Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay
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1 Scope and Application

1.1 Method Description
Method 546 is a procedure for the determination of “total” microcystins (MC) and nodularins (NOD) in finished drinking water and in ambient water using enzyme-linked immunosorbent assay (ELISA). The term “Total microcystins and nodularins” is defined as the sum of the congener-independent, intracellular and extracellular microcystin and nodularin that is measurable in a sample. Method 546 measures the total concentration based on detection of a characteristic feature common to microcystin and nodularin congeners (structural variants), specifically, the Adda amino acid side chain: \((4E,6E)-3\)-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid \((\text{Fischer et al., 2001})\). To ensure comparability between laboratories, the ELISA is calibrated against one congener, MC-LR \((\text{CASRN 101043-37-2})\).

1.2 Required Operator Expertise
This method is intended for use by analysts skilled in the performance of ELISA and in the interpretation of the associated data. The analyst should be familiar with the calibration model used for indirect competitive ELISA, the four-parameter logistic fit, which is described in Section 3.3.

1.3 Supporting Data

1.3.1 Single-Laboratory Lowest Concentration Minimum Reporting Levels (LCMRLs)
LCMRLs for MC-LR are presented in Section 17, Table 1. The LCMRL is the lowest spiking concentration such that the probability of spike recovery in the 50 to 150% range is at least 99%. Laboratories using this method are not required to determine LCMRLs, but they must demonstrate that the Minimum Reporting Level (MRL) \((\text{Sect. 3.11})\) for MC-LR meets the requirements described in Section 9.1.3.

1.3.2 Single-Laboratory Precision and Accuracy Data
Precision and accuracy data for MC-LR fortified in reagent water, drinking water, and ambient water are presented in Section 17, Table 2, Table 3, and Table 4.

1.4 Method Flexibility
The Adda-ELISA technique must be used for the congener-independent detection of microcystins and nodularins. An indirect competitive assay must be used. Changes may not be made to sample collection, preservation, and storage procedures \((\text{Sect. 8})\), or to the quality control (QC) requirements \((\text{Sect. 9})\).

2 Summary of the Method
This method is based on the 96-well microtiter plate format. In these wells, microcystins and nodularins in the samples and a microcystin-protein analogue immobilized in the wells compete for the binding sites of a primary detection antibody in solution. After a wash step, an enzyme-conjugate is added to the wells and binds to the primary antibody in an inverse relationship to the original concentration of microcystins and nodularins in the sample. After a second wash step, tetramethylbenzidine substrate is added to develop color via an enzyme-mediated reaction. After a set period, an acidic solution is added to each well to stop color generation. Finally, the absorbance of each well is measured using a plate reader. The concentration of microcystins and nodularins is calculated using a four-parameter logistic calibration curve.
3 Definitions

3.1 Analysis Batch
Standards, samples, and quality control elements are assayed on a single 96-well plate using identical lots of reagents and wells. Each plate by definition is an Analysis Batch, regardless of the number of wells included. Quality control samples must be analyzed in each Analysis Batch at the frequencies prescribed in Section 9.2. Each Analysis Batch includes the following elements:

- Calibration standards
- Laboratory Reagent Blanks
- Low-Range Calibration Verification standard
- Laboratory Fortified Blanks
- Field samples (drinking water and ambient water)
- Laboratory Fortified Sample Matrix and Laboratory Fortified Sample Matrix Duplicates

3.1.1 Well Replicates
Within the Analysis Batch, this method requires each calibration standard, field sample, and QC sample to be assayed in two wells. These two wells are called well replicates. Two values are associated with each well replicate: an absorbance measured by the plate reader, and a concentration calculated from this absorbance.

3.1.2 Use of Well Replicate Absorbance Values
For each set of well replicates, the percent coefficient of variation (%CV) is calculated from the two absorbance values. The %CV of the absorbance values for calibration standards must meet QC criteria stated in Section 10.2. The %CV of the absorbance values for all field and QC samples must meet the limits stated in Section 9.2.1.

3.1.3 Use of Well Replicate Concentrations
For each set of well replicates, the mean is calculated from the two concentration values. The mean concentration must be used for reporting field sample results. The mean must be used in all method calculations and for evaluating results against QC limits.

3.2 Calibration Standards
Solutions of MC-LR provided in the ELISA kit or prepared in the laboratory that are appropriate for the measurement range of the ELISA kit.

3.3 Calibration Curve
The calibration points are modelled using a four-parameter logistic function (Sect. 3.3.1), relating concentration (x-axis) to the measured absorbance in the wells (y-axis). A calibration curve generated during method development is presented in Section 17, Figure 1. Note the inverse relationship between concentration and response. The zero calibration standard gives the highest absorbance and the highest calibration standard gives the lowest absorbance. Note also that the slope, or sensitivity, of the ELISA response is greatest in the middle of the curve and tends toward zero slope at extreme low and high concentrations. For a more detailed explanation of the four-parameter calibration model, see Maciel (1985) and Sasaki (web resource).
3.3.1 Four-Parameter Logistic Equation

\[
y = \frac{(a - d)}{1 + (\frac{x}{c})^b} + d
\]

\[
y = \text{absorbance}
\]
\[
x = \text{concentration}
\]
\[
a = \text{absorbance at the bottom plateau}
\]
\[
b = \text{slope related term at the inflection point}
\]
\[
c = \text{concentration at the inflection point} = EC_{50}
\]
\[
d = \text{absorbance at the top plateau}
\]

The coefficients, \(a\), \(b\), \(c\), and \(d\), are calculated by the data reduction software using regression analysis.

3.4 EC_{50}

The EC_{50} is the concentration of microcystin that yields an absorbance halfway between the bottom plateau of the calibration curve (coefficient \(a\)) and the top plateau (coefficient \(d\)). The EC_{50} is the concentration at the inflection point (Section 17, Figure 1), and is in the center of the most reliable measurement range (i.e., the greatest slope) of the ELISA. The EC_{50} is determined and used as described in Sections 3.4.1 and 3.4.2.

3.4.1 Determining the EC_{50}

For each calibration curve, the EC_{50} is equal to the coefficient, \(c\), of the four-parameter logistic fit. The EC_{50} is found on the calibration report generated by the plate reader.

