Environmental Technology Verification Program
Advanced Monitoring Systems Center

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
Verification of
Enzyme-Linked Immunosorbent Assay (ELISA) Test Kits for the
Quantitative Determination of Endocrine Disrupting Compounds
(EDCs) in Aqueous Phase Samples

June 5, 2008

Prepared by

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SECTION A PROJECT MANAGEMENT</td>
<td>7</td>
</tr>
<tr>
<td>A1 VERIFICATION TEST ORGANIZATION</td>
<td>7</td>
</tr>
<tr>
<td>A2 BACKGROUND</td>
<td>14</td>
</tr>
<tr>
<td>A3 VERIFICATION TEST DESCRIPTION AND SCHEDULE</td>
<td>15</td>
</tr>
<tr>
<td>A4 QUALITY OBJECTIVES AND CRITERIA FOR MEASUREMENT DATA</td>
<td>17</td>
</tr>
<tr>
<td>A5 SPECIAL TRAINING/CERTIFICATION</td>
<td>18</td>
</tr>
<tr>
<td>A6 DOCUMENTATION AND RECORDS</td>
<td>18</td>
</tr>
<tr>
<td>SECTION B MEASUREMENT AND DATA ACQUISITION</td>
<td>23</td>
</tr>
<tr>
<td>B1 EXPERIMENTAL DESIGN</td>
<td>23</td>
</tr>
<tr>
<td>B2 SAMPLING REQUIREMENTS</td>
<td>33</td>
</tr>
<tr>
<td>B3 SAMPLE HANDLING AND CUSTODY REQUIREMENTS</td>
<td>34</td>
</tr>
<tr>
<td>B4 QUALITY CONTROL</td>
<td>34</td>
</tr>
<tr>
<td>B5 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE</td>
<td>35</td>
</tr>
<tr>
<td>B6 CALIBRATION/VERIFICATION OF TEST PROCEDURES</td>
<td>35</td>
</tr>
<tr>
<td>B7 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES</td>
<td>36</td>
</tr>
<tr>
<td>B8 NON-DIRECT MEASUREMENTS</td>
<td>37</td>
</tr>
<tr>
<td>B9 DATA MANAGEMENT</td>
<td>37</td>
</tr>
<tr>
<td>SECTION C ASSESSMENT AND OVERSIGHT</td>
<td>39</td>
</tr>
<tr>
<td>C1 ASSESSMENTS AND RESPONSE ACTIONS</td>
<td>39</td>
</tr>
<tr>
<td>C2 REPORTS TO MANAGEMENT</td>
<td>42</td>
</tr>
<tr>
<td>SECTION D DATA VALIDATION AND USABILITY</td>
<td>43</td>
</tr>
<tr>
<td>D1 DATA REVIEW, VALIDATION, AND VERIFICATION REQUIREMENTS</td>
<td>43</td>
</tr>
<tr>
<td>D2 VALIDATION AND VERIFICATION METHODS</td>
<td>43</td>
</tr>
<tr>
<td>D3 RECONCILIATION WITH USER REQUIREMENTS</td>
<td>44</td>
</tr>
<tr>
<td>SECTION E REFERENCES</td>
<td>46</td>
</tr>
<tr>
<td>E1 REFERENCES</td>
<td>46</td>
</tr>
<tr>
<td>APPENDICES</td>
<td></td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Organization Chart for the Verification Test................................................................. 8

List of Tables

Table 1. ELISA Test Kit Evaluation Responsibilities for Each Participating Laboratory .......... 14
Table 2. Planned Verification Test Schedule.................................................................................. 16
Table 3. Criteria for Critical Measurements for Reference Method............................................. 19
Table 4. Summary of Data Recording Process............................................................................. 22
Table 5. Target Analytes............................................................................................................... 23
ETV Advanced Monitoring Systems Center

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

June 5, 2008

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Company ________________________________

Date ______________________________________
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SECTION A
PROJECT MANAGEMENT

A1 VERIFICATION TEST ORGANIZATION

This test/quality assurance (QA) plan describes evaluating the performance of enzyme-linked immunosorbent assay (ELISA) test kits for the determination of endocrine disrupting compounds (EDCs) in water through a verification test. The verification test will be conducted under the auspices of the U.S. Environmental Protection Agency (EPA) through the Environmental Technology Verification (ETV) Program. It will be performed under the ETV Advanced Monitoring Systems (AMS) Center which is managed by Battelle through a cooperative agreement with EPA. The scope of the AMS Center covers verification of monitoring technologies for contaminants and natural species in air, water, and soil. Testing of the enzyme-linked immunosorbent assays (ELISAs) will be conducted with multiple collaborating laboratories, including EPA Office of Research and Development (ORD) National Risk Management Research Laboratory (NRMRL), EPA ORD National Exposure Research Laboratory (NERL), EPA Region 3, EPA Region 5, and the United States Geological Survey (USGS). Staff from these participating laboratories will operate the technologies during the verification test and also conduct the reference analyses. The laboratory participation will be coordinated by EPA NRMRL, in collaboration with Battelle. Each participating vendor will provide Battelle or EPA NRMRL with their respective technology and will train the participating laboratory staff in their technology use as needed.

The organization chart in Figure 1 identifies the responsibilities of the organizations and individuals associated with the verification test. Roles and responsibilities are defined further below. Quality Assurance (QA) oversight will be provided by the Battelle Quality Manager and also by the EPA NRMRL Director of QA or her designee, at her discretion.
Figure 1. Organization Chart for the Verification Test
(dotted lines indicate indirect reporting)
A1.1  Battelle

Dr. Stephanie Buehler is the AMS Center's Verification Test Coordinator for this test. In this role, Dr. Buehler will have overall responsibility for ensuring that the technical, schedule, and cost goals established for the verification test are met. Specifically, Dr. Buehler or her designee will:

- Prepare the draft test/QA plan, verification reports, and verification statements.
- Revise the draft test/QA plan, verification reports, and verification statements in response to reviewers’ comments.
- Establish a budget for the verification test and manage the effort to ensure the budget is not exceeded.
- Assemble a team of qualified technical staff to conduct the verification test.
- Assist Eric Kleiner of EPA NRMRL as needed in directing the team (Battelle, EPA, and USGS staff) in performing the verification test in accordance with this test/QA plan.
- Ensure that all quality procedures specified in this test/QA plan and in the AMS Center Quality Management Plan\(^1\) (QMP) are followed.
- Serve as a point of contact for vendor representatives.
- Ensure that confidentiality of sensitive vendor information is maintained.
- Assist vendors as needed during verification testing.
- Become familiar with the operation and maintenance of the technologies through instruction by the vendors, if needed.
- Respond to any issues raised in assessment reports, audits, or from test staff observations, and institute corrective action as necessary.
- Coordinate distribution of the final test/QA plan, verification reports, and verification statements.

