# Ohio EPA Total (Extracellular and Intracellular) Microcystins - ADDA by ELISA Analytical Methodology

Version ~~2.0~~2.1

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## 1. SCOPE AND APPLICATION

This method is used for the determination of total (extracellular and intracellular) microcystins – ADDA in surface water, ground water and finished drinking water.

Reporting Limit (RL): 0.30 µg/L

## 2. SUMMARY OF METHOD

The Ohio EPA Total (Extracellular and Intracellular) Microcystins – ADDA by ELISA Analytical Methodology[1] is an immunoassay for the detection of microcystins in water samples. This test is an indirect competitive ELISA allowing the congener-independent detection of microcystins and nodularins. It is based on the recognition of microcystins, nodularins and their congeners by specific antibodies. Microcystins, nodularins and their congeners when present in a sample and a microcystin-protein analogue immobilized on the plate compete for the binding sites of antibodies in solution. 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 the microcystins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using a microplate reader at 450 nm.

## 3. SAMPLE COLLECTION AND PRESERVATION

3.1 A minimum of 100 mL should be collected in a glass or polyethylene terephthalate glycol (PETG) container.

**NOTE (1)**: Samples treated with chlorine or any other oxidizer (e.g. $KMnO_4$, $NaMnO_4$) must be quenched ~~with~~. Typically, 10 mg sodium thiosulfate or ascorbic acid added per 100 mL of sample during collection ~~sufficent~~is sufficient.

**NOTE (2)**: Cleaning of approved sample collection containers is acceptable as long as the laboratory can demonstrate effectiveness of the cleaning procedure by collecting and analyzing reagent water in 5% per batch of the cleaned containers. The reagent water results must be less than the reporting limit. The laboratory must maintain these records.

3.2 All samples must be protected from sunlight and cooled on ice at 0-4°C immediately after collection and maintained at 0-4º C until analysis. Samples must be analyzed within 5 days from the time of collection. Holding time can be increased by freezing the sample within 5 days of collection. When freezing, allow adequate volume for expansion and place the sample container on its side to prevent breakage.

3.3 Field Duplicate (FD) Sample Collection: Collect enough volume in a container to split into two separate sample containers of at least 100 mL. Vigorously shake the initial volume of sample prior to pouring it into the two separate collection containers. All quenching requirements must be followed (NOTE 1).

## 4. INTERFERENCES

Due to the high variability of compounds found in water samples, test interferences caused by

matrix effects cannot be completely ruled out. Ohio EPA continues to work with U.S. EPA and other experts to identify and provide more guidance on potential interferences.

## 5. SAFETY

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.

5.2 Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of ~~Material~~ Safety Data Sheets (~~M~~SDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

## 6. APPARATUS

6.1 Class 'A' Volumetric Flask: 500 mL

~~6.2 Log books~~

6.~~3~~2 Micropipette ~~with disposable plastic tips:~~capable of 10 to 100 µL

6.~~4~~3 Multi-channel pipette: 50-300 µL

6.~~5~~4 Stepping pipette: 100-500 µL (optional)

6.~~6~~5 Pipette tips~~: 1-200 µL~~ appropriate for the pipette

6.~~7~~6 Multi-channel pipette reagent reservoir minimum 50 mL capacity

6.~~8~~7 Microplate reader capable of analyzing at 450 nm

6.~~9~~8 Glass vials: 4 mL

6.~~1~~09 Glass vials: 40 mL

6.1~~1~~0 Syringe filters: 0.45 µm pore size (Glass Fiber)

6.1~~2~~1 ~~Glass Gastight~~ Luer-lock syringes: 5 mL

6.1~~3~~2 ELISA sealing Teflon tape

6.1~~4~~3 Freezer

6.1~~5~~4 Refrigerator capable of 4ºC

6.1~~6~~5 Chlorine meter

6.1~~7~~6 pH Test Strips (pH 0 – 14)

6.1~~8~~7 Hydrochloric Acid Solution (0.1N)

6.1~~9~~8 Sodium Hydroxide Solution (0.1N)

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6.~~20~~19 Water Bath capable of ~~(~~35ºC~~)~~

6.20 Automated assay system (optional)

## 7. REAGENTS

7.1 Liquid disinfectant

7.2 Analysis Kit (capable of analyzing all microcystin congeners with the ADDA structure). Store kit according to manufacturer's instructions. Standards and reagents may be used until the manufacturer's expiration date ~~on the box~~.