3.4.2 Use of the EC_{50}

Because the EC_{50} is in the center of the most reliable measurement range, guidance for fortifying QC samples is based on this value. For this method, QC samples requiring fortification with MC-LR should have concentrations near the EC_{50}. These QC samples include Laboratory Fortified Blanks and Laboratory Fortified Sample Matrix.

3.5 Laboratory Fortified Blank (LFB)

An aliquot of reagent water to which a known quantity of MC-LR is added. The LFB is lysed and filtered to match the analytical procedure for field samples. The LFB is used during the IDC to verify method performance for precision and accuracy. The LFB is also a required QC element with each Analysis Batch. The results of the LFB verify method performance in the absence of sample matrix.

3.6 Laboratory Fortified Sample Matrix (LFSM)

An aliquot of a field sample to which a known quantity of MC-LR is added. The purpose of the LFSM is to determine whether the sample matrix contributes bias to the analytical results.

3.7 Laboratory Fortified Sample Matrix Duplicate (LFSMD)

A second aliquot of the field sample used to prepare the LFSM that is fortified and assayed in the same Analysis Batch as the LFSM. The LFSMD is used to verify method precision in both ambient water and drinking water matrixes.
3.8 **Laboratory Reagent Blank (LRB)**
An aliquot of reagent water that is lysed and filtered to match the sample processing procedure. The
LRB is used to determine if microcystins or other interferences are introduced from the sample
containers, sample processing equipment, or the reagents used in the assay.

3.9 **Lowest Concentration Minimum Reporting Level (LCMRL)**
The single-laboratory LCMRL is the lowest spiking concentration such that the probability of spike
recovery in the 50 to 150% range is at least 99% *(U.S. EPA, 2004 and U.S. EPA, 2010)*.

3.10 **Low-Range Calibration Verification (Low-CV)**
The Low-CV is a calibration standard with a concentration equal to, or less than, the MRL. The purpose
of the Low-CV is to confirm the accuracy of the calibration at concentrations near the MRL.

3.11 **Minimum Reporting Level (MRL)**
The minimum concentration that can be reported by a laboratory as a quantified value for total
microcystins and nodularins in a sample following analysis. This concentration must meet the criteria
defined in **Section 9.1.3** and must be no lower than the concentration of the lowest calibration standard.

3.12 **Primary Dilution Standard (PDS)**
A solution of MC-LR in methanol prepared from the MC-LR Stock Standard Solution. The PDS solutions
are used to fortify QC samples (LFB, LFSM, and LFSMD).

3.13 **Quality Control Sample (QCS)**
A solution containing MC-LR at a known concentration that is obtained from a source different from the
source of calibration standards. The purpose of the QCS is to verify the accuracy of the primary
 calibration standards.

3.14 **Reagent Water**
Purified water that does not contain any measurable quantity of microcystins, nodularins, or interfering
compounds at or above one-half of the MRL.

3.15 **Stock Standard Solution**
A concentrated standard in methanol that is prepared in the laboratory from purified MC-LR or that is
purchased from a commercial source with a certificate of analysis.

4 **Interferences**

4.1 **General Considerations**
The accuracy of the ELISA procedure is dependent upon analyst technique, the accuracy of pipetted
volumes, and consistent incubation periods across the wells of each plate.

4.2 **Assay Drift**
Assay drift refers to systematic rather than random imprecision in measured analyte concentrations, the
magnitude of which depends on the sample position within the plate. A possible cause for assay drift is
slight differences in incubation times as reagents are added sequentially across the plate *(Davies, 2005)*.
Identical control samples distributed throughout the plate are necessary to detect assay drift. This
method includes QC measures to evaluate assay drift. During the IDC, laboratories must assay five LRBs
distributed across the plate and each Analysis Batch must include two LRBs placed on opposite sides of
the plate. Because the LRBs give absorbance values near the upper plateau of the calibration curve, the calculated LRB concentrations are sensitive to slight changes in the measured absorbance. If the distributed LRBs pass the QC limit of one-half the MRL, then plate drift is minimal and under control.

4.3 Sample Matrix Effects in Ambient Water
During method development, a positive bias of approximately 30% in ambient water from a single source was observed. Two ambient waters from other sources did not cause matrix bias.

4.4 Sample Matrix Effects in Drinking Water
During method development, six drinking water sources were evaluated for matrix effects. Of these, three exhibited positive bias within a range of 12 to 15%. Three sources did not cause matrix bias.

4.5 Cross Contamination
This method covers the analysis of samples collected from both ambient water and drinking water. To avoid cross contamination, segregate glass syringes used to filter ambient water, which may contain high levels of microcystins, from those used to filter drinking water. Alternately, use plastic, disposable syringes. Thoroughly clean glass sample containers if they are reused. Do not reuse septa from bottles containing ambient water samples.

4.6 Interpretation of Results
The results reported by this method represent total microcystins and nodularins based on Adda ELISA calibrated with MC-LR.

5 Safety
Each reagent used in these procedures should be treated as a potential health hazard and exposure to these materials should be minimized. Each laboratory is responsible for maintaining an awareness of OSHA regulations regarding safe handling of any chemicals used in this method. A reference file of Safety Data Sheets should be made available to all personnel involved in the analysis. The primary hazard when conducting the procedures in this method is exposure to cyanotoxins in samples and concentrated standards. Handle samples and standards using appropriate personal protective equipment.

6 Equipment and Supplies
References to specific brands and catalog numbers are included as examples only and do not imply endorsement of the products. Such reference does not preclude the use of other vendors or manufacturers. Specific references are intended to represent suitable specifications for the items.

6.1 Adda ELISA Test Kits
Indirect competitive assay based on detection of the Adda epitope (Abraxis Part Number 520011OH, or equivalent). Automated systems for processing the 96-well plate may be used.

6.2 Sample Containers
Amber glass bottles fitted with polytetrafluoroethylene (PTFE)-lined screw caps. The single use of sample containers is recommended. Good laboratory practices for cleaning are expected to be followed if sample bottles are reused. Do not use bottles treated at a high temperature in a muffle furnace (400 °C and above) as a cleaning procedure. Studies conducted during method development indicate a
tendency for microcystins and nodularins to adsorb to the surface of glassware repeatedly cleaned by heating in a muffle furnace.

6.3 Plate Reader
Microtiter plate reader and associated software capable of reading absorbance at 450 nm and constructing a calibration curve using a four-parameter logistic function (Sect. 3.3.1).