Ms. Amy Dindal is Battelle’s Manager for the AMS Center. As such, Ms. Dindal will oversee the various stages of verification testing. Ms. Dindal will:

- Review the draft and final test/QA plan.
- Review the draft and final verification reports and verification statements.
Ensure that necessary Battelle resources, including staff and facilities, are committed to the verification test.

Ensure that confidentiality of sensitive vendor information is maintained.

Support Dr. Buehler in responding to any issues raised in assessment reports and audits.

Maintain communication with EPA’s technical and quality managers.

Issue a stop work order if Battelle or EPA QA staff discovers adverse findings that will compromise test results.

Mr. Zachary Willenberg is Battelle’s Quality Manager for the AMS Center.

Mr. Willenberg or his designee will:

- Review the draft and final test/QA plan.
- Conduct a technical systems audit at least once during the verification test.
- Audit at least 10% of the verification data.
- Prepare and distribute an assessment report for each audit.
- Verify implementation of any necessary corrective action.
- Request that Battelle’s AMS Center Manager issue a stop work order if audits indicate that data quality is being compromised.
- Provide a summary of the QA/QC activities and results for the verification reports.
- Review the draft and final verification reports and verification statements.

### A1.2 Technology Vendors

The responsibilities of the technology vendors are as follows:

- Review and provide comments on the draft test/QA plan.
- Accept (by signature of a company representative) the final test/QA plan prior to test initiation.
- Provide their technology for evaluation during the verification test.
- Provide all other equipment/supplies/reagents/consumables needed to operate their technology for the duration of the verification test.
Supply training on the use of the technology, and provide written consent and instructions
for test staff to carry out verification testing, including written instructions for routine
operation of their technology, as necessary.
Provide maintenance and repair support for their technology, on-site if necessary,
throughout the duration of the verification test.
Review and provide comments on the draft verification report and statement for their
respective technology.

A1.3 EPA - AMS Center

EPA’s responsibilities in the AMS Center are based on the requirements stated in the
“Environmental Technology Verification Program Quality Management Plan” (EPA QMP).2 The
roles of specific EPA staff are as follows:
Ms. Lauren Drees is the EPA NRMRL Director of QA. For the verification test,
Ms. Drees or her designee will:
Review the draft test/QA plan.
Perform at her option one external technical systems audit during the verification test.
Notify the EPA AMS Center Project Officer of the need for a stop work order if the
external audit indicates that data quality is being compromised.
Prepare and distribute an assessment report summarizing results of the external audit.
Review draft verification reports and verification statements.

Mr. Douglas Grosse is EPA’s Project Officer for the AMS Center. Mr. Grosse will:
Review the draft test/QA plan.
Approve the final test/QA plan.
Review the draft verification reports and verification statements.
Oversee the EPA review process for the test/QA plan, verification reports, and
verification statements.
Coordinate the submission of verification reports and verification statements for final
EPA approval.
• Contact the AMS Center Manager to issue a stop work order if an EPA assessment indicates that data quality is being compromised.

A1.4 EPA – NRMRL

This test will be conducted in collaboration with EPA’s NRMRL laboratory in Cincinnati, OH, who will be providing in-kind support for this test. Mr. Eric Kleiner will be the EPA NRMRL Coordinator, responsible for coordinating the testing at the EPA and USGS participating laboratories as well as coordination with Battelle. The responsibilities from EPA’s NRMRL laboratory include:

- Review the draft test/QA plan.
- Prepare all split samples and reference samples for all phases of the study.
- Ship split samples to the participating laboratories.
- Coordinate sample analyses among the participating laboratories and ensure that all participants adhere to the agreed upon schedules.
- Implement the test/QA plan and ensure that all QA procedures described are followed.
- Plan reference analyses for all tested EDCs.
- Conduct analyses on all 17-β-Estradiol (E2), 17-α-ethynylestradiol (EE2), and alkylphenol (AP) ELISA test kits as well as gas chromatography-mass spectrometry (GC-MS) reference analysis on specified hormone (E2 and EE2) test samples.
- Coordinate QA/QC efforts of individual participating laboratories.
- Record observations about the operation of the ELISA test kits at the NRMRL facilities throughout the test.
- Coordinate, receive, and compile results data from the participating laboratories.
- Interpret the resulting data from the test kits, in collaboration with the Verification Test Coordinator, as necessary.
- Provide raw data and data analyses results to the Verification Test Coordinator.
- Review the draft verification reports and verification statements.
A1.5 EPA – NERL Cincinnati

Scientists at EPA NERL in Cincinnati, OH will be providing in-kind support for this test. The responsibilities from EPA NERL-Cincinnati staff include:

- Conduct analyses on all EE2 ELISA test kits.
- Provide raw data and data analyses results to the Mr. Kleiner.

A1.6 EPA – NERL Las Vegas

Scientists at EPA NERL in Las Vegas, NV will be providing in-kind support for this test. The responsibilities from EPA NERL-LV include:

- Conduct analyses on all AP ELISA test kit.
- Provide raw data and data analyses results to Mr. Kleiner.

A1.7 EPA – Region 3

Scientists in EPA Region 3 will be providing in-kind support for this test. The responsibilities from EPA Region 3 staff include:

- Conduct analyses on all E2 and EE2 ELISA test kits.
- Provide raw data and data analyses results to Mr. Kleiner.