7.3 DPD free chlorine reagent

7.4 Dechlorination agent: Sodium thiosulfate or Ascorbic acid

7.5 Reagent Water: Water free of contaminants

7.6 Saturated sodium chloride solution (optional): Dissolve 300 g of sodium chloride in 900 mL reagent water.

## 8. SAMPLE PREPARATION

8.1 Disinfect the work area.

8.2 Sample pH must be adjusted within the range of 5-11. Samples with pH levels outside of this range may produce inaccurate (falsely low) results and must be adjusted as necessary using hydrochloric acid (HCl) or sodium hydroxide (NaOH) solutions, prior to analysis.

**NOTE (3):** Pour off a portion of sample and use it to test pH.

8.3 Samples treated with chlorine: Check the sample for residual chlorine. If residual chlorine is greater than 0.1 mg/L, add 10 mg of quenching agent (sodium thiosulfate or ascorbic acid) per 100 mL of sample. Samples treated with any other oxidizer must also be checked for sufficient quenching. Any sample not sufficiently quenched must be appropriately qualified (CL) and noted in the final report. For all qualifier definitions, see Section 13.

## 9. SAMPLE LYSING PROCEDURE BY FREEZE/THAW

9.1 Shake the sample and pour 20 mL of the sample into two separate 40 mL vials to begin the three freeze/thaw lysing cycles. The second 40 mL vial may be analyzed as a laboratory duplicate (LD). Otherwise store second vial as needed for future analysis.

9.2 Place vials in the freezer until completely frozen (To speed up the process, vial(s) may be immersed in a saturated sodium chloride solution or dry ice).

9.3 Once sample is completely frozen, remove from freezer and thaw (To speed up the process, vial(s) may be immersed in a 35ºC water bath until it is completely thawed).

9.4 Repeat steps 9.2 and 9.3 two more times.

9.5 Filter 2 mL of sample into two 5 mL vials. Samples are ready for immediate analysis.



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Freeze remainder of samples on their side, to be analyzed at a later date.

**NOTE (4):** If sample is frozen to extend hold time, once the sample is thawed it has undergone the first freeze/thaw cycle.

## 10. INITIAL DEMONSTRATION OF CAPABILITY

A Method Detection Limit (MDL) study for the Microcystins – ADDA test kit must be calculated annually by each analyst, when a new analyst begins work or whenever a change in analytical performance caused by either a change in instrument hardware or operating conditions would dictate the MDL must be recalculated.

In order for a laboratory to maintain a reporting limit of at least 0.30 µg/L, each analyst must demonstrate the capability to achieve an MDL of < 0.30 µg/L.

MDLs must be established for microcystins using a standard with a concentration one to ten times the reporting limit (0.30 µg/L). To calculate the MDL value, take seven replicate aliquots of the standard and process them through the entire analytical method. Once the results for the seven replicates have been obtained, calculate the MDL as follows:

$$MDL = (t)*(SD_R)$$

Where: t = Student's t value for a 99% confidence interval and a standard deviation estimate with n-1 degrees of freedom (t = 3.143 for the seven replicates)

$SD_R$ = Standard deviation of the replicate aliquot analyses

The MDL study will be valid if the resulting value of the MDL is no more than ten times lower than the replicate standard concentration level and does not exceed the replicate standard concentration level. Save and print a copy of each MDL study as part of the laboratory's record maintenance protocol.

**NOTE (5):** Ohio EPA can provide additional guidance on completing an MDL study and all calculations upon request.

## 11. ANALYSIS

The accuracy of ELISA analysis is highly dependent upon analyst technique, adequate storage conditions of the test kit, pipetting sequence, accuracy of reagent volumes and maintenance of constant/optimum laboratory temperature during the analysis. The ELISA analysis is a time sensitive procedure. Care must be taken to ensure the reagent addition steps are completed in an efficient manner and incubation times are followed according to manufacturer's instructions.

**NOTE (6):** The assay procedure must be performed away from direct sun light.

11.1 Verify kit standards and reagents are used prior to the expiration date.

11.2 Bring samples and standards to room temperature prior to analysis.

11.3 Follow manufacturer's instructions provided with the individual Microcystins – ADDA kit for calibration, quality control (QC) and sample analysis procedures.

11.4 Sample analyses resulting in a higher concentration than the highest standard in the calibration curve must be diluted within the calibration range and reanalyzed to obtain

accurate results. If a sample is diluted, the final values must be calculated by multiplying the result by the proper dilution factor. Samples may not be diluted in the well plate.

11.5 Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol.

## 12. QUALITY CONTROL (QC) AND DATA REPORTING

The Ohio EPA requires at a minimum the following program specific analytical QC requirements be met.