6.4 Adhesive Plate Covers
Clear adhesive film for sealing wells during the incubation steps of the assay, VWR Catalog Number 60941-120.

6.5 Pipette for Loading Wells
Adjustable or fixed-volume manual pipette with 50 microliter capacity and manufacturer-recommended pipette tips. A manual pipette is recommended for addition of standards, field samples, and QC samples to the wells.

6.6 Repeating Pipette
Repeating pipette with 5 mL capacity disposable tips, HandyStep® electronic (Wertheim, Germany). An electronic repeating pipette is recommended for addition of antibody, enzyme conjugate, substrate, and stop solution to the wells.

6.7 Multi-Channel Pipette
Eight-channel pipette with 250 µL capacity per channel and polypropylene pipette tips. An eight-channel pipette is recommended for addition of wash solution to the wells during the washing steps.

6.8 Basin for Wash Solution
Plastic basin designed for multi-channel pipettes, VWR Catalog Number 21007-970. A basin with 55 mL capacity is recommended.

6.9 Syringes for Filtering Samples after Lysing
Glass: gas-tight Luer-lock, 5 mL capacity, Hamilton Co. Model Number 100STLL. Plastic: Luer-lock, 3 mL capacity, polypropylene barrels with polyethylene plungers, Thermo Fisher Scientific, Inc., Catalog Number S7515-3.

6.10 Syringe Filters for Filtering Samples after Lysing
25 mm glass fiber, 1.2 µm pore size, polypropylene housing, Environmental Express (Charleston, South Carolina) Catalog Number SF012G. 25 mm glass fiber, 0.45 µm pore size, polypropylene housing, GE Healthcare Life Sciences/Whatman (Marlborough, MA) Catalog Number 6894-2504.

6.11 Fifteen Milliliter to Forty Milliliter Vials
Borosilicate glass, clear or amber, with PTFE-lined closures. Vials with capacity in this range are recommended for the lysing procedure. Do not use bottles treated in a muffle furnace at a high temperature as a cleaning procedure. See caution in Section 6.2.

6.12 Four Milliliter Vials
Borosilicate glass, with PTFE-lined closures. Four milliliter vials are recommended for receiving and storing sample filtrate after lysing.
7  Reagents and Standards

7.1  Reducing Agent for Residual Chlorine
Sodium thiosulfate (CASRN 7772-98-7) is used to reduce residual chlorine in drinking water samples at the time of collection.

7.2  Stock and PDS Solvent
Methanol (CASRN 67-56-1) is used to reconstitute MC-LR if this material is purchased as a solid, and to dilute the MC-LR stock to prepare PDS solutions.

7.3  MC-LR Stock and PDS Solutions
Obtain MC-LR (CASRN 101043-37-2) as a solution with a concentration of at least 10 µg/mL or as the neat material. Reconstitute the neat material in methanol to obtain a stock concentration of at least 10 µg/mL. Dilute the MC-LR stock with methanol to prepare PDS solutions. Select MC-LR concentrations for PDS solutions such that at least 5 µL are used to fortify QC samples, or to prepare calibration standards. More than one PDS concentration may be necessary to meet this requirement.

7.4  Calibration Standards
Calibration standards supplied in the ELISA kits are recommended. Calibration standards prepared in the laboratory are permitted. Laboratories should use appropriate QC practices to determine when standards need to be replaced.

8  Sample Collection, Preservation, and Storage

8.1  Sample Collection for Drinking Water
Prior to shipment to the field, add sodium thiosulfate to each sample bottle. The final concentration of sodium thiosulfate in the sample should be 100 mg/L. Do not dilute sodium thiosulfate in water when preparing sample bottles. The reducing agent must be added to the empty bottle in solid form. In the field, open the tap and allow the system to flush for approximately 5 minutes. Fill each bottle, taking care not to flush out the sodium thiosulfate, and invert several times to mix the sample with the reducing agent.

8.2  Sample Volume
Collect enough sample to meet the requirements in this method. Considerations for sample size are sufficient volume for preparing QC samples and appropriate volume for frozen storage.

8.3  Prohibition Regarding Ascorbic Acid
Do not use ascorbic acid to reduce chlorine in drinking water samples. During studies to evaluate analyte stability during transport and storage, the authors discovered that microcystins degrade in the presence of ascorbic acid.

8.4  Sample Collection for Ambient Water
The addition of sodium thiosulfate is not required for ambient water samples, but may be added if the laboratory chooses to prepare only one type of sample container.

8.5  Sample Shipment and Storage
Samples must be chilled during shipment and must not exceed 10 °C during the first 48 hours after collection. Freeze samples upon arrival at the laboratory.
8.5.1 Verification upon Receipt

8.5.1.1 Temperature
Samples must be confirmed to be at, or below, 10 °C when they are received in the laboratory. A temperature of greater than 10 °C is acceptable if transit time is short and the samples do not have sufficient time to chill. In this case, examine the ice packs in the cooler. If they remain frozen, the samples are valid.

8.5.1.2 Residual Chlorine
For drinking water, analyze one sample from each cooler using common assays for total residual chlorine, for example, the \(N,N\)-diethyl-p-phenylenediamine (DPD)-colorimetric technique. The total chlorine concentration should be less than the detection limit of the assay. A duplicate sample may be collected for conducting the residual chlorine assay.

8.5.2 Considerations for Frozen Storage
Use clear, or amber, borosilicate glass bottles with PTFE-lined septa. Select bottle capacity and sample volume to prevent breakage of bottles during freezing. Plan ahead to retain enough volume for preparing QC samples as required in this method.

8.6 Sample Holding Time
Analyze samples as soon as possible. Samples that are collected and stored as described in Section 8 must be analyzed within 14 days of collection. Fourteen days is set as a safe holding time based on the empirical evidence: during method development, degradation of microcystins in two drinking water matrices was observed after three weeks of frozen storage (Sect. 13.2.3 and Table 9).

8.7 Storage of Samples after Lysing
Samples may be filtered and assayed any time after lysing if within 14 days of collection. If not assayed immediately, store lysed samples by freezing in glass vials with PTFE-faced septa, for example, 1 mL of lysed and filtered sample held in a 4 mL vial.