A1.8 EPA – Region 5

Scientists in EPA Region 5 will be providing in-kind support for this test. The responsibilities from EPA’s Region 5 staff include:

- Conduct analyses on all AP ELISA test kits.
- Conduct GC-MS AP reference analyses.
- Provide raw data, QC data, and data analyses results to Mr. Kleiner, including those from the GC-MS analyses.
A1.9 USGS – Kansas

Scientists at the USGS laboratory in Kansas will be providing in-kind support for this test. The responsibilities of the USGS-Kansas staff include:

- Conduct analyses on all E2 ELISA test kits.
- Provide raw data and data analyses results to Mr. Kleiner.

Table 1 provides a summary of the test kit evaluation responsibilities for each participating laboratory described in Section A1.

Table 1. ELISA Test Kit Evaluation Responsibilities for Each Participating Laboratory

<table>
<thead>
<tr>
<th>Responsibility</th>
<th>NRMRL</th>
<th>NERL-Cinc</th>
<th>Region 3</th>
<th>NERL-LV</th>
<th>Region 5</th>
<th>USGS-KS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Collection, Processing and Distribution</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Kit Evaluation – E2 magnetic particle ELISA</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Kit Evaluation – E2 96 well ELISA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Test Kit Evaluation – EE2 96 well ELISA</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Kit Evaluation – AP 96 well ELISA</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Measurement - E2, EE2 GC-MS</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference Measurement - AP GC-MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

A2 BACKGROUND

The ETV Program’s AMS Center conducts third-party performance testing of commercially available technologies that detect or monitor natural species or contaminants in air, water, and soil. The purpose of ETV is to provide objective and quality assured performance data on environmental technologies, so that users, developers, regulators, and consultants can make informed decisions about purchasing and applying these technologies. Stakeholder committees of buyers and users of such technologies recommend technology categories, and technologies within those categories, as priorities for testing.

EDCs are of increasing concern throughout the country. Several EPA Regions (such as Region 3, Region 5, and Region 8) have undertaken activities to monitor for these compounds.
Several states are considering including monitoring for EDCs in their regulatory programs (Pennsylvania Department of Environmental Protection collaborative monitoring program with USGS and State of California National Pollutant Discharge Elimination System program). There is great public concern regarding the potential of EDCs to interfere with both human, domestic animal, and wildlife reproduction, as manifest in the numerous reports in the peer reviewed literature and the public press.

Presently, GC-MS, high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) are the primary methods being used for detecting these compounds. GC-MS was primarily used early on, making it one of the more established methods for detecting these compounds, so GC-MS will serve as the reference method for this test. Immunoassay techniques, particularly enzyme-linked immunosorbent assay (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.

A3 VERIFICATION TEST DESCRIPTION AND SCHEDULE

A3.1 Summary of Technology Category and Verification Test

Technologies to be evaluated in this verification test are ELISA test kits. The basis of immunoassay analytical detection is the capability of antibodies to specifically recognize and form stable complexes with antigens. Immunoassays employ antibodies as analytical reagents. While both chemical and manufacturer specific, ELISA kits operate on the basis of competition between an enzyme conjugate and the chemical in the sample for a limited number of binding sites on the antibody coated plate. 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 chemical in the sample, the less color reaction produced and recorded using a standard microtiter plate reader or tube photometer.

This technology could be a beneficial technique for the EPA regional offices and EPA ORD as well as state and local clients in that the ELISA technique is potentially much faster and more cost effective than traditional GC-MS and LC-MS methods. The cost of analyzing samples for one analyte by ELISA can range from $17 to $25 per sample, depending on the number of replicates run, while GC-MS analysis of samples can cost between $500 and $900 per sample.
Also, the analysis time for ELISA is typically one day, as compared to GC-MS, which is typically 2 to 3 weeks.

This verification test will be an evaluation of commercially available ELISA kits. These ELISA kits will be used to quantitate, per the manufacturer protocols, three EDCs, 17-β-Estradiol (E2), 17-α-Ethynylestradiol (EE2), and Nonylphenol (NP), in four different matrices. EPA and USGS laboratories will use the ELISA kits (according to Table 1) to quantitate triplicate spiked samples for hormones (E2 and EE2) and/or NP, which will be prepared and shipped by EPA NRMRL. Note that NP is the target analyte of interest, but the ELISA test kits are targeted for the general class of AP, which includes NP. The test will be conducted in four phases, each phase being a different aqueous matrix.

### A3.2 Verification Test Schedule

Table 2 shows the planned schedule of testing and data analysis/reporting activities to be conducted in this verification test.

<table>
<thead>
<tr>
<th>Month</th>
<th>Testing Activities</th>
<th>Data Analysis and Reporting</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 2008</td>
<td>Phase I</td>
<td>Review and compile test data records as they become available.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Review and summarize verification staff observations.</td>
</tr>
<tr>
<td>July 2008</td>
<td>Phase II</td>
<td>See Phase I</td>
</tr>
<tr>
<td>August 2008</td>
<td>Phase III</td>
<td>See Phase I</td>
</tr>
<tr>
<td>September 2008</td>
<td>Phase IV</td>
<td>Prepare report templates and complete common sections of reports.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Begin evaluating and analyzing available data generated during testing.</td>
</tr>
<tr>
<td>October 2008</td>
<td>Prepare draft verification reports and statements</td>
<td>Complete draft reports and submit for vendor and peer review</td>
</tr>
<tr>
<td>November 2008</td>
<td>Prepare final verification reports and statements</td>
<td>Revise draft reports and submit final reports for EPA approval</td>
</tr>
</tbody>
</table>
A3.3 Test Site

This project will be a collaborative effort between EPA NRMRL, EPA NERL, EPA Region 3, EPA Region 5, USGS, and the Battelle AMS Center, as outlined in Section A1. As discussed in Section A1, the verification test will be conducted in various EPA and USGS laboratories with different combinations of test kits and samples being evaluated at different laboratories (as outlined in Table 1).

A3.4 Health and Safety

Participating laboratories will conduct all verification testing and reference measurements following the safety and health protocols in place for the laboratory and facilities. This includes maintaining a safe work environment and a current awareness of handling potentially toxic chemicals. Exposure to potentially toxic chemicals will be minimized, appropriate personal protective equipment will be worn, and safe laboratory practices will be followed.

