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 250 variants of Microcystin have been isolated. The most common variant is Microcystin-LR. Other common Microcystin variants include YR, RR, 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 led 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.

Performance Data

Test sensitivity: The detection limit for this assay, based on MC-LR, is 0.10 ppb (µg/L).

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.

Selectivity: The assay exhibits very good cross-reactivity with all cyanobacterial cyclic peptide toxin congeners tested to date.

Samples Recoveries and Assay Precision:

Recovery

Four (4) groundwater samples were spiked with various levels of Microcystin-LR and assayed using the ABRAXIS® Microcystins (ADDA)-DM Assay:

Spike Level (ppb)	Recovery Mean (ppb)	Recovery Std.Dev. (ppb)	Recovery (%)
0.25	0.237	0.019	95
0.50	0.480	0.036	96
1.0	0.959	0.024	96
2.0	1.919	0.067	96
Average			96

Precision

Control	1	2	3
Replicates	5	5	5
Days	3	3	3
n	15	15	15
Mean (ppb)	0.25	0.99	2.9
% CV (within)	5.3	3.6	3.0
% CV (between)	6.5	5.4	4.0

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

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

References

(1) A. Zeck, M. G. Weller, D. Bursill, R. Niessner: Genetic Microcystin Immunoassay Based on Monoclonal Antibodies Against Adda. Analyst 126(11), 2001, 2002-2007.
(2) Meriluoto J, Spoof L, Codd GA. Handbook of Cyanobacterial Monitoring and Cyanotoxin Analysis. Chichester, UK: John Wiley & Sons; 2016.
(3) Worldwide Patenting PCT WO 01/18059 A2.
(4) U.S. Patent Number 6,967,240.
(5) U.S. Patent Number 9,739,777.

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Date these instructions are effective : 05/01/2025

Version: 05

ABRAXIS® Microcystins (ADDA)-DM ELISA Microtiter Plate

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

Product No. 522015

1. General Description

The ABRAXIS® Microcystins (ADDA)-DM (direct monoclonal) ELISA is an immunoassay for the quantitative and sensitive detection of Microcystins and Nodularins in water samples, benthic mats, and shellfish (bivalves). This test is suitable for the quantitative and/or qualitative detection of Microcystins and Nodularins in water samples **[please refer to the appropriate technical bulletins for sample collection, handling, and treatment of drinking (treated and untreated) and recreational water samples]**. Please see section D, below, for the sample preparation procedure for bivalves and the Cylindrospermopsin and Microcystins in Benthic Mats technical bulletin for the sample preparation procedure for benthic mat samples. If necessary, positive samples can be confirmed by HPLC, protein phosphatase assay, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Microcystins. 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 ABRAXIS® Microcystins (ADDA)-DM 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 Microcystins and Nodularins. It is based on the recognition of Microcystins, Nodularins, and their congeners by a monoclonal antibody. Toxin, when present in a sample, and a Microcystins-HRP analogue compete for the binding sites of anti-Microcystins antibodies in solution. The anti-Microcystins antibodies are then bound by a second antibody (goat anti-mouse) 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 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 ABRAXIS® Microcystins (ADDA)-DM 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 might 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 Microcystins (ADDA)-DM assay results: calcium sulfate, magnesium sulfate, sodium chloride, magnesium chloride, sodium nitrate, potassium phosphate, calcium chloride, manganese sulfate, and aluminum oxide up to 10,000 ppm; copper chloride, sodium fluoride, sodium thiosulfate, ferric sulfate, and zinc sulfate up to 1,000 ppm; humic acid 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 Microcystins in Brackish Water or Seawater Sample Preparation for the ABRAXIS® Microcystins (ADDA)-DM ELISA Technical Bulletin (available upon request).

