Importance of Cylindrospermopsin 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. Cylindrospermopsin is a toxin produced by several different strains of cyanobacteria (blue-green algae) and has been found in fresh water throughout the world. Certain strains of *Cylindrospermopsis raciborskii* (found in Australia, Hungary, and the United States), *Umezakia natans* (found in Japan), and *Aphanizomenon ovalisporum* (found in Australia and Israel) have been found to produce Cylindrospermopsin. The production of Cylindrospermopsin seems to be strain specific rather than species specific.

Acute poisoning of humans and animals constitutes the most obvious problem from toxic cyanobacterial blooms and, in several cases, has led to death. Human exposure to Cylindrospermopsin can occur through ingestion of contaminated water or food (fish) or during recreational activities in which water is swallowed. Dermal contact with Cylindrospermopsin may occur during showering or bathing, or during recreational activities such as swimming or boating. These toxins mediate their toxicity by inhibiting liver function and are potent inhibitors of protein synthesis and glutathione, leading to cell death.

To protect against adverse health effects, the U.S. Environmental Protection Agency (EPA) has established guidelines for Cylindrospermosin in drinking water:

-For children pre-school age and younger (less than six years old), 0.7 µg/L (ppb)

-For school-age children and adults, 3.0 µg/L (ppb)

Performance Data

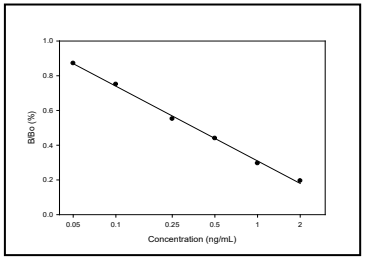
Test sensitivity: The detection limit for this assay is 0.040 ppb (µg/L).

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

Specificity: This ELISA recognizes Cylindrospermopsin and related compounds with varying degrees:

Cylindrospermopsin	100%
Deoxy-Cylindrospermopsin	112%
7-Epi-Cylindrospermopsin	157%

Standard Curve:



Samples: A sample correlation between the ELISA and HPLC methods showed a good correlation.

Recovery

Four (4) groundwater samples were spiked with various levels of Cylindrospermopsin and assayed using the Eurofins AbraxisCylindrospermopsin Assay:

Spike Level (ppb)	Mean (ppb)	Std.Dev. (ppb)	Recovery (%)
		Recovery	
0.1	0.101	0.019	101
0.25	0.269	0.026	108
0.5	0.514	0.038	103
1.0	0.982	0.113	98
Average			103

Precision

Control	1	2	3
Replicates	3	3	3
Days	3	3	3
n	9	9	9
Mean (ppb)	0.198	0.501	1.01
% CV (within assay)	6.2	4.3	5.2
% CV (between assay)	8.3	5.3	4.9

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Cylindrospermopsin ELISA (Microtiter Plate)

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Enzyme-Linked Immunosorbent Assay for the Determination of Cylindrospermopsin in Water

Product No. 522011

1. General Description

The Eurofins Abraxis Cylindrospermopsin ELISA is an immunoassay for the quantitative and sensitive detection of Cylindrospermopsin in water samples. No additional sample preparation is required prior to analysis. If necessary, positive samples can be confirmed by HPLC or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Cylindrospermopsin. 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 Cylindrospermopsin 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 for the detection of Cylindrospermopsin. It is based on the recognition of Cylindrospermopsin by specific antibodies. Cylindrospermopsin, when present in a sample, and a Cylindrospermopsin-HRP analogue compete for the binding sites of rabbit anti-Cylindrospermopsin antibodies in solution. The anti-Cylindrospermopsin antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the wells of 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 Cylindrospermopsin 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 Cylindrospermopsin 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.

The presence of the following substances were found to have no significant effect on the Cylindrospermopsin assay results: aluminum oxide, calcium chloride, calcium sulfate, manganese sulfate, magnesium sulfate, magnesium chloride, sodium chloride, and potassium phosphate up to 10,000 ppm; sodium thiosulfate, sodium nitrate, and zinc sulfate up to 1,000 ppm; humic acid and ferric sulfate up to 100 ppm; copper chloride up to 10 ppm; Lugol's solution up to 0.01%.

