September 2009

Environmental Technology Verification Report

ABRAXIS ECOLOGENIA®
ETHYNYLESTRADIOL (EE2) 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® ETHYNYLESTRADIOL (EE2) MICROPLATE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TEST KITS

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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 and has been approved for publication. 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 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: Mark Mills and Scott Jacobs, U.S. EPA NRMRL, Cincinnati, OH; Jennifer Gundersen, Dave Russell, Ronald Landy, Annie Hilliard, John Curry, 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, USGS, Kansas; Jim Lazorchak, Tirumuru Reddy, and Dan Bender, U.S. EPA 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, NOAA, and Marion Kelly, U.S. EPA, for their review of this verification report.

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List of Abbreviations

AMS	Advanced Monitoring Systems
°C	degrees Celsius
COC	chain of custody
DI	deionized
E1	estrone
E2	17-β-estradiol
E3	estriol
EDC	endocrine-disrupting compound
EE2	17-α-ethynylestradiol
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
MB	method blank
MDL	method detection limit
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
ppb	parts per billion
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 ETV 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 EPA's National Risk Management Research Laboratory (NRMRL) and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of the Abraxis Ecologenia® Ethynylestradiol (EE2) 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® Ethynylestradiol (EE2) Microplate ELISA test kit. The following is a description of the test kit, based on information provided by the vendor. The EE2 microplate ELISA test kit applies the principle of ELISA to determine EE2 in water samples. The EE2 microplate ELISA kit uses a colorimetric procedure to detect EE2.

The standards, samples, and an enzyme-labeled EE2 conjugate are added to a disposable microtiter plate (uncoated) and mixed. 100 microliter (µL) aliquots of the mixture are then added to antibody (monoclonal anti-EE2) coated wells in a 96-well microplate. At this point a competitive reaction occurs between the ethynylestradiol which may be in the sample and the enzyme-labeled ethynylestradiol 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 reagent from the ethynylestradiol and labeled ethynylestradiol that remain bound to the antibodies on the plate, in proportion to their original concentrations. 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 ethynylestradiol concentration is determined by measuring the absorbance of the sample solution with a photometer (450 nm) and comparing it to the absorbance of standards.

Figure 2-1. Abraxis Ecologenia® Ethynylestradiol (EE2) Microplate ELISA Test Kit

The EE2 microplate ELISA test kit (Figure 2-1) contains a 96-well microplate coated with ethynylestradiol antibody (mouse anti-ethynylestradiol), two vials of lyophilized horseradish peroxidase-labeled ethynylestradiol analog, two 7 mL vials of buffer solution, five 1.5 mL vials of ethynylestradiol standard concentrations (0, 0.05, 0.15, 0.5, 3.0 parts per billion (ppb)) 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 microtiter plate, and one adhesive plate cover.

The EE2 microplate ELISA test kit measures 7 by 5 by 4 ½ inches. Final results and calibration curves are printed from the photometric analyzer or sent directly to a laboratory computer. List price is $699 for a 96-test kit. Other materials that are required but are not provided with the EE2 microplate ELISA test kit are pipettes, and a plate photometer capable of reading at 450 nanometer (nm). These materials can be purchased separately or rented.

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 particle. 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) National Risk Management Research Laboratory (NRMRL), EPA ORD National Exposure Research Laboratory (NERL), and EPA Region 3. 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 evaluated the Abraxis EE2 microplate ELISA test kit to quantitate 17-α-ethynylestradiol (EE2) in four different water matrices, per the manufacturer protocols. More

detailed information on the uses of the EDC tested is provided in Table 3-1. EPA laboratories used the Abraxis Ecologenia® EE2 microplate ELISA kit (according to Table 3-2) to quantitate triplicate spiked samples for hormones (EE2), which were prepared and shipped to Region 3 by EPA NRMRL. The test was conducted in four phases from June to September 2008, with each phase being a different aqueous matrix. As the more established method for detecting these compounds, GC-MS served as the reference method[2] for this test.

Table 3-1. Target Analytes

Analyte	Synonyms	CAS#	Use
17α-Ethynyl-1,3,5(10)-estratriene-3,17b-diol	17-α-Ethynylestradiol (EE2)	57-63-6	Synthetic estrogen found in birth control

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

Responsibility	NRMRL	NERL	Region 3
Sample Collection, Processing and Distribution	√		
Test Kit Evaluation – EE2 96 well ELISA	√	√	√
Reference Measurement - EE2 GC-MS	√		

The Abraxis EE2 microplate ELISA test kit was verified by evaluating the following parameters:

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

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 EE2 microplate ELISA test kit were conducted in three different collaborating laboratories by the laboratory staff. These laboratories were: EPA Region 3; EPA NRMRL, Cincinnati, OH; and EPA NERL, Cincinnati, OH.