9 Quality Control

QC requirements include the IDC, and QC elements associated with each Analysis Batch. This section describes each QC parameter, its required frequency, and the performance criteria that must be met in order to satisfy EPA data quality objectives. These QC requirements are considered the minimum acceptable QC protocol. Laboratories are encouraged to institute additional QC practices to meet their specific needs.

9.1 Initial Demonstration of Capability (IDC)
The IDC must be successfully performed prior to analyzing field samples. The IDC includes four determinations: demonstration of precision and accuracy, demonstration of acceptable system background, MRL confirmation, and a second-source verification of the calibration standards (Quality Control Sample). The IDC requirements are outlined in Section 17, Table 10.

When conducting the IDC, the analyst must meet the calibration requirements specified in Section 10. The lowest calibration standard used to establish the initial calibration must be at, or below, the concentration representing the MRL. The four determinations necessary to complete the IDC may be included in a single Analysis Batch, i.e., processed on a single ELISA plate.
9.1.1 Demonstration of Precision and Accuracy
Prepare seven replicate LFBs (Section 3.6) by fortifying each with MC-LR at 0.50 µg/L. Sodium thiosulfate must be added as described in Section 8.1. Lyse, filter, and assay (Section 11) the LFBs in a single Analysis Batch (Sect. 3.1). For this Analysis Batch, include the LRBs prepared in Section 9.1.2 to fulfill the LRB QC requirement. A Low-CV (Sect. 9.2.3) is also required. The percent relative standard deviation (%RSD) for MC-LR in the seven LFB replicates must be less than, or equal to, 15%. The mean recovery for the seven replicates must be greater than, or equal to, 70% and less than, or equal to, 130%.

\[
Average \% \ Recovery = \frac{Average \ Measured \ Concentration}{Fortified \ Concentration} \times 100
\]

\[
%RSD = \frac{Standard \ Deviation \ of \ Measured \ Concentrations}{Mean \ Concentration} \times 100
\]

9.1.2 Demonstration of Acceptable System Background
In the same Analysis Batch constructed for the precision and accuracy IDC, lyse, filter, and assay five LRBs (Section 3.8). The LRBs must contain sodium thiosulfate. The LRBs must be distributed throughout the plate. The result obtained for each LRB must be less than one-half of the MRL.

9.1.3 Minimum Reporting Level (MRL) Confirmation
Establish a target concentration for the MRL based on the intended use of the method. Confirm the MRL following the procedure outlined below.

9.1.3.1 Prepare and Assay MRL Samples
Fortify seven replicate LFBs with MC-LR at, or below, the proposed MRL concentration. The LFBs must contain sodium thiosulfate as specified in Section 8.1. Lyse, filter, and assay the samples in a single Analysis Batch. The Analysis Batch must include two LRBs (Sect. 9.2.2) and a Low-CV.

9.1.3.2 Calculate MRL Statistics
Calculate the mean and standard deviation of the seven replicates. Determine the Half Range for the Prediction Interval of Results (HR_{PIR}) using the following equation:

\[
HR_{PIR} = 3.963s
\]

Where,

s is the standard deviation and 3.963 is a constant value for seven replicates (U.S. EPA, 2004).

Calculate the Upper and Lower Limits for the Prediction Interval of Results (PIR = Mean ± HR_{PIR}) as shown below.

\[
Upper \ PIR \ Limit = \frac{Mean + HR_{PIR}}{Fortified \ Concentration} \times 100\%
\]

\[
Lower \ PIR \ Limit = \frac{Mean - HR_{PIR}}{Fortified \ Concentration} \times 100\%
\]

These equations are only defined for seven replicate samples.
9.1.3.3  **MRL Acceptance Criteria**
The MRL is confirmed if the *Upper PIR Limit* is less than, or equal to, 150% and the *Lower PIR Limit* is greater than, or equal to, 50%. If these criteria are not met, the MRL may be set too low and the confirmation should either be repeated, or the MRL established and confirmed at a higher concentration.

9.1.4  **Quality Control Sample (QCS)**
Assay a mid-level QCS, prepared as described in Section 9.2.6, to confirm the accuracy of the primary calibration standards.

9.2  **Analysis Batch QC Requirements**
This section describes the QC elements that must be included with each Analysis Batch (Sect. 3.1). The Analysis Batch QC requirements are summarized in Section 17, Table 11.

9.2.1  **Criterion for Replicate Wells**
All field and QC samples are added to at least two wells (Sect. 11.3). See Section 3.1.1 for a definition of well replicates. The %CV of the absorbance values measured for the well replicates must be less than, or equal to, 15%. Calculate the %CV as follows:

\[
\%CV = \frac{\text{Standard Deviation of Absorbances}}{\text{Mean Absorbance}} \times 100\%
\]

If the %CV exceeds 15% for a field sample or QC sample (Low-CV, LRB, LFB, LFSM, and LFSMD), then that sample is invalid. Note that the well replicates of calibration standards must meet a different set of criteria for %CV. These criteria are found in Section 10.2.

9.2.2  **Laboratory Reagent Blank (LRB)**
For each Analysis Batch, prepare, lyse, and filter one LRB. The LRB must contain sodium thiosulfate if drinking water samples are included in the Analysis Batch. Assay the LRB in duplicate by placing one pair of well replicates on opposite sides of the plate (four total wells). The total microcystin and nodularin concentration in each LRB must be less than one-half the MRL. If the concentration is equal to, or greater than, this level, then any samples that yielded a positive result in the Analysis Batch are invalid. Subtracting blank values from sample results is not permitted.

9.2.3  **Low Calibration Verification (Low-CV)**
With each Analysis Batch, assay one Low-CV. The Low-CV is a calibration standard prepared at a concentration equal to, or less than, the MRL. A calibration standard from the kit may be used. Do not add sodium thiosulfate, do not lyse, and do not filter the Low-CV. The assayed concentration in the Low-CV must be greater than, or equal to, 50% and less than, or equal to, 150% of the true value. If the result fails this criterion, then the entire Analysis Batch is invalid.

9.2.4  **Laboratory Fortified Blank**
At least two LFBs, fortified at an identical concentration, are required with each Analysis Batch. Add sodium thiosulfate if drinking water samples are included in the Analysis Batch. Fortify the LFB near the EC50 of the calibration curve. Lyse and filter each LFB in a separate vial. See Section 3.4.1 for help determining the EC50.
9.2.4.1 Acceptance Criterion for LFBs
The percent recovery for each LFB must be greater than, or equal to, 60% and less than, or equal to, 140% of the true value. If either LFB fails this criterion, then the entire Analysis Batch is invalid.