A4 QUALITY OBJECTIVES AND CRITERIA FOR MEASUREMENT DATA

In performing the verification test, Battelle and all participating laboratory staff will follow the technical and QA procedures specified in this test/QA plan and will comply with the data quality requirements in the AMS Center QMP. The primary objective of this verification test is to evaluate whether or not commercially available ELISA kits are able to accurately and reliably measure environmental samples in different matrices for selected EDCs in comparison to GC-MS methods. Additionally, this verification test will document verification testing staff observations to assess other performance characteristics of the ELISA test kits. Below is a discussion of the quality objectives and the criteria for measurement data that have been established to assure that the test objectives are met.
A4.1 Quality Objectives

Data quality objectives indicate the minimum data quality required to meet the ELISA test kit verification objectives. Data quality objectives for this verification test include those related to the reference method performance, those related to the ELISA test kit technology performance, as well as those related to documenting verification testing staff observations. Quantitative and qualitative measurement quality objectives have been established for this test to define required data quality for reference measurement data. Table 3 presents the acceptance criteria for the reference method critical measurements. The reference method measurement quality will be assured by adherence to these criteria. Any data or measurement quality criteria associated with the operation of the ELISA test kit technologies for critical measurements will be provided by the vendor. The Battelle Quality Manager or his designee will perform a technical systems audit (TSA) at least once during this verification test to review these QA/QC requirements. The EPA NRMRL Director of QA or her designee also may conduct an independent TSA, at her discretion.

A5 SPECIAL TRAINING/CERTIFICATION

Documentation of training related to technology testing, data analysis, and reporting is maintained for all Battelle technical staff in training files at their respective Battelle location. Similar documentation of technical staff from laboratories conducting testing during this verification test will reside at their respective laboratory, as appropriate. The Battelle Quality Manager may verify the presence of appropriate training records for all participating technical staff prior to the start of testing. Staff that operate and/or maintain an evaluated technology during the verification test may be trained by the technology vendor prior to the start of testing, as needed. If such training occurs, Battelle or NRMRL will document this training with a consent form, signed by the vendor that states which specific staff have been trained and determined by the vendor to be competent in operation of the vendor’s technology.

A6 DOCUMENTATION AND RECORDS

The records for this verification test will include the test/QA plan, sample chain-of-custody forms, laboratory record books (LRB), data collection forms, electronic files (both raw
data and spreadsheets of sample results or statistical calculations), and the final verification reports and verification statements. Each laboratory will provide analytical results and supporting data in electronic format. The electronic copy data report will contain the final analytical results for each field sample plus the quality control sample data required in Table 4, where applicable. The electronic copy deliverables will also consist of final report tables and a complete data package that enables traceability of final reported data to the raw data.

Table 3. Critical Measurements for Reference Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Minimum Acceptance Criteria</th>
<th>Corrective Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method Procedural Blank (MB)</td>
<td>Target analytes &lt; 5 x method detection limit (MDL)</td>
<td>Reprocess (extract) sample batch. If batch cannot be re-processed; flag all field sample data that are &gt;RL, unless the concentrations in the related samples are &gt;5X the concentrations detected in the MB. In this case no flag is required.</td>
</tr>
<tr>
<td>Instrument Solvent Blank</td>
<td>Target analytes &lt; lowest calibration standard</td>
<td>Review data and analysis for possible sources of contamination. Reanalyze and/or document corrective action. Data must be flagged.</td>
</tr>
<tr>
<td>Surrogate Spike (SIS)</td>
<td>60-120% recovery or as specified in method standard operating procedures (SOPs)</td>
<td>Review data. Discuss with EPA NRMRL Coordinator. Reanalyze, re-extract, and/or document corrective action and deviations.</td>
</tr>
<tr>
<td>Laboratory Duplicate (Relative Percent Difference – RPD)</td>
<td>Target compounds ≤ 30% RPD (Concentration must be &gt; 2x MDL)</td>
<td>Review data to assess impact of matrix. If other QC data are acceptable, then flag associated QC data. If QC data are not affected by matrix failure, then re-process duplicate. If not possible, then notify client and flag associated QC data.</td>
</tr>
</tbody>
</table>
Data packages will include any of the following elements that are applicable to the analysis:

- Data package narrative
- Final data report tables
- List of current RLs and MDLs for the preparation and analysis methods used for sample processing
- Entire package of sample custody documentation, including sample receipt form
- Sample processing records
- ELISA test kits results
- GC-MS results and records, including:
  - Instrument tuning
  - Calibration data
  - Calibration verifications
  - Surrogate recoveries
  - Internal standard response and retention times
  - All QC data required by the analytical method
  - Run logs
  - Raw data

The lead laboratory staff will receive and review the data packages submitted by their respective laboratories for completeness, acceptability, and compliance with the requirements of this TQAP before submitting them to the EPA NRMRL Coordinator. EPA NRMRL in collaboration with EPA Region 3 will prepare a draft data summary. This summary will include:

- Calibration data for all ELISA and GC-MS analysis.
- Analytical results and statistical evaluation of analytical results.
- QA results to validate the quality and usability of the data generated.

The data summary and all of the test records will be submitted to the Battelle Verification Test Coordinator for review. All records received by the Verification Test Coordinator will be maintained in the Verification Test Coordinator’s office until the completion of the verification report at which time the records will be transferred to permanent storage at Battelle’s Records Management Office at the conclusion of the verification test. All Battelle LRBs are stored indefinitely, either by the Verification Test Coordinator or Battelle’s Records Management
Office. EPA will be notified before disposal of any files. Table 4 has further details regarding the data recording practices and responsibilities.

EPA NRMRL will also keep a copy of all final verification test files, although the official data records for the verification test will be maintained by Battelle (as described above). Once the study is complete, all files associated with the test including project management files and the draft data summary, will be archived by EPA NRMRL for at least 3 years. Laboratory raw data and supporting equipment records will be archived by each participating organization for at least three years. The location (e.g., specific personal computer, server, or media type and storage location) of final versions of the electronic files will be noted in the test records.

