May 2011

Environmental Technology Verification Report

ZEU INMUNOTEC MICROCYSTIN TEST KIT: MICROCYSTEST

Prepared by
Battelle

Battelle
The Business of Innovation

Under a cooperative agreement with

EPA **U.S. Environmental Protection Agency**

ETV ETV ETV

May 2011

Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

ZEU-INMUNOTEC, S.L.
MICROCYSTIN TEST KIT:
MICROCYSTEST

by
Ryan James, Anne Gregg, and Amy Dindal, Battelle
John McKernan, U.S. EPA

Notice

The U.S. Environmental Protection Agency, through its Office of Research and Development, funded and managed, or partially funded and collaborated in, the research described herein. It has been subjected to the Agency's peer and administrative review. Any opinions expressed in this report are those of the author(s) and do not necessarily reflect the views of the Agency, therefore, no official endorsement should be inferred. Any mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Foreword

The EPA is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permitters, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six environmental technology centers. Information about each of these centers can be found on the Internet at http://www.epa.gov/etv/.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at http://www.epa.gov/etv/centers/center1.html.

Acknowledgments

The authors wish to acknowledge the contribution of the many individuals that made this verification testing possible. Quality assurance (QA) oversight was provided by Michelle Henderson, U.S. EPA, and Zachary Willenberg, Battelle. We thank David Schumacher of the Nebraska Department of Environmental Quality and Robert Waters from the New York, Suffolk County Department of Health Services for their support of this verification by providing recreational water samples, and also for peer review of the test/QA plan. We acknowledge the support from Daniel Snow and David Cassada from the University of Nebraska Water Sciences Laboratory for developing and validating the reference method in addition to analyzing the samples for the verification. Finally, we want to thank Andrew Lincoff, U.S. EPA and Daniel Snow of the University of Nebraska for their review of the test/QA plan and this verification report.

Contents

Tables

Figures

List of Abbreviations

ADQ	audit of data quality
AMS	Advanced Monitoring Systems
^{o}C	degrees Celsius
CCV	continuing calibration verification
CR	cross reactivity
CV	coefficient of variation
DQI	data quality indicator
DQO	data quality objective
DI	Deionized
EPA	Environmental Protection Agency
ETV	Environmental Technology Verification
LFM	laboratory fortified matrix
LC-MS-MS	liquid chromatography tandem mass spectrometry
LOQ	Limit of quantification
MB	method blank
MDL	method detection limit
mg/L	milligram per liter
mL	Milliliter
nm	Nanometer
NDEQ	Nebraska Department of Environmental Quality
NRC	National Research Council
NRMRL	National Risk Management Research Laboratory
OD	optical density
ppb	parts per billion
%D	percent different
PEA	performance evaluation audit
PP	protein phosphatases
PPIA	Protein Phosphatase Inhibition Assay
pNPP	p-nitrophenylphospate
pNP	p-nitrophenol
PT	performance test
QA	quality assurance
QAO	quality assurance officer
QC	quality control
QMP	quality management plan
%R	percent recovery
r^2	coefficient of determination
RB	reagent blank
RW	recreational water
RPD	relative percent difference
RSD	relative standard deviation
SD	standard deviation
SOP	standard operating procedure

SPE	solid phase extraction
TFA	trifluoroacetic acid
TQAP	Test/Quality Assurance Plan
TSA	technical systems audit
µg/L	microgram per liter
WSL	Water Sciences Laboratory

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permitters; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible. The definition of ETV verification is to establish or prove the truth of the performance of a technology under specific, pre-determined criteria or protocols and a strong quality management system. The highest-quality data are assured through implementation of the ETV Quality Management Plan. ETV does not endorse, certify, or approve technologies.

The EPA's National Risk Management Research Laboratory (NRMRL) and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under the ETV program. The AMS Center recently evaluated the performance of the MicroCystest Plate Kit offered by ZEU-INMUNOTEC.

Chapter 2
Technology Description

This verification report provides results for the verification testing of the ZEU-INMUNOTEC MicroCystest Plate Kit, based on information provided by the vendor. The information provided below was not verified in this test.

The MicroCystest test is based on protein phosphatase inhibition assay (PPIA) and designed to detect and quantify microcystins in water. The toxicity of microcystins is associated with the inhibition of protein phosphatases (PP) 1 and 2A in the liver cells. MicroCystest is therefore able to detect the potential toxicity caused by microcystins, as the kit measures the activity of the PP2A enzyme in samples possibly contaminated with these toxins. PP2A is capable of hydrolysing a chromogenic substrate like pNPP (p-nitrophenylphospate) to pNP (p-nitrophenol), which can be detected at 405 nanometers (nm). Samples containing microcystins will inhibit the enzyme proportionally to the amount of toxin contained in the sample. The test will respond to all congeners of microcystins present in the sample (more than 80 congeners are known to exist). The final concentration of microcystin can be calculated using a standard curve obtained from the standards included in the kit, expressed as μg/L microcystin-LR equivalents.

The MicroCystest kit can be used with drinking water and recreational water samples. To summarize, direct analysis of water (filtered or unfiltered) measures dissolved microcystins. Then the filtered cellular residue is treated with methanol, trifluoroacetic acid (TFA), and Tween 20™ and centrifuged. The resulting solution is diluted and analyzed to measure the intracellular microcystin and combined with the dissolved microcystin to determine the total microcystins. The kits include ready-to-use standards and all reagents needed in the assay. A spectrophotometer with 405 nm filter is required for results interpretation.

A maximum of 44 samples can be run with one 96-well kit; however if 11 or less samples are required, each kit can be split a maximum of four times, because four individual vials of phosphatase are provided. Each sample and standard is tested in duplicate and a standard curve must be analyzed in every run.

The MicroCystest is shown in Figure 1 and measures 6.1 x 4.3 x 4.3 inches (15.5 x 11.0 x 10.8 centimeters). The cost is $450 per 96-well plate kit. Other materials and equipment not provided with the kits are pipettes, pipette tips, a photometer capable of reading at 405 nm, and the supplies needed for filtering and lysing the sample.

Figure 1. MicroCystest Plate Kit

Chapter 3
Test Design and Procedures

3.1 Test Overview

This verification test was conducted according to procedures specified in the Test/Quality Assurance Plan for Verification of Microcystin Test Kits[1] (TQAP) and adhered to the quality system defined in the ETV AMS Center Quality Management Plan (QMP)[2]. As indicated in the test/QA plan, the testing conducted satisfied EPA QA Category III requirements. The test/QA plan and/or this verification report were reviewed by:

- Andrew Lincoff, U.S. EPA
- Daniel Snow of the University of Nebraska
- Robert Waters, New York Suffolk County Department of Health Services.

Evaluating microcystin test kits was identified by the AMS Center stakeholders as a priority area in 2005. With stakeholder input to the design, reference method selection, and submission of recreational waters to be evaluated, the test assessed the performance of microcystin test kits relative to key verification parameters including accuracy, precision, and method detection limit (MDL). This verification test took place from July 26 through August 12, 2010. The reference analysis was performed the week of August 16, 2010.

3.2 Experimental Design

The objective of this verification test was to evaluate the performance of the microcystin test kits against a known concentration of each microcystin variant in ASTM International Type II deionized (DI) water, as well as microcystin variants in unknown proportions from recreational water (RW) samples. Battelle conducted this verification test with recreational samples provided from the Nebraska Department of Environmental Quality (NDEQ), with the University of Nebraska Water Sciences Laboratory (WSL) providing reference analyses. The technologies were used to analyze a variety of water samples spiked with the variants microcystin-LR, microcystin-LA, and microcystin-RR. Because none of the technologies tested can specify between the different variants, the samples were spiked with individual variants. The quantitative results from the ZEU microcystin test kit were compared to the results from the reference method by calculating percent difference between the results. The reference method for microcystin was based on direct injection liquid chromatography tandem mass spectrometry (LC-MS-MS)[3] for the determination of microcystins. To attain lower levels of detection, a sample preparation method was developed by the WSL to extract the microcystins from the water samples and concentrate the samples using solid phase extraction (SPE)[4]. The Zeu MicroCystest kit provided a quantitative determination of microcystins by evaluating:

- Accuracy - comparison of test kit results (samples prepared in DI water) to results from a reference method;
- Precision – repeatability of test kit results from three sample replicates analyzed in DI water, matrix interference, and recreational water samples;
- Linearity – determination of whether or not the test kit response increases in direct proportion to the known concentration of microcystin;

- Method detection limit - the lowest quantity of toxin that can be distinguished from the absence of that toxin (a blank value) at a 95% confidence level;
- Inter-kit lot reproducibility – determination of whether or not the test kit response is significantly different between two different lots of calibration standards within the kits;
- Matrix interference – evaluation of the effect of natural recreational water matrices and chlorophyll-a on the results of the test kits; and
- Operational and sustainability factors – such as general operation, data acquisition, setup, and consumables.

Test kits were operated according to the vendor's instructions by a vendor-trained Battelle technician. Water samples were tested according to the kit instructions, and in compliance with the microcystin TQAP.

3.3 Test Procedures

The ability of each microcystin test kit to determine the concentration of microcystin was challenged using quality control (QC) samples, performance test (PT) samples and RW samples. These sample results were also compared to reference method results. Table 1 presents the test samples analyzed during this verification test.

QC, PT, and RW samples were prepared by Battelle technical staff the day before testing began. The test samples were prepared in glass volumetric flasks and stored in amber glass vials at 4 ^{o}C ± 3 ^{o}C until use. The reference samples that were prepared from the test solutions were stored in amber glass bottles at < -10^{o}C until analysis approximately two weeks later. Replicate samples for the test kits were taken from the same sample bottle. The QC, PT, and RW samples were prepared blindly for the operator by coding the sample labels to ensure the results were not influenced by the operator's knowledge of the sample concentration and variant.

Because the reference method is specific to individual microcystins, PT samples for each of the three different variants were combined into a volumetric flask and brought up to a known volume with DI water before being sent to the reference laboratory. Then the calculated dilution factor was applied to the reference method result to determine the PT sample concentration of each variant. The RW samples were sent for reference analysis without dilution.

3.3.1 QC Samples

Reagent blank (RB) samples were prepared from DI water and exposed to identical handling and analysis procedures as other prepared samples, including the addition of all reagents. These samples were used to ensure that no sources of contamination were introduced in the sample handling and analysis procedures. At least 10% of all the prepared samples were RBs.

