September 2010

Environmental Technology Verification Report

ABRAXIS ECOLOGENIA® ALKYLPHENOL (AP) MICROPLATE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TEST KITS

Prepared by
Battelle

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EPA U.S. Environmental Protection Agency

ETV ETV ETV

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ETV Advanced Monitoring Systems Center

ABRAXIS ECOLOGENIA®
ALKYLPHENOL (AP) MICROPLATE
ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)
TEST KITS

By

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Notice

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Foreword

The U.S. Environmental Protection Agency (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 support of all those who helped plan and conduct the verification test, analyze the data, and prepare this report. We sincerely appreciate the involvement and support of all staff from the participating laboratories who conducted testing as part of this verification test. In particular, we would like to thank the following staff for their contribution in conducting this verification at their respective laboratories: Marc Mills, U.S. EPA National Risk Management Research Laboratory (NRMRL), Cincinnati, OH; Dave Russell, Ronald Landy, Anching Tang, and Martin Lazarus, U.S. EPA Region 3 Fort Meade, MD; Dennis Wesolowski, Larry Zintek, and Charles Steiner, U.S. EPA Region 5 Chicago, IL; Mike Meyer, Keith Loftin, Larry Barber, and James Gray, United States Geological Survey (USGS), Lawrence, KS; Jim Lazorchak, Tirumuru Reddy, and Dan Bender, U.S. EPA National Exposure Research Laboratory (NERL), Cincinnati, OH; and Jeanette Van Emon, U.S. EPA NERL, Las Vegas, NV. Finally, we would like to thank Lisa Olsen, USGS; Paul Pennington, National Oceanic and Atmospheric Administration; and Marion Kelly, U.S. EPA, for their review of this verification report. Quality assurance oversight was provided by Michelle Henderson, U.S. EPA, and Zachary Willenberg, Battelle.

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List of Abbreviations

AMS	Advanced Monitoring Systems
AP	alkylphenol
APE	alkylphenol ester
°C	degrees Celsius
COC	chain of custody
DI	deionized
DMSO	dimethyl sulfoxide
EDC	endocrine-disrupting compound
ELISA	enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
GC-MS	gas chromatography–mass spectrometry
GFF	glass fiber filter
HPLC	high performance liquid chromatography
L	liter
LC-MS	liquid chromatography–mass spectrometry
MDL	method detection limit
mL	milliliter
µg/L	micrograms per liter
µL	microliter
µm	micron
NERL	EPA ORD National Exposure Research Laboratory
ng	nanogram
nm	nanometer
NP	nonylphenol
NRMRL	EPA ORD National Risk Management Research Laboratory
ORD	EPA Office of Research and Development
PE	performance evaluation
QA	quality assurance
QC	quality control
QMP	quality management plan
rpm	revolutions per minute
RSD	relative standard deviation
S	standard deviation
SOP	standard operating procedure

SPE	solid phase extraction
TSA	technical systems audit
USGS	United States Geological Survey
v/v	volume/volume
WWTP	wastewater treatment plant

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification Program (ETV) 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 ETV Advanced Monitoring Systems (AMS) Center is operated by Battelle under a cooperative agreement with EPA's NRMRL. The AMS Center recently evaluated the performance of the Abraxis Ecologenia Alkylphenol (AP) Microplate Enzyme-Linked Immunosorbent Assay (ELISA) Test Kit for determining endocrine-disrupting compounds (EDCs) in water.

Chapter 2
Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This report provides results for the verification testing of the Abraxis Ecologenia AP Microplate ELISA test kit (referred to as the AP ELISA test kit in this report). The following is a description of the test kit, based on information provided by the vendor.

The Abraxis Ecologenia AP ELISA test kit applies the principle of ELISA to determine APs in water samples. The AP ELISA test kit uses a colorimetric procedure to detect alkylphenols. The AP ELISA test kit measures multiple APs at different percent reactivities. Nonylphenol has the highest reactivity (100%). APs other than nonylphenol were not tested for precision, bias, or matrix effects in this verification, but were evaluated for cross-reactivity with nonylphenol.

The standards and samples, and an enzyme-labeled alkylphenol conjugate are added to a disposable microtiter plate (uncoated), and mixed. One hundred microliter (μL) aliquots of the mixture are then transferred from the 96-well uncoated microtiter plate to a 96-well microplate with antibody (monoclonal anti-alkylphenol) coated wells. At this point a competitive reaction occurs between the alkylphenol which may be in the sample and the enzyme labeled alkylphenol for a finite number of antibody binding sites. The reaction is allowed to continue for sixty (60) minutes. At the end of the incubation period, the plate contents are decanted to remove the unbound reagents from the alkylphenol and labeled alkylphenol that remain bound to the antibodies on the plate, in proportion to their original concentration. After decanting, the plate is washed with washing solution. A substrate is then added and enzymatically converted from a colorless to a blue solution, after an incubation period, the reaction is stopped by the addition of diluted acid. The alkylphenol concentration is determined by measuring the absorbance of the sample solution with a photometer (450 nanometer (nm)) and comparing it to the absorbance of standards.

Figure 2- 1. Abraxis Ecologenia AP Microplate ELISA Test Kit

The alkylphenol ELISA Kit (Figure 2-1) contains a 96-well microplate coated with alkylphenol antibody (mouse anti-alkylphenol), two vials of lyophilized horseradish peroxidase-labeled alkylphenol analog, two 7 milliliter (mL) vials of buffer solution, five 1.5 mL vials of alkylphenol standards at concentrations of 0, 50, 200, 1000, 5000 micrograms

per liter (μg/L) with preservatives and stabilizers, a 15 mL bottle of hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine solution in an organic base, a 15 mL bottle of diluted acid, a 50 mL bottle of 6X concentrated washing buffer, one uncoated microplate, and one adhesive plate cover.

Other materials that are required but are not provided with the Alkylphenol ELISA Kit are pipettes and a plate photometer capable of reading at 450 nm. These materials can be purchased separately or rented.

The Alkylphenol ELISA Kit is 7 by 5 by 4 ½ inches. Final results and calibration curves are printed out on the photometric analyzer or sent directly to a lab computer. List price is $750 for a 96-test kit.