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 EE2, 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 EE2 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 EE2 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 EE2 were taken for each matrix for each phase. These GC-MS measurements were made to determine if any measureable amounts of EE2 might exist in the sample matrix prior to the addition of any sample spikes. If a detectable concentration was found, this concentration was then added to the spiked amount of EE2 to calculate the total concentration for all spiked samples of a particular phase. Specific concentrations of EE2, 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. For Phase III, 1.47 ng/L of EE2 was found in the background matrix sample. 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 EE2 microplate test kit were compared to each other and compared to GC-MS results.

The Abraxis EE2 microplate 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 directed by the manufacturer of the test kit, were used for the four different matrices. Each sample was analyzed 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 prior to SPE. Calibration and maintenance of the technology reader (i.e., microplate reader) was performed as specified by the vendor.

A US EPA NRMRL GC-MS standard operating procedure (SOP) was followed for reference measurements.[2] The GC-MS methods for estrone (E1), E2, estriol (E3), EE2, testosterone, dihydrotestosterone, androstenedione, and progesterone operated within a concentration range of

2-50 ng/L. Samples for the GC-MS methods went through an extraction step to concentrate (or dilute, depending upon the samples) 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 hormone samples, including the glass carboy, were cleaned and silanized using a procedure included in the test/QA plan.[1] All samples were thoroughly mixed and were thus assumed to contain the same concentration. Samples were spiked with EE2 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 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. Holding times of hormone samples are currently unknown; therefore, all samples were either analyzed or solvent exchanged within 24 hours of receipt to reduce error associated with analyte degradation during sample holding. 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 analyses 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 laboratory in Cincinnati, Ohio. The water was spiked with EE2 to a concentration of 10 ng/L of EE2. 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 4L sample for each reference laboratory. DI water blanks were also prepared and shipped in separate 500 mL bottles. The blank samples were analyzed after SPE but only in two wells (or test tubes) on the kits as opposed to three wells (or test tubes) for all other samples. Before spiking, the DI water was sampled and analyzed by GC-MS to confirm the background levels of EE2. Samples of the spiked mixtures were taken and the concentrations of these samples and blank were determined using the Abraxis EE2 microplate 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 the background levels of EE2. Background levels of EE2 measured in the samples were added to the spiked concentration of EE2 once results were obtained. Next, a cleaned, 20 L, glass carboy was used to collect 20 L of the stream water, which was then spiked to contain a 10 ng/L concentration of EE2. 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 filled with DI water at the same time as the stream water in the 20 L carboys.

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 the background levels of EE2. In a cleaned, 20 L, glass carboy, 20 L of WWTP effluent was be prepared containing 10 ng/L of EE2. 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.

3.3.1.4 Phase IV Samples

Grab samples of influent wastewater were collected in three, clean, five gallon bucket 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 to confirm the background levels of EE2. In a cleaned, 20 L, glass carboy, 20 L of WWTP influent was prepared containing 10 ng/L concentration of EE2. 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

The ELISA test kit users followed simple cleanup procedures as directed in the vendor's instructions. The 2.5 L sample was split into three 500 mL aliquots. Each of the three aliquots was analyzed by utilizing glass fiber filter (GFF) cleanup and SPE. Each aliquot sample was transferred in triplicate to the 96-well microplate for quantification, per the test kit protocols. The cleanup procedures are described below.

Each sample for ELISA analysis was filtered through a 1 μm GFF. After filtering, three 500 mL aliquots were removed from the filtered sample for SPE clean-up. These three aliquots were treated as three independent samples. SPE directions entitled "Extractions for EE2 from Water Sample for 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 1 μm glass fiber filter.
2. Rinse a C18 cartridge with 5 mL of methanol and then 10 mL of distilled water at a flow rate not exceeding 20 mL/min (preconditioning).
3. Pour the filtered sample through the C18 SPE cartridge at a flow rate, no faster than 20 mL/min.
4. Wash the cartridge with 5 mL of distilled water (up to 20 mL/min). Keep suctioning for about a minute to dry the cartridge.
5. Wash the cartridge with 5 mL of hexane (up to 20 mL/min).
6. Elute the analyte with 5 mL of dichloromethane at a rate, no faster than 3mL/min.
7. Evaporate the solvent with nitrogen gas to dryness.
8. Add 1 mL of 100% methanol to the residue and stir the mixture with a vortex mixer. To adjust the content to 10% methanol (volume/volume (v/v)) add 9 mL of distilled water for a total volume of 10 mL.

