THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

TECHNOLOGY TYPE:	**MICROCYSTIN TEST KIT**
APPLICATION:	**RECREATIONAL WATER MICROCYSTIN DETECTION**
TECHNOLOGY NAME:	**Microcystin ADDA ELISA Test Kit**
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ETV Joint Verification Statement

The U.S. Environmental Protection Agency (EPA) has established the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative or improved 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. Information and ETV documents are available at www.epa.gov/etv.

ETV works in partnership with recognized standards and testing organizations, with stakeholder groups (consisting of buyers, vendor organizations, and permitters), and with 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 and 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 Advanced Monitoring Systems (AMS) Center, one of six verification centers under ETV, is operated by Battelle in cooperation with EPA's National Risk Management Research Laboratory. The AMS Center evaluated the performance of microcystin test kits for water monitoring. This verification statement provides a summary of the test results for the Abraxis Microcystin ADDA enzyme-linked immunosorbent assay (ELISA) Test Kit.

VERIFICATION TEST DESCRIPTION

This verification test of the Abraxis Microcystin ADDA ELISA Test Kit was conducted from July 26 through August 12, 2010 at Battelle laboratories in Columbus, OH. Reference analyses by liquid chromatography tandem

mass spectrometry (LC-MS/MS) were performed the week of August 16, 2010 by the University of Nebraska Water Sciences Laboratory.

The objective of this verification test was to evaluate the performance of the microcystin test kit in analyzing known concentrations of microcystin in ASTM International Type II deionized (DI) water and in natural recreational water (RW) samples. The technology was used to analyze a variety of water samples for the variants microcystin-LR, microcystin-LA, and microcystin-RR. Because the technology cannot specify between the more than 80 microcystin variants, the samples prepared for this test were spiked with three individual variants. The Microcystin ADDA ELISA Test Kit provided a quantitative determination of microcystins and was evaluated in terms of:

- 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 RW 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 RW matrices and chlorophyll-*a* on the results of the test kits; and
- Operational and sustainability factors - general operation, data acquisition, setup, consumables, etc.

Each microcystin test kit was operated according to the vendor's instructions by a vendor-trained Battelle technician. Samples and calibration standards were analyzed in duplicate and positive and negative controls were analyzed at the vendor-specified frequency.

The ability of the Abraxis ADDA Microcystin Test Kit to determine the concentration of microcystin was challenged using quality control (QC) samples, performance test (PT) samples and RW samples. 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 °C ± 3 °C until use. The reference samples that were prepared from the test solutions were stored in amber glass bottles at < -10°C. 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.

Unlike many contaminants, certified microcystin standards are not commercially available. In planning this verification test, multiple sources of standards were investigated. With agreement from the stakeholders, all vendors and the EPA project officer, the standards used for this verification were purchased from the most reputable sources (LR and RR from Canadian National Research Council and LA from Abraxis), based on a Performance Evaluation Audit, and used for both the testing solutions and the reference method calibration.

QA oversight of verification testing was provided by Battelle and EPA. Battelle QA staff conducted technical systems audits of both the laboratory and field testing, and Battelle QA staff conducted a data quality audit of at least 10% of the test data. This verification statement, the full report on which it is based, and the test/QA plan for this verification test are available at www.epa.gov/etv/centers/center1.html.

TECHNOLOGY DESCRIPTION

Following is a description of the Abraxis ADDA Test Kit, based on information provided by the vendor. The information provided below was not verified in this test.

The ADDA Test Kit is an ELISA for the congener independent determination of microcystins and nodularins in water samples. The assay utilizes polyclonal antibodies that have been raised against the Microcystin ADDA ELISA Test Kit moiety of the molecule, allowing for the detection of numerous microcystin and nodularin variants (over 80 variants are currently known) in drinking, surface, and groundwater at levels below World Health Organization (WHO) guidelines.

The test is an indirect competitive ELISA and is based on the recognition of microcystins, nodularins and their variants by a polyclonal sheep antibody. When present in a sample, microcystins and nodularins compete with a microcystins-protein analog that is immobilized on wells of a microtiter plate for the binding sites of antibodies in solution. After a washing step, a second antibody(Horseradish Peroxidase) is added and incubated. After a washing step and addition of a substrate/chromogen solution, a color signal is generated. The intensity of the color is inversely proportional to the concentration of the microcystins/nodularins present in the sample. The color reaction is stopped after a specified time and analyzed using a plate photometer to obtain the optical density (OD) at a wavelength of 450 nanometers (nm).