Chapter 3
Test Design and Procedures

3.1 Introduction

This verification test was conducted according to procedures specified in the *Test/QA Plan for Verification of Enzyme-Linked Immunosorbent Assay (ELISA) Test Kits for the Quantitative Determination of Endocrine Disrupting Compounds (EDCs) in Aqueous Phase Samples*.[1] Deviations to the test/QA plan were made due to unanticipated circumstances. As such, the test procedures described in this chapter are a complete description of the actual test conditions.

Because of their potential to interfere with human, domestic animal, and wildlife reproduction, EDCs are of increasing concern throughout the country. Several EPA Regions have undertaken activities to monitor for these compounds, and several states are considering including monitoring for EDCs in their regulatory programs. Presently, gas chromatography–mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), and liquid chromatography–mass spectrometry (LC-MS) are being used for detecting these compounds. However, immunoassay techniques, particularly ELISA, are becoming increasingly popular in the field of environmental analysis due to their high sensitivity, ease of use, short analysis time, and cost-effectiveness.

Immunoassay analytical detection is based on the capability of antibodies to specifically recognize and form stable complexes with antigens. Immunoassays employ antibodies as analytical reagents. In ELISA test kits, an enzyme conjugate competes with the chemical in the sample for a limited number of binding sites on the antibody coated plate or particles. The extent of color development is inversely proportional to the amount of chemical in the sample or standard. The higher the concentration of a specific steroid or other EDC in the sample, the less color reaction produced and recorded using a plate reader or tube photometer.

Testing was conducted with multiple collaborating laboratories, specifically the EPA Office of Research and Development (ORD) NRMRL, EPA ORD National Exposure Research Laboratory (NERL) in Las Vegas, and EPA Region 5. The laboratory participation was coordinated by EPA NRMRL, in collaboration with Battelle. Laboratory names are removed, and simply stated as "Laboratory (or Lab) 1, 2, and 3" in the test results section, since inter-laboratory comparison was not an objective of this report.

This verification test was conducted in four phases to evaluate the ability of the Abraxis AP ELISA test kit to quantitate a specific AP, nonylphenol (NP), in four different water matrices

(see 3.3 Test Procedures), per the manufacturer protocols. More detailed information on the EDC tested is provided in Table 3-1. EPA laboratories used the Abraxis AP ELISA kit (according to Table 3-2) to quantitate triplicate spiked samples for NP, which were prepared and shipped by EPA NRMRL. Note that NP was the target analyte of interest, but the AP ELISA test kit was developed for the general class of AP, which includes NP and other compounds. As the more established method for detecting these compounds, GC-MS served as the reference method[2] for this test.

Table 3-1. Target Analyte

Analyte	Synonyms	CAS#	Use
Nonylphenol	NP	84852-15-3	Detergents, surfactants, emulsifiers, wetting agents, cosmetics, cleaners, pesticides, paints

Table 3-2. ELISA Test Kit Evaluation Responsibilities for Each Participating Laboratory

Responsibility	NRMRL	NERL-LV	Region 5
Sample Collection, Processing and Distribution	√		
Test Kit Evaluation – AP 96-well ELISA	√	√	√
Reference Measurement - AP GC-MS			√

The Abraxis Ecologenia AP Microplate ELISA test kit was verified by evaluating the following parameters:

- Precision
- Percent bias
- Matrix effects
- Operational factors.

Verification of the system was conducted from June to September 2008. Precision was determined by measuring the relative standard deviation of average concentration values as reported by the test kit. Percent bias was determined as positive or negative, with positive values indicating that ELISA concentration was higher than the reference method and negative values indicating that it was lower. Matrix effects were determined by comparing the percent bias measurements for Phase I deionized (DI) water samples to the percent bias measurements for the Phase II through IV matrix-water samples.

Operational factors were determined based on documented observations of the testing staff and the Verification Test Coordinator. Operational factors were described qualitatively, not quantitatively; therefore, no statistical approaches were applied to the operational factors.

3.2 Test Facilities

Laboratory analyses of the AP ELISA test kit were conducted in three different collaborating laboratories by the laboratory staff. These laboratories were: EPA Region 5 in Chicago, EPA ORD NERL laboratory in Las Vegas, and the EPA ORD NRMRL laboratory in Cincinnati, OH. Reference measurements for AP were performed at EPA Region 5.

3.3 Test Procedures

This verification test was conducted in four phases. Phase I consisted of a clean water sample (DI water) spiked with a single concentration of NP, split into single samples, and submitted to the ELISA kit users in each collaborating laboratory to measure the concentration, in triplicate. The split sample, as well as the un-spiked, matrix background sample, were also simultaneously sent for reference GC-MS analysis of NP, alkylphenol ethoxylates (APEs), and for various compounds which are known to cause cross-reactivity with some ELISA kits. Phase II consisted of environmental surface water samples subjected to the same spiking and splitting process as Phase I. Phase III consisted of a complex matrix of wastewater treatment plant (WWTP) effluent samples subjected to the same spiking and splitting process as Phase I and II. Phase IV consisted of a complex matrix of WWTP influent samples, spiked and split as in previous phases. Details on the sample matrices, spiking levels, and spiking procedures for each phase are provided in Section 3.3.1. All NP spike concentrations used in each phase of this verification test were based on real-world concentrations found in environmental samples, per the procedure described in the test/QA plan.[1]

Background concentrations of NP were measured by GC-MS for each matrix for each phase. These measurements were made to determine if any measureable amounts of NP might exist in the sample matrix prior to the addition of any spikes. If a detectable concentration was found, this concentration was then added to the spiked amount of NP to calculate the total concentration for all spiked samples of a particular phase. Specific concentrations of NP, as presented in Section 3.3.1, were spiked into the sample matrix for each phase, regardless of any background concentrations of these compounds that may have been present in the collected water. Background concentrations of NP were not detected except for Phase IV where 8,800 nanograms per liter (ng/L) NP were found in the background matrix samples. The nominal concentration of each sample was then calculated using the measured background concentration and the expected spiked concentrations for each phase. The ELISA kit results from the various laboratories for the Abraxis AP ELISA test kit were compared to each other and compared to GC-MS results.

