. Rep.* **6**, 32958; doi: 10.1038/srep32958 (2016).

(3) Henrik Harms et al., *Bioorg. Med. Chem. Lett.* **26** (2016) 4960-4965.

(4) Linda Tonk et al., Production of cyanopeptolins, anabaenopeptins, and microcystins by the the harmful cyanobacteria *Anabaena* 90 and *Microcystis* PCC 7806. *Harmful Algae* **8** (2009) 219-224.

(5) Lisa Kamp et al., The effects of water sample treatment, preparation, and storage prior to cyanotoxin analysis for cylindrospermopsin, microcystin and saxitoxin. *Chemico-Biological Interactions* **246** (2016) 45-51.

†QuikLyse™ reagents may be used in a method of U.S. Patent 9,739,777

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R071118

Anabaenopeptins ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the
Determination of Anabaenopeptin in Water Samples

Product No. 520070

1. General Description

The Abraxis Anabaenopeptins ELISA is an immunoassay for the quantitative and sensitive detection of Anabaenopeptins in water samples. This test is suitable for the quantitative and/or qualitative detection of Anabaenopeptins in water samples (refer to Sample Collection and Handling, Section C). If necessary, positive samples can be confirmed by LC/MS, protein phosphatase assay, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Anabaenopeptins. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

3. Storage and Stability

The Anabaenopeptins ELISA 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. Consult state, local, and federal regulations for proper disposal of all reagents.

4. Test Principle

The test is a direct competitive ELISA for the detection of Anabaenopeptins. It is based on the recognition of Anabaenopeptins by specific antibodies. Toxin, when present in a sample, and an Anabaenopeptins-HRP analogue compete for the binding sites of the rabbit anti-Anabaenopeptins antibodies in solution. The anti-Anabaenopeptins antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the wells of the microtiter plate. After a washing step and an addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Anabaenopeptins 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 Anabaenopeptins ELISA, Possible Test Interference

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

Samples containing methanol must be diluted to a concentration < 5% methanol to avoid matrix effects.

No matrix effects have been observed with seawater samples.

No matrix effects have been observed with samples which have been treated with sodium thiosulfate at concentrations ≤ 1 mg/mL or ascorbic acid at concentrations ≤ 1 mg/mL.

Mistakes in handling the test can 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, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct or indirect sunlight.

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

A. Materials Provided

1. Microtiter plate (12 X 8 strips) coated with a secondary antibody (goat anti-rabbit)
2. Standards (6): 0, 0.10, 0.25, 0.50, 1.0, 2.0 ppb, 1 mL each
3. Control: 0.75 ± 0.185 ppb, 1 mL
4. Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank (LRB) and for dilution of samples above the range of the standard curve
5. Antibody Solution, 6 mL
6. Anabaenopeptins-HRP Enzyme Conjugate Solution, 6 mL
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use, see Test Preparation (Section E)
8. Substrate (Color) Solution (TMB), 16 mL
9. Stop Solution, 12 mL

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

1. Micro-pipettes with disposable plastic tips (20-200 µL)
2. Multi-channel pipette (50-300 µL), stepper pipette (50-300 µL), or electronic repeating pipette with disposable plastic tips
3. Deionized or distilled water
4. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section E)
5. Graduated cylinder
6. Paper towels or equivalent absorbent material
7. Timer
8. Tape or parafilm
9. Microtiter plate reader (wavelength 450 nm)
10. Microtiter plate washer (optional)

C. Sample Collection and Handling

Collect water samples in **glass or PETG** containers. Use of other types of plastic collection and/or storage containers may result in adsorptive loss of Anabaenopeptins, producing inaccurate (falsely low) results. Drinking water samples should be treated with sodium thiosulfate or ascorbic acid immediately after collection. If samples must be held for longer periods (up to 5 days), samples should be stored refrigerated. For storage periods greater than 5 days, samples should be stored frozen.

If Anabaenopeptins concentration (free and cell bound) is required, an appropriate cell lysing procedure (freeze and thaw, QuikLyse™†, 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. Please see the appropriate sample preparation technical bulletin (available upon request) for additional information on cell lysis.*

Samples may be filtered prior to analysis using glass fiber filters (Environmental Express 1.2 µm syringe filters (Environmental Express part number SF012G) are recommended). If determining Anabaenopeptins concentration, samples should be lysed prior to filtration to prevent the removal of cell-bound Anabaenopeptins, which would cause inaccurate (falsely low) results. *Note: The use of alternate filter types (non-glass fiber filters) may produce falsely low sample results, as Anabaenopeptins may bind to the filter material, removing it from the sample.*

D. Notes and Precautions

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary.

The use of a multi-channel, stepping, or electronic repeating pipette is recommended for the addition of the antibody, enzyme conjugate, substrate (color), 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, substrate (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.

E. Test Preparation

1. Allow the reagents and samples to reach ambient temperature before use.
2. Remove the number of microtiter plate strips required from the resealable pouch. The remaining strips are stored in the pouch with the desiccant (tightly sealed).
3. The standards, control, sample diluent, antibody, enzyme conjugate, substrate (color), and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer (5X) 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 and mix thoroughly.

F. 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	Samp2									
B	Std 0	Std 4	Samp2									
C	Std 1	Std 5	etc.									
D	Std 1	Std 5	etc.									
E	Std 2	Contr.										
F	Std 2	Contr.										
G	Std 3	Samp1										
H	Std 3	Samp1										

G. Assay Procedure

1. Add **50 µL of the standards, 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 HRP enzyme conjugate** solution to the individual wells successively using a multi-channel, stepping, or electronic repeating pipette.
3. Add **50 µL of the 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 30 seconds. Be careful not to spill the contents. Incubate the strips for **60 minutes** at room temperature.
4. 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. Please 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 **150 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 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 microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

H. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 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 the vertical linear (y) axis versus the corresponding Anabaenopeptins concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb of Anabaenopeptins by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Anabaenopeptins than standard 1 (0.10 ppb) should be reported as containing < 0.10 ppb of Anabaenopeptins. Samples showing a higher concentration than standard 5 (2.0 ppb) must be diluted using the provided Sample Diluent buffer 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 Anabaenopeptins greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Anabaenopeptins less than that standard.

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