All written records must be in ink. Any corrections to notebook entries, or changes in recorded data, must be made with a single line through the original entry. The correction is then to be entered, initialed, and dated by the person making the correction. In all cases, strict confidentiality of data from each vendor’s technology, and strict separation of data from different vendors’ technology, will be maintained. Separate files (including manual records, printouts, and/or electronic data files) will be kept for each technology.
## Table 4. Summary of Data Recording Process

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

B1  EXPERIMENTAL DESIGN

This verification test will be an evaluation of four commercially available ELISA kits to quantitate, per the manufacturer protocols, three EDCs, including 17-β-Estradiol (E2), 17-α-Ethynylestradiol (EE2), or Nonylphenol (NP). More detailed information on the uses of the EDCs to be tested is provided in Table 5. Note that NP is the target analyte of interest, but the ELISA test kits are targeted for the general class of AP, which includes NP. Kits to be evaluated may consist of varying methods of operation, such as 96-well microtiter plate kits and magnetic particle kits. The participating laboratories will use the ELISA kits to quantitate triplicate spiked samples for hormones (E2 and EE2) and/or NP, which will be prepared and shipped by EPA NRMRL. The test will be conducted in four phases, each phase evaluating a different aqueous matrix.

Table 5. Target Analytes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Synonyms</th>
<th>CAS#</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>(17β)-Estra-1,3,5(10)-triene-3,17-diol</td>
<td>17-β-Estradiol (E2)</td>
<td>50-28-2</td>
<td>Naturally occurring hormone</td>
</tr>
<tr>
<td>17α-Ethynyl-1,3,5(10)-estratriene-3,17b-diol</td>
<td>17-α-Ethynylestradiol (EE2)</td>
<td>57-63-6</td>
<td>Synthetic estrogen found in birth control</td>
</tr>
<tr>
<td>Nonylphenol</td>
<td>NP</td>
<td>84852-15-3</td>
<td>Detergents, surfactants, emulsifiers, wetting agents, cosmetics, cleaners, pesticides, paints</td>
</tr>
</tbody>
</table>

ELISA kits are meant to be user friendly and easy to use following step-by-step instructions. They are designed to be compound specific, only measuring the compound that it was made to detect. Each kit will be evaluated on its ability to quantitate the compound that it is designed to detect and how well the kit results compare to GC-MS results of the same samples. Some ELISA kits may react with compounds similar to the target compound, known as cross-
reactivity. During each phase of the study, background levels of the kits target compounds will be measured by GC-MS in that particular matrix as well as some of the compounds with which the kits have cross-reactivity, as stated by the vendor. For some of the compounds for which there is cross-reactivity, there may be no established analytical methods available by GC-MS to detect these compounds at the concentrations and in the matrices evaluated per this test/QA plan. Blank samples of each matrix (i.e., not spiked with E2, EE2, or NP) will also be evaluated on the ELISA test kits to determine if any of the cross-reactive compounds present in the matrix (as determined through GC-MS analysis) might be interfering with the test kit results.

Each kit will be operated following the manufacturer’s instructions, which may include the use of a glass fiber filter (GFF) and solid-phase extraction (SPE). The purpose of SPE is to clean as well as concentrate the sample in order to get the sample concentration into the kit’s analytical range. The ELISA test kits often do not come with GFFs and SPEs. Thus, this equipment will be purchased by NRMRL and used with the test kits, as appropriate. The same GFF and SPE will be used across laboratories and throughout testing.

This verification test will be done in four phases. Phase I will consist of a clean water sample (de-ionized (DI) super-Q water) being spiked with a single concentration of E2, EE2, and NP, split into single samples, and submitted to the ELISA kit users to measure the concentration, in triplicate. The split sample, as well as the un-spiked, matrix background sample, will also be simultaneously sent for reference GC-MS analysis of E2, EE2, NP, and alkylphenol ethoxylates (APEs), which are known to cause cross-reactivity with some of the ELISA kits. Phase II will consist of environmental surface water samples going through the same process as Phase I. Phase III will consist of a more complex matrix of wastewater treatment plant (WWTP) effluent samples going through the same process. Phase IV will consist of even a more complex matrix of WWTP influent samples going through the same process. Details on the sample matrices, spiking levels, and spiking procedures for each Phase are provided in Section B1.1. All spike concentrations used in each phase of this verification test are based on real-world concentrations found in environmental samples. Specific concentrations of E2, EE2, and NP, as presented in Section B1.1, will be spiked into the sample matrix for each phase, regardless of any background concentrations of these compounds that may be present in the collected water. The spiked concentrations will be added to any background hormone concentrations that are found via GC-MS analysis of matrix blanks to get the total expected hormone concentration for a given sample.
The ELISA kit results from the various laboratories for a given kit will be compared to each other and compared to GC-MS results for repeatability, reproducibility, and percent bias.

ELISA test kit technologies will be tested only in a laboratory under controlled conditions; no field testing will take place during this verification test. This will allow comparison of the technology results to a reference method under a specified set of conditions. The analyses will be performed according to the vendor’s recommended procedures as described in the user’s instructions or manual, or during training provided to the operators. Similarly, calibration and maintenance of the technology readers (i.e., microplate readers and photometers) will be performed as specified by the vendor. Results from the technologies being verified will be recorded manually by the operator on appropriate data sheets or captured in an electronic data system and then transferred manually or electronically for further data workup. Qualitative characteristics of each technology such as ease of use will be assessed through observations made by the operators throughout the verification test. The results from each technology will be reported individually. No direct comparison will be made between technologies from different vendors, but each vendor’s technology will undergo similar testing so it is convenient for end users to evaluate the ETV testing results.

B1.1 Test Procedures

This section describes specific test procedures for this verification test of ELISA test kits. Specific step-by-step analysis procedures will be provided in each kit’s instructions for the ELISA test kits and in standard operating procedures (SOPs) for the GC-MS methods.

The GC-MS methods for hormones will measure for E1, E2, E3, EE2, testosterone, dihydrotestosterone, androstenedione, and progesterone with a concentration range of 2-50ng/L. The GC-MS methods for APs will include NP (conc. range 250-4000ug/L), nonylphenol monoethoxylate (conc. range 500-8000ug/L), nonylphenol diethoxylate (1000-16000ug/L), octylphenol (conc. range 50-800ug/L), and bisphenol A (50-800ug/L). Samples for the hormone and AP GC-MS methods will go through an extraction step to concentrate (or dilute) the sample into the method analytical range.