The Abraxis AP ELISA test kit was tested only under laboratory controlled conditions, as opposed to field conditions which would have been more variable. The analyses were performed according to the vendor's recommended procedures as described in the user's manual. Simple cleanup procedures (as noted in the following sentences), as directed by the manufacturer of the test kit, were used for the four different matrices. Each sample was analyzed both directly (prior to cleanup) and after solid phase extraction (SPE) cleanup using the procedure detailed in the kit instructions and provided in Section 3.3.2. Each sample for ELISA analysis was filtered through a 1 micron (μm) glass fiber filter (GFF) prior to direct analysis and SPE. Calibration and maintenance of the photometer was performed as specified by the vendor.

A US EPA Region 5 GC-MS standard operating procedure (SOP) was followed for reference measurements.[2] Samples for the GC-MS methods went through an extraction step to concentrate (or dilute, depending upon the sample) to ensure the samples were within the method's analytical range.[2] The procedures for preparing, storing, and analyzing the test samples are provided below.

3.3.1 Test Sample Collection and Preparation

All sample bottles and glassware associated with NP samples were new and did not need additional silanization. The glass carboy for NP sample preparation was cleaned for reused using a standard procedure[1] and did not require additional silanization. All samples were thoroughly mixed and were thus assumed to contain the same concentration. Samples were spiked with NP as one large stock solution and then split into smaller sub-samples in bottles. All sample bottles were amber glass to prevent photodegradation of the analytes. All samples were prepared and shipped by NRMRL, immediately after being made, in coolers on ice or freezer packs to maintain a temperature at or below 4 degrees Celsius (^{o}C) temperature. When samples were received by each laboratory, the condition of the samples, i.e., temperature, broken bottles etc., was noted by the receiving laboratory operator and the samples were then immediately placed in a refrigerator at 4^{o}C until analyzed. All samples were either analyzed or solvent exchanged within 24 hours of receipt to reduce error associated with analyte degradation during sample holding. Because of logistical issues at Laboratory 2, samples were unable to be extracted or analyzed within the first 24 hours after receipt of the sample. All laboratories performing quantitative analysis, ELISA or GC-MS, received split samples from the same bulk sample. Each laboratory that participated in the ELISA analysis received one 2.5 L spiked sample plus one 500 mL DI water method blank. The laboratory that performed the reference analysis received one 4 L spiked sample and one 1 L DI water method blank to be processed by the GC-MS method.

3.3.1.1 Phase I Samples

A sample of DI water was collected in a cleaned, 20 L, glass carboy from the USEPA NRMRL laboratory in Cincinnati, Ohio. The water was spiked with NP to a concentration of 32.5µg/L. This concentration was selected because it is on the higher end of the range of concentrations expected to be encountered in a real-world situation and is representative of the anticipated mid-range of the test kit. The carboy was thoroughly mixed, by inserting a stir bar and stirring on a stir plate at 300 revolutions per minute (rpm) for 2 hours, to ensure homogeneous concentrations of the analyte throughout the carboy. One 2.5 L spiked sample was collected for each participating laboratory as well as one 4 L sample for each reference laboratory. DI water blanks were also prepared and shipped in separate 500 mL bottles. The blank samples were analyzed both directly and after SPE but only in two wells on the kits as opposed to three wells for all other samples because of limited space. Before spiking, the DI water was sampled and analyzed by GC-MS to confirm that there were no background levels of NP. Samples of the spiked mixtures were taken and the concentrations of these matrix samples and DI water method blank were determined using the Abraxis AP ELISA test kit and GC-MS.

3.3.1.2 Phase II Samples

Grab samples of stream water were collected in three, clean, five gallon buckets from the South Hasha Tributary to Eastfork Lake in Clermont County, Ohio. The tributary was accessed from where it crosses Williamsburg-Bantam Road. Before the stream water was spiked, a single sample of the collected stream water was taken, split into triplicate aliquots, and analyzed by GC-MS to confirm there were no background levels of NP. Next, a cleaned, 20 L, glass carboy was used to collect 20 L of the stream water, which was then spiked to contain a 32.5 μg/L concentration of NP. The carboy was thoroughly mixed by inserting a stir bar and stirring on a stir plate at 300 rpm for 2 hours, to ensure homogeneous concentration of the analyte throughout the carboy. Split samples were taken, as noted for Phase I. DI water method blanks were prepared by filling 20 L carboys with DI water at the same time as the stream water.

3.3.1.3 Phase III Samples

Grab samples of final effluent wastewater were collected in three, clean, five gallon buckets from the Metropolitan Sewer District of Greater Cincinnati in Hamilton County, Ohio. After the sample was transported back to the NRMRL laboratory, the effluent was measured and then transferred into a clean, 20 L carboy. Before spiking, a single sample of the effluent was taken, split into triplicate aliquots, and analyzed by GC-MS to confirm there were no background levels of NP. In a cleaned, 20 L, glass carboy, 20 L of WWTP effluent was prepared containing 32.5 μg/L of NP. The carboy was thoroughly mixed by inserting a stir bar and stirring on a stir plate at 300 rpm for 2 hours to ensure homogeneous concentration of the analyte throughout the carboy. Split samples were collected as noted for Phase I.

3.3.1.4 Phase IV Samples

Grab samples of influent wastewater were collected in three, clean, five gallon buckets from the Metropolitan Sewer District of Greater Cincinnati in Hamilton County, Ohio. After the sample was transported back to the NRMRL laboratory, the influent was measured and transferred into a 20 L carboy. Before spiking, a single sample of the influent was taken, split into triplicate aliquots, and analyzed by GC-MS and the background levels of NP were determined to be 8,800 ng/L (8.8 μg/L). Background levels of NP measured in the samples were added to the spiked concentration of NP once results were obtained. In a cleaned, 20 L glass carboy, 20 L of WWTP influent was prepared containing 32.5 μg/L concentration of NP (for a total concentration with background of 41.3 μg/L). The carboy was thoroughly mixed by inserting a stir bar and stirring on a stir plate at 300 rpm for 2 hours to ensure homogeneous concentration of the analyte throughout the carboy. Split samples were collected as noted in Phase I.

3.3.2 Test Sample Analysis Procedure

Prior to cleanup, the 2.5 L sample was split into three 500 mL aliquots. Each of the three aliquots was analyzed by direct analysis utilizing only GFF cleanup and by utilizing GFF cleanup and SPE. Each aliquot sample was transferred in triplicate to the ELISA kits for quantification, per the test kit protocols. The ELISA test kit users followed simple cleanup procedures as directed in the vendor's instructions. The cleanup procedures are described below.