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

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

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

6. Working Instructions

A. Materials Provided

1. Microtiter plate (12 X 8 strips) coated with a second antibody (goat anti-mouse)
2. Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 1.5 mL each
3. Control: 0.75 ± 0.185 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. Microcystins-HRP Conjugate Solution, 6 mL
6. Microcystins-DM Antibody Solution (monoclonal anti-Microcystins), 6 mL
7. ABRAXIS® 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. Paper towels or equivalent absorbent material
6. Timer
7. Tape or parafilm
8. Microtiter plate reader (wavelength 450 nm)
9. Microtiter plate washer (optional)

C. Water Sample Collection and Handling

Collect water samples in **glass or PETG** containers and test within 24 hours. Use of other types of plastic containers may result in adsorptive loss of Microcystins, producing inaccurate (falsely low) results. Drinking water samples should be treated with sodium thiosulfate immediately after collection (refer to appropriate technical bulletin). 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 (freeze and thaw, sonication, ABRAXIS® 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 for additional information on cell lysis.*

Samples may be filtered prior to analysis using glass fiber filters. 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. Also, please note that some glass fiber filters are manufactured using a process which may cause interference which would cause inaccurate (falsely high) results. To avoid this potential bias in sample results, a total volume of at least 10 mL should be passed through the glass fiber filter, with the first 5 mL of filtered sample being discarded and the second 5 mL collected for testing (please see the Microcystins sample filtration technical bulletin for additional information on sample filtration). 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.

D. Bivalve Sample Preparation (3.75 ng/g to 125 ng/g range of detection in bivalve tissues)

Additional materials required: 60% methanol solution (add 120 mL of methanol to 80 mL deionized or distilled water, mix thoroughly), micropipette (200-1000 µL) with tips, scale, temperature controlled water bath (60°C), 10 mL disposable syringes, 0.45 µm PES membrane with pre-filter syringe filters (Environmental Express PN SF145E only; GSD PN 706045), centrifuge capable of 3000 rpm, glass vials with Teflon-lined caps (4 mL and 20 mL), 10 mL glass graduated cylinder, vortex mixer, blender.

Note: *Microcystins may bind to plastic; minimize extract contact with any plastic materials.*

1. Homogenize shucked, uncooked bivalve sample with blender. (Blender must be thoroughly cleaned between samples to avoid cross-contamination which may cause inaccurate sample results.)
2. Weigh 1 g of sample into 20 mL glass vial.
3. Add 2 mL of 60% methanol to vial. Vortex thoroughly. Place vial in 60°C water bath for 30 minutes.
4. Centrifuge sample at 3000 rpm for 10 minutes.
5. Transfer supernatant to graduated cylinder. (Graduated cylinder must be thoroughly cleaned between samples.)
6. Repeat steps 3 through 5 once, pooling the two supernatants in the graduated cylinder. Use 60% methanol to bring extract to 5 mL final volume (for 1:5 sample extraction/dilution). Vortex thoroughly.
7. Filter extract into a clean glass vial using syringe filter. Extract may be stored frozen for later analysis or diluted for analysis by adding 200 µL of sample extract to 800 µL of sample diluent (an additional 1:5 dilution, for a 1:25 total sample dilution in the final extract) and vortex thoroughly. (***Note:*** Extracts must be at the final 1:25 dilution prior to analysis to remove matrix interference; analysis of sample extracts at lesser dilutions will produce inaccurate results.)

E. Notes and Precautions

Micro-pipetting equipment and pipette tips for pipetting the standards and 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, 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 only use the reagents and standards from one kit lot in one test, as they have been adjusted in combination.

F. 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), enzyme conjugate, antibody, substrate, and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the ABRAXIS® 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.

G. 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-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										

H. Assay Procedure

1. **Add 100 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 conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. **Add 50 µL of the antibody solution** to the individual wells successively using a multi-channel pipette or a stepping 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 90 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 pipette or a stepping 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 pipette or a stepping pipette.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

I. 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 of 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 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 of Microcystins by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Gold Standard Diagnostics upon request. Bivalve sample results must be multiplied by a factor of 25 to account for the sample extraction/dilution.

The concentrations of samples are determined using the standard curve run with each test. Water samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Microcystins. Bivalve samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing <3.75 ng/g of Microcystins. Water or bivalve samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted and reanalyzed to obtain accurate quantitative results. The concentration of the positive control provided should be 0.75 ± 0.185 ppb.