After the SPE column, the EE2 samples were reconstituted with 10 mL of a 10% methanol solution. For the spiked samples, this process effectively increased the overall spike concentration 50 times to an expected level of 490 ng/L. All reconstituted samples were applied 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 EE2 microplate ELISA test kit that were followed during this verification test are provided below.

The Abraxis ELISA EE2 96 well microtiter plate 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 add methanol to obtain a final methanol concentration of 10% (v/v).
2. Reconstitute the antigen-enzyme conjugate powder with buffer solution.
3. Mix 100 μL of conjugate solution and 100 μL of EE2 standard (or sample) in each well of the uncoated plate. 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 color solution 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 EE2 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 EE2 microplate ELISA test kit results to those of the GC/MS reference method for EE2. Samples analyzed for each phase included 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 method blanks (MBs), instrument solvent blanks, and surrogate spikes, as indicated in the test/QA plan.[1] Method blank samples were analyzed to ensure that no sources of contamination were present. If the analysis of a 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. D_4-EE2 was used as a surrogate standard for the GC/MS analysis of EE2 in the samples. No levels of EE2 were detected in any of the reference method blank samples.

Surrogate recoveries in Phase I – IV samples varied across phases. Phase I surrogate recoveries ranged from 59 – 96% and averaged 85 ± 10% across 13 samples. All recoveries were considered in the acceptable range. Phase II recoveries ranged from 77 to 155% and averaged

132 ± 25% over 11 samples. Surrogate recoveries for six of the samples were outside of the acceptable range. Compared to the surrogates, the peak shapes for the target analytes were good and the baselines were clean in the chromatogram. Phase III surrogate recoveries ranged from 154% to 197% and averaged 176 ± 14% over 8 samples. Surrogate recoveries for all samples were outside of the acceptable range for Phase III. Phase IV surrogate recoveries ranged from 61% to 93% and averaged 76 ± 10% over 11 samples. Surrogate recoveries for all Phase IV samples were within the acceptable range.

4.1.2 Test Kit Method Blanks

Method blank samples were run in duplicate after SPE clean-up with each set of samples for all four phases. 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 Abraxis EE2 microplate ELISA test kit has a stated method detection limit (MDL) of 0.05 – 3 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 EE2 microplate test kit was evaluated by three laboratories (see Table 3-2). Concentrations of EE2 were not detected in any of the method blank samples from two of the participating laboratories. For the NERL - Cincinnati results, levels of EE2 above the MDL were not detected in Phase II – IV method blanks. However, during the analysis of Phase I samples, concentrations of EE2 above the MDL were found for one of the two replicates in one set (the method blank analyzed after SPE) of duplicate method blanks for one test kit. Two EE2 microplate ELISA test kits were evaluated by this laboratory for each phase of testing. The second test kit operated during Phase I did not show any detectable levels of EE2 in any of the method blanks analyzed. Averaging the duplicate samples for the after-SPE method blanks in the first test kit put the overall EE2 concentration below the MDL. All method blanks for this phase came from the same initial sample.

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 below.

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 EE2 prepared from a different source other 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 E2 concentrations divided by the expected concentration. The EE2 PE audit samples were within 30 % of the expected concentration for Phases I and II but were outside of this range for Phases III and IV (112% more and 43% less, respectively). The PE audit sample results for Phase III were similar to the GC-MS sample results for Phase III. This could have impacted the comparison of the ELISA test kit results to the GC-MS data for Phase III. No adjustments were made to the standards nor were PE audit samples reanalyzed based on these results.

Table 4-1. PE Audit Sample Results

Phase	Expected Concentration (ng/L)	Actual Concentration (ng/L)	% Error
I	10	8.27	-17
II	10	9.47	-5
III	10	21.2	112
IV	10	5.73	-43

4.2.2 Technical Systems Audit

The Battelle Quality Manager performed a TSA twice during this verification test. Battelle conducted TSAs at the Cincinnati, OH facilities on July 23-24, 2008 and at the Fort Meade, MD facility on July 31, 2008. Both laboratories participating in Cincinnati, OH, were audited. 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 in the 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,[1] 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 known spike concentration as well as any detected background levels of EE2 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%.

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 EE2 microplate 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 EE2 microplate ELISA test kit. Two of the laboratories (Lab 1 and Lab 2) ran identical samples on two separate test kits (kit "a" and kit "b"). Lab 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 EE2 found using the EE2 microplate ELISA test kit for all analyses. RSD values are also presented across all results for each phase.