3.3.2 PT Samples

PT samples were used to verify the accuracy, precision, linearity, MDL, and inter-kit lot reproducibility of the test kits. All PT samples were prepared at Battelle using DI water as the water source. PT samples were individually spiked with microcystin-LR, microcystin-LA, and microcystin-RR and analyzed in triplicate. The concentration levels were 0.10 (LR only), 0.50, 1.0, 2.0, and 4.0 (RR only) ppb to evaluate the full dynamic range of the test kits for these variants. The cross-reactivity (CR) of the response of the variants caused the variants to be analyzed at various concentration levels. EPA Guidelines[5] were followed to estimate the MDL

of the quantitative test kits. In doing so, a solution with a concentration five times the vendor's reported detection limit for each variant was used. A minimum of seven replicate analyses of this solution were made individually for each variant to obtain precision data with which to determine the MDL.

Table 1. Summary of Test Samples

Type of Sample	Microcystin Variant	Microcystin Concentration (ppb)	Replicates	Total Number of Samples per Test Kit
QC Samples- Laboratory Reagent Blank (RB)	none	0	3	10% of total test samples, 2
PT Samples - DI Water	LR	0.10, 0.50,1.0, 2.0	3	12
	LA	0.50, 1.0, 2.0	3	9
	RR	0.50, 1.0, 2.0, 4.0	3	12
	LR	5 times the vendor stated MDL	7	7
	LA	5 times the vendor stated MDL	7	7
	RR	5 times the vendor stated MDL	7	7
PT Samples - RW Matrix Interference Samples: RW sample and tenfold dilution	LR	2.0 *	3	6
	LA	2.0 *	3	6
	RR	2.0 *	3	6
PT Samples - Chlorophyll-*a* Matrix Interference Samples: Chlorophyll-*a* sample and tenfold dilution	LR	2.0 *	3	6
	LA	2.0 *	3	6
	RR	2.0 *	3	6
PT Samples - Inter-kit lot reproducibility	A second set of vendor provided calibration standards from a different lot analyzed following the vendor's procedure			
RW Samples- Through freeze-thaw lysing procedure	Unknown	three samples >20, three samples >10, three samples ND	3	27
RW Samples- vendor recommended lysing procedure	Unknown	three samples at unknown concentrations	3	9

*concentration that is within the calibration range of the test kit

Additional performance testing was conducted to verify the impact of possible matrix interferences. Two types of possible matrix interferences, RW water and chlorophyll-*a*, were tested. Testing was performed using a RW sample with a low level of native microcystin concentration (based on information from NDEQ). This RW sample was serially diluted by a factor of 10 with DI water to provide a less concentrated level of the RW matrix. Then both the original RW sample and diluted RW samples were fortified with 2.0 ppb of microcystin LR, LA,

or RR. The spike level chosen was dependent on the detection range of the kit. The test kit results in each of the matrices were compared to determine the impact of the matrix concentration on the test kit results. In addition, the results from the matrix samples were compared with the PT sample in DI water of the same microcystin concentration.

To evaluate the effect of chlorophyll-*a* as a possible interference, the test was designed for a DI water sample fortified with 10 milligram/Liter (mg/L) of chlorophyll-*a* (Sigma Aldrich, Cat # C5753-5MG chlorophyll-*a* from spinach) to be prepared by adding a known amount of chlorophyll-*a* into a volumetric flask and diluting to volume. Because ZEU-INMUNOTEC recommends their specific lysing procedure, the chlorophyll-*a* interference sample preparation was modified. For this test kit, the chlorophyll-*a* was spiked into the filter extraction solvent as if the water sample had already been filtered and the filter had already been extracted by the solvent. Then the samples went through the rest of the procedure. The solvent samples were spiked at 10 and 1.0 mg/L chlorophyll-a. Then each of these concentration levels was fortified with 2.0 ppb of microcystin-LR, LA, or RR. The test kit results in each of the matrices were compared to determine the impact of the chlorophyll-*a*.

Lastly, the calibration standards provided with the microcystin test kits from different lots could cause variability in the results across test kits. Therefore, two separate lots of calibration standards were analyzed using the kits and compared to determine the inter-kit lot reproducibility.

3.3.3 RW Samples

RW samples were obtained from lakes in and around Lincoln, Nebraska to assess kit performance in recreational waters. The procedure for collecting and preparing the samples for verification testing and reference analysis is described in the NDEQ standard operating procedure for microcystin analysis (SOP# SWS-2320.1A)[6]. In summary, staff from NDEQ collected the water samples from lakes where there is a potential for human exposure to microcystins. The RW samples were collected in brown plastic bottles with head space remaining and returned to the laboratory where they were frozen and thawed three times to lyse the cyanobacteria and free the microcystin into solution, making it available for analysis. Then the samples were split for verification testing and reference analysis. Using analytical data generated by NDEQ, samples used for ETV testing were selected from lakes that had both detectable and not-detectable microcystin concentrations. Because not all possible variants are monitored by the reference method, there could be a discrepancy between the test kit results and the total microcystin determined by the reference method.

As previously discussed in Chapter 2, the MicroCystest contains a specific lysing procedure to analyze for microcystin. For this test kit, three of the RW samples were split before the freeze-thaw process to compare the results using the two lysing procedures. The MicroCystest was used to analyze the three RW samples with and without the freeze-thaw lysing. One of the three RW sample extracts from the ZEU-INMUNOTEC lysing procedure was analyzed by the reference method to compare the lysing process and the entire test procedure.

Chapter 4
Quality Assurance/Quality Control

QA/QC procedures were performed in accordance with the AMS Center QMP and the TQAP for this verification test. QA level III, Applied Research was specified for this test by the EPA Project Officer. These procedures and results are described in the following subchapters.

4.1 Reference Method Quality Control

To ensure that this verification test provided suitable data for a robust evaluation of performance, a variety of data quality objectives (DQOs) were established for this test. The DQOs indicated the minimum quality of data required to meet the objectives of the verification test. The DQOs were quantitatively defined in terms of specific data quality indicators (DQIs) and their acceptance criteria. The quality of the reference method measurements were assured by adherence to these DQI criteria and the requirements of the reference methods, including the calibration and QA/QC requirements of the method. Blank samples were required to generate results below the detection limit and the Laboratory Fortified Matrix (LFM), duplicate, and Performance Evaluation Audit (PEA) sample results were required to be within 30% of the expected results. Continuing calibration verification (CCV) standards were required to be within 20% of the expected result. Battelle visited the reference laboratory prior to initiation of the reference analysis and audited the data package provided by the reference laboratory following analysis. More details about the audits are provided in Section 4.2. Table 2 presents these DQIs and the reference method QC sample results. A total of 22 samples were analyzed by the reference method; 17 were extracted prior to analysis, and five were analyzed by direct injection. One sample duplicate was processed with the 17 extracted samples to assess the DQI. No sample duplicate was included for samples analyzed via direct injection.

The calibration of the LC-MS/MS method was verified by the analysis of a CCV at a minimum of every 10 samples. All of the calibration standards were used as CCVs and were interspersed throughout the run every five samples. The percent recoveries (*%R*) of CCVs were calculated from the following equation:

$$\%R = \frac{C_s}{s} \times 100 \quad (1)$$

where C_s is the measured concentration of the CCV and, s is the spiked concentration. If the CCV analysis differed by more than 20% from the true value of the standard (i.e., %R values outside of the acceptance window of 80 to120%), the instrument was recalibrated before continuing the analysis. As shown in Table 3, all reference CCV analyses were within the required range.

Spiked samples were analyzed to assess the efficiency of the extraction method. There was a LFM spike performed every 20 samples and this was assessed by calculating the spike percent recovery (*%Rs*) as below.

$$\%Rs = \frac{C_s - C}{s} \times 100 \quad (2)$$

C_s is the measured concentration of the spiked sample, C is the measured concentration of the unspiked samples, and s is the spiked concentration. The spike %R was required to be within 30% of the spiked amount. The two LFM sample results were within this range for all three of the variants.

The relative percent difference (*RPD*) of the duplicate sample analysis was calculated from the following equation.

$$RPD = \frac{|C - C_D|}{(C + C_D)/2} \times 100 \quad (3)$$

C is the concentration of the sample analysis, and C_D is the concentration of the duplicate sample analysis. If the *RPD* was greater than 30%, then the extraction method and the analytical methods were investigated. Reference method CCV RPD results are provided in Table 2. Reference method precision of laboratory samples was not determined because the duplicate extraction was performed on the reagent blank sample.

Table 2. DQIs and Summary of Reference Method QC Results

DQI	Method of Assessment (Frequency)	Acceptance Criteria for Microcystins	Results	
Performance Evaluation Audit (PEA)	PEA Samples (Once before testing begins)	70% - 130% Recovery	See Tables 4 and 5 in Section 4.2.1	
Method contamination check	Method Blank (MB) (Once every 20 samples)	< Lowest Calibration Standard	ND for all three variants	
Method Calibration Check	Continuing Calibration Verification (CCV) (Once every 5 samples)	80% - 120% Recovery	See Table 3	
Method precision	Laboratory Duplicates (Once every 20 samples)	< 30% Difference	See Table 3	
Method accuracy	Laboratory Fortified Matrix (LFM) Spikes (Once every 20 samples)	70% - 130% Recovery	LRM 1 93% LR 79% LA 97% RR	LRM 2 103% LR 105% LA 88% RR

Table 3. Summary of Reference Method CCV Percent Recoveries and Method Precision

CCV Conc. (ppb)	Variant % Recovery			Variant RPD		
	LR	LA	RR	LR	LA	RR
10	99.5	98.2	96.1	NA	NA	NA
30	109	104	112	12%	7%	13%
30	96.5	97.1	98.7			
60	97.6	94.2	93.5	5%	14%	14%
60	103	109	108			
75	98.7	91.8	101	NA	NA	NA

4.2 Audits

Three types of audits were performed during the verification test: a performance evaluation audit (PEA), a technical systems audit (TSA) of the verification test procedures, and an audit of data quality (ADQ). Audit procedures are described further below.

