Importance of Microcystins/Nodularins Determination

Most of the world's population relies on surface freshwaters as its primary source of drinking water. The drinking water industry is constantly challenged with surface water contaminants that must be removed to protect human health. Toxic blooms of cyanobacteria (blue-green algae) are an emerging issue in the U.S. and the world because of increased source water nutrient pollution caused by eutrophication. Microcystins and Nodularins are cyclic toxin peptides. Microcystins (several structural variants or congeners are found) have been found in fresh water throughout the world and are produced by the genus *Microcystis, Anabaena, Oscillatoria, Nostoc, Anabaenopsis,* and terrestrial *Hapalosiphon.* Nodularins are produced by the genus *Nodularia* and are found in marine and brackish water. To date, approximately 80 variants of microcystin have been isolated. The most common variant is microcystin-LR. Other common microcystin variants include LA, YR, RR, and LW.

Acute poisoning of humans and animals constitutes the most obvious problem from toxic cyanobacterial blooms, and in several cases has lead to death. These toxins mediate their toxicity by inhibiting liver function and are potent inhibitors of the serine/threonine protein phosphatases; therefore they may act as tumor promoters. To protect consumers from adverse health effects caused by these toxins, the WHO has proposed a provisional upper limit for microcystin-LR of 1.0 ppb (ng/L) in drinking water.

Human poisonings have often been suspected in the past but not confirmed due to lack of appropriate analytical techniques. In 1996, an episode of human intoxication by microcystins was first confirmed by Azevedo et al., after an outbreak of acute liver failure that resulted in the deaths of 76 patients at two dialysis centers in Caruaru, Brazil.

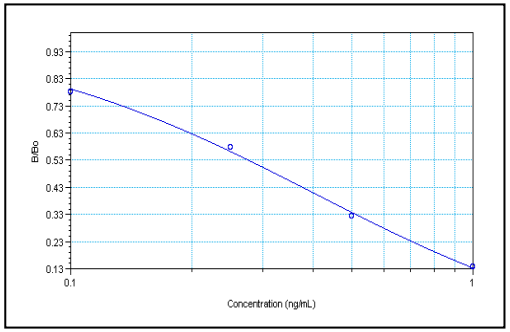
Performance Data

Test sensitivity: The detection limit for this assay based on MC-LR in serum is 0.40 ppb (µg/L).

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

Serum Recovery: Sample recovery: 70-130%.

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



Specificity

The cross-reactivity of the Abraxis Microcystins ELISA for various Microcystins congeners:

Compound	X-reactivity (%)
Microcystin-LR	100
dmMC-LR	104
Microcystin-LW	102
Microcystin-LY	92
Nodularins	78
Microcystin-YR	76
Microcystin-LF	72
RM-[Leu1]MCLY	68
Microcystin-RR	67
Microcystin-LA	66
N-hemi-ADDA	38
ADDA	15
D-Phenylalanine	NR
L-Phenylalanine	NR
DL-Phenylalanine	NR

NR = no reactivity up to 1000 ppb

References

(1) A. Zeck, M.G. Weller, D. Bursill, R. Niessner: Generic Microcystin Immunoassay Based on Monoclonal Antibodies Against Adda. Analyst 126(11), 2001, 2002-2007.
(2) Worldwide Patenting PCT WO 01/18059 A2.
(3) U.S. Patent Number 6,967,240.
(4) J. Chen, P. Xie, L. Li, J. Xu: First Identification of the Hepatotoxic Microcystins in the Serum of a Chronically Exposed Human Population Together with Indication of Hepatocellular Damage. Toxicological Sciences. 108(1), 2009, 81-89.

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R031317

Microcystins ELISA for Serum (Microtiter Plate)

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

Product No. 522031

1. General Description

The Abraxis Microcystins ELISA for Serum is an immunoassay for the quantitative and sensitive detection of Microcystins and Nodularins in human serum samples. For additional serum types (canine, etc.), please see the appropriate technical bulletin. A sample preparation and dilution is required prior to analysis. Positive samples should be confirmed by HPLC, protein phosphatase assay, or other conventional methods.

NOTE: This assay is intended for Research Use Only and not for in vitro diagnostic use.

2. Safety Instructions

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 Microcystins ELISA for Serum 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 which detects the presence of Microcystins and Nodularins in human serum. It is based on the recognition of Microcystins, Nodularins, and their congeners by a monoclonal antibody. Microcystins, Nodularins, and their congeners, when present in a sample, and a Microcystins-HRP analogue compete for the binding sites of anti-Microcystins antibodies in solution. The Microcystins antibodies are then bound by a second antibody (goat anti-mouse) immobilized on the plate. After a washing step and the addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of the Microcystins/Nodularins 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 ELISA, Possible Test Interference

Only serum samples should be analyzed in the Microcystins ELISA for Serum. Plasma samples should not be used, as interference from clotting factors found in plasma will cause inaccurate results.

Due to the high variability of compounds that might be found in human serum samples, test interferences caused by matrix effects cannot be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources of such errors can include:
Inadequate storage conditions of the test kit, wrong 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 sunlight.