The Microcystin ADDA ELISA Test Kit is not able to distinguish the difference between two microcystin variants. Results from the Microcystin ADDA ELISA Test Kit are calibrated with respect to the microcystin-LR variant. However, other microcystin variants are known (based on information provided by Abraxis) to react to different extents with the antibodies used for detection; this is referred to as the cross reactivity (CR) of the variant. For this verification test, Microcystin ADDA ELISA Test Kit results for LR were reported from the calibration curve, results for the LA variant were reported as 125% of the kit results (based on the CR value of 125%), and the RR variant was reported as 91% of the test kit results.

VERIFICATION RESULTS

The verification of the Abraxis Microcystin ADDA ELISA Test Kit is summarized by the parameters described in Table 1.

Table 1. Abraxis Microcystin ADDA ELISA Test Kit Performance Summary

Verification Parameters	LR	LA	RR
Accuracy (range of %D)			
0.10 ppb	-13% to 2%		
0.50 ppb	-9% to 40%	27% to 109%	42% to 64%
1.0 ppb	19% to 58%	77% to 133%	98% to 123%
2.0 ppb	-45% to 39%	69% to 113%	23% to 69%
4.0 ppb	-6% and 3%	105% and 113%	11% to 50%
Precision (range of %RSD)	5% to 45% (7 of 9 samples < 16%)	3% to 25%	4% to 16%
Precision (RW samples)	3% to 47%, all except 2 RSDs were < 12%		
Linearity (y=)	0.933x + 0.087 r^2=0.906	2.66x - 0.220 r^2=0.990	1.18x + 0.168 r^2=0.961
Method Detection Limit (ppb)	0.137	0.218	0.047

Inter-kit lot reproducibility. Calibration standards from two different lots were measured and the relative percent difference (RPD) of the resulting ODs ranged from 0% to 13%, with eight of the 12 being less than 6%.

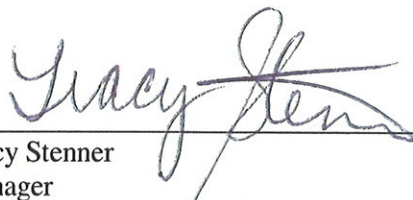
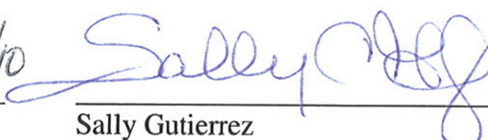
Matrix Interference. Matrix interference effects were assessed by using a t-test to compare results from samples made by spiking undiluted and diluted interference matrices with the PT sample results at 2.0 ppb spiked concentration. For chlorophyll-*a* and RW matrices, three out of 16 comparisons resulted in statistically significant differences (two comparisons could not be performed because there were only two replicate results for the LR spiked undiluted RW samples): 1) between the 2.0 ppb LA spike into DI water and the 2.0 ppb LA spike into chlorophyll-*a* at 1.0 mg/L ($p = 0.005$); 2) between the 2.0 ppb LA spike into DI water and the 2.0 ppb LA spike into 10 mg/L chlorophyll-*a* ($p = 0.006$); and 3) between the RR spikes into undiluted and diluted RW ($p = 0.01$). Given that the molecular basis on which the test kits operate is well-characterized and understood from the

literature, these results were unexpected. Two variants (LR and LA) demonstrated an interference effect but the third variant (RR) did not. This could have been caused by a number of factors, such as chlorophyll-*a* source and stability. However, due to the limited number of replicates and low power of this study, additional testing would be required to provide a better understanding as to whether there is a matrix interference due to chlorophyll-*a*, or another variable not investigated in this verification testing.

Recreational Water (RW). Because the reference method did not measure all possible microcystin variants, no quantitative comparison was made between the Microcystin ADDA ELISA Test Kit and the reference method results. The reference data were converted into LR-equivalents according to the Microcystin ADDA ELISA Test Kit cross reactivity for the variants. In general, the samples that were determined to have higher total concentrations by the Microcystin ADDA ELISA Test Kit had higher total concentrations as determined by the reference method. All of the Microcystin ADDA ELISA Test Kit total microcystin results were greater than the reference method results, which was consistent with the likelihood that all of the microcystins were not being measured by the reference method.

Operational Factors. The test kit operator reported that the Microcystin ADDA ELISA Test Kit was easy to use. Solution or sample preparation was minimal, mostly involving diluting the samples that were initially above the quantification range. The procedure included three incubation periods that totaled 2.5 hours. Previous knowledge or training on the use of micro-pipettes and or multi-channel pipettes with 96-well plates is recommended for consistent readings. A spectrophotometer plate reader is necessary for obtaining the spectrophotometric readings that are then analyzed using any commercial ELISA evaluation program (four-parameter is recommended by the vendor). Once the analysis was complete, the remaining solutions were disposed in the trash in accordance with local regulations.

