Importance of Microcystins/Nodularins 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. Microcystins and Nodularins are cyclic toxin peptides. Microcystins (of which there are many structural variants, or congeners) have been found in fresh water throughout the world. To date, approximately 80 variants of Microcystins have been isolated. The most common variant is Microcystin-LR. Other common Microcystin variants include LA, YR, RR, LF, and LW. These toxins are produced by many types of cyanobacteria (blue-green algae), including *Microcystis, Anabaena, Oscillatoria, Nostoc, Anabaenopsis,* and terrestrial *Hapalosiphon*. Nodularins are produced by the genus *Nodularia* and are found in marine and brackish water.

Acute poisoning of humans and animals constitutes the most obvious problem from toxic cyanobacterial blooms, and in several cases has lead to death. Human and animal exposure to these toxins occurs most frequently through ingestion of water, through drinking or during recreational activities in which water is swallowed. These toxins mediate their toxicity by inhibiting liver function and are potent inhibitors of the serine/threonine protein phosphatases, and therefore may act as tumor promoters.

To protect consumers from adverse health effects caused by these toxins, the World Health Organization (WHO) has proposed a provisional upper limit for Microcystin-LR of 1.0 ppb (µg/L) in drinking water. In June 2015, USEPA issued Health Advisories (HAs) for Microcystins in drinking water of 0.3 ppb for children pre-school age and younger (less than six years of age) and 1.6 ppb for school age children through adults.

Performance Data

Test sensitivity: The detection limit, based on Microcystins-LR, (90% B/B_0) is approximately 0.09 ppb (µg/L). The middle of the test (50% B/B_0) is approximately 0.719 ng/mL. Determinations closer to the middle of the calibration curve give the most accurate results.

Test reproducibility: Precision:

Control	1	2	3
Replicates	5	5	5
Days	3	3	3
n	15	15	15
Mean (ppb)	0.518	1.590	3.134
%CV (within assay)	11.1	8.0	11.3
%CV (between assay)	6.5	5.4	7.6

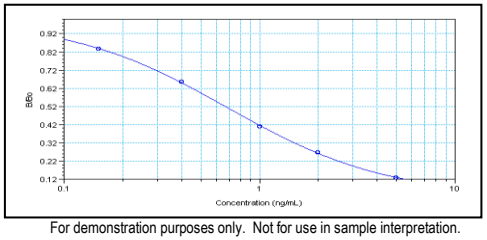
Recoveries:

Level	Mean	%CV	%Recovery
0.25	0.247	14.1	98.8
0.50	0.527	9.1	105.4
1.50	1.560	9.9	104.0
3.00	3.179	9.2	106.0

Specificity: Cross-reactivity of the Abraxis Microcystins Tube Kit for various congeners:

Microcystin-LR	100%
Nodularins	104%
Microcystin-RR	75%
Microcystin-LA	64%
Microcystin-LW	64%
Microcystin-YR	58%
Microcystin-LF	42%

Standard Curve:



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R062218

Microcystins Tube Kit

Enzyme-Linked Immunosorbent Assay for the Determination of Microcystins and Nodularins in Water Samples

Product No. 520012A

1. General Description

The Abraxis Microcystins Tube Kit is an immunoassay for the quantitative and sensitive screening of Microcystins and Nodularins in water samples. This test is suitable for the quantitative and/or qualitative screening of Microcystins and Nodularins in drinking and recreational water samples (please refer to Sample Collection and Handling, section C). 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 Microcystins. In addition, the substrate solution contains tetramethylbenzidine 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 Microcystins Tube 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 based on the recognition of Microcystins, Nodularins, and their congeners by specific antibodies (see Performance Data). The Microcystins, when present in a sample, and a Microcystins-enzyme conjugate compete for the binding sites of rabbit anti-Microcystins antibodies in solution. The Microcystins antibodies are then bound by a second antibody (anti-rabbit) immobilized on the test tube. 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 Microcystins 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 Microcystins Tube Kit, Possible Test Interference

Although many organic and inorganic compounds commonly found in 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.

Samples containing methanol must be diluted to a concentration < 40% 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 104.6%.

No matrix effects have been observed with samples that have been treated with sodium thiosulfate at concentrations ≤ 1 mg/mL.

Mistakes in handling the test also 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, 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, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.