9.2.5 Laboratory Fortified Sample Matrix (LFSM) and Laboratory Fortified Sample Matrix Duplicate (LFSMD)
One LFSM and LFSMD set is required with each Analysis Batch in a drinking water sample. If more than 20 drinking water samples are present in the Analysis Batch, two sets are required. One LFSM and LFSMD set is required with each Analysis Batch in an ambient water sample. If more than 20 ambient water samples are present in the Analysis Batch, two sets are required. The native background concentration in the sample matrix must be determined in a separate field sample. Over time, distribute LFSMs among the various drinking water and ambient water sources that the laboratory receives.

9.2.5.1 Prepare the LFSM and LFSMD
Three separate aliquots of a field sample are required for determining the native background concentration, and for preparing the LFSM and the LFSMD. Prepare the LFSM and LFSMD by fortifying two aliquots of the same field sample with an appropriate amount of MC-LR. If frozen, thaw the samples. Mix thoroughly to homogenize the sample before distributing into the three vials. Choose a spiking concentration such that the total microcystin and nodularin result will fall near the EC50 of the calibration curve, and fortify at least twice the native concentration, if known. Lyse and filter the samples, or if the samples were frozen, complete two more cycles of lysing.

9.2.5.2 Special Considerations for Ambient Water
If the concentration in ambient water is unknown, randomly select samples and fortify with approximately 1.0 µg/L MC-LR. If the initial concentration in the randomly selected LFSMs is high, the fortified sample result may fall outside of the range of the ELISA calibration, or fail to meet the requirement of fortifying at a concentration at least twice the native value. In these cases, the QC results are deemed unusable and may be discarded. However, the laboratory should attempt to collect valid LFSM data over time for representative ambient waters.

9.2.5.3 Calculate the Mean Percent Recovery
Calculate the mean percent recovery (%R) for each LFSM and LFSMD set using the equation:

\[ \%R = \frac{(A - B)}{C} \times 100\% \]

Where,
A = mean measured concentration of the LFSM and LFSMD,
B = measured concentration in the unfortified sample, and
C = fortification concentration.

In order to obtain meaningful percent recovery results, correct the mean value of the LFSM and LFSMD for the native concentration in the unfortified sample, even if the native value is less than the MRL.

9.2.5.4 Evaluate Recovery for Fortified Matrix
The mean percent recovery for each LFSM and LFSMD set should be greater than, or equal to, 60% and less than, or equal to, 140% of the true value. If the percent recovery falls outside this range, and the laboratory performance is in control for the LFBs within the same Analysis Batch, the recovery may be matrix biased. Qualify the result for the sample from which the LFSM was prepared as “suspect–matrix”. 
9.2.5.5 Calculate the RPD for the LFSM and LFSMD
Calculate the relative percent difference (RPD) using the equation:

\[ RPD = \frac{|LFSMD - LFSM|}{(LFSMD + LFSM)/2} \times 100\% \]

9.2.5.6 Evaluate the RPD for Fortified Matrix
The RPD for each LFSM and LFSMD set should be less than, or equal to, 40%. If the RPD falls outside this range, and the laboratory performance is in control for the LFBs within the same Analysis Batch, the precision may be matrix biased. Qualify the result for the sample from which the LFSMD was prepared as “suspect–matrix”.

9.2.6 Quality Control Sample (QCS)
A QCS (Sect. 3.13) must be assayed during the IDC, and then with each new lot of calibration standards. The MC-LR used for the QCS must be obtained from a source independent from the source of the calibration standards. Prepare the QCS in reagent water near the EC50. A QCS supplied with the ELISA kit may be used if these criteria are met. The percent recovery of MC-LR in the QCS must be greater than, or equal to, 70% and less than, or equal to, 130%.

10 Calibration

10.1 Calibration Procedure
A calibration is required with each Analysis Batch. Use the concentrations stated in the kit instructions. Do not add additional calibration levels or eliminate any levels. Laboratories may prepare calibration standards in house; however, the number of levels and concentrations must match those in the original kit. Each calibration standard must be added to at least two wells. The lowest calibration standard must be at, or below, the concentration of the MRL.

10.2 Calibration Acceptance Criteria
The calibration curve is validated by evaluating the %CV of the absorbance values for the well replicates representing each calibration level, and the correlation coefficient of the four-parameter logistic curve. Calculate the %CV (Equation 9.2.1) for each of the paired absorbance values, including the “zero” standard. The %CV for each pair must be less than, or equal to, 10%. However, one pair is allowed to exceed 10% providing the %CV is less than, or equal to, 15%. The square of the correlation coefficient \( r^2 \) of the four-parameter curve must be greater than, or equal to, 0.98.

If the calibration fails the %CV limits or \( r^2 \) is less than 0.98, then the entire Analysis Batch is invalid. Assay the samples in a subsequent Analysis Batch. Freeze the filtered samples if this Analysis Batch cannot be completed on the same day as the original attempt. Each sample must be within the 14-day holding time for the repeat assay.

11 Procedure
This section describes the procedure for preparing samples and processing the microtiter plate to conduct the ELISA. Fortify QC samples prior to the lysing step (LFBs, and LFSM and LFSMD sets). If samples were frozen, it is acceptable to fortify after this first lysing cycle. See Section 9.2 for the frequency and procedure for preparing fortified samples.
11.1 Lysing by Freeze-Thaw
Mix thoroughly and immediately transfer 5 to 10 mL of each field sample into a 40 mL vial to begin three freeze-thaw cycles. If the sample was previously frozen, only two freeze-thaw cycles are needed. Smaller vials may be used, but reduce the sample volume to less than 25% of vial capacity. Ensure samples are completely frozen and completely thawed during each cycle. Thaw samples at approximately 35 °C in a water bath and mix after each cycle.

11.2 Filtering the Sample
Filter 1 to 2 mL of each lysed sample into a 4 mL vial using a glass-fiber syringe filter.

11.3 Conducting the ELISA
Follow the manufacturer's instructions for adding samples and reagents to the plate. Fill two wells with each calibration standard, field sample, and QC sample. Use Table 11 as a summary guide for constructing the Analysis Batch to ensure that all required QC elements are included. Drinking water and ambient water samples may be assayed in the same Analysis Batch.