Each sample for ELISA analysis was filtered through a 1μm GFF prior to direct analysis on the AP ELISA test kit and for SPE clean-up. After filtering, one 712 μL aliquot was removed and 8μL dimethyl sulfoxide (DMSO) and 80μL methanol were added. This aliquot mixture was transferred to three wells (100μL for each well). Once all aliquots were removed for direct analysis, three 500 mL aliquots were removed from the filtered sample for SPE. These three aliquots were treated as three independent samples. SPE directions entitled "Flowchart: AP ELISA" which were based on the vendor's protocols and summarized by EPA NRMRL, were followed.[1] The SPE protocol consists of the following steps:

1. Filter 500 mL of the sample, or the remainder of liquid in the sample bottle noting the volume for later calculation, through 1μm glass fiber filter.
2. Rinse a C18 SPE cartridge (preconditioning) with 10 mL of dichloromethane (up to 20 mL/min), 5 mL of methanol (up to 20 mL/min), and 5 mL of distilled water (up to 20 mL/min).
3. Pour the filtered sample through the C18 SPE cartridge at a flow rate, no faster than 10 mL/min.
4. Wash the C18 SPE cartridge with 5 mL of distilled water (up to 20 mL/min) and 5 mL distilled water/methanol = 1:1 (up to 20 mL/min).
5. Dry the C18 SPE cartridge by vacuuming for 45 minutes.
6. Elute the analyte with 6 mL of dichloromethane (3-5 mL/min).
7. Evaporate the solvent with nitrogen gas to dryness which must be done under 30 ºC or lower temperature.
8. Dissolve the residue in 1% DMSO and 10% methanol solution by adding 0.4 mL of DMSO and 4 mL of methanol to the residue and then add 35.6mL of distilled water for a total volume of 40 mL.

Coming off the C18 SPE cartridge, the NP sample is concentrated 50 times that of the original concentration. As noted in the steps above, samples were reconstituted with 40mL of 1% DMSO 10% methanol solution. For the spiked samples, this process effectively produced an expected level of 375,000 ng/L (375 μg/L). All reconstituted samples were transferred to three wells (100 μL for each well) according to the manufacturer's instructions. Samples were quantified by reading their photometric responses at a wavelength of 450 nm using a plate reader following the manufacturer's instructions. The general steps for operating the Abraxis AP ELISA test kit that were followed during this verification test are provided below.

The Abraxis AP ELISA test kit assay procedure consists of the following steps:

1. Take the kit out of the refrigerator approximately half an hour before use and let come to room temperature (18-25^{o}C). Filter the sample through a 1μm glass fiber filter and then add methanol and DMSO to obtain a final methanol concentration of 10% volume/volume (v/v) and DMSO concentration of 1% (v/v).
2. Dilute the AP standard concentrate 10 fold with methanol solution (methanol/distilled water=0.8 mL/8.2 mL) to prepare designated concentrations of AP standards, from 5 μg/L to 500 μg/L.
3. Reconstitute the enzyme-labeled AP conjugate powder with 7 mL out of 8 mL of buffer solution. 4. Mix 100 μL of conjugate solution and 100 μL of NP standard (or sample) in each well of uncoated microplate. Dispense the conjugate solution first then add standard solution of sample. Each standard will be added to two wells while each sample will be added to four wells.

4. Dispense 100 μL aliquots of the standard/sample and conjugate mixture into each coated well of the microplate. Incubate it for 60 minutes at room temperature (18-25^{o}C).
5. Dilute wash solution (6-fold concentration) in 5 times of its volume of distilled water to prepare a wash solution.
6. Rinse each microplate well with approximately 300 μL of the wash solution and repeat the step twice more. Firmly tap out the plate on a lint-free paper towel to remove solution from the microplate.
7. Dispense 100 μL of the coloring reagent into each well and incubate it for 30 minutes at room temperature (18-25^{o}C). Then, add 100 μL of stop solution to terminate reaction.
8. Measure the absorbance at 450 nm for each standard solution and generate a standard curve. The quantity of APs in the sample is then calculated from an absorbance reading and interpolation from the standard curve.

Chapter 4
Quality Assurance/Quality Control

QA/Quality Control (QC) procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[3] and the test/QA plan for this verification test.[1] Test procedures were as stated in the test/QA plan[1]; however deviations to the test/QA plan were made due to unanticipated circumstances. As such, the test procedures described in Chapter 3 are a complete description of the actual test conditions. The statistical calculations intended for analysis of the test kit results were also changed. This deviation is further described in Chapter 5. This change had no impact on the quality of the results. QA/QC procedures and results are described below.

4.1 Quality Control Samples

Steps taken to maintain the quality of data collected during this verification test included analyzing specific quality control samples for both the reference method (GC-MS) and the test kits.

4.1.1 GC-MS Method Blank and Surrogate Spike Results

This verification test included a comparison of the Abraxis AP ELISA test kit results to those of the GC-MS reference method for NP. Samples analyzed for each phase included performance evaluation (PE) samples, test samples, background samples, and blank samples. The quality of the reference measurements was evaluated by adherence to the requirements of the GC-MS method for this compound, including requirements for reference method blanks (MBs), instrument solvent blanks, and surrogate spikes, as indicated in the test/QA plan[1]. Reference method blank samples were analyzed to ensure that no sources of contamination were present. If the analysis of a reference method blank sample indicated a concentration above five times the method detection limit, contamination was suspected. Any contamination source(s) were corrected and samples were reanalyzed or flagged before proceeding with the analyses. Surrogate spikes were also included in each sample. Average acceptable recoveries for these samples were between 60 and 140%. Samples outside of the acceptable range were generally flagged and/or reanalyzed. n-Nonylphenol was used as a surrogate standard for the GC-MS analysis of NP in the samples. No levels of NP were detected in any of the reference method blank samples.