4.2.1 Performance Evaluation Audit

A PEA was conducted to assess the quality of the reference measurements made in this verification test. National Institute of Standards and Technology traceable standards of microcystin are not available; however, the Canadian National Research Council (NRC) offers standards that have gone through the most validation of any commercially available standards and were recognized by the vendors and stakeholders reviewing the TQAP as the most reliable standards. The microcystin-LA variant was not available through the Canadian NRC and therefore was obtained from Abraxis. The approach of using the microcystin-LA variant standard from Abraxis was approved by all participating vendors prior to use. The standards obtained from both sources were prepared at 50 ppb in DI water and sent blindly to the reference laboratory for analysis. These PEA samples were analyzed directly (i.e., without additional preparation) and were in the mid-level of the calibration range of the reference method. The stock solutions used to prepare the calibration standards by the reference laboratory were prepared by dissolving neat standards (not solutions) obtained from EMD Biosciences (microcystin-LR), Sigma Aldrich (microcystin-LA), and ENZO Life Sciences (microcystin-RR). The results from the analyses are presented in Table 4.

Table 4. PEA Results: Analytical Comparison of Microcystin Standards

Standard Source	# of Replicates	Analysis Date	MC-LR (% Recovery)	MC-LA (% Recovery)	MC-RR (% Recovery)
NRC Canada	2	27-May	150% ± 3%	Not available	192% ± 1%
	8	9-Jun	135% ± 7%	Not available	194% ± 12%
Abraxis	2	27-May	129% ± 2%	86% ± 2%	144% ± 0%
	8	9-Jun	121% ± 6%	86% ± 5%	153% ± 10%

Shading indicates results outside acceptable 30% tolerance based on TQAP

The recoveries of the NRC and Abraxis standards revealed that the reference laboratory method, using the standards from alternate sources, were outside the acceptance range of ±30%. It was then discussed with the stakeholders, and accepted by the vendors and the EPA Project Officer, that the reference laboratory use the two available NRC standards (LR and RR) as well as LA

from Abraxis for preparing the reference method calibration solutions. It is not a common practice for calibration standards and test solutions to be generated from the same source. However, since the objective was to generate comparable vendor and reference data, it was deemed necessary and appropriate for this verification test due to the difficulties in obtaining certified microcystin standards.

To achieve the low detection limits required to analyze the test samples, an SPE extraction method was also developed and used by the reference laboratory for samples expected to be below 5.0 ppb. The MDL of this method was determined from extraction and analysis of eight solutions of LR, LA, and RR at 0.38 ppb. The reference method MDLs for LR, LA, and RR were determined to be 0.10 ppb, 0.14 ppb, and 0.13 ppb, respectively. Appendix A is the memo from the reference laboratory presenting the MDL data.

A second PEA was performed to evaluate the extraction method efficiency and the analytical method at a lower concentration relevant for this verification test. Battelle provided WSL with a blind spiked DI sample at 0.25 ppb that was extracted in triplicate. The results from the second PEA are presented in Table 5.

Table 5. PEA Results: Evaluation of Extracted Low Level Water Sample

	LR		LA		RR	
0.25 ppb Spiked Sample	**Conc. (ppb)**	**% Recovery**	**Conc. (ppb)**	**% Recovery**	**Conc. (ppb)**	**% Recovery**
Replicate 1	0.23	92%	0.21	84%	0.24	96%
Replicate 2	0.25	100%	0.23	92%	0.22	88%
Replicate 3	0.23	92%	0.22	88%	0.26	100%
Average	0.24	95%	0.22	88%	0.24	96%
Standard Deviation	0.01	5%	0.01	4%	0.02	8%

4.2.2 Technical Systems Audit

Battelle's Quality Assurance Officer (QAO) conducted a TSA to ensure that the verification test was being conducted in accordance with the TQAP and the AMS Center QMP. As part of the TSA, test procedures were compared to those specified in the TQAP, and data acquisition and handling procedures as well as the reference method procedures were reviewed. Two observations on storage of test records and sample handling and custody were documented and submitted to the Battelle Verification Test Coordinator for response. The observations from the TSA were addressed and documented as necessary. The conclusion of the TSA was that verification testing was performed according to the TQAP. TSA records are permanently stored with the QAO.

4.2.3 Data Quality Audit

Two ADQs were performed for this verification test. The first was for the data collected on the first day of testing and the second was on the complete data package generated during verification test preparation and execution. During the audits, test kit data were reviewed and verified for completeness, accuracy and traceability.

Because the EPA Project Officer designated this as an EPA Category III verification test, at least 10% of the data acquired were audited. The QAO traced the data from the initial acquisition,

through reduction and statistical analysis, to final reporting to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

Observations and findings (mostly related to test record documentation) were reported and submitted to the Battelle Verification Test Coordinator after the TSA and all observations were addressed prior to the submission of this final report.

Chapter 5
Statistical Methods

The statistical methods used to evaluate the quantitative performance factors listed in Section 3.2 are presented in this chapter. Qualitative observations were also used to evaluate verification test data.

The microcystin test kits being verified report total microcystin and are also calibrated against microcystin-LR. Because of this, the kit data were converted from microcystin-LR equivalents to compare the test kit results to the reference method results for all PT samples. Using cross reactivity data provided by each vendor (specific to each test kit), the microcystin-LR equivalents were converted to microcystin concentration by variant as follows:

$$C_{\text{by variant}} = \frac{C_{\text{LR equiv}}}{CR} \tag{4}$$

where $C_{LR\ equiv}$ is the test kit result in equivalents of microcystin-LR and *CR* is the mass-based cross reactivity of the variant.[7]

For the RW samples, each variant identified through analysis by the reference method was converted to LR-equivalents, and added together to calculate the total microcystins. The total microcystin-LR equivalents from the RW reference analyses were compared to the total microcystin results from the test kits. Because not all possible variants are monitored by the reference method, there could be a discrepancy between the test kit results and the total microcystin determined by the reference method.

5.1 Accuracy

Accuracy of the test kits verified was assessed relative to the results obtained from the reference analyses. The results for each set of analyses were expressed in terms of a percent difference (*%D*) as calculated from the following equation:

$$\%D = \frac{C_T - C_R}{C_R} \times 100 \tag{5}$$

where C_T is the microcystin-LR equivalent results from the test kits being verified and C_R is the concentration as determined by the reference method.

5.2 Linearity

Linearity was determined by linear regression with the toxin concentration measured by the reference method as the independent variable, and the test kit result being verified as the dependent variable. Linearity was expressed in terms of the slope, intercept, and the coefficient of determination (r^2). In addition, plots of the observed and predicted concentration values were constructed to depict the linearity for each variant of microcystin being tested.

5.3 Precision

The standard deviation (*S*) of the results for the replicate samples were calculated and used as a measure of test kit precision at each concentration. *S* was calculated from the following equation:

$$S = \left[\frac{1}{n-1} \sum_{k=1}^{n} (C_k - \overline{C})^2 \right]^{1/2} \quad (6)$$

where n is the number of replicate samples, C_k is the concentration measure for the k[th] sample, and $\overline{C}$ is the average concentration of the replicate samples. The kit precision at each concentration was reported in terms of the relative standard deviation (*RSD*) presented below.

$$RSD = \left| \frac{S}{\overline{C}} \right| \times 100 \quad (7)$$

5.4 Method Detection Limit

MDL was determined by seven replicate analyses of a fortified sample with the toxin concentration of five times the vendor's estimated detection limit. The MDL was calculated from the following equation:

$$MDL = t \times S \quad (8)$$

where t is the Student's value for a 95% confidence level, and S is the standard deviation of the replicate samples.

5.5 Inter-Kit Lot Reproducibility

Inter-kit lot reproducibility was assessed by calculating the RPD (Equation 3) between optical density (OD) results that are given to compare between the lots of calibration standards.

5.6 Matrix Effects

Matrix interference effects also were assessed by using a t-test to compare the microcystin test kit results generated from samples made by spiking undiluted and diluted interference matrices with the PT sample results at the same spiked concentration (either 2 or 4 ppb spike concentration). Each paired t-test was performed using the replicate data from each type of sample. The null hypothesis is that there is no difference between the two sets of data. Therefore, the resulting probability (p)-value gives the likelihood of observing a difference as large as is seen in the data, or a larger difference, if the null hypothesis were true. Therefore, at the 95% confidence interval, p-values less than 0.05 will indicate there is evidence against the null hypothesis being true and therefore a significant difference between the two sets of data. Since the number of replicates was predetermined by the test kit instructions and TQAP, power and sample size calculations were not conducted for this assessment.

Chapter 6
Test Results for the ZEU-INMUNOTEC MicroCystest Kit

The following sections provide the results of the quantitative and qualitative evaluations of this verification test for the ZEU-INMUNOTEC MicroCystest Kit.

6.1 ZEU-INMUNOTEC MicroCystest Kit Summary

The MicroCystest requires that each standard and sample be analyzed in duplicate and then the raw data output from the plate reader software reports a mean concentration of the duplicate analyses. Therefore, a sample indicated in Table 1 to have three replicates corresponded to six wells being filled as part of the MicroCystest. Each MicroCystest plate contains four calibration solutions. Following the analysis method, the plate reader measures the absorbance of the wells containing the calibration solutions at a wavelength of 405 nm and the calibration curve is generated based on the OD of each well. These results are plotted against concentrations using a vendor-provided spreadsheet that generated a four parameter curve to quantify the rest of the samples.

If the MicroCystest determined a result to be either above or below the calibration range, an "out of range" result was indicated and the sample was either diluted into the linear range or reported as being less than the limit of quantification (<LOQ). The MicroCystest does not routinely include positive or negative controls in the test kit. However, positive controls were provided by the vendor during training. Because the positive control samples were available, they were subsequently utilized for quality control during the test.

6.2 Test Kit QC Samples

As described in Section 3.3.1, the QC samples analyzed with the MicroCystest included RB samples. Ten percent of all samples analyzed were RB samples, and the results were used to verify that no contamination was introduced during sample handling. All RB sample results were reported as below the LOQ for the MicroCystest (0.25 ppb), as presented in Table 6. Two RB samples were analyzed by the reference method and were determined to be below the LOQ for all three variants.

Table 6. RB Sample Results for the ZEU-INMUNOTEC MicroCystest Kit

QC Sample ID	Plate	Mean Concentration (ppb)
RB 1	1	< LOQ
RB 2	1	< LOQ
RB 3	1	< LOQ
RB 4	3	< LOQ
RB 5	3	< LOQ
RB 6	3	< LOQ

Other quality control samples of the MicroCystest include calibration standards; however, this test kit does not routinely include positive or negative controls. Positive controls were received from the vendor for the training before testing began. Even though a positive control is not specified by the vendor, the technician analyzed a 0.70 ppb positive control at the end of each MicroCystest plate to ensure the proper technique was used by the technician. At least one positive control was analyzed at the end of each plate and in some instances when space allowed, additional positive controls were analyzed. As shown in Table 7, the percent recovery results ranged from 78% to 113% recovery. All but two coefficients of variation (CV) results were below 10%. The exceptions were Plate 1 (30%) and Plate 6 (12%). Since no acceptance criteria were provided by the vendor, no results were rejected or rerun based on these data.