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

A. Materials Provided

1. Microtiter Plate coated with a second antibody (goat anti-mouse)
2. Serum Matrix Microcystins Standards (5): 0, 0.1, 0.25, 0.5, 1.0 ppb, 1 mL each
3. Serum Treatment Reagent A, 30 mL
4. Serum Treatment Reagent B, 1 mL
5. Monoclonal Anti-Microcystins in Serum Antibody Solution, 6 mL
6. Microcystins in Serum-HRP Conjugate Solution, 6 mL
7. Sample Diluent, 30 mL, for dilution of samples (see Sample Preparation, Section E)
8. Wash Solution (5X) Concentrate, 100 mL, must be diluted prior to use (see Test Preparation, Section F)
9. Color Solution (TMB), 16 mL
10. Stop Solution, 12 mL

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

1. Micro-pipettes with disposable plastic tips (50-250 µL)
2. Multi-channel pipette (50-300 µL), stepper pipette (50-300 µL), or electronic repeating pipette with disposable plastic tips
3. 4 mL glass vials with Teflon-lined caps
4. Microcentrifuge Tubes
5. Microcentrifuge
6. Overhead tube rotator or equivalent
7. Microtiter plate washer (optional)
8. Microtiter plate reader (wavelength 450 nm)

C. Sample Collection and Handling

Microcystins will bind to many types of plastic, therefore, **blood samples must be collected in glass or PETG collection tubes**. Use of plastic collection tubes other than PETG may result in adsorptive loss of Microcystins, producing inaccurate (falsely low) results. Blood samples which are collected using plastic collection tubes other than PETG should be transferred into glass or PETG containers immediately after collection; allowing blood samples to remain in non-PETG plastic vials for preparation of the serum fraction may result in loss of analyte and should be avoided in order to obtain accurate results.

As sample extraction requires the use of a plastic microcentrifuge tube, to which analyte could bind with prolonged exposure, it is recommended that after transferring samples into the microcentrifuge tubes, they are placed directly into the centrifuge and, once centrifuging is completed, the supernatant is immediately transferred into a clean glass vial.

D. 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 of the standard solutions and samples 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 use only the reagents and standards from one kit lot in one test, as they have been adjusted in combination. Carefully read and understand the instructions and precautions given in this insert before proceeding.

E. Sample Preparation

1. Add 500 µL of serum sample to an appropriately labeled 4 mL glass vial.
2. Add 500 µL of Serum Treatment Reagent A.
3. Add 20 µL of Serum Treatment Reagent B. Vortex thoroughly. Mix using an overhead tube rotator for 15 minutes.
4. Transfer treated sample to an appropriately labeled microcentrifuge tube and immediately centrifuge for 10 minutes at 10,000 g. A waxy precipitate will be visible at the bottom of the microcentrifuge tube and the supernatant should be clear (although not colorless). If the supernatant is not clear, centrifuge for an additional 10 minutes. Immediately pipette the supernatant into a clean, appropriately labeled 4 mL glass vial.
   Note: Contact time between samples and plastic microcentrifuge tubes should be minimized to prevent adsorptive loss of analyte which would produce inaccurate (falsely low) results (see section D, Notes and Precautions, for additional information on appropriate sample containers).
5. Add 250 µL of Sample Diluent to a second clean 4 mL glass vial. Add 250 µL of the treated serum to the Sample Diluent. Vortex thoroughly. The sample is then ready for analysis (see Assay Procedure, Section H).

The ELISA result will be multiplied by a factor of 4 to obtain the final Microcystins concentration in the sample. Samples showing lower concentrations than standard 1 (0.1 ppb) should not be multiplied by the factor but should be reported as containing < 0.4 ppb. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted and re-analyzed.

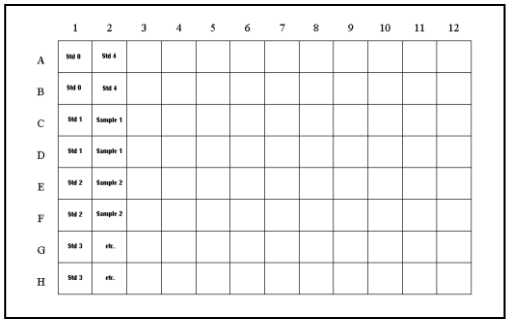
F. Test Preparation

1. Allow the microtiter plate and the reagents to reach room 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, antibody, enzyme conjugate, substrate and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Solution (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-Sd4: Standards
Sample 1, Sample 2, etc.: Samples



H. Assay Procedure

1. Add 100 µL of the standard solutions and treated 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 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 for 30 minutes at room temperature.
3. Add 50 µL of the conjugate 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 for 90 minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips three times using the 1X washing buffer solution. Please use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 150 µL of color solution to the wells 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 for 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 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 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 samples will then yield levels in ppb of Microcystins by interpolation using the standard curve. These values must then be multiplied by a factor of 4 to obtain the final Microcystins concentration. 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 lower concentrations of Microcystins than Standard 1 (0.1 ppb) should not be multiplied by the factor but should be reported as containing < 0.4 ppb. Samples showing a higher concentration than Standard 4 (1.0 ppb) must be diluted further to obtain accurate results.