The listed price for the Microcystin ADDA ELISA Test Kit at the time of the verification test was $440. The kit has a 12-month shelf life when received, and should be stored at 4 to 8 °C. Of the 96 wells on one plate, 16 wells are needed for calibration and control samples. The remaining 80 wells are for sample analyses that are performed in duplicate. Other consumables not included in the kit are pipettes, pipette tips, and distilled or DI water that can be supplied by the vendor.

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ADDITIONAL INFORMATION AND COMMENTS

Accuracy. Unlike many contaminants, certified reference standards are not commercially available. Several sources of standards were investigated and in our experience they can vary greatly in concentration and purity. The ETV study was conducted using a Microcystin-LR source from Canada, Abraxis standards are prepared using Microcystin-LR obtained from Dr. Carmichael. Our comparison of the 2 toxin sources indicate that both standard sources are within 20% of each other.

Another factor that needs to be considered when comparing results is that the reference method used in this study (LC-MS-MS) is not nearly as sensitive as the ELISA, therefore a SPE concentration step had to be performed with every sample. SPE extraction tended to give lower recoveries.

The combination of the standard source and lower SPE recoveries with the reference method lead to differences in accuracy between the instrumental (reference) and ELISA methods.

Table 2A. Abraxis ADDA Test Kit Performance Summary, Calculation based on Theoretical Values

Verification Parameters	LR	LA	RR
Accuracy (range of %D)			
0.1 ppb	-13% to 2%		
0.5 ppb	-24% to 18%	1% to 67%	8% to 25%
1 ppb	-1% to 31%	24% to 63%	7% to 20%
2 ppb	-46% to 32%	44% to 81%	1% to 35%
4 ppb	-5% and -13%	54% and 60%	-2% to 20%
Precision (range of %RSD)	5% to 45% (7 of 9 samples < 16%)	3% to 25%	4% to 16%
Precision (RW samples)	3% to 47%, all except 2 RSDs were < 12%		
Linearity (y=)	0.933x + 0.0865 r^2=0.906	2.6582x - 0.22 r^2=0.990	1.1801x + 0.1684 r^2=0.961
Method Detection Limit (ppb)	0.137	0.218	0.047

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. For chlorophyll-a and RW matrices, three out of 16 comparisons resulted in statistically significant differences (two comparisons could not be performed because there were only two replicate results for the LR spiked undiluted RW samples): 1) between the 2.0 ppb LA spike into DI water and the 2.0 ppb LA spike into chlorophyll-a at 1.0 mg/L ($p = 0.005$); 2) between the 2.0 ppb LA spike into DI water and the 2.0 ppb LA spike into chlorophyll-a at 10 mg/L ($p = 0.006$); and 3) between the RR spikes into undiluted and diluted RW ($p = 0.01$). *[Given that the molecular basis on which the test kit operates is well characterized and understood from the literature, these results were unexpected. Two variants (LR and LA) demonstrated interference effect but the third variant (RR) did not. This could have been caused by a number of factors, such as chlorophyll-a, stability, etc. In addition the chlorophyll-a used in the ETV study is insoluble and precipitated into a glob at the bottom of the vial when diluted in water. However, due to the limited number of replicates and low power of this study,*

additional testing would be required to provide a better understanding as to whether there is a matrix interference due to chlorophyll-a, or another variable not investigated in this verification testing].

Based on the additional studies listed in Table 15A, **no interference of chlorophyll-a** can be found.

Table 15A. Chlorophyll-a Interferent Sample Results for the Abraxis ADDA Test Kit

Variant	Sample Description	Mean Kit Results: LR Equivalents (ppb)	Corrected Conc. by Variant (ppb)
LR	2.0ppb LR in DI	2.171	2.171
	2.0ppb LR in 1mg/mL Chlorophyll a in DI	1.710	1.710
	2.0ppb LR in 10mg/mL Chlorophyll a in DI	2.146	2.146
	2.0ppb LR in 100mg/mL Chlorophyll a in DI	2.268	2.268
RR	2.0ppb RR in DI	1.807	1.986
	2.0ppb RR in 1mg/mL Chlorophyll a in DI	1.606	1.765
	2.0ppb RR in 10mg/mL Chlorophyll a in DI	1.732	1.903
	2.0ppb RR in 100mg/mL Chlorophyll a in DI	1.659	1.823
LA	2.0ppb LA in DI	3.764	3.011
	2.0ppb LA in 1mg/mL Chlorophyll a in DI	3.216	2.573
	2.0ppb LA in 10mg/mL Chlorophyll a in DI	3.788	3.030
	2.0ppb LA in 100mg/mL Chlorophyll a in DI	3.446	2.757

Additional Factors. The Battelle **operator conducting the verification study** has 10 years of laboratory experience, but **was not experienced with ELISA analysis**. Our experience indicates that familiarity with ELISA and additional ELISA assay experience greatly increases performance, this will manifest in better precision and accuracy of results.

Fernando Rubio 11/24/2010

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Date