Surrogate recoveries in Phase I – IV samples varied across phases. All samples were analyzed once using the reference analysis. Phase I surrogate recoveries ranged from 70% to 104% and

averaged 91 ± 15% across the four samples. Phase II surrogate recoveries ranged from 70% to 83% and averaged 78 ± 6% across the four samples. All recoveries for Phase I and II surrogate samples were considered to be in the acceptable range. Phase III surrogate recoveries ranged from 50% to 84% and averaged 64 ± 15% across the four samples. Phase IV surrogate recoveries ranged from 49% to 73% and averaged 56 ± 12% across the four samples. Surrogate recovery for the Phase IV PE sample was considered outside of the acceptable range, exceeding the lower control limit. The identification of the analyte was deemed acceptable but it was noted that the reported value for this sample may be biased low. The actual value for this sample is expected to be greater than the reported value. However, no changes were made to the reference analysis results. Surrogate recovery for the matrix background sample was considered outside of the acceptable range, exceeding the lower control limit. The identification of the analyte was deemed acceptable and it was noted that the reported value for this sample is an estimate. No adjustments were made to the reference analysis results used for statistical analyses.

4.1.2 Test Kit Method Blanks

Test kit method blank samples were run in duplicate using both direct analysis and after SPE clean-up with each set of samples for all four phases. Test kit method blank samples were unspiked DI water. Because concentrations for samples analyzed with the test kit are calculated based on the interpolations from a curve constructed from the standards run with each batch of samples, it is possible to obtain concentration values for all samples. However, the AP ELISA test kit has a stated method detection limit (MDL) of 5 μg/L. Based on this MDL, it is assumed that sample concentrations lower than this level cannot be reliably determined or reported. Thus, any samples, including method blank samples, with concentrations lower than the manufacturer's stated MDL were considered non-detects.

The Abraxis AP ELISA test kit was evaluated by three laboratories (see Table 3-2). Concentrations of NP were not detected in any of the method blank samples from one of the participating laboratories. Method blank samples evaluated by the NERL – Las Vegas lab in all phases were all found to contain levels of NP above the MDL. Analyses using the Abraxis AP ELISA test kit by the EPA NRMRL laboratory showed detectable levels of NP in all method blank samples evaluated in Phases II – IV. For Phase I samples, concentrations of NP above the MDL were found for one of the two replicates in one set (the method blank analyzed after SPE) of duplicate method blank samples. Averaging the duplicate samples for the after-SPE method blank samples put the overall NP concentration below the MDL. Method blank samples for direct analysis contained detectable levels of NP. It is not clear what caused the apparent detectable levels of NP across two of the participating laboratories. The monitored NPs were not detected in any of the method blank samples sent for analysis by the reference method. No adjustments were made to the data used for statistical analyses.

4.2 Audits

Three types of audits were performed during the verification test: a PE audit of the reference method measurements (GC-MS analyses), a technical systems audit (TSA) of the verification test performance, and a data quality audit. Audit procedures are described further in the following sections.

4.2.1 Performance Evaluation Audit

A PE audit was conducted to assess the quality of the reference method measurements (GC-MS analyses) made in this verification test. The reference method PE audit was performed by supplying an independent second standard solution of NP prepared from a different source than that used in verification testing. The PE audit samples were analyzed in the same manner as all other samples and the analytical results for the PE audit samples were compared to the nominal concentration. The target criterion for this PE audit was agreement of the analytical result within 30% of the expected concentration. This audit was performed once during each phase of testing. Table 4-1 shows the percent error results for the PE samples for each phase. The percent error was calculated based on the difference between the actual and expected NP concentrations divided by the expected concentration. All of the NP PE samples had measured concentrations that were 50-53% less than the expected concentration for all phases. No adjustments were made to the standards nor were PE audit samples reanalyzed based on these results.

Table 4-1. PE Audit Sample Results

	Expected Concentration (ng/L)	Actual Concentration (ng/L)	% Error
Phase I	30000	14000	-53
Phase II	30000	14000	-53
Phase IIII	30000	14000	-53
Phase IV	30000	15000	-50

4.2.2 Technical Systems Audit

The Battelle Quality Manager performed a TSA twice during this verification test. Because the testing was taking place in multiple laboratories across the country, Battelle's Quality Manager visited only two laboratories for in-person TSAs. Battelle conducted TSAs at the Cincinnati, OH facility on July 23-24, 2008 and at the Fort Meade, MD facility on July 31, 2008. All TSA findings were reported to the Verification Test Coordinator.

The purpose of this audit was to ensure that the verification test was being performed in accordance with the AMS Center QMP,[3] the test/QA plan,[1] and the GC-MS SOP[2] used during this verification test. In the TSA, the Battelle Quality Manager reviewed the reference methods used, compared actual test procedures to those specified or referenced in test/QA plan, and reviewed data acquisition and handling procedures. The Battelle Quality Manager also toured the laboratory where verification and reference testing were taking place, inspected sample chain of custody (COC) documentation, reviewed technology-specific record books, checked standard certifications and technology data acquisition procedures, and conferred with technical staff. A TSA report was prepared, including a statement of findings and the actions taken to address any adverse findings, and a copy of Battelle's TSA report was sent to the EPA AMS Center QA Manager. No adverse findings were reported. The TSA findings were communicated to technical staff at the time of the audit.

4.2.3 Data Quality Audit

At least 10% of the data acquired during the verification test were audited. Battelle's Quality Manager 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. Minor transcription errors and errors due to rounding were identified and corrected before the results were used for the calculations described in Chapter 5.

4.3 QA/QC Reporting

Each audit was documented in accordance with Section 3.3.4 of the AMS Center QMP. [3] Once the audit reports were prepared, the Battelle Verification Test Coordinator ensured that a response was provided for each adverse finding or potential problem and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were submitted to the EPA.

4.4 Data Review

Records generated in the verification test received an independent internal review before these records were used to calculate, evaluate, or report verification results. Table 4-2 summarizes the types of data recorded. Data were reviewed by a Battelle technical staff member involved in the verification test. The person performing the review added his/her initials and the date to a hard copy of the record being reviewed.