Table 7. Positive Control Sample Results for the MicroCystest

Positive Control ID	Plate	Mean Concentration (ppb)	CV (%)	Percent Recovery (%)
1	1	0.55	30	78%
2a	2	0.65	7.3	93%
2b	2	0.75	1.2	110%
3	3	0.62	3.8	89%
4	4	0.79	2.6	110%
5a	5	0.66	0.80	95%
5b	5	0.60	4.5	86%
6	6	0.56	12	81%

6.3 PT Samples

Tables 8, 9, and 10 present the results for the PT samples for the three variants of microcystin used during this verification test. In addition, the tables present the sample concentration corrected for the microcystin inhibitory ability, the reference method results and the accuracy results by variant for the PT samples prepared in DI water. All samples were analyzed in triplicate.

6.3.1 Accuracy

Tables 8, 9, and 10 also present the accuracy results for the MicroCystest, expressed as %D when calculated with the theoretical spike concentration and the reference method concentration. As shown in Equation 5 (Section 5.1), the reference method value was used for calculation of accuracy.

Table 8. ZEU-INMUNOTEC MicroCystest Sample Results and Reference Method Results for LR

Sample Description	Kit Results: LR Equivalents (ppb)	Accuracy by LR Equivalents for Theoretical Concentration (% Difference)	Accuracy by LR Equivalents for Reference Concentration (% Difference)	Reference Concentration (ppb)
0.10 LR	< LOQ	NA	NA	0.10
	0.33	230%	230%	
	0.38	280%	280%	
Avg ± SD	NC[1]	NC	NC	
0.50 LR	0.62	23%	47%	0.42
	0.50	1%	20%	
	0.65	31%	55%	
Avg ± SD	0.59 ± 0.08	18% ± 16%	41% ± 19%	
1.0 LR	1.8	75%	110%	0.83
	1.7	69%	100%	
	1.5	54%	85%	
Avg ± SD	1.7± 0.10	66% ± 11%	100% ± 13%	
2.0 LR	2.8	41%	48%	1.9
	2.7	37%	44%	
	2.6	28%	35%	
Avg ± SD	2.7 ± 0.10	35% ± 7%	43% ± 7%	

[1] NC=Not calculated because of one out of three <LOQ results

Table 9. ZEU-INMUNOTEC MicroCystest Sample Results and Reference Method Results for LA

Sample Description	Kit Results: LR Equivalents (ppb)	Accuracy by LR Equivalents for Theoretical Concentration (% Difference)	Accuracy by LR Equivalents for Reference Concentration (% Difference)	Reference Concentration (ppb)
0.50 LA	0.63	26%	58%	0.40
	0.57	14%	43%	
	0.60	20%	50%	
Avg ± SD	0.60 ± 0.03	18% ± 16%	50% ± 8%	
1.0 LA	1.5	49%	110%	0.70
	1.4	41%	100%	
	1.4	38%	97%	
Avg ± SD	1.4 ± 0.10	43% ± 6%	100% ± 8%	
2.0 LA	2.5	25%	47%	1.7
	2.6	31%	54%	
	2.6	28%	50%	
Avg ± SD	2.6 ± 0.10	28% ± 3%	50% ± 4%	

Table 10. ZEU-INMUNOTEC MicroCystest Sample Results and Reference Method Results for RR

Sample Description	Kit Results: LR Equivalents (ppb)	Accuracy by LR Equivalents for Theoretical Concentration (% Difference)	Accuracy by LR Equivalents for Reference Concentration (% Difference)	Reference Concentration (ppb)
0.50 RR	0.45	-11%	17%	0.38
	0.78	56%	110%	
	0.61	21%	59%	
Avg ± SD	0.61 ± 0.17	22% ± 33%	61% ± 44%	
1.0 RR	1.7	70%	220%	0.54
	1.7	72%	220%	
	1.7	65%	210%	
Avg ± SD	1.7 ± 0.10	69% ± 4%	210% ± 7%	
2.0 RR	2.0	-1%	24%	1.6
	2.2	8%	35%	
	2.2	9%	36%	
Avg ± SD	2.1 ± 0.10	5% ± 5%	32% ± 6%	
4.0 RR	2.4	-40%	-24%	3.2
	2.4	-41%	-26%	
	2.3	-42%	-27%	
Avg ± SD	2.4 ± 0.10	-41% ± 1%	`-26% ± 1%	

For the LR spiked samples, the reference method results ranged from 0% to 17% less than the target concentration. For LR, the percent difference ranged from 20% to 280%, with overall average percent difference values ranging from 41% to 260% between the MicroCystest and the reference method. For the 0.10 ppb samples, only two of the three replicate MicroCystest samples were detectable. These results were just above the LOQ and the %D was 230% and 280%, corresponding to an absolute maximum difference from the reference concentration of 0.28 ppb. For the 0.50 ppb samples, the %D ranged from 20% to 55%, but the absolute difference from the reference concentration was no more than 0.23 ppb. For the 1.0 ppb samples, the %D ranged from 85% to 110%, corresponding to a maximum absolute difference from the reference concentration was 0.87 ppb. Similarly, for the 2.0 ppb samples, the %D ranged from 35% to 48% and the maximum absolute difference from the reference concentration was 0.92 ppb. For LR, the %D when compared to the theoretical spike concentration ranged from 1% to 280% with the overall average %D values ranging from 18% to 230%.

For the LA spiked samples, the reference method results were approximately 15% to 33% lower than the spike value. For LA, the percent difference ranged from 43% to 110%, with overall average percent difference values ranging from 50% to 100%. For the 0.50 ppb samples, the %D ranged from 43% to 58%, but the absolute difference from the reference concentration was no more than 0.23 ppb. For the 1.0 ppb samples, the %D ranged from 97% to 110%, corresponding to a maximum absolute difference from the reference concentration was 0.79 ppb. Finally, for the 2.0 ppb samples, the %D ranged from 47% to 54% and the maximum absolute difference from the reference concentration was 0.92 ppb. For LA, the %D when compared to the

theoretical spike concentration ranged from 14% to 49% with the overall average %D values ranging from 18% to 43%.

For the RR spiked samples, the reference method results were approximately 20% 46% lower than the spike value. For RR, the percent difference ranged from -27% to 220%, with overall average percent difference values ranging from -26% to 210%. For the 0.50 ppb samples, the %D ranged from 17% to 110%, corresponding to an absolute maximum difference from the reference concentration of 0.40 ppb. For the 1.0 ppb samples, the %D ranged from 210% to 220% and the maximum absolute difference from the reference concentration was 1.2 ppb. For the 2.0 ppb samples, the %D ranged from 24% to 36% corresponding to a maximum absolute difference from the reference concentration of 0.58 ppb. For the 4.0 ppb samples, the %D ranged from -24% to -27%, corresponding to a maximum absolute difference from the reference concentration of 0.86 ppb. For RR, the %D when compared to the theoretical spike concentration ranged from -42% to 72% with the overall average %D values ranging from -41% to 69%.

6.3.2 Precision

Precision results for the MicroCystest are presented in Table 11. The RSD was determined as a percentage according to Equation 7 (Section 5.3) for all DI water, matrix interference and recreational water samples. The RSDs ranged from 1% to 13% for the LR variant (mean 5.2%) For LA, the RSDs ranged from 1% to 10% (mean 4.2%) and from 1% to 27% for the RR variant (mean 6.8%); however, seven of the eight sample sets had RSDs lower than 15%. The precision for the RW samples ranged from 1% to 6% (mean 3.9%). The overall average of all RSDs was 5%, with a minimum of 1% and a maximum of 27%.

Table 11. ZEU-INMUNOTEC MicroCystest Precision Results

Variant	Sample Concentration in DI	Precision (%RSD)
LR	0.10 ppb	12%
	0.50 ppb	13%
	1.0 ppb	6%
	2.0 ppb	5%
	2.0 ppb LR in 1.0 mg/L Chlorophyll-*a* DI	2%
	2.0 ppb LR in 10 mg/L Chlorophyll-*a* DI	4%
	2.0 ppb LR in 10x dilution of RW Matrix	1%
	2.0 ppb LR in RW Matrix	1%
LA	0.50 ppb	5%
	1.0 ppb	4%
	2.0 ppb	2%
	2.0 ppb LA in 1.0 mg/L Chlorophyll-*a* DI	4%
	2.0 ppb LA in 10 mg/L Chlorophyll-*a* DI	1%
	2.0 ppb LA in 10x dilution of RW Matrix	10%
	2.0 ppb LA in RW Matrix	3%
RR	0.50 ppb	27%
	1.0 ppb	2%
	2.0 ppb	5%
	4.0 ppb	2%
	2.0 ppb RR in 1.0 mg/L Chlorophyll-*a* DI	2%
	2.0 ppb RR in 10 mg/L Chlorophyll-*a* DI	15%
	2.0 ppb RR in 10x dilution of RW Matrix	1%
	2.0 ppb RR in RW Matrix	6%
Unknown	RW 1 (20x dilution)	2%
	RW 2 (20x dilution)	3%
	RW 3 (20x dilution)	4%
	RW 4 (4x dilution)	3%
	RW 5 (4x dilution)	1%
	RW 6 (2x dilution)	6%
	RW 7	NA
	RW 8	6%
	RW 9	6%

NA - Result was less than the LOQ so no calculation of RSD

6.3.3 Linearity

The linearity of the MicroCystest measurements was assessed by performing a linear regression of the MicroCystest kit results against the reference method results for the four PT samples ranging from 0.10 ppb to 2.0 ppb of microcystin LR in DI water, three PT samples ranging from 0.50 ppb to 2.0 ppb for microcystin LA, and four PT samples ranging from 0.50 ppb to 4.0 ppb RR in DI water. Figures 2, 3, and 4 present the results of the linear regressions for LR, LA, and RR respectively. The slope, intercept, and coefficient of determination (r^2) for each regression equation are shown on the charts. The linear regressions compared to the reference method results had coefficients of determination of 0.95for LR, 0.95 for LA, and 0.63 for RR. In general, LR looked to generate data that was reasonably linear while LA and RR seem to indicate a log-normal relationship between the reference method concentration and the MicroCystest measurements.