Extensive cleanup procedures of complex matrix samples can be very time consuming and therefore it would be beneficial to simplify the process. This test will evaluate ELISA kit
performance utilizing only simple cleanup procedures, as directed by the manufacturer of the kit, for the four different matrices for each EDC of concern. Each sample will be analyzed both directly (prior to cleanup) and after SPE cleanup using the procedure detailed in the kit instructions and provided below. DIR (for direct) or SPE (for solid-phase extraction) will be added to the beginning of each sample name to distinguish them from each other. Each sample for ELISA analysis will be filtered through a 1μm glass fiber filter prior to direct analysis and SPE.

All sample bottles and glassware associated with hormone samples, including the glass carboy, will be cleaned and silanized using a standard procedure (see Appendix A). All sample bottles associated with NP samples will be new and will not need additional silanization. The glass carboy for NP sample preparation will be cleaned for reuse using a standard procedure (see Appendix B) and will not require additional silanization.

**Phase I:** DI super-Q water will be spiked by EPA NRMRL with a mixture of E2 and EE2 at a known concentration, while separate DI super-Q water will be spiked with nonylphenol (NP) at a known concentration. The estrogens and NP will be spiked in separate water samples due to differing cleanup/pre-concentration methods needed for these compounds. In one cleaned 20L, glass carboy, 20L of DI water will be prepared containing 10ng/L of E2 and 10ng/L of EE2. In a second, cleaned, 20L, glass carboy, 20L of de-ionized water will be prepared containing 30μg/L of NP. These concentrations were selected because they are on the higher end of the range of concentrations expected to be encountered in a real-world situation and are representative of the anticipated mid-range of the test kits. Both carboys will be 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 each analyte throughout the carboy. These sample names will be DI-ES-LAB for hormones and DI-NP-LAB for APs. “LAB” will be replaced with the unique lab name. Laboratory names for labeling will be as follows: EPA NRMRL will be NR, EPA NERL Cincinnati will be NC, EPA NERL Las Vegas will be NV, USGS will be GS, EPA Region 3 will be R3, and EPA Region 5 will be R5. DI super-Q water blanks will also be prepared and shipped in separate 500mL bottles. The blank sample will be analyzed both directly (DIR) and after SPE (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 will
be sampled and analyzed by GC-MS to confirm the background levels of E2, EE2, and NP. These sample names will be DI-ES-BKG for hormones and DI-NP-BKG for APs. Samples of the spiked mixtures will be taken and the concentrations of these samples and blank will be determined using immunoassay kits and the GC-MS methods.

There will be multiple users to analyze the spiked samples using the appropriate kits for E2, EE2 and AP, although only three of the six participating laboratories will use a particular kit, depending on the kit (see Table 1 for laboratory responsibilities). Two of the three laboratories will run duplicate kits while the third lab, EPA NRMRL, will run only one kit. Once samples are prepared and spiked by EPA NRMRL, the samples will be preserved on ice and shipped immediately to the appropriate users for analysis within 24 hours of receiving the sample.

The various “clean matrix” DI super-Q water samples will be spiked by personnel at EPA NRMRL as one large stock solution, split into smaller sub-samples, and then shipped on ice or freezer packs at 4°C to the appropriate laboratories for ELISA and GC-MS analysis. All sample bottles will be amber glass to prevent photodegradation of the analytes. Each laboratory that is participating in the ELISA analysis for a particular analyte will receive one 2.5L sample plus one 500mL DI water method blank. Each laboratory that is participating in the GC-MS analysis for a particular analyte will receive one 4L sample and one 1L DI water method blank to be processed by the GC-MS method. Note that the sample volumes required for the ELISA and GC-MS analyses are 500 mL and 1L, respectively. Since samples will be analyzed in triplicate, the total volume of sample provided (2.5L and 4L) will be larger than required, but this will be done to utilize convenient bottle sizes and to provide additional sample for re-analysis should that be necessary. The sample names for blanks will be DI-ES-BLK-1 for hormones and DI-NP-BLK-1 for APs. The DI water method blanks will be filled with DI super-Q water from the same source and at the same time as the DI water for the samples. The users will follow simple cleanup procedures as directed in the vendor’s instructions. Kit operators will receive one 2.5L sample to be split into three 500mL aliquots. Each of those three aliquots will be analyzed by direct analysis utilizing only GFF cleanup, and by utilizing GFF cleanup and SPE. Each aliquot sample will be applied in triplicate to the ELISA kits for quantification, per the test kit protocols. Tests will be run within the appropriate concentration range of the specific ELISA kit and GC-MS methods.
Each sample for ELISA analysis will be filtered through a 1μm GFF prior to direct analysis and SPE. After filtering the estrogen (ES) sample, remove one 1260μL aliquot and add 140μL of methanol. This aliquot mixture will be used for direct analysis on the 96 well microtiter style E2 and EE2 kits and applied directly to three wells (100μL for each well) on each kit. There will be some leftover aliquot volume. Also, one 1600μL aliquot will be removed from the estrogen (ES) sample to be applied directly to three tubes (250μL for each well) in each magnetic particle style kit. There will be some leftover aliquot volume. These sample names will be DIR-DI-ES. Also filter the NP sample through a 1μm glass fiber filter. After filtering, remove one 712μL aliquot and add 8μL DMSO and 80μL methanol. This aliquot mixture will be used on the 96 well microtiter AP kit and applied directly to three wells (100μL for each well) in the 96 well microtiter style AP kit. There will be some leftover aliquot volume. Once all aliquots are removed for direct analysis, remove three 500mL aliquots for solid phase extraction (SPE). These three aliquots will be treated as three independent samples. Follow SPE directions entitled “Extractions for EE2 from Water Sample for ELISA” (see Appendix C) for hormone samples and “Flowchart: AP ELISA” (see Appendix D) for NP samples. Estrogen samples for use on 96 well microtiter estrogen kits will be reconstituted with 10mL of a 10% methanol solution (first 1mL methanol, vortex, then 9mL DI water). Estrogen samples for use on the E2 magnetic particle kit, remove 20uL from the aforementioned reconstitution and add this 20uL to 1980uL of DI water (total volume 2mL) for the E2 magnetic particle kits. There will be leftover aliquot volume. NP samples will be reconstituted with 40mL of 1% DMSO 10% methanol solution. All reconstituted samples will be applied to three wells (100μL for each well) on each 96 well microtiter style kit or three tubes (250μL for each tube) on each magnetic particle style kit according to the manufacturer’s instructions. These sample names will be SPE-DI-1, 2, and 3. Samples on ELISA kits will be quantified by reading kit at 450 nm using a plate reader for the 96 well kits and tube style spectrophotometer for the magnetic particle kits following the manufacturer’s instructions.