Samples containing methanol must be diluted to a concentration ≤ 20% methanol to avoid matrix effects.

Seawater samples must also be diluted to a concentration ≤ 20% to avoid matrix effects. Alternately, if a lower detection limit is required, interfering compounds can be removed from seawater or brackish water samples prior to analysis. Please see the Cylindrospermopsin in Brackish Water or Seawater Sample Preparation Technical Bulletin (available upon request).

No matrix effects have been observed with samples which have been treated with sodium thiosulfate or ascorbic acid at concentrations up to and including 1 mg/mL. Please see Sample Collection and Handling (Section C) for additional information on sample collection, preservation, and storage.

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).

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 second antibody (goat anti-rabbit)
2. Standards (7): 0, 0.05, 0.10, 0.25, 0.50, 1.0, 2.0 ppb, 1.5 mL each
3. Control: 0.75 ± 0.15 ppb, 1.5 mL, prepared from a secondary source, for use as a Quality Control Standard (QCS)
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. Cylindrospermopsin-HRP Conjugate Solution *(Vortex before use.)*, 6 mL
6. Antibody Solution (rabbit anti-Cylindrospermopsin), 6 mL
7. Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section E)
8. Substrate (Color) Solution (TMB), 12 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 (10-300 µL), stepper pipette (10-300 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)
10. Microtiter plate washer (optional)

C. Sample Collection and Handling

Water samples should be collected in glass, polyethylene terephthalate glycol (PETG), high density polyethylene (HDPE), polycarbonate (PC), polypropylene (PP), or polystyrene (PS) containers. Samples can be stored refrigerated for up to 5 days. If samples must be held for greater than 5 days, samples should be stored frozen.

Finished (treated) drinking water samples must be preserved (quenched) with sodium thiosulfate or ascorbic acid immediately after collection to remove residual chlorine. ***Samples can be quenched with sodium thiosulfate or ascorbic acid at concentrations up to and including 1 mg/mL (higher concentrations will cause interference with the assay).***

The quenching of residual chlorine is necessary for treated water samples only. Raw (untreated) drinking water samples (samples not treated with chlorine) do not require additional reagents at the time of collection.

The pH tolerance range of the assay for samples is between pH 4 and pH 11. Drinking water samples treated with ascorbic acid (0.1 mg/mL) and sodium bisulfate (1 mg/mL) according to EPA Method 545 will be below this range and must be adjusted (between pH 4 and pH 7 is recommended) to avoid interference with the assay.

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 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 package 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 closed).
3. The standards, control, sample diluent (LRB), antibody, enzyme conjugate, substrate, 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.
5. The stop solution must be handled with care as it contains diluted H_2SO_4.

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.

Std0-Std6: Standards
Contr.: Control (QCS)
LRB: Laboratory Reagent Blank
Samp1, Samp2, etc.: Samples

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

G. Assay Procedure

1. Add **50 µL of the standards, control (QCS), LRB, or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. ***Vortex the enzyme conjugate solution.*** Add **50 µL of the 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 **45 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 **four 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 **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 **30-45 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 Cylindrospermopsin concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control (QCS), LRB, and samples will then yield levels in ppb of Cylindrospermopsin by interpolation using the standard curve. Results can also be determined by using a spreadsheet macro available from Eurofins Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Cylindrospermopsin than standard 1 (0.05 ppb) should be reported as containing < 0.05 ppb of Cylindrospermopsin. Samples showing a higher concentration than standard 6 (2.0 ppb) must be diluted to obtain accurate results. The concentration of the positive control (QCS) provided should be 0.75 ± 0.15 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 Cylindrospermopsin greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Cylindrospermopsin less than that standard.

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

I. References

(1) Cylindrospermopsin, Review of Toxicological Literature. Prepared by Integrated Laboratory Systems for Scott Masten, National Institute of Health Sciences, RTP, NC. Contract Number N01-ES-65402, December 2000.