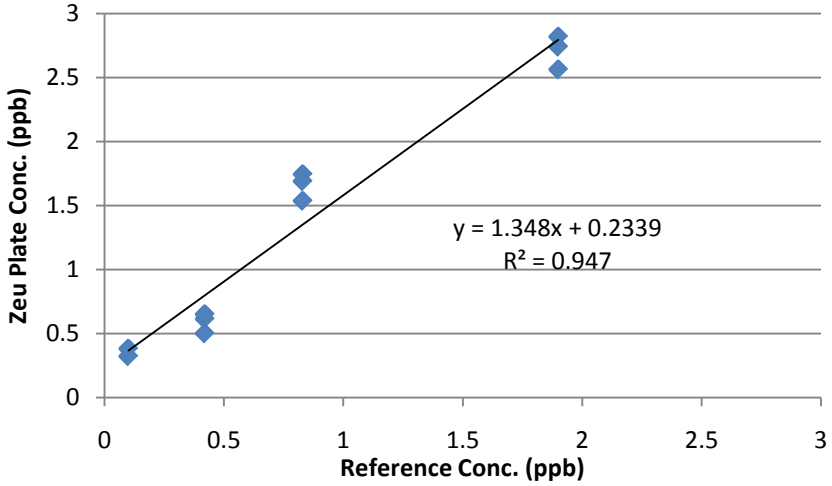

Figure 2. Linearity for the ZEU-INMUNOTEC MicroCystest for LR

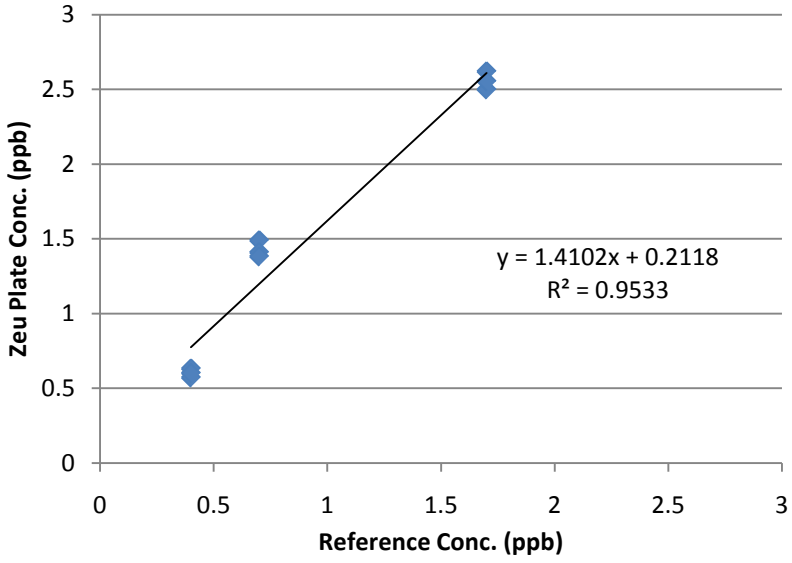

Figure 3. Linearity for the ZEU-INMUNOTEC MicroCystest for LA

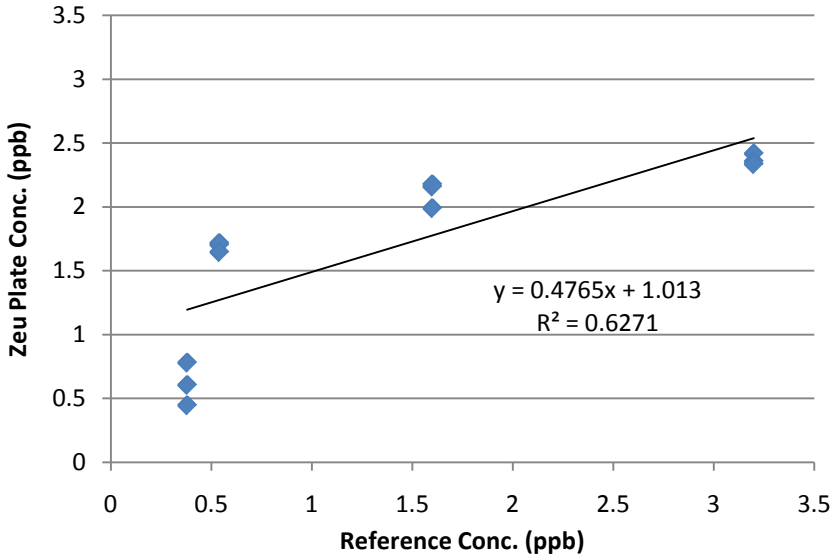

Figure 4. Linearity for the ZEU-INMUNOTEC MicroCystest for RR

6.3.4 Method Detection Limit

The MDL was assessed by analyzing at least seven replicates of a sample spiked at approximately five times the vendor-stated detection limit for the microcystin test kit (which was 0.25 ppb). Table 12 lists the replicate results; the %CV of the duplicate MicroCystest analysis for each replicate, the standard deviations for the replicate results, and shows the calculated MDLs for the three variants. The calculated MDL values were 0.24, 0.17, and 0.61 ppb for LR, LA, and RR respectively. The increased variability in the RR results was unexpected because the sample bottle, plate, analysis method, and operator were the same. It is possible that the increased MDL was due to an operational issue.

Table 12. Detection Limit Results for the ZEU-INMUNOTEC MicroCystest

Variant	LR		LA		RR	
Sample Concentration (ppb)	**Mean Conc. (ppb)**	**%CV**	**Mean Conc. (ppb LR Equivalents)**	**%CV**	**Mean Conc. (ppb LR Equivalents)**	**%CV**
1.3	2.1	2.6	1.9	8.2	1.6	20
1.3	2.3	1.7	1.9	3.0	1.4	2.5
1.3	2.4	1.1	1.8	1.9	1.5	5.0
1.3	2.4	1.2	1.7	17	1.9	18
1.3	2.2	7.7	1.9	0.70	2.1	0.20
1.3	2.2	4.3	1.9	3.1	2.2	4.1
1.3	2.1	1.5	1.9	1.2	1.9	12
Standard Deviation	0.13		0.09		0.31	
t (n=7)	1.9		1.9		1.9	
MDL	**0.24**		**0.17**		**0.61**	

6.3.5 Inter-Kit Lot Reproducibility

Two sets of kit calibration standards were analyzed on the sample plate to determine whether or not the calibration standards from different lots were similar. The data are presented in Table 13. The OD values were compared by calculation of the RPD between each pair of OD measurements. All RPDs except one were less than 9% with the highest RPD value at 25%.

Table 13. Inter-kit Lot Comparison of Kit Calibration Standards for the ZEU-INMUNOTEC MicroCystest

	OD Values (ppb)		
Standard (ppb)	**Set A**	**Set B**	**RPD**
0.25	1.63	1.56	4%
	1.37	1.50	9%
0.50	1.13	1.19	5%
	1.16	1.20	3%
1.0	0.826	0.641	25%
	0.696	0.699	0%
2.5	0.355	0.344	3%
	0.375	0.351	7%

6.3.6 Matrix Effect

Matrix interference effects were assessed by using a t-test to compare the MicroCystest results generated from samples made by spiking undiluted and diluted interference matrices with the PT sample results at the same concentration. The two possible interfering matrices included a RW sample both undiluted and after undergoing a tenfold dilution and chlorophyll-*a* at 10 mg/L and 1.0 mg/L. Tables 14 and 15 provide the MicroCystest sample results for the RW matrix interference samples and chlorophyll-*a* interference samples, respectively, including the average and SD for each sample. Because this comparison is made to evaluate only the impact of the matrix on the sample result, LR equivalents are used.

Each paired t-test was performed using the replicate data from each type of sample. The null hypothesis is that there is no difference between the two sets of data. The resulting probability (p)-value gives the likelihood of observing a difference as large as is seen in the data, or a larger difference, if the null hypothesis were true. Therefore, at the 95% confidence interval, p-values less than 0.05 will indicate there is evidence against the null hypothesis being true, and therefore a significant difference between the two sets of data exists.

Table 16 summarizes the results of a paired t-test for both sets of interference data by showing the p-values associated with each of the applicable comparisons across both types of possible interfering matrices. Across both the chlorophyll-*a* and RW results, none of the 18 comparisons were determined to be statistically different. Therefore, the interferences tested during this verification test did not affect the performance of the MicroCystest. There are p-values from 18 tests reported in Table 16 and none them is smaller than 0.05. At a significance level of 5%, we would expect one test out of every 20 to have a p-value below 0.05 just by chance, even if the null hypothesis were true in each case. A formal multiple comparisons adjustment is not needed here because a performance standard is not being evaluated as this is more of an exploratory test

to determine if there is any difference caused by the matrix. However, a conservative Bonferroni correction, for example, would set the p-value associated with a significant result at 0.05 divided by 18, corresponding to a p-value of 0.0028 for the individual tests.