A. Reagents and Materials Provided

1. 40 test tubes coated with a secondary antibody (anti-rabbit), in a resealable aluminum pouch
2. Microcystins-HRP Enzyme Conjugate, 25 mL
3. Anti-Microcystins Antibody Solution, 25 mL
4. Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 4 mL each
5. Control at 0.75 ± 0.19 ppb, 4 mL
6. HA Control at 0.3 ± 0.07 ppb, 4 mL
7. Wash Solution (100X) Concentrate, 25 mL, must be diluted before use, see Test Preparation (Section D)
8. Substrate (Color) Solution (TMB), 25 mL
9. Stop Solution, 25 mL (handle with care)

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

1. Micro-pipettes with disposable plastic tips (500 µL)
2. Stepper pipette with disposable plastic tips (12.5 mL-50 mL)
3. Test tube rack capable of holding 12 mm test tubes *securely*
4. Photometer capable of reading 12 mm tubes at 450 nm
5. Vortex mixer
6. Deionized or distilled water
7. Paper towels or equivalent absorbent material
8. Timer

C. Sample Collection and Handling

Collect water samples in glass or PETG containers and test within 24 hours. Drinking water samples should be treated with sodium thiosulfate (up to 1 mg/mL) immediately after collection to remove residual chlorine. 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 total Microcystins concentration (free and cell bound) is required, an appropriate cell lysing procedure (three freeze and thaw cycles are recommended) must be performed prior to analysis. *Note: The use of sonication in cell lysing can negatively affect toxin concentrations, producing falsely low sample results.*

Samples may be filtered prior to analysis using glass fiber filters (Environmental Express 1.2 µm syringe filters, part number SF012G, are recommended). If determining total Microcystins concentration, samples should be lysed prior to filtration to prevent the removal of cell-bound Microcystins, 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 Microcystins may bind to the filter material, removing it from the sample.*

D. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. In order to equalize the incubation periods across the entire assay, a stepping pipette is recommended for adding the enzyme conjugate, antibody, diluted wash, substrate, and stop solutions. Please only use the coated tubes, reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the coated test tubes, reagents, and samples to ambient temperature before use.
2. After removing the required number of coated test tubes from the aluminum pouch, seal the remaining test tubes in the pouch with the desiccant.
3. The conjugate, standard solutions, antibody, substrate and stop solutions are ready to use and do not require any further dilutions.
4. After adjusting to ambient temperature *(Note: Some salts may settle out upon refrigeration, but will redissolve when adjusted to ambient temperature and mixed thoroughly.)*, dilute the Wash Solution (100X) Concentrate at a ratio of 1:100 with deionized or distilled water (i.e., 1 mL of Wash Solution Concentrate added to 100 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.
6. After analysis, store the remaining kit components in the refrigerator (4-8°C).

E. Procedural Notes and Precautions

As with all immunoassays, a consistent technique is the key to optimal performance. To obtain the greatest precision, be sure to treat each tube in an identical manner.

Add reagents directly to the bottom of the tube while avoiding contact between the reagents and the pipette tip. This will help assure consistent quantities of reagent in the test mixture.

Avoid cross-contamination and carryover of reagents by using clean pipette tips for each standard/sample addition and by avoiding contact between reagent droplets on the tubes and pipette tips.

Avoid foam formation during vortexing.

F. Assay Procedure

1. Remove the required number of coated test tubes from the re-sealable aluminum pouch. Place the tubes in a rack capable of holding 12 mm test tubes *securely* and label appropriately. Analysis in duplicate is recommended.
2. Add **500 µL of enzyme conjugate solution** to the coated tubes successively using a stepping pipette.
3. Add **500 µL of the standard solutions, controls,** or **samples** into the appropriate coated tubes.
4. Add 500 µL of **antibody solution** to the coated tubes successively using a stepping pipette. Carefully vortex the tubes at a low speed for 1 to 2 seconds or swirl the tubes rapidly allowing the contents to mix and being careful not to spill or splash the contents.
5. Incubate the tubes for 20 minutes at room temperature.
6. Decant the contents of the tubes by vigorously shaking into a sink. Blot the inverted tubes on absorbent paper towels. Flood the tubes with **5 mL of diluted (1X) washing buffer solution**, decant by shaking vigorously into a sink, and blot the inverted tubes on absorbent paper towels. Repeat four times for a total of **five washes**.
7. Add **500 µL of substrate (color) solution** to the tubes. Carefully vortex the tubes at a low speed for 1 to 2 seconds or swirl the tubes rapidly, being careful not to spill or splash the contents.
8. Incubate the tubes for 20 minutes at room temperature. Protect the tubes from direct or indirect sunlight.
9. Add **500 µL of stop solution** to the tubes in the same sequence as for the substrate solution.
10. Read the absorbance at 450 nm using a test tube 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 such as 4-Parameter. Results can be determined using a spreadsheet macro (4-Parameter) available from Abraxis upon request. 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 Microcystins concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb (ng/mL) of Microcystins by interpolation using the standard curve.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Microcystins. Samples showing a higher concentration than standard 5 (5.0 ppb) should be reported as containing > 5.0 ppb Microcystins or must be diluted to obtain accurate results. Samples maybe diluted in deionized or distilled water if necessary.

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 Microcystins greater than the concentration of that standard. Samples with higher absorbances than a standard will have concentrations of Microcystins less than that standard.

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