11.3.1 Color Development Step
If processing plates manually, use a technique that ensures each well is incubated with substrate for exactly the same period. To accomplish this, add stop solution in the same sequence and at the same rate as the addition of substrate.

11.3.2 Reading the Plate
Measure the color by reading the absorbance at 450 nanometers using a microtiter plate reader.

12 Data Analysis and Calculations

12.1 Quantitation
A four-parameter logistic curve fit must be used (Sect. 3.3). Other curve-fitting models are not permitted. Calculate the sample concentration for each well using the multipoint calibration. For each field and QC sample, average the two concentration values from each well. Use this mean to report sample results and to evaluate QC results against acceptance limits. Report only those values that fall between the MRL and the highest calibration standard. Final results should be rounded to two significant figures.

12.2 Exceeding the Calibration Range
If a result exceeds the range of the calibration curve, dilute the sample with reagent water. Based on the estimated concentration, select a dilution factor that results in a diluted sample concentration near the EC_{50} of the calibration curve. The concentration in the diluted sample must fall between the MRL and the highest calibration standard. Analyze the diluted sample in a subsequent Analysis Batch. Incorporate the dilution factor into the final concentration calculations. Report the dilution factor with the sample result.

13 Method Performance

13.1 Precision, Accuracy, and LCMRL Results
Tables for these data are presented in Section 17. The single-laboratory LCMRL, based on the response of MC-LR, is presented in Table 1. Single-laboratory precision and accuracy data are presented at low and high concentrations within the measurement range: near the MRL (Table 2), and at a mid-range
concentration (Table 3). Three matrixes were studied: reagent water, lake water, and drinking water processed from lake water. Precision and accuracy results in reagent water at a concentration near the EC_{50} are presented in Table 4.

13.2 Analyte Stability Studies
The stability of microcystins and nodularins was studied in both ambient water and drinking water. Because MC-LR is only one of many possible congeners, separate experiments were designed to evaluate the stability of MC-LR and a more representative mixture of microcystins and nodularins. These experiments are described in the sections that follow.

13.2.1 Experimental Conditions
Each matrix was fortified with MC-LR and the representative mixture of seven congeners for a total of four experimental conditions:

- Drinking water fortified with 0.50 µg/L MC-LR
- Ambient water fortified with 0.50 µg/L MC-LR
- Drinking water fortified with 0.35 µg/L of a representative microcystin and nodularin mixture
- Ambient water fortified with 0.35 µg/L of a representative microcystin and nodularin mixture

Ambient water was obtained from a lake known to experience algal blooms and used to supply a drinking water treatment plant. Drinking water was obtained from a treatment plant fed by a lake known to experience algal blooms. Drinking water samples were inoculated with microbial rich water from an impacted surface source to incorporate an additional challenge to the efficacy of the storage procedure. A bulk sample was prepared for each experimental condition, then distributed into separate 40 mL vials for storage. The ELISA was calibrated with MC-LR.

13.2.2 Composition of the Mixture of Congeners used in the Experiments
Seven microcystin and nodularin congeners were used: MC-LR, RR, LW, YR, LA, 3-desmethylated-MC-RR (3-dm-MC-RR) and NOD-R. Individual congener concentrations were 0.050 µg/L. The six microcystins were selected to represent a range of chemistries for microcystin congeners known to occur in ambient water in terms of polarity, ionic charge and backbone methylation. In addition, MC-LW contains tryptophan (W) which is highly susceptible to oxidation, and MC-YR contains tyrosine (Y) which is moderately susceptible to oxidation. NOD-R was included to represent nodularin congeners.

13.2.3 Storage Conditions, Duration, and Results
All samples were preserved and stored as required in this method. Seven samples from each experimental set were analyzed after 0, 2, 7, 15, and 22 days of storage. For drinking water, analyte recovery at each time point was calculated by comparing the mean concentration measured in the stored samples to the mean concentration measured in five LFBs fortified at the same concentration and prepared on the day of assay. This approach cancels other factors contributing to the variability in the sample response and isolates the experimental variable of storage time. For ambient water, the percent change in analyte concentration at each time point was calculated by comparing the mean of the stored samples to the mean of seven identical ambient samples analyzed at T-zero (zero storage time). This alternate means of assessing stability was necessary because the ELISA response was biased by the matrix in this particular lake water compared to calibration standards.
15 Table 5 presents the results for drinking water fortified with MC-LR, and Table 6 presents the results for ambient water fortified with MC-LR. Table 7 presents the results for drinking water fortified with the mixture of congeners, and Table 8 presents the results for ambient water fortified with the mixture of congeners.

13.2.4 Degradation of Microcystins and Nodularins during Storage
Microcystins and nodularins degraded during frozen storage in two drinking waters studied during method development. These data (Table 9) are included in the method to support the 14-day holding time recommendation.

13.2.4.1 Experimental Design
Each matrix was fortified with a representative mixture of congeners (Sect. 13.2.2) for a total of two experimental conditions:

- Drinking water from central Ohio fortified at 0.35 µg/L
- Drinking water from eastern Pennsylvania fortified at 0.35 µg/L

A bulk sample was prepared for each experimental condition, then distributed into separate 40 mL vials for storage. All samples were preserved and stored as required in this method. Seven samples from each experimental set were analyzed after 0, 6, 9, 16, 23, 43, and 91 days of storage. Analyte recovery at each time point was calculated by comparing the mean concentration measured in the stored samples to the mean concentration measured in seven LFBs fortified at the same concentration and prepared on the day of assay. The ELISA was calibrated with the same MC and NOD mixture used to fortify the drinking water samples and the LFBs.

As an additional control, LFBs fortified at the same concentration as the study samples were stored under identical conditions. Three vials from the frozen LFB pool were sacrificed at each sampling event to evaluate stability in the absence of matrix.

13.2.4.2 Experimental Results
Table 9 presents the results of the analyte stability study during which degradation was observed. Microcystin recovery in the two drinking water matrixes is near 70 percent after three weeks of frozen storage. These losses appear to occur during frozen storage and not as a result of the process of freezing. Note that the T-zero samples were lysed (frozen and thawed three times) and no losses were detected. In addition, the LFB controls do not exhibit analyte loss, ruling out the sample container as a contributing factor. These facts point to the matrix as the cause for analyte degradation during storage.