Table 14. RW Matrix Interference Sample Results for the ZEU-INMUNOTEC MicroCystest

Variant	Sample Description	Mean Kit Results: LR Equivalents (ppb)	Average Result (ppb)	SD
Unknown	Unspiked RW Matrix (RW 9)	0.69 0.77 0.69	0.72	0.05
LR	2.0 ppb LR in DI	2.8 2.7 2.6	2.7	0.10
	2.0 ppb LR in tenfold dilution of RW Matrix	2.7 2.7 2.7	2.7	0
	2.0 ppb LR in RW Matrix	2.7 2.7 2.7	2.7	0.10
LA	2.0 ppb LA in DI	2.5 2.6 2.6	2.6	0.10
	2.0 ppb LA in tenfold dilution of RW Matrix	2.5 2.5 2.1	2.4	0.20
	2.0 ppb LA in RW Matrix	2.6 2.6 2.7	2.6	0.10
RR	2.0 ppb RR in DI	2.0 2.2 2.2	2.1	0.10
	2.0 ppb RR in tenfold dilution of RW Matrix	2.2 2.3 2.2	2.2	0.10
	2.0 ppb RR in RW Matrix	2.4 2.7 2.4	2.5	0.20

Table 15. Chlorophyll-*a* Interferent Sample Results for the ZEU-INMUNOTEC MicroCystest

Variant	Sample Description	Mean Kit Results: LR Equivalents (ppb)	Average Result (ppb)	SD
LR	2.0 ppb LR in DI	2.8 2.7 2.6	2.7	0.10
	2.0 ppb LR in 1.0 mg/L Chlorophyll-a DI	2.6 2.5 2.6	2.6	0.10
	2.0 ppb LR in 10 mg/L Chlorophyll-a DI	2.6 2.6 2.8	2.7	0.10
LA	2.0 ppb LA in DI	2.5 2.6 2.6	2.6	0.10
	2.0 ppb LA in 1.0 mg/L Chlorophyll-a DI	2.6 2.5 2.4	2.5	0.10
	2.0 ppb LA in 10 mg/L Chlorophyll-a DI	2.5 2.4 2.5	2.5	0
RR	2.0 ppb RR in DI	2.0 2.2 2.2	2.1	0.10
	2.0 ppb RR in 1.0 mg/L Chlorophyll-a DI	2.0 2.0 2.0	2.0	0
	2.0 ppb RR in 10 mg/L Chlorophyll-a DI	1.9 2.0 2.5	2.1	0.30

Table 16. Statistical Comparisons between Interference Samples for the ZEU-INMUNOTEC MicroCystest

	p-value (D-different, ND-not different)		
Description of Comparison	**LR**	**LA**	**RR**
2.0 ppb in DI compared with 2.0 ppb in tenfold dilution of RW	0.962 (ND)	0.369 (ND)	0.137(ND)
2.0 ppb in DI compared with 2.0 ppb in undiluted RW	0.890 (ND)	0.258 (ND)	0.031 (ND)
2.0 ppb in undiluted RW compared with tenfold dilution of RW	0.526 (ND)	0.308 (ND)	0.073 (ND)
2.0 ppb in DI compared with 2.0 ppb in 1.0 mg/L Chlorophyll-*a* DI	0.170 (ND)	0.617 (ND)	0.218 (ND)
2.0 ppb in DI compared with 2.0 ppb in 10 mg/L Chlorophyll-*a* DI	0.729 (ND)	0.132 (ND)	0.848 (ND)
2.0 ppb in 1.0 mg/L Chlorophyll-*a* DI compared with 2.0 ppb in 10 mg/L Chlorophyll-*a* DI	0.300 (ND)	0.490 (ND)	0.505 (ND)

6.4 RW Sample Results

Table 17 presents the RW results for the MicroCystest and the reference analysis. The concentrations were determined by the reference method for only three of the approximately 80 variants that are naturally occurring in recreational waters. The total microcystins measured by the MicroCystest may have other variants present that would not have been detected by the reference method. Therefore, testing included only a qualitative comparison between the MicroCystest and the reference method results. In general, the samples that were determined to have higher total concentrations by the MicroCystest had higher total concentrations with the reference method as well. All of the MicroCystest total microcystin results were greater than the reference method results, which was limited to quantifying three of the ~80 known variants. However, the results of the MicroCystest were usually within a factor of four of the reference method, indicating that the LR, LA, and RR variants make up a considerable proportion of the microcystins that are measurable by the MicroCystest.

RW 6, 7, and 8 were lysed using the freeze thaw method used with the other RW samples. In addition, an aliquot of each was removed before the lysing to follow the procedure in the MicroCystest Kit. The dissolved (unfiltered RW 6), filtrate (filtered RW 6), and intracellular partitions (solid algae collected on filter to be lysed) of the RW samples were analyzed by the Microcystest and the intracellular portion of RW 6 was also analyzed by the reference method. The results of these samples are presented in Table 18.

Because RW6 sample had detectable levels of microcystin in each sample fraction (dissolved, filtrate, and intracellular), the RW 6 sample illustrated the use of the Zeu lysing method. The RW 6 dissolved and liquid filtrate results should represent the same amount of dissolved microcystin as one is just the filtered form of the other. The results were consistent as the RW 6 dissolved and filtrate fractions produced concentrations of 0.31 and 0.35 ppb, respectively. The RW intracellular represented the microcystins bound in the algae cells. The result from the MicroCystest was 2.0 ppb. Lastly, the RW 6 freeze-thaw result of 2.6 ppb represented the total amount to microcystin released from the algal cells during the freeze-thaw cycling in

combination with the already dissolved microcystin. As would be expected, the freeze-thaw result was similar to the total result of the MicroCystest RW 6 dissolved result of 0.31 ppb and the MicroCystest RW 6 intracellular result of 2.0 ppb for a total microcystin concentration of approximately 2.4 ppb. The total determined by the reference method (using freeze-thaw lysing) was 2.0 ppb and the total determined by the reference method (using the MicroCystest lysing method) was 1.0 ppb. Therefore, when comparing the MicroCystest results, the lysing procedure produced very similar results, but with the reference method the results were different by 1.0 ppb.

Table 17. Recreational Water Sample Results for the ZEU-INMUNOTEC MicroCystest

	Test Kit Results (ppb)					Reference Results (ppb)			
Sample Description	**Kit Results: LR Equivalents (ppb)**	**Dilution Factor**	**Corrected Conc. (ppb)**	**Average Conc. (ppb)**	**Standard Deviation (ppb)**	**LR**	**LA**	**RR**	**Total**
RW 1 (20x dilution)	2.0	20	40	41	0.65	9.6	1.8	19	30
	2.1	20	41						
	2.1	20	41						
RW 2 (20x dilution)	2.1	20	41	40	1.3	7.2	2.2	6.3	16
	2.0	20	39						
RW 3 (20x dilution)	1.9	20	38	40	1.7	7.6	<0.1	2.7	10
	2.1	20	42						
	2.0	20	40						
RW 4 (4x dilution)	1.3	4	5.1	4.9	0.13	<0.1	3.5	<0.1	3.5
	1.2	4	4.9						
	1.2	4	4.8						
RW 5 (4x dilution)	1.6	4	6.3	6.2	0.09	3.1	0.15	0.40	3.6
	1.5	4	6.1						
	1.6	4	6.3						
RW 6 freeze thaw (2x dilution)	1.2	2	2.5	2.6	0.16	1.8	<0.1	0.26	2.01
	1.4	2	2.7						
RW 7 freeze thaw	<LOQ	1	<LOQ	<LOQ	NA	<0.1	<0.1	<0.1	<0.1
	<LOQ	1	<LOQ						
RW 8- freeze thaw	0.66	1	0.66	0.69	0.04	0.27	<0.1	0.12	0.39
	0.71	1	0.71						
RW 9 (RW Matrix)	0.69	1	0.69	0.72	0.05	0.18	<0.1	<0.1	0.18
	0.77	1	0.77						
	0.69	1	0.69						

NA – Standard Deviation was not calculated because sample results were less than the LOQ

Table 18. RW Lysing Extract Sample Results for the ZEU-INMUNOTEC MicroCystest

Sample Description	Test Kit Results (ppb)					Reference Results (ppb)			
	Kit Results: LR Equivalents (ppb)	Dilution Factor	CR Corrected Conc. (ppb)	Average Conc. (ppb)	Standard Deviation (ppb)	LR	LA	RR	Total
RW 6 dissolved	0.33	1	0.33	0.31	0.04	NA	NA	NA	NA
	0.28	1	0.28						
	<LOQ	1	<LOQ						
RW 6 filtrate	0.25	1	0.25	0.35	0.10	NA	NA	NA	NA
	0.44	1	0.44						
	0.37	1	0.37						
RW 6 intracellular	2.0	1	2.0	2.0	0.03	0.99	<0.1	<0.1	0.99
	2.1	1	2.1						
	2.0	1	2.0						
RW 6 freeze thaw (2x dilution)	1.2	2	2.5	2.6	0.16	1.8	<0.1	0.26	2.0
	1.4	2	2.7						
RW 7 dissolved	<LOQ	1	<LOQ	0.23	NC	NA	NA	NA	NA
	<LOQ	1	<LOQ						
	0.23	1	0.23						
RW 7 filtrate	<LOQ	1	<LOQ	<LOQ	<LOQ	NA	NA	NA	NA
	<LOQ	1	<LOQ						
	<LOQ	1	<LOQ						
RW 7 intracellular	<LOQ	1	<LOQ	<LOQ	<LOQ	NA	NA	NA	NA
	<LOQ	1	<LOQ						
	<LOQ	1	<LOQ						
RW 7 freeze thaw	<LOQ	1	<LOQ	<LOQ	<LOQ	<0.1	<0.1	<0.1	<0.1
	<LOQ	1	<LOQ						
RW 8 dissolved	<LOQ	1	<LOQ	0.28	NC	NA	NA	NA	NA
	0.28	1	0.28						
	<LOQ	1	<LOQ						
RW 8 filtrate	<LOQ	1	<LOQ	<LOQ	<LOQ	NA	NA	NA	NA
	<LOQ	1	<LOQ						
	<LOQ	1	<LOQ						
RW 8 intracellular	0.57	1	0.57	0.59	0.03	NA	NA	NA	NA
	0.63	1	0.63						
	0.58	1	0.58						
RW 8- freeze thaw	0.66	1	0.66	0.69	0.04	0.27	<0.1	0.12	0.39
	0.71	1	0.71						

NC – Standard deviation was not calculated because sample results were less than the LOQ.
NA – For reference method results, NA indicates that the sample was not analyzed through the reference method.

6.5 Operational Factors

During testing activities, the technical operators were instructed to fill out an Ease of Use Questionnaire. This section summarizes these observations as well as other operational considerations about the technology.

6.5.1 Ease of Use

The test kit operator reported that the MicroCystest was easy to use. The brochure explains the extraction and analyses procedure clearly. Solution preparation involves hydrating the phosphatase buffer and gently shaking the solution for an hour. The procedure includes one 30-minute incubation period at 37 ^{o}C. Previous knowledge or training on the use of micro-pipettes and or multi-channel pipettes with 96-well plates is recommended for consistent readings. The Battelle operator trained by the vendor had experience with pipetting and 10+ years of laboratory experience. A spectrophotometer plate reader is necessary for obtaining the spectrophotometric readings at 405 nm that are then analyzed using any commercial plate reading evaluation program (a four-parameter plate reading program is recommended by the vendor). The lysing procedure was not included in the ease of use evaluation of the test kit.

6.5.2 Cost and Consumables

Once the analysis is complete, the remaining solutions and well contents were disposed of according to local regulations.

