THE ENVIRONMENTAL TECHNOLOGY VERIFICATION PROGRAM

TECHNOLOGY TYPE:	**MICROCYSTIN TEST KIT**
APPLICATION:	**RECREATIONAL WATER MICROCYSTIN DETECTION**
TECHNOLOGY NAME:	**MicroCystest Plate Kit**
COMPANY:	**ZEU-INMUNOTEC, S.L.**
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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 ZEU-INMUNOTEC MicroCystest Plate Kit.

VERIFICATION TEST DESCRIPTION

This verification test of the MicroCystest 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 microcystin test kit performance 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 MicroCystest provided a quantitative determination of microcystins and was evaluated in terms of:

- Accuracy - comparison of test kit results (samples prepared in DI) 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 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.

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 MicroCystest 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 ^{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. 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 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 the 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

The following is a description of the MicroCystest, based on information provided by the vendor. The information provided below was not verified in this test.

MicroCystest is a toxicity test based on a protein phosphatase inhibition assay (PPIA), 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 potentially 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 nm. Samples containing microcystins will inhibit the enzyme proportionally to the amount of toxin contained in the sample. The test quantifies the toxicity of all microcystins

present in the sample (more than 80 microcystins have been described to date) and compares toxin concentration to acceptable levels. The final concentration of microcystins can be calculated using a standard curve obtained from the standards included in the kit, expressed as μg/L of microcystin-LR.

The MicroCystest kit allows for both dissolved and intracellular microcystin. 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 kit includes ready-to-use standards and all reagents needed in the assay. A spectrophotometer with 405 nm filter is required for results interpretation.

The kit offers a great flexibility, as a maximum of 44 samples can be run with one 96-well kit; however if 11 or fewer 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.

VERIFICATION RESULTS

The verification of the Zeu MicroCystest is summarized by the parameters described in Table 1.

Table 1. Zeu MicroCystest Performance Summary

Verification Parameters	LR	LA	RR
Accuracy (ppb, range of %D (mean%))*			
0.10	230% and 280% (230%)		
0.50	20% - 55% (41%)	43% - 58% (50%)	17% - 110% (61%)
1.0	85% - 110% (100%)	97% - 110% (100%)	200% - 220% (210%)
2.0	35% - 48% (43%)	47% - 54% (50%)	24% - 36% (32%)
4.0			-27% to -24% (-26%)
Precision (range of %RSD (mean))	1% - 13% (5%)	1% - 10% (4%)	1% - 27% (7%)
Precision (RW samples (mean))	1% to 6% (4%)		
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 ppb spiked concentration. 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 did not affect the performance of the MicroCystest.

Recreational Water (RW). In general, samples determined to have higher total concentrations by the MicroCystest 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 was consistent with the likelihood that all of the microcystins were not being measured by the reference method because it measured only 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 constitute a considerable proportion of the microcystins

measured by the MicroCystest. In addition to the freeze-thaw method of lysing algae cells to release microcystins, a Zeu-specific lysing technique was conducted. 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. A spectrophotometer plate reader is necessary for obtaining the spectrophotometric readings at 405 nm that are also analyzed using a commercial plate reading evaluation program (SoftMax Pro was recommended by the vendor). Once the analysis was complete, the remaining solutions were disposed in the trash in accordance with local regulations.

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 wells are needed for calibration samples. The remaining 88 wells are for sample analyses that are performed in duplicate (44 total samples). Other consumables not included in the kit are pipettes, pipette tips, DI water and the lysing procedure materials. The price for the MicroCystest at the time of the verification test was $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 nanometers, and the supplies needed for filtering and lysing of the sample.

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