14 Pollution Prevention
For information about pollution prevention applicable to laboratory operations described in this method, consult: Less is Better, Guide to Minimizing Waste in Laboratories, a web-based resource available from the American Chemical Society at www.acs.org.

15 Waste Management
The Agency requires that laboratory waste management practices be consistent with all applicable rules and regulations, and that laboratories protect the air, water, and land by minimizing and controlling all
releases from fume hoods and bench operations. In addition, compliance is required with any sewage discharge permits and regulations, particularly the hazardous waste identification rules and land disposal restrictions.

16 References


Ohio EPA Division of Environmental Services. Ohio EPA Total (Extracellular and Intracellular) Microcystins – ADDA by ELISA Analytical Methodology; Method 701.0 Version 2.2 (and previous versions); Ohio EPA: Reynoldsburg, OH, November 2015.


U.S. EPA. Statistical Protocol for the Determination of the Single-Laboratory Lowest Concentration Minimum Reporting Level (LCMRL) and Validation of Laboratory Performance at or Below the Minimum Reporting Level (MRL); EPA 815-R-05-006; Office of Water: Cincinnati, OH, November 2004.

Tables, Figures, and Method Performance Data

Data presented in this section were obtained using Abraxis, Inc., “Microcystins/Nodularins (Adda) ELISA” kits, Part Number 520011OH. Samples were added to the 96-well plate using manual pipettes. Antibody, enzyme conjugate, substrate, and stop solution were added using an electronic stepping pipette. Absorbance was measured using an 8-channel microplate reader (Abraxis, Inc., Model 4300).

Table 1. Lowest Concentration Minimum Reporting Limit for MC-LR

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fortification Levels, µg/L</th>
<th>LCMRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-LR</td>
<td>0.0, 0.10, 0.15, 0.20, 0.25, 0.30&lt;sup&gt;a&lt;/sup&gt;, 0.35, 0.40</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> The authors confirmed the MRL at 0.30 µg/L using the statistical calculations in Section 9.1.3.

Table 2. Accuracy and Precision near the MRL for MC-LR, Seven Replicates

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Fortification, µg/L</th>
<th>Native Concentration, µg/L</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent water</td>
<td>0.30</td>
<td>n/a</td>
<td>105</td>
<td>8.8</td>
</tr>
<tr>
<td>Drinking water&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.30</td>
<td>0.037</td>
<td>117</td>
<td>12</td>
</tr>
<tr>
<td>Ambient water&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.30</td>
<td>0.063</td>
<td>110</td>
<td>6.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Central KY, total organic carbon = 3.0 mg/L, pH = 7.55, free chlorine = 0.89 mg/L, total chlorine = 1.17 mg/L, hardness = 126 mg/L as CaCO₃.

<sup>b</sup> Southern Ohio lake, pH = 7.97

Table 3. Accuracy and Precision for MC-LR at a Mid-Range Concentration

<table>
<thead>
<tr>
<th>Matrix, n=10</th>
<th>Fortification, µg/L</th>
<th>Native Concentration, µg/L</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent water</td>
<td>2.2</td>
<td>n/a</td>
<td>93.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Drinking water&lt;sup&gt;a&lt;/sup&gt;, n=7</td>
<td>2.2</td>
<td>0.037</td>
<td>107</td>
<td>5.1</td>
</tr>
<tr>
<td>Ambient water&lt;sup&gt;b&lt;/sup&gt;, n=7</td>
<td>1.5</td>
<td>0.063</td>
<td>96.8</td>
<td>9.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Central KY, total organic carbon = 3.0 mg/L, pH = 7.55, free chlorine = 0.89 mg/L, total chlorine = 1.17 mg/L, hardness = 126 mg/L as CaCO₃.

<sup>b</sup> Southern Ohio lake, pH = 7.97

Table 4. Accuracy and Precision near the EC₅₀ for MC-LR in Reagent Water, Seven Replicates

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Fortification, µg/L</th>
<th>% Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent water</td>
<td>0.50</td>
<td>115</td>
<td>5.4</td>
</tr>
</tbody>
</table>
Table 5. Stability of MC-LR Fortified at 0.50 µg/L in Drinking Water

<table>
<thead>
<tr>
<th>Storage Time, days:</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery b</td>
<td>91.8</td>
<td>107</td>
<td>98.9</td>
<td>109</td>
<td>110</td>
</tr>
</tbody>
</table>

a. Central KY, total organic carbon = 3.0 mg/L, pH = 7.41, free chlorine = 1.25 mg/L, total chlorine = 1.60 mg/L, hardness = 134 mg/L as CaCO₃.

b. % Recovery = (mean concentration of seven fortified tap water samples stored for X days) / (mean concentration of five Laboratory Fortified Blanks at 0.50 µg/L prepared on the day of assay) * 100%; where X is the storage time.

Table 6. Stability of MC-LR Fortified at 0.50 µg/L in Ambient Water

<table>
<thead>
<tr>
<th>Storage Time, days:</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units: µg/L</td>
<td>0.689</td>
<td>-18.6</td>
<td>14</td>
<td>1.5</td>
<td>-12</td>
</tr>
<tr>
<td>% Change</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Southern Ohio lake, pH = 7.97

b. % Change from initial, analyzed concentration = (mean concentration of seven samples stored for X days – mean concentration of seven samples analyzed on Day-zero) / (mean concentration of seven samples analyzed on Day-zero) * 100%; where X is the storage time.

Table 7. Stability of a MC and NOD Mixture Fortified at 0.35 µg/L in Drinking Water

<table>
<thead>
<tr>
<th>Storage Time, days:</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery b</td>
<td>108</td>
<td>109</td>
<td>104</td>
<td>123</td>
<td>109</td>
</tr>
</tbody>
</table>

a. Central KY, total organic carbon = 3.0 mg/L, pH = 7.41, free chlorine = 1.25 mg/L, total chlorine = 1.60 mg/L, hardness = 134 mg/L as CaCO₃.

b. % Recovery = (mean concentration of seven fortified tap water samples stored for X days) / (mean concentration of five Laboratory Fortified Blanks at 0.35 µg/L prepared on the day of assay) * 100%; where X is the storage time.