RSDs ranged from 6 to 72% for SPE analysis. The RSDs among all analyses were the same for Phases II and IV (41%). For Phase I which was performed with DI water, the overall RSD was 67%. This RSD was approximately 2.5 times that for Phase III, which had the lowest RSD at 25%. There was no discernable trend in RSD when going from less (i.e., Phase I – DI water) to presumably more challenging phases (i.e., Phase IV – wastewater influent).

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

		Average Conc (ng/L)	RSD	Overall Average Conc (ng/L)	RSD	Expected Phase Conc (ng/L)
Phase I	Lab 1 kit a	778	64%			
	Lab 1 kit b	499	50%			
	Lab 2 kit a	163	14%	508	67%	490
	Lab 2 kit b	467	15%			
	Lab 3	out of range[b]				
Phase II	Lab 1 kit a	587	9%			
	Lab 1 kit b	947	29%			
	Lab 2 kit a	466	16%	672	41%	490
	Lab 2 kit b	505	21%			
	Lab 3	856	39%			
Phase III	Lab 1 kit a	688	11%			
	Lab 1 kit b	370	23%			
	Lab 2 kit a	589	9%	525	25%	564
	Lab 2 kit b	495	13%			
	Lab 3	485	22%			
Phase IV	Lab 1 kit a	401	6%			
	Lab 1 kit b	508	21%			
	Lab 2 kit a	446	72%	437	41%	490
	Lab 2 kit b	573	8%			
	Lab 3	303	35%			

[a] The average concentration and RSD are based on all replicates within the detectable range of the test kit.
[b] Test kit results were below the lower end of the test kit's range.

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 concentration is higher than the reference method, while a negative percent bias indicates that the ELISA test kit concentrations are lower than the reference method. Table 6-2 presents the percent bias results.

Phase III percent bias results were consistently negative among the test kit analyses. For Phases I, II, and IV, the percent bias results were mixed. Phase IV percent bias results were all positive except for Lab 3, which was negative. For Phase II, the bias was negative for Lab 2 and positive for Lab 3. Within these agreements, the percent bias results were different for kits a and b. Kit a had significantly higher percent bias for both Labs 1 and 2 than Kit b in Phase I. For Phase II, Lab 1 results indicated a positive and negative bias of approximately the same magnitude for the two kits tested. Only Lab 2 results produced a percent bias absolute value of <5%.

Table 6-2. ELISA Test Kit Percent Bias vs. GC-MS

	Phase I	Phase II	Phase III	Phase IV
Lab 1 kit a	66	-25	-28	8
Lab 1 kit b	7	20	-61	37
Lab 2 kit a	-65	-41	-38	20
Lab 2 kit b	0	-36	-48	54
Lab 3	out of range[a]	9	-49	-18

[a] Test kit results were below the lower end of the test kit's range.

For comparison, average concentrations, RSD, and percent bias for the GC-MS measurements with regard to the expected concentration are presented in Table 6-3 for each phase. RSD values were less than 30% for all phases. Phases I and IV GC-MS results were lower than the expected concentration, but the percent bias was within 30% of the expected value. Percent bias results for Phases II and III were positive and more than two times higher (based on absolute values) than for Phase IV. These results demonstrate that the GC-MS results were biased high (in Phases II and III) and low (in Phases I and IV) as compared to the expected concentration.

Table 6-3. GC-MS Average Concentration, RSD, and Percent Bias Results

Phase	Average Conc (ng/L)	RSD	% Bias (vs. Expected Conc)
I	9.37	4%	-4
II	15.72	1%	61
III	19.08	11%	69
IV	7.43	6%	-24

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 EE2. A positive percent bias indicates that the ELISA test kit concentration is higher than the expected spike concentration, while a negative percent bias indicates that the ELISA test kit concentrations are lower than the expected spike concentration. Table 6-4 presents the percent bias results. No false negatives were observed during this verification test.

Table 6-4. ELISA Test Kit Percent Bias vs. Expected Spike Concentration

	Phase I	Phase II	Phase III	Phase IV
Lab 1 kit a	59	20	22	-18
Lab 1 kit b	2	93	-34	4
Lab 2 kit a	-67	-5	4	-9
Lab 2 kit b	-5	3	-12	17
Lab 3	out of range[a]	75	-14	-38

[a] Test kit results were below the lower end of the test kit's range.