Phase II: Surface water will be collected from a stream in Ohio that is known to be free of E2, EE2, and APs. Before the surface water is spiked, a single sample of the collected surface water will be taken, split into triplicate aliquots, and analyzed by GC-MS to confirm the background levels of E2, EE2, and NP. (Background levels of analytes measured will later be added to the spiked concentration of analytes, if necessary, once results are obtained.) After the
background sample is taken, in a cleaned, 20L, glass carboy, 20L of the stream water will be prepared containing 10ng/L concentration of E2 and 10ng/L concentration of EE2. In a second, cleaned, 20L, glass carboy, 20L of the clean stream water will be prepared containing 30μg/L concentration of NP. Both carboys will be 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 will be taken. The DI water method blanks will be filled with DI water at the same time as the stream water in the 20L carboys. The same testing will be performed as in Phase I.

**Phase III:** Effluent discharges will be collected from a WWTP in Cincinnati, Ohio. Before spiking, a single sample of the effluent will be taken, split into triplicate aliquots, and analyzed by GC-MS to confirm the background levels of E2, EE2, and NP. In a cleaned, 20L, glass carboy, 20L of WWTP effluent will be prepared containing 10ng/L of E2 and 10ng/L of EE2. In a second, cleaned, 20L, glass carboy, 20L of the effluent water will be prepared containing 30μg/L concentration of NP. The carboys will be 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 will be collected. The same testing will be performed as in Phases I and II.

**Phase IV:** Influent samples will be collected from a WWTP in Cincinnati, Ohio. Before spiking, a single sample of the influent will be taken, split into triplicate aliquots, and analyzed by GC-MS to confirm the background levels of E2, EE2, and NP. In a cleaned, 20L, glass carboy, 20L of WWTP influent will be prepared containing 10ng/L concentration of E2 and 10ng/L concentration of EE2. In a second, cleaned, 20L, glass carboy, 20L of the influent water will be prepared containing 30μg/L concentration of NP. The carboys will be 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 will be collected. The same testing will be performed as in Phases I, II, and III.
B1.2 Statistical Analysis

The statistical methods and calculations used for evaluating quantitative performance parameters are described in the following sections. The performance of the ELISA test kit will be quantified by calculating measures of repeatability, reproducibility, and bias.

B.1.2.1 Repeatability

The performance of the ELISA test kit will be quantified by calculating measures of repeatability, reproducibility, and bias. Repeatability is a measure of the random variation that affects the precision of a method. As defined in this verification, it is a measure of the random variation between ELISA test kits measuring the same spiked sample within the same laboratory. Repeatability will be calculated in each phase using the following equation,

\[
\text{Repeatability} = \sum_{j=1}^{2} \sum_{i=1}^{2} (x_{ji} - \bar{x}_{j,.})^2
\]

(1)

where \( j = 1, 2 \) denotes the laboratory and \( i = 1, 2 \) denotes the ELISA test kit within laboratory, \( x_{ij} \) is the ELISA concentration for the \( j^{th} \) laboratory and the \( i^{th} \) test kit, and \( \bar{x}_{j,.} \) is the ELISA mean concentration of the \( j^{th} \) laboratory.

B1.2.2 Reproducibility

Reproducibility is another measure of the random variation that affects the precision of a method. As defined in this investigation, reproducibility is a measure of the random variation between laboratories measuring the same spiked sample. Reproducibility will be calculated by the following equation,

\[
\text{Reproducibility} = \sum_{j=1}^{3} n_j (\bar{x}_{j,.} - \bar{x}_{,.})^2
\]

(2)

where \( j = 1, 2, 3 \) denotes the laboratory, \( \bar{x}_{j,.} \) is the ELISA mean concentration for the \( j^{th} \) laboratory, \( \bar{x}_{,.} \) is the overall ELISA mean concentration of the three laboratories, and \( n_j \) is the number of measurements for the \( j^{th} \) laboratory.
The technology precision for each sample will be reported in terms of the relative standard deviation (RSD), which will be calculated by the standard deviation of the measurements over the average.

**B1.2.3 Percent Bias**

Bias is a systematic error that will cause measurements to err in one direction, either to high or low. A positive % Bias will indicate the ELISA concentration is higher than the reference method and a negative % Bias will indicate the ELISA concentration is lower than the reference method. Bias will be calculated for each measurement in each phase for each kit using the following equation,

\[
\text{% Bias} = \left[ \frac{x_{ij}}{y_n} - 1 \right] \times 100
\]  

(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, \( x_{ij} \) 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.

**B.1.2.4 Matrix Effects**

Matrix effects will be determined 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 will be determined as described in B.1.2.3. T-tests may be applied to the data to help determine whether the differences in percent bias measurements between the PT and environmental samples are significant. Matrix effects will also be considered using the percent bias results.

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

General observations on potential cross-reactivity will be documented. Blank samples of each matrix will be evaluated by GC-MS to determine background levels of the compounds with which the kits have cross-reactivity, as stated by the vendor. For some of compounds for which there is cross-reactivity, there may be no established analytical methods available by GC-MS to
detect these compounds at the concentrations and in the matrices evaluated per this test/QA plan. Blank samples of each matrix (i.e., not spiked with E2, EE2, or NP) will also be evaluated on the ELISA test kits to determine if any of the cross-reactive compounds present in the matrix (as determined through GC-MS analysis) might be interfering with the test kit results.

Percent recovery results will be presented on a per sample or per phase basis. Percent recovery results may be analyzed to provide another measure of accuracy to the end user.