The kit has a 6-month shelf life as received, and should be stored at 4 – 8 ^{o}C. Of the 96-wells on one plate, eight are needed for calibration samples. The remaining 88 are for sample analyses that are performed in duplicate (44 total samples). Other equipment and consumables not included in the kit are pipettes, pipette tips, DI water, a photometer capable of reading at 405 nanometers, and the supplies needed for filtering and lysing of the sample. The price for the MicroCystest at the time of the verification was $450 per 96-well plate kit.

Chapter 7
Performance Summary for the ZEU-INMUNOTEC MicroCystest

The verification of the ZEU-INMUNOTEC MicroCystest is summarized by the parameters described in Table 19.

Table 19. ZEU-INMUNOTEC MicroCystest Performance Summary

Verification Parameters	LR	LA	RR
Accuracy (ppb, range of %Difference)			
0.10	230% and 280%		
0.50	20% - 55%	43% - 58%	17% - 105%
1.0	85% - 110%	97% - 110%	210% - 220%
2.0	35% - 48%	47% - 54%	24% - 36%
4.0			-27% to -24%
Precision (range of %RSD)	1% - 13%	1% - 10%	1% - 27%
Precision (RW samples)	1% to 6%		
Linearity (y=)	1.4x + 0.23 $r^2 = 0.95$	1.4x + 0.21 $r^2 = 0.95$	0.48x + 1.0 $r^2 = 0.63$
Method Detection Limit (ppb)	0.24	0.17	0.61

Inter-kit lot reproducibility. Calibration standards from two different lots were measured and all of the RPDs except one were less than 9% with the highest RPD value at 25%.

Matrix Interference. Matrix interference effects were assessed by using a t-test to compare results from samples made by spiking undiluted and diluted interference matrices with the PT sample results at 2.0 ppb spiked concentration. Across both the chlorophyll-*a* and RW results, none of the 18 comparisons were determined to be statistically different (at low power), and therefore, the interferences tested during this verification did not affect the performance of the MicroCystest.

Recreational Water (RW). In general, the samples that were determined to have higher total concentrations by the MicroCystest also had higher total concentrations as determined by the reference method. All of the MicroCystest total microcystin results were greater than the reference method results, which were consistent with the likelihood that all of the microcystins were not being measured by the reference method that is limited to measuring three variants. However, the results of the MicroCystest were usually within a factor of three or four of the reference method, indicating that the LR, LA, and RR variants are common in the RW samples tested, making up more than a quarter of the microcystins measurable by the MicroCystest.

In addition to the freeze-thaw method of lysing algae cells to release microcystins, a Zeu-specific lysing technique was verified. Three RW samples were analyzed using both lysing approaches and the results reported.

Operational Factors. The test kit operator reported that the MicroCystest was easy to use. The brochure explains the extraction and analyses procedure clearly. Solution preparation involves hydrating the phosphatase buffer and gently shaking the solution for an hour. The procedure

includes one 30-minute incubation period at 37 ^{o}C. Previous knowledge or training on the use of micro-pipettes and or multi-channel pipettes with 96-well plates is recommended for consistent readings. The Battelle operator trained by the vendor had experience with pipetting and 10+ years of laboratory experience. A spectrophotometer plate reader is necessary for obtaining the spectrophotometric readings at 405 nm that are then analyzed using a commercial plate reading evaluation program. The lysing procedure was not included in the ease of use evaluation of the test kit. Once the analysis was complete, the remaining solutions and well contents were disposed of in the regular laboratory trash. Since waste disposal requirements vary from state-to-state, the reader is encouraged to consult with state government agency for proper waste disposal requirements.

According to the vendor, the kit has a 6-month shelf life as received and should be stored at 4 – 8 ^{o}C. Of the 96-wells on one plate, eight are needed for calibration samples. The remaining 88 are for sample analyses that are performed in duplicate (44 total samples). Other equipment and consumables not included in the kit are pipettes, pipette tips, DI water, a photometer capable of reading at 405 nanometers, and the supplies needed for filtering and lysing of the sample. The price for the MicroCystest at the time of the verification test was $450 per 96-well plate kit.

Chapter 8
References

1. *Test/Quality Assurance Plan for Verification of Microcystin Test KitsTest/Quality Assurance Plan for Verification of Microcystin Test Kits.* U.S. Environmental Technology Verification Program, Battelle, July 2010.
2. *Quality Management Plan for the ETV Advanced Monitoring Systems Center, Version 7.* U.S. Environmental Technology Verification Program, Battelle, November 2008.
3. Cong, L.H., B.; Chen, Q.; Lu, B.; Zhang, J.; Ren, Y., *Determination of trace amount of microcystins in water samples using liquid chromatography coupled with triple quadrupole mass spectrometry. Anal. Chim. Acta*, 2006. **569** (1-2): p. 157-168.
4. Cassada, D., *Standard Operating Procedure (SOP) Determination of algaltoxin residues in water extracts by solid phase extraction (SPE), liquid chromatography (LC)-atmospheric pressure electrospray ionization tandem mass spectrometry (MS/MS)*. July, 2010, Water Sciences Laboratory, University of Nebraska.
5. *"Guidelines Establishing Test Procedures for the Analysis of Pollutants."*, USEPA, Editor. 2000, U.S. Code of Federal Regulations.
6. *SOP# SWS-2320.1A: Microcystin Analysis Using the Abraxis ELISA (Enzyme-Linked Immuno-Sorbent Assay) Method.* Nebraska Department of Environmental Quality.
7. Loftin, K.A., et al., *Comparison of Two Cell Lysis Procedures for Recovery of Microcystins in Water Samples from Silver Lake in Dover, Delaware, with Microcystin Producing Cyanobacterial Accumulations*, in *USGS Open-File Report 2008 -1341*. 2008. p. 9.

APPENDIX A
Reference Laboratory Method Detection Limit Memo

July 14, 2010

To: Anne Gregg and Ryan James, Battelle Laboratories

From: Daniel Snow and David Cassada, UNL Water Sciences Laboratory

Re: Summary of Microcystin SPE method validation – July 13-14, 2010

Microcystins LA, LR and RR were spiked into water and extracted using solid phase extraction (SPE) to evaluate method accuracy and precision, and method detection limits. The method described in Cong et al. 2006 was modified to allow for extraction of a larger sample by using higher capacity polymeric (Waters Oasis, HLB) SPE cartridges. Briefly, 400-milliliter (mL) of purified reagent water was fortified with 1500 µL of a diluted mixed stock (0.1 ng/µL) obtained from Battelle to produce 0.375 µg/L of each analyte. Nodularin (1600 µL of a 0.10 ng/µL solution) was also added to produce a concentration of 0.40 µg/L. Eight 50 mL portions of this fortified water were weighed into 125 mL amber glass bottles and each portion separately spiked with 100 µL of the enkephalin-Leu internal standard (IS) solution (0.1 ng/µL) to give a concentration of 2.0 µg/L. A single method blank was prepared by spiking with IS and surrogate only.

After capping and shaking each solution to equilibrate, samples were drawn under vacuum through pre-conditioned 200 mg Oasis HLB SPE cartridges at a rate of approximately 10 mL/min. When the sample had completely passed through the cartridge, it was allowed to air-dry under vacuum, removed from the extraction apparatus and prepared for elution. Ten (10) milliliters of high purity methanol (Fisher Optima Grade) were used to elute analyte, IS and surrogate compounds from the cartridges. The methanol was evaporated under nitrogen to approximately 0.4 mL and the extracts transferred to low volume inserts for analysis on the LCQ ion trap tandem LC/MS system. Calibration solutions (5, 10, 30, 60 and 75 ng/mL) were prepared in water from the same mixed stock as the spiking solutions. A table summarizing the results of the validation is copied below (Table A-1.).

A second 10 mL aliquot of methanol was passed through 4 of SPE cartridges and collected separately to check for completeness of analyte elution. These second aliquots were reduced to the same 0.40 mL volume as the MDL eluents and analyzed. The resulting absolute areas of the analyte, surrogate, and internal standard peaks obtained were approximately 1% of the areas obtained in the first portion. This suggests that lower elution volumes can result in decreased analyte recovery.

References

Cong, L.; Huang, B.; Chen, Q.; Lu, B.; Zhang, J.; Ren, Y. (2006) Determination of trace amount of microcystins in water samples using liquid chromatography coupled with triple quadrupole mass spectrometry. *Anal. Chim. Acta*, **569** (1-2), 157-168.

Table A-1. Average, standard deviation, method detection limits (MDL = S x tN-1) and recoveries of microcystins obtained from extraction and analysis of eight fortified reagent water (0.375 µg/L) samples.

50 mL sample Aliquot	Amount obtained (ng)				Concentration (µg/L)			
	Nodularin	**MC-RR**	**MC-LR**	**MC-LA**	**Nodularin**	**MC-RR**	**MC-LR**	**MC-LA**
MDL 1	23.994	19.679	18.084	19.913	0.480	0.394	0.362	0.398
MDL 2	24.647	19.661	21.985	21.752	0.493	0.393	0.440	0.435
MDL 3	22.716	17.660	20.524	18.404	0.454	0.353	0.410	0.368
MDL 4	23.157	19.715	21.022	20.304	0.463	0.394	0.420	0.406
MDL 5	26.361	19.731	20.462	21.182	0.527	0.395	0.409	0.424
MDL 6	19.618	18.214	18.322	18.393	0.392	0.364	0.366	0.368
MDL 7	20.254	14.533	20.046	21.490	0.405	0.291	0.401	0.430
MDL 8	19.889	15.247	17.518	14.614	0.398	0.305	0.350	0.292
AVG	22.580	18.055	19.745	19.507	0.452	0.361	0.395	0.390
STD DEV	2.460	2.113	1.586	2.360	0.049	0.042	0.032	0.047
MDL	**7.371**	**6.333**	**4.753**	**7.072**	**0.147**	**0.127**	**0.095**	**0.141**
%REC	**112.9**	**96.3**	**105.3**	**104.0**	**112.9**	**96.3**	**105.3**	**104.0**
Expected value	20.0	18.75	18.75	18.75	0.4	0.375	0.375	0.375