Percent bias results varied for the EE2 microplate ELISA test kit. There was a mixture of positive and negative percent bias for all phases and within multiple test kits used by one laboratory. No consistent trends were apparent for any phase or laboratory. Percent bias results were negative for all samples in Phase IV except for that from Lab 1 kit b, which had a positive bias. Discrepancies between the percent bias for duplicate test kits operated by the same laboratory were apparent for both Lab 1 and 2. Bias for one kit was 2 to 22 times that for the other kit operated by the same laboratory. The highest percent bias results were found in Phase II samples for different laboratories. A positive percent bias, indicating that the EE2 microplate ELISA test kit results were higher than the expected spiked concentration, of 93% and 75% were found. The remaining percent bias results for Phase II were some of the lowest found across all phases. In comparing the results of Table 6-4 to Table 6-2 where the test kit results were compared to the GC-MS results, in general the test kits results were closer to the expected concentrations than they were to the GC-MS results. However, bias results for Phase I were similar in both cases.

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 6-5 presents these results.

Table 6-5. Percent Recovery

		Phase I	Phase II	Phase III	Phase IV
% Recovery	Lab 1 kit a	159	120	122	82
	Lab 1 kit b	102	193	66	104
	Lab 2 kit a	33	95	104	91
	Lab 2 kit b	95	103	88	117
	Lab 3	no data	175	86	62
	Average	**97**	**137**	**93**	**91**

Average percent recoveries for Phases I, III, and IV for the Abraxis EE2 microplate ELISA test kit were 97%, 93%, and 91%, respectively. Phase II had an average percent recovery of 137%. Recoveries across all phases ranged from 62% to 120% except for four samples, where the recoveries were outside this range at 33%, 156%, 175%, and 193%. Two of these outlier recoveries were in Phase I and two were in Phase II. There was variability in the percent recovery across phases within a laboratory's results. None of the three analyses provided consistently high or low percent recoveries. The average percent recoveries for all phases were within the range of acceptable recoveries for the GC-MS reference method (60% - 130%).

Some ELISA kits will react with compounds similar to the target compound, known as cross-reactivity. The EE2 microplate ELISA 96 well hormone kit for EE2 is compound specific with minimal cross reactivity (<0.2% for all cross-reactive compound). Thus, an evaluation of cross-reactivity was not performed for this test kit.

6.4 Operational Factors

In general, training is needed to effectively and properly operate the 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 turn over. 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 (at least half an hour) was required prior to the introduction of the sample to allow all reagents time to come to room temperature before using them. Calibrated pipettes, disposable test tubes, and a plate reader 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 $699. 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 EE2 microplate ELISA test kit to detect EE2 in water was evaluated using four different water matrices. The test kit was operated by three different laboratories with the use of SPE cleanup. The test kit results were evaluated against the expected spike concentrations and the reference measurements of the same samples made using GC-MS.

Relative standard deviations (RSDs) ranged from 6 to 72% for SPE analysis. The RSDs among all analyses were the same for Phases II and IV (41%). For Phase I which was performed with DI water, the overall RSD was 67%. This RSD was approximately 2.5 times that for Phase III, which had the lowest RSD at 25%. There was no discernable trend in RSD when going from less (i.e., Phase I – DI water) to presumably more challenging phases (i.e., Phase IV – wastewater influent).

Percent bias, as compared to the GC-MS reference analysis results, for Phases I, II, and IV, was mixed. Phase III percent bias results were consistently negative among the test kit analyses. There was a mixture of positive and negative bias for Phases I, II, and IV, and within multiple test kits used by one laboratory. No consistent trends were apparent among the analyses.

No false negatives were observed during this verification test. Average percent recoveries of EE2 for Phases I, III, and IV were 91%, 93%, and 97%, respectively. Phase II had an average percent recovery of 137%. Recoveries across all phases ranged from 62 to 120%. None of the three analyses provided consistently high or low percent recoveries. The percent recoveries for all phases were within the range of acceptable recoveries for the GC-MS reference method (60% - 130%).

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 (at least half an hour) was required prior to the introduction of the sample to allow all reagents time to come to room temperature before using them. Calibrated pipettes, disposable test tubes, and a plate reader 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 $699.

Chapter 8
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1. *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,* Battelle, Columbus, Ohio, July 1, 2008.

2. US EPA NRMRL Standard Operating Procedure (SOP) for the Analysis of Steroid Hormones in Aqueous Samples, QA ID 503-P3-0, 09/29/05.

3. *Quality Management Plan (QMP) for the ETV Advanced Monitoring Systems Center*, Version 6.0, U.S. EPA Environmental Technology Verification Program, Battelle, Columbus, Ohio, November 2005.