Table 8. Stability of a MC and NOD Mixture Fortified at 0.35 µg/L in Ambient Water

<table>
<thead>
<tr>
<th>Storage Time, days:</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>15</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units: µg/L</td>
<td>0.673</td>
<td>-19.5</td>
<td>-0.42</td>
<td>13</td>
<td>-11</td>
</tr>
<tr>
<td>% Change</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Southern Ohio lake, pH = 7.97

b. % Change from initial, analyzed concentration = (mean concentration of seven samples stored for X days – mean concentration of seven samples analyzed on Day-zero) / (mean concentration of seven samples analyzed on Day-zero) * 100%; where X is the storage time.
Table 9. Stability of a MC and NOD Mixture Fortified at 0.35 µg/L in Drinking Watera

<table>
<thead>
<tr>
<th>Storage Time, days:</th>
<th>0</th>
<th>6</th>
<th>9</th>
<th>16</th>
<th>23</th>
<th>43</th>
<th>91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking water processed from lake waterb, % Recovery:</td>
<td>107</td>
<td>89.6</td>
<td>86.9</td>
<td>85.8</td>
<td>72.6</td>
<td>75.3</td>
<td>63.7</td>
</tr>
<tr>
<td>Drinking water processed from ground waterc, % Recovery:</td>
<td>99.6</td>
<td>78.0</td>
<td>88.6</td>
<td>77.6</td>
<td>70.1</td>
<td>71.4</td>
<td>72.4</td>
</tr>
<tr>
<td>Laboratory Fortified Blanks (LFBs) stored identically to matrix samplesd:</td>
<td>119</td>
<td>94.5</td>
<td>118</td>
<td>105</td>
<td>115</td>
<td>110</td>
<td></td>
</tr>
</tbody>
</table>

a. % Recovery = (mean concentration of seven fortified tap water samples stored for X days) / (mean concentration of seven Laboratory Fortified Blanks at 0.35 µg/L prepared on the day of assay) * 100%; where X is the storage time.
b. Central OH, total organic carbon = 1.2 mg/L, pH = 9.77, total chlorine = 1.68 mg/L, hardness = 127 mg/L as CaCO3.
c. Eastern PA, total organic carbon = 0.69 mg/L, pH = 7.37, Hardness = 193 mg/L as CaCO3.
d. % Recovery = (mean concentration of three LFBs stored for X days) / (mean concentration of seven Laboratory Fortified Blanks at 0.35 µg/L prepared on the day of assay) * 100%; where X is the storage time.

Table 10. Initial Demonstration of Capability (IDC) QC Requirements

<table>
<thead>
<tr>
<th>Method Reference</th>
<th>Requirement</th>
<th>Specification</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1.1</td>
<td>Demonstration of precision and accuracy</td>
<td>Lyse and assay 7 replicate Laboratory Fortified Blanks (LFBs) at 0.50 µg/L.</td>
<td>Percent relative standard deviation ≤15%. Mean percent recovery ≥70% and ≤130%.</td>
</tr>
<tr>
<td>9.1.2</td>
<td>Demonstration of acceptable system background</td>
<td>Lyse and assay 5 Laboratory Reagent Blanks (LRBs) distributed throughout a plate.</td>
<td>MC concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.</td>
</tr>
<tr>
<td>9.1.3</td>
<td>MRL confirmation</td>
<td>Fortify and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR meet the recovery criteria.</td>
<td>Upper PIR ≤150% Lower PIR ≥50%</td>
</tr>
<tr>
<td>9.1.4</td>
<td>Quality Control Sample (QCS)</td>
<td>Prepare a QCS near the EC50 with MC-LR from a source independent from the calibration standards.</td>
<td>Percent recovery ≥70% and ≤130% of the true value</td>
</tr>
<tr>
<td>Method Reference</td>
<td>Requirement</td>
<td>Specification and Frequency</td>
<td>Acceptance Criteria</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>ELISA Calibration</td>
<td>Use kit-recommended levels and concentrations. Two well replicates (Sect. 3.1.1) per standard.</td>
<td>%CV of absorbance ≤10%; ≤15% allowed for 1 pair. ( r^2 \geq 0.98 )</td>
</tr>
<tr>
<td>9.2.1</td>
<td>Well replicates</td>
<td>Assay field and QC samples in two wells.</td>
<td>Sample invalid if %CV of absorbance values &gt;15%</td>
</tr>
<tr>
<td>9.2.2</td>
<td>Laboratory Reagent Blank (LRB)</td>
<td>Lyse one LRB per Analysis Batch. Assay in duplicate on opposite sides of the plate.</td>
<td>MC/NOD concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.</td>
</tr>
<tr>
<td>9.2.3</td>
<td>Low Calibration Verification (Low-CV)</td>
<td>Calibration standard at, or below, the MRL concentration. One per Analysis Batch.</td>
<td>Percent recovery ≥50% and ≤150% of the true value</td>
</tr>
<tr>
<td>9.2.4</td>
<td>Laboratory Fortified Blank (LFB)</td>
<td>Reagent water fortified near the EC(_{50}). Lyse and assay 2 per Analysis Batch.</td>
<td>Percent recovery for each LFB &gt;60% and ≤140% of the true value</td>
</tr>
<tr>
<td>9.2.5</td>
<td>Laboratory Fortified Sample Matrix (LFSM) and LFSM Duplicate</td>
<td>Fortify near the EC(_{50}) and twice native concentration. One set in Analysis Batches containing drinking water; two if 20 or more field samples. One set in Analysis Batches containing ambient water; two if 20 or more field samples.</td>
<td>Mean percent recovery of LFSM and LFSMD pair ≥60% and ≤140%. Relative percent difference (RPD) ≤40%. Qualify results for samples failing these limits as “suspect–matrix”.</td>
</tr>
<tr>
<td>9.2.6</td>
<td>Quality Control Sample (QCS)</td>
<td>Assay 1 QCS for each new lot of calibration standards. Prepare the QCS near the EC(_{50}) with MC-LR from a source independent of the calibration standards.</td>
<td>Percent recovery ≥70% and ≤130% of the true value</td>
</tr>
</tbody>
</table>
Figure 1. ELISA calibration curve with calculated EC$_{50}$. Note: x-axis uses a logarithmic scale.