APPENDIX B
ZEU-INMUNOTEC MicroCystest Raw Data

Table B-1. ZEU-INMUNOTEC MicroCystest Raw Data

Sample Description	Variant	Mean Conc. (ppb)	Standard Deviation (ppb)	CV%
Reagent Blank	RB	Range?	Range?	Range?
Reagent Blank	RB	Range?	Range?	Range?
Reagent Blank	RB	Range?	Range?	Range?
Reagent Blank	RB	0.151	0.046	30.1
Reagent Blank	RB	0.137	0.01	7.1
Reagent Blank	RB	0.127	0.034	26.7
Positive Control 1	LR	0.548	0.165	30.2
Positive Control 2a	LR	0.651	0.048	7.3
Positive Control 2b	LR	0.751	0.009	1.2
Positive Control 3	LR	0.621	0.024	3.8
Positive Control 4	LR	0.794	0.021	2.6
Positive Control 5a	LR	0.662	0.005	0.8
Positive Control 5b	LR	0.604	0.027	4.5
Positive Control 6	LR	0.564	0.069	12.2
Std 0 Diff Lot	LR	0.128	0.031	23.9
Std 0.1 Diff Lot	LR	0.236	0.022	9.2
Std 0.3 Diff Lot	LR	0.457	0.005	1.1
Std 0.8 Diff Lot	LR	1.2	0.099	8.3
Std 2.5 Diff Lot	LR	2.636	0.039	1.5
0.1 LR	LR	Range?	Range?	Range?
0.1 LR	LR	0.325	0	0
0.1 LR	LR	0.384	0	0
0.5 LA	LA	0.631	0.006	0.9
0.5 LA	LA	0.571	0.016	2.8
0.5 LA	LA	0.601	0.017	2.8
0.5 LR	LR	0.616	0.028	4.6
0.5 LR	LR	0.503	0.032	6.3
0.5 LR	LR	0.653	0.026	4
0.5 RR	RR	0.446	0.024	5.3
0.5 RR	RR	0.78	0.045	5.8
0.5 RR	RR	0.605	0.039	6.4
1.0 LA	LA	1.489	0.025	1.7
1.0 LA	LA	1.409	0.107	7.6
1.0 LA	LA	1.381	0.043	3.1
1.0 LR	LR	1.746	0.065	3.7
1.0 LR	LR	1.691	0.012	0.7

Table B-1. ZEU-INMUNOTEC MicroCystest Raw Data Continued				
Sample Description	**Variant**	**Mean Conc. (ppb)**	**Standard Deviation (ppb)**	**CV%**
1.0 LR	LR	1.538	0.028	1.8
1.0 RR	RR	1.703	0.04	2.3
1.0 RR	RR	1.717	0.013	0.7
1.0 RR	RR	1.646	0.047	2.9
2.0 LA	LA	2.498	0.108	4.3
2.0 LA	LA	2.619	0.028	1.1
2.0 LA	LA	2.553	0.013	0.5
2.0 LR	LR	2.82	0.091	3.2
2.0 LR	LR	2.741	0.081	3
2.0 LR	LR	2.564	0.117	4.6
2.0 RR	RR	1.99	0.269	13.5
2.0 RR	RR	2.159	0.031	1.4
2.0 RR	RR	2.177	0.018	0.8
4.0 RR	RR	2.418	0.001	0
4.0 RR	RR	2.357	0.118	5
4.0 RR	RR	2.335	0.022	0.9
1.25 LA	LA	1.924	0.158	8.2
1.25 LA	LA	1.941	0.058	3
1.25 LA	LA	1.831	0.035	1.9
1.25 LA	LA	1.694	0.283	16.7
1.25 LA	LA	1.92	0.014	0.7
1.25 LA	LA	1.893	0.059	3.1
1.25 LA	LA	1.867	0.022	1.2
1.25 LR	LR	2.087	0.054	2.6
1.25 LR	LR	2.348	0.041	1.7
1.25 LR	LR	2.398	0.027	1.1
1.25 LR	LR	2.394	0.029	1.2
1.25 LR	LR	2.238	0.173	7.7
1.25 LR	LR	2.192	0.094	4.3
1.25 LR	LR	2.135	0.033	1.5
1.25 RR	RR	1.593	0.314	19.7
1.25 RR	RR	1.38	0.035	2.5
1.25 RR	RR	1.492	0.075	5
1.25 RR	RR	1.883	0.339	18
1.25 RR	RR	2.093	0.003	0.2
1.25 RR	RR	2.218	0.091	4.1
1.25 RR	RR	1.868	0.216	11.6
2 ppb Chloro LA	LA	2.46	0.113	4.6
2 ppb Chloro LA	LA	2.439	0.008	0.3
2 ppb Chloro LA	LA	2.463	0.186	7.6
2 ppb Chloro LA 10x	LA	2.606	0.065	2.5
2 ppb Chloro LA 10x	LA	2.532	0.03	1.2

Table B-1. ZEU-INMUNOTEC MicroCystest Raw Data Continued

Sample Description	Variant	Mean Conc. (ppb)	Standard Deviation (ppb)	CV%
2 ppb Chloro LA 10x	LA	2.39	0.001	0.1
2 ppb Chloro LR	LR	2.567	0.078	3
2 ppb Chloro LR	LR	2.642	0.017	0.7
2 ppb Chloro LR	LR	2.759	0.249	9
2 ppb Chloro LR 10x	LR	2.606	0.019	0.7
2 ppb Chloro LR 10x	LR	2.506	0.064	2.6
2 ppb Chloro LR 10x	LR	2.556	0.075	2.9
2 ppb Chloro RR	RR	1.909	0.001	0.1
2 ppb Chloro RR	RR	2.014	0.019	1
2 ppb Chloro RR	RR	2.498	0.574	23
2 ppb Chloro RR 10x	RR	2	0.209	10.5
2 ppb Chloro RR 10x	RR	1.979	0.146	7.4
2 ppb Chloro RR 10x	RR	2.042	0.073	3.6
2 ppb Matrix LA	LA	2.584	0.026	1
2 ppb Matrix LA	LA	2.608	0.017	0.6
2 ppb Matrix LA	LA	2.722	0.034	1.3
2 ppb Matrix LA 10x	LA	2.536	0.073	2.9
2 ppb Matrix LA 10x	LA	2.53	0.011	0.5
2 ppb Matrix LA 10x	LA	g2.129	0.07	3.3
2 ppb Matrix LR	LR	2.74	0.049	1.8
2 ppb Matrix LR	LR	2.697	0.104	3.9
2 ppb Matrix LR	LR	2.723	0.019	0.7
2 ppb Matrix LR 10x	LR	2.681	0.121	4.5
2 ppb Matrix LR 10x	LR	2.692	0.016	0.6
2 ppb Matrix LR 10x	LR	2.737	0.028	1
2 ppb Matrix RR	RR	2.365	0.037	1.6
2 ppb Matrix RR	RR	2.664	0.306	11.5
2 ppb Matrix RR	RR	2.443	0.042	1.7
2 ppb Matrix RR 10x	RR	2.217	0.094	4.3
2 ppb Matrix RR 10x	RR	2.272	0.054	2.4
2 ppb Matrix RR 10x	RR	2.222	0.019	0.8
RW1 (20x dil)	Unknown	2.007	0.049	2.4
RW1 (20x dil)	Unknown	2.05	0.064	3.1
RW1 (20x dil)	Unknown	2.071	0.097	4.7
RW 2 (20x dil)	Unknown	2.054	0.076	3.7
RW 2 (20x dil)	Unknown	1.964	0.056	2.9
RW 2 (20x dil)	Unknown	1.493	0.496	33.2
RW 3 (20x dil)	Unknown	1.923	0.046	2.4
RW 3 (20x dil)	Unknown	2.089	0.11	5.3
RW 3 (20x dil)	Unknown	1.985	0.008	0.4
RW 4 (4x dil)	Unknown	1.264	0.096	7.6
RW 4 (4x dil)	Unknown	1.224	0.03	2.4

Table B-1. ZEU-INMUNOTEC MicroCystest Raw Data Continued

Sample Description	Variant	Mean Conc. (ppb)	Standard Deviation (ppb)	CV%
RW 4 (4x dil)	Unknown	1.202	0.018	1.5
RW 5 (4x dil)	Unknown	1.572	0.112	7.1
RW 5 (4x dil)	Unknown	1.53	0.081	5.3
RW 5 (4x dil)	Unknown	1.568	0.026	1.7
RW 6 (2x dil)	Unknown	1.248	0.192	15.4
RW 6 (2x dil)	Unknown	1.363	0.001	0.1
RW6 dissolved	Unknown	0.334	0.064	19.1
RW6 dissolved	Unknown	0.283	0	0
RW6 dissolved	Unknown	Range?	Range?	Range?
RW6 filtrate	Unknown	0.25	0	0
RW6 filtrate	Unknown	0.443	0	0
RW6 filtrate	Unknown	0.369	0.056	15.2
RW6 intracellular	Unknown	2.016	0.079	3.9
RW6 intracellular	Unknown	2.065	0.05	2.4
RW6 intracellular	Unknown	2.025	0.04	2
RW7	Unknown	0.102	0	0
RW7	Unknown	Range?	Range?	Range?
RW7 dissolved	Unknown	Range?	Range?	Range?
RW7 dissolved	Unknown	Range?	Range?	Range?
RW7 dissolved	Unknown	0.228	0	0
RW7 filtrate	Unknown	Range?	Range?	Range?
RW7 filtrate	Unknown	Range?	Range?	Range?
RW7 filtrate	Unknown	Range?	Range?	Range?
RW7 intracellular	Unknown	Range?	Range?	Range?
RW7 intracellular	Unknown	Range?	Range?	Range?
RW7 intracellular	Unknown	0.092	0	0
RW8	Unknown	0.66	0.006	0.9
RW8	Unknown	0.714	0.032	4.5
RW8 dissolved	Unknown	Range?	Range?	Range?
RW8 dissolved	Unknown	0.281	0.009	3.4
RW8 dissolved	Unknown	Range?	Range?	Range?
RW8 filtrate	Unknown	Range?	Range?	Range?
RW8 filtrate	Unknown	Range?	Range?	Range?
RW8 filtrate	Unknown	Range?	Range?	Range?
RW8 intracellular	Unknown	0.574	0.013	2.2
RW8 intracellular	Unknown	0.632	0.022	3.4
RW8 intracellular	Unknown	0.577	0.046	7.9
RW9	Unknown	0.69	0.024	3.5
RW9	Unknown	0.77	0.005	0.6
RW9	Unknown	0.694	0.087	12.6