Table 4-2. Summary of Data Recording Process

Data Recorded	Where Recorded	How Often Recorded	By Whom	Disposition of Data
Dates times and details of test events	Laboratory record books or data recording forms, or electronically	Start/end of test procedure, and at each change of a test parameter or change of technology status	Participating laboratories	Used to organize and check test results; manually incorporated into data spreadsheets as necessary
Technology calibration information	Laboratory record books, data recording forms, or electronically	At technology reader calibration or recalibration, as applicable	Participating laboratories	Incorporated into verification report as necessary
Technology readings	Recorded electronically or manually by the operator or electronically by the technology reader, as appropriate	Each sample and QC analysis	Participating laboratories	Converted to or manually entered into spreadsheets for statistical analysis or comparisons
Sample collection and reference method analysis procedures, calibrations, etc.	Laboratory record books, chain-of-custody, electronically, or other data recording forms	Throughout sampling and analysis processes	Participating laboratories	Retained as documentation of sample collection or reference method performance
Reference method results	Electronically from reference measurement technology	Every sample or QC analysis	Participating laboratories	Transferred to spreadsheets for calculation of results and statistical analysis or comparisons

Chapter 5
Statistical Methods

The statistical methods used to evaluate the quantitative performance factors listed in Section 3.1 are presented in this chapter. Qualitative observations were also used to evaluate verification test data.

Per the test/QA plan, [1] repeatability and reproducibility were intended to be calculated as performance parameters for this verification test. However, after further discussion with EPA, and in agreement with EPA, it was determined that higher level summary statistics provided a better synopsis of the test kit results. Thus, the mean and relative standard deviations (precision) were calculated for the test kit results.

5.1 Precision

The standard deviation (S) of the results for the replicate analyses of the same sample was calculated as follows:

$$S = [\frac{1}{n-1}\sum_{k=1}^{n}(M_2 - \overline{M})^2]^{1/2} \quad (1)$$

where n is the number of replicate samples, M_k is the ELISA test kit measurement for the k^{th} sample, and M is the average ELISA test kit measurement of the replicate samples. The precision for each sample is reported in terms of the relative standard deviation (RSD), which was calculated as follows:

$$RSD(\%) = \left|\frac{S}{\overline{M}}\right| \times 100 \quad (2)$$

The RSD was calculated for each laboratory that participated in the verification test and for each test kit that was tested. The RSD was also calculated across all laboratories and test kits for each phase of testing.

5.2 Percent Bias

Percent bias was calculated as a percentage for each measurement in each phase for each kit using Equation 3:

$$\% \text{ Bias} = [\ \frac{x_{ji}}{y_n} - 1\ ] \times 100 \quad (3)$$

where j = 1, 2, 3 denotes the laboratory, i = 1, 2 denotes the ELISA test kit within laboratory, n = 1, 2 denotes the reference method, x_{ji} is the ELISA concentration for the j^{th} laboratory and the i^{th} test kit, y_n is the concentration of the reference method GC-MS or the concentration of the spike. Ideally percent bias results will be within ±25%.

5.3 Matrix Effects

Matrix effects were examined by comparing the percent bias measurements for the Phase I DI water samples to the percent bias measurements for the Phase II – IV samples. Percent bias was determined as described in Section 5.2.

General observations of potential matrix effects, such as false negatives, if observed, were documented but were not used in statistical calculations. False negatives were defined as a negative (zero) response in a sample that is spiked with contaminant at a detectable concentration.

General observations on potential cross-reactivity were documented. Blank samples of each matrix were evaluated by GC-MS to determine background levels of the compounds with which the kits have cross-reactivity, as stated by the vendor.

Percent recovery results were calculated on a per-sample and per-phase basis and were based on the expected spiked concentration of the analyte in each sample matrix. Percent recovery was calculated using the Equation 4:

$$\% \text{ Recovery} = \frac{A}{E} \times 100 \quad (4)$$

Where A is the actual ELISA test kit measurement and E is the expected concentration. The expected concentration includes the expected spike concentration to which was added any detected background levels of NP in the matrix water (see Section 3.3). Percent recovery results are presented to provide another measure of test kit performance to the end user. Ideal percent recovery values are near 100%.

A comparison of the ELISA results generated with and without the use of SPE cleanup was also performed. This evaluated whether the use of the more involved SPE cleanup procedure was necessary/warranted with the ELISA test kits. Percent bias calculations based on the actual and expected spike concentrations and a t-test were used to evaluate these results.

5.4 Operational Factors

Operational factors were determined based on documented observations of the testing staff. Operational factors are described qualitatively, not quantitatively; therefore, no statistical approaches were applied to the operational factors.

Chapter 6
Test Results

The results of the verification tests of the Abraxis AP ELISA test kit are presented below for each of the performance parameters.

6.1 Precision

The relative standard deviation (RSD) is used as a means of evaluating the precision of the ELISA test kit. Three laboratories operated the AP ELISA test kit. One laboratory (Lab 1) ran identical samples on two separate test kits (kit "a" and kit "b"). Labs 2 and 3 ran a single kit. Table 6-1 presents the resulting RSD for each participating laboratory and test kit along with the overall average concentrations per phase of NP found using the Abraxis AP ELISA test kit for all analyses. RSD values are also presented across all results for each phase. Expected NP concentrations for both direct and SPE analysis are presented for each phase in Table 6-2 as a comparison to the actual test kit results in Table 6-1. Expected concentrations were also used to calculate percent recoveries (see Section 6.3).

Overall RSDs by phase for the test kit ranged from 35 to 59% for direct analysis and 60 to 66% for SPE analysis. The RSDs were quite similar across all phases of the SPE analysis, with only a maximum of a 6% difference between any two phases. Phases I, II, and IV has almost identical RSD while Phase III had the highest RSD (66%). RSDs for the direct analysis were similar, with Phases I, II, and IV having similar RSDs and Phase III having the highest direct analysis RSD (59%). For both direct and SPE analysis, Phase IV (which was performed with wastewater influent) had the lowest RSD.

An RSD of greater than 35% for at least triplicate samples is considered by the vendor to be indicative of unreliable data. It is possible that operator error or inexperience could have lead to such high variability. The vendor trained staff on the operation of the test kit, but these trained staff were, in some cases, not available for the verification test because of testing delays and staff turnover. Therefore, staff that operated the test kits during the verification test may not have been trained by the vendor. Other factors must also be considered, such as the performance of the test kit itself.