12.1 Analyze all calibration standards, QC standards and samples in at least two well replicates. The mean of the well replicates must be used in all analytical calculations and reporting of sample results.

12.2 The curve generation must include a calibration concentration point ≤ 0.30 µg/L (RL).

12.3 Calibration curve Correlation Coefficient (R) must be > 0.990 or calibration curve Coefficient of Determination ($R^2$) > 0.980 to be acceptable.

12.4 Coefficient of Variation (%CV) for well replicate absorbance values for calibration standards and QC standards should be < 10%. If %CV for more than one calibration standard is > 10%, the analytical run is not acceptable. Corrective action and reanalysis of the sample batch is required.

**NOTE (7):** An analytical run may be accepted if %CV for only one calibration standard is > 10% but < 15% as long as all other calibration standards in the analytical run are < 10%.

Calculate %CV as follows:

$\%CV = (SD_A/Mean_A)*100$

Where: $SD_A$ = Standard deviation of well replicate absorbances
$Mean_A$ = Mean of well replicate absorbances

12.5 %CV for replicate absorbance values for samples must be < 15%, if the value is > 15% then reanalyze or qualify the results with the appropriate qualifier (J) and noted in the final report.

12.6 Laboratory Reagent Blank (LRB)/Diluent Blank: An LRB must be analyzed with each batch of samples to verify laboratory and reagents are free of contaminants. Values exceeding the reporting limit require corrective action and reanalysis of the sample batch. If reanalysis is not possible, the results must be appropriately qualified (B) and noted in the final report.

12.7 Low Calibration Range Check (LCRC): An LCRC must be analyzed with each batch of samples to verify accuracy of the calibration curve near the reporting limit. The LCRC may be one of the curve calibration points and the concentration must be ≥ 0.30 µg/L and ≤ 0.50 µg/L. Acceptance limits must be within ±40% of the true value. LCRC values exceeding the acceptance limits require corrective action and reanalysis of the sample batch. If reanalysis is not possible, all sample concentration results less than an acceptable QCS analyzed in the same batch must be appropriately qualified (J) and noted in the final report.

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12.8 Quality Control Standard (QCS): A secondary source QCS must be analyzed with each batch of samples to verify the concentration of the calibration curve. If a QCS is already included in the kit, it may be used if it has a different lot number than the calibration standards and was prepared from a separate primary stock. Acceptance limits must be within ±25% of true value. QCS values exceeding the acceptance limits require corrective action and reanalysis of the sample batch. If reanalysis is not possible, the results must be appropriately qualified (J) and noted in the final report.

12.9 Laboratory Duplicate (LD): LDs must be analyzed on 5% of samples in any analytical batch and both results must be reported to the Ohio EPA. The Relative Percent Difference (RPD) must be calculated and records must be maintained.

Calculate RPD as follows:

$RPD = [(D_2 – D_1)/D_{Avg}]*100$

Where: $D_1$ = Laboratory Duplicate 1
$D_2$ = Laboratory Duplicate 2
$D_{Avg}$ = Average of Laboratory Duplicates 1and 2

12.10 Field Duplicates (FD): FDs should be analyzed on 5% of samples in any analytical batch and both results should be reported. The RPD must be calculated and records must be maintained.

Calculate RPD as follows:

$RPD = [(D_2 – D_1)/D_{Avg}]*100$

Where: $D_1$ = Field Duplicate 1
$D_2$ = Field Duplicate 2
$D_{Avg}$ = Average of Field Duplicates 1 and 2

12.11 Samples not analyzed within the required holding time should be recollected. If recollection is not possible, the results must be appropriately qualified (PT) and noted in the final report.

12.12 Samples received with residual chlorine concentrations greater than the acceptable limit indicate ineffective quenching and should be recollected. If recollection is not possible, the results must be appropriately qualified (CL) and noted in the final report.

## 13. QUALIFIERS

B Analytical result is estimated. Analyte was detected in associated reagent blank as well as the samples.

CL Analytical result is estimated due to ineffective quenching.

J Analyte was positively identified; the associated numerical value is estimated.

PT The reported result is estimated because the sample was not analyzed within required holding time.

[1]The Ohio EPA Total (Extracellular and Intracellular) Microcystins - ADDA by ELISA Analytical Methodology in raw and finished drinking water samples has been developed in conjunction with U.S. EPA, public water system laboratories, Ohio State University-Stone Laboratory and other



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national experts.  Ohio EPA will continue to work with these groups to evaluate and revise the method as additional information becomes available.

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