Table 6-1. ELISA Test Kit Average NP Concentration and Relative Standard Deviation (RSD) Results[a]

		Direct[a]				SPE[a]			
		Average Conc (ng/L)	RSD	Average Conc (ng/L)	RSD	Average Conc (ng/L)	RSD	Average Conc (ng/L)	RSD
Phase I	Lab 1 kit a	9998	37%			55412	67%		
	Lab 1 kit b	18399	17%			102867	57%		
	Lab 2	8869	81%	13854	47%	68191	55%	97795	61%
	Lab 3	18149	25%			164710	21%		
Phase II	Lab 1 kit a	16983	21%			189998	42%		
	Lab 1 kit b	13996	19%			116302	33%		
	Lab 2	9072	86%	15771	48%	66782	59%	169329	60%
	Lab 3	23032	32%			304234	5%		
Phase III	Lab 1 kit a	6032	42%			170466	19%		
	Lab 1 kit b	17834	2%			29199	8%		
	Lab 2	17429	105%	12021	59%	41294	32%	114828	66%
	Lab 3	10394	14%			189810	12%		
Phase IV	Lab 1 kit a	18704	26%			301618	31%		
	Lab 1 kit b	22283	12%			269332	15%		
	Lab 2	12822	89%	20997	35%	69455	50%	250914	60%
	Lab 3	25639	29%			498549[b]	4%		

[a] The average concentration and RSD are based on all replicates within the detectable range of the test kit for direct measurements (i.e., no cleanup) and for analyses which included SPE cleanup.
[b] Concentration was close to the upper MDL (500,000 ng/L) of the test kit.

Table 6-2. Expected NP Concentrations for Each Phase

	Expected Concentration (ng/L)	
Phase	Direct	SPE
I	32500	406250
II	32500	406250
III	32500	406250
IV	41300	516250

6.2 Percent Bias

Bias is a systematic error that causes measurements to err in one direction, either high or low. For this section, percent bias was calculated relative to the GC-MS reference method results. A positive percent bias indicates that the ELISA test kit NP concentration is higher than the reference method, while a negative percent bias indicates that the ELISA test kit NP concentrations are lower than the reference method. Table 6-3 presents the percent bias results.

Table 6-3. ELISA Test Kit Percent Bias vs. GC-MS

	Phase I		Phase II		Phase III		Phase IV	
	DIRECT	SPE	DIRECT	SPE	DIRECT	SPE	DIRECT	SPE
Lab 1 kit a	-58	-82	-11	-20	-70	-32	10	42
Lab 1 kit b	-23	-66	-26	-51	-11	-88	31	27
Lab 2	-63	-77	-52	-72	-13	-83	-25	-67
Lab 3	-24	-45	21	28	-48	-24	51	135

Percent bias results were negative for Labs 1 and 2 across Phases I through III. For Phase IV, Labs 1 and 3 percent bias was positive while Lab 2 was negative, for both direct and SPE analysis. In general, a larger bias was shown for samples that used the SPE clean-up vs. direct analysis in all phases. Lab 3 percent bias was mixed across all phases but was consistent between clean-up procedures for a given phase (e.g., Lab 3 bias was negative for direct and SPE analysis in Phase I). The Lab 3 Phase IV bias for SPE analysis was the largest bias found from any laboratory and across any phase.

For comparison, average NP concentrations and percent bias for the GC-MS measurements with regard to the expected concentration are presented in Table 6-4 for each phase. Percent bias results were negative across all phases and varied from within 26% to 59% of the expected concentration. These results demonstrate that the GC-MS results were biased low in all phases as compared to the expected concentration, with Phase IV having the lowest percent bias. Given the large negative bias of the GC-MS results, a comparison of the ELISA test kit results to those from the GC-MS analysis is likely not representative of the ELISA test kit performance. However, the GC-MS bias does not explain the test kit bias in Table 6-3 in all cases.

Table 6-4. GC-MS Average NP Concentration and Percent Bias Results by Phase

Phase	Average Conc (ng/L)	% Bias (vs. Expected Conc)
I	24000	-26
II	19000	-42
III	20000	-38
IV	17000	-59

6.3 Matrix Effects

To understand how the matrix of each phase of testing might have affected the results, percent bias and percent recovery were calculated for the test kit results in comparison to the expected spiked concentration of NP. A positive percent bias indicates that the ELISA test kit NP concentration is higher than the expected spike concentration, while a negative percent bias indicates that the ELISA test kit NP concentrations are lower than the expected spike concentration. Table 6-5 presents the percent bias results. No false negatives were observed during this verification test.

Table 6-5. ELISA Test Kit Percent Bias vs. Expected Spike Concentration by Phase

	Phase I		Phase II		Phase III		Phase IV	
	DIRECT	SPE	DIRECT	SPE	DIRECT	SPE	DIRECT	SPE
Lab 1 kit a	-69	-86	-48	-53	-81	-58	-55	-42
Lab 1 kit b	-43	-75	-57	-71	-45	-93	-46	-48
Lab 2	-73	-83	-72	-84	-46	-90	-69	-87
Lab 3	-44	-59	-29	-25	-68	-53	-38	-3

All samples across all laboratories had negative percent bias for Phases I – IV. This bias ranged from -3% to -93%. Though percent bias results for both kits were negative in all cases, the magnitude of this negative bias was often quite different between test kit a and b for Lab 1. In some cases, the negative bias for one kit was up to two times that of the other. Percent bias results for Phase IV were similar between direct and SPE analysis for Labs 1 and 2. Phase II results showed a similar relationship. The Phase II SPE percent bias results were similar across Labs 1 and 2. The Lab 3 percent bias results for Phases II, III, and IV were less negative (biased higher) for the SPE analysis as compared to the direct analysis. This relationship is the clearest in Phase IV, where the direct analysis for Lab 3 produced a -38% bias while the SPE analysis produced a -3% bias.

As another measure of accuracy, percent recovery results, comparing the test kit results against the expected spiked concentration, were also calculated on a per sample and per phase average basis. Table 6-6 presents these results.

Table 6-6. Percent Recovery by Phase

	LAB	Phase I		Phase II		Phase III		Phase IV	
		DIRECT	SPE	DIRECT	SPE	DIRECT	SPE	DIRECT	SPE
% Recovery	Lab 1 kit a	31	14	52	47	19	42	45	58
	Lab 1 kit b	57	25	43	29	55	7	54	52
	Lab 2	27	17	28	16	54	10	31	13
	Lab 3	56	41	71	75	32	47	62	97
Average		**43**	**24**	**49**	**42**	**40**	**27**	**48**	**55**

Average percent recoveries of NP by phase using the AP ELISA test kit were 40 to 48% for direct analysis and 24 to 55% for SPE analysis. Percent recoveries for samples analyzed by each laboratory ranged from 7% to 97%. Within a particular phase, average percent recoveries were less for SPE than direct analysis. This also generally holds true for the samples for Labs 1 and 2. For Lab 3, percent recoveries for SPE analysis were higher in Phases II through IV then for the direct analysis in these phases. Only Phase I of the Lab 3 analysis showed percent recoveries for the SPE analysis as lower than the direct analysis recoveries. During the SPE process, the evaporation step of the eluant (dichloromethane) can be very difficult to properly perform

without correct training and experience, as NP can easily evaporate during this step. This might account for the low recoveries after SPE. However, this cannot and has not been verified. Percent recoveries for kit a and kit b for Lab 1 generally did not agree well with each other. The average percent recoveries for all phases were below acceptable levels for the GC-MS reference method (60-140%).

A comparison of the ELISA results generated with (SPE) and without the use of SPE (direct) was performed. Throughout this section, comparisons between results generated through direct analysis and with the use of SPE have been explored using the percent bias and percent recovery results. For this discussion, comparison of the two techniques will be determined through the use of a nonparametric test. Nonparametric tests were conducted using the percent bias spike results. Nonparametric tests were conducted on each phase. In all cases, the nonparametric results indicated that the median differences in biases found using results generated with (SPE) and without the use of SPE (direct) were not statistically significantly greater than zero.

Some ELISA kits will react with compounds similar to the target compound, known as cross-reactivity. The Abraxis AP ELISA test kit will react with a known percent reactivity to multiple alkylphenol ethoxylates (APEs). During each phase of the study, some of the compounds with which the test kit has cross-reactivity were measured alongside background levels of the kit's target compound in that matrix by GC-MS. For some of the compounds for which there is known cross-reactivity with the Abraxis AP ELISA test kit, there are no established analytical methods available by GC-MS at these concentrations and in these matrices. Therefore some bias will have to be accepted from influence of cross-reactive compounds that cannot be identified via GC-MS; however, this bias is expected to be in the positive direction (i.e., increasing expected NP concentrations). According to the test kit instructions, there is expected to be minimal bias from these compounds compared to the primary target compound. Table 6-7 lists concentrations found in each matrix blank sample from each phase along with the known percent reactivities for the cross-reactive compounds to the AP ELISA test kit that were measured during this verification test. Unfortunately, matrix blank samples used for background analysis were not analyzed on the Abraxis AP ELISA test kit. Because of this, the potential for cross-reactive compounds present in the matrix for each phase to interfere with the test kit results cannot be truly evaluated. However, because of the significant negative bias of the results, it can be assumed that cross-reactivity was not a problem with the test kit.

Table 6-7. Concentrations of Cross-Reactive Compounds by Phase[a]

	GC-MS Concentration (ng/L)				
Cross-Reactive Compounds	Phase I	Phase II	Phase III	Phase IV	Cross Reactivity (%)
Nonylphenol Diethoxylate (NP2EO)	ND	ND	ND	ND	2.1
Octylphenol	ND	ND	ND	2400	96
Nonylphenol Monoethoxylate	ND	ND	ND	20000	1.2

[a]ND = not detected

6.5 Operational Factors

In general, training is needed to effectively and properly operate ELISA test kits. The vendor trained staff on the operation of the test kit, but these trained staff were, in some cases, not available for the verification test because of testing delays and staff turnover. Therefore, staff that operated the test kits during the verification test may not have been trained by the vendor.

Operational concerns or issues were not reported from any of the three participating laboratories. The test kit instructions were readily followed by each of the operators. Preparation time was required prior to the introduction of the sample to allow all reagents time to come to room temperature before using them. Calibrated pipettes and a photometer capable of reading at 450 nm are required for the operation of the test kit, but are not supplied with the test kit. Any GFF or SPE equipment used with the samples was also not supplied with the test kit.

Each purchased test kit is capable of conducting 96 tests and costs $750. For comparison, GC-MS analyses of these samples are estimated to cost between $500 and $900 per sample. [1]

Chapter 7
Performance Summary

The ability of the Abraxis AP ELISA test kit to detect NP in water was evaluated using four different water matrices. The test kit was operated by three different laboratories with and without the use of SPE. The test kit results were evaluated against the expected spike concentrations and the reference measurements of the same samples made using GC-MS.

RSDs ranged from 35 to 59% for direct analysis and 60 to 66% for SPE analysis. The RSDs were quite similar across all phases of the SPE analysis, with only a maximum of a 6% difference between any two phases. Phases I, II, and IV has almost identical RSD while Phase III had the highest RSD (66%). RSDs for the direct analysis were similar, with Phases I, II, and IV having similar RSDs and Phase III having the highest direct analysis RSD (59%). For both direct and SPE analysis, Phase IV (which was performed with wastewater influent) had the lowest RSD.

Percent bias, as compared to the GC-MS reference analysis results, was negative for Labs 1 and 2 across Phases I – III. For Phase IV, Labs 1 and 3 percent bias was positive while Lab 2 was negative, for both direct and SPE analysis. In general, a larger bias was shown for samples that used the SPE clean-up vs. direct analysis in all phases. Percent bias, as compared to the expected spiked NP concentration, was negative for all laboratories across all phases. This bias ranged from -3% to -93%. T-test results indicated that the bias found using results generated with and without the use of SPE were not statistically different ($p > 0.05$).

No false negatives were observed during this verification test. Average percent recoveries of NP were 40 to 48% for direct analysis and 24 to 55% for SPE analysis. Individual percent recoveries for samples analyzed by each laboratory ranged from 7% to 97%. Within a particular phase, average percent recoveries were less for SPE than direct analysis. The average percent recoveries for all phases were below acceptable levels for the GC-MS reference method (60% - 140%).

Operational concerns or issues were not reported from any of the three participating laboratories. The test kit instructions were readily followed by each of the operators. Preparation time was required prior to the introduction of the sample to allow all reagents time to come to room temperature before using them. Calibrated pipettes and a photometer capable of reading at 450 nm are required for the operation of the test kit, but are not supplied with the test kit. Any GFF or SPE equipment used with the samples was also not supplied with the test kit. Each purchased test kit is capable of conducting 96 tests and costs $750.

Chapter 8
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