A comparison of the ELISA results generated with and without the use of SPE will also be performed. This will evaluate whether the use of the more involved cleanup procedure was necessary/warranted with the ELISA test kits. Percent bias and/or a t-test will be used to evaluate these results.

**B1.2.5 Operational Factors**

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

**B1.3 Reporting**

The data obtained in the verification test will be compiled separately for each technology, and the data evaluations will be applied to each technology’s data set without reference to any other technology. At no time will data from different technologies be intercompared or ranked. Following completion of the data evaluations, a draft verification report and verification statement will be prepared for each test kit stating the verification test procedures and documenting the performance observed. For example, descriptions of the data acquisition procedures, consumables used, repairs and maintenance needed, and the nature of any problems will be presented in the draft report. Each report will briefly describe the ETV Program, the AMS Center, and the procedures used in verification testing. The results of the verification test will be stated quantitatively, without comparison to any other technology tested, or comment on the acceptability of the technology’s performance. Each draft verification report will be submitted for review by the technology vendor and by EPA and other peer reviewers. Comments on the draft report will be addressed in revisions of the report. The peer review comments and
responses will be tabulated to document the peer review process. The reporting and review process will be conducted according to the requirements of the AMS Center QMP.¹

B2 SAMPLING REQUIREMENTS

B2.1 Sample Collection, Storage and Shipment

All samples are assumed to contain the same concentration due to thorough mixing of each carboy. All samples will be shipped by NRMRL, immediately after being made, in coolers on ice or freezer packs to maintain a 4°C temperature. When samples are received by each laboratory, the condition of the samples, i.e., temperature, broken bottles etc., will be noted by the receiving laboratory operator and then immediately placed in a refrigerator at 4°C until analyzed. Holding times of hormones and AP samples are currently unknown, therefore, all samples will be analyzed, or at a minimum solvent exchanged, within 24 hours of receipt. All laboratories performing quantitative analysis, ELISA or GC-MS, will receive split samples from the same bulk sample and will analyze them within 24 hours to reduce error associated with analyte degradation during sample holding. This shipping, receiving, and holding time procedure described in this section applies to all phases of this verification test.

Phase I: During Phase I, a 40L grab sample of Super Q water will be collected in two cleaned, 20L, glass carboys from the USEPA laboratory in Cincinnati, Ohio. One carboy will be used for spiking with hormones and the other will be used for spiking with NP.

Phase II: During Phase II, a grab sample of stream water will be collected in three, clean, five gallon buckets from the South Hasha Tributary to Eastfork Lake in Clermont County, Ohio. The tributary will be accessed from where it crosses Williamsburg-Bantam Road. Once the sample is transported back to the laboratory, 40L of the stream water will be measured out and transferred into the two, clean, 20L carboys.

Phase III: During Phase III, a grab sample of final effluent wastewater will be collected in three, clean, five gallon buckets from the Metropolitan Sewer District of Greater Cincinnati in Hamilton County, Ohio. Once the sample is transported back to the NRMRL laboratory, 40L of the effluent will be measured out and then transferred into the two, clean, 20L carboys. Amber glass will be used to ensure that the WWT samples do not degrade due to light exposure.
Phase IV: During Phase IV, a grab sample of influent wastewater will be collected in three, clean, five gallon bucket from the Metropolitan Sewer District of Greater Cincinnati in Hamilton County, Ohio. Once the sample is transported back to the NRMRL laboratory, 40L of the influent will be measured out and transferred into the two 20L carboys. Effluent water samples will be collected in 2.5L amber glass containers from the carboys to be shipped by NRMRL to all participating laboratories. Amber glass will be used to ensure that the WWT samples do not degrade due to light exposure.

B3 SAMPLE HANDLING AND CUSTODY REQUIREMENTS

All samples produced by EPA NRMRL will be packaged and shipped by NRMRL to the respective laboratories for analysis, one shipment for each phase. All samples will be shipped in coolers on ice or freezer packs to maintain a 4 ± 2°C temperature. When samples are received by each laboratory, the condition of the samples, i.e., temperature, broken bottles etc. will be noted and then immediately placed in a refrigerator at 4 ± 2°C until analyzed. Chain-of-custody (COC) records will be initiated at EPA NRMRL and transferred with the samples to analysts. The grab sample collected will be placed in shipping containers with adequate amounts of ice or freezer packs to maintain samples at 4 ± 2°C. All samples received at each analyst laboratory from NRMRL will be inspected, logged, and immediately placed in cold storage (4°C refrigeration) upon receipt. Any problems observed with the samples upon receipt (e.g. leaking, broken, outside of temperature, missing chain-of-custody, missing sample labels, etc.) will be noted in the sample log book and those notes transferred to NRMRL.

B4 QUALITY CONTROL

Steps will be taken to maintain the quality of data collected during this verification test. This will include analyzing specific quality control samples (QCS) at a regular frequency. QCSs will include standard solutions, prepared as described in the ELISA test kit instructions. Standard solutions help to assess the proper performance of the test kit and also are essential in estimating the concentration of a given test sample. Method blank samples (DI water) will also be evaluated with each batch of hormone and AP samples for each phase of this test. MB samples will be used to help ensure that no sources of contamination are introduced in the
sample handling and analysis procedures. MB samples will be analyzed directly on the ELISA test kits as well as after they have gone through any preparation procedures (filter and SPE clean-up) to verify that any clean-up procedures used are not introducing any potential contamination into the sample. QCSs producing results that do not meet the anticipated results specified by the vendor will be reanalyzed and corrective action taken if needed to ensure that test sample results are not affected. Corrective actions may include reanalyzing samples to verify that the technology has been operated properly.

Quality controls and corrective actions for any reference analyses conducted are provided in Table 3. MB samples will also be included in the GC-MS analysis to ensure that no sources of contamination have been introduced. Blanks for GC-MS analysis will include instrument blanks, laboratory blanks, method (procedural) blanks, and DI water blanks.

B5 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The equipment used by the participating laboratories will be tested, inspected, and maintained as per the standard operating procedures of the respective laboratory and/or the manufacturer’s recommendations, or those stated in this TQAP, so as to meet the performance requirements established in this document. When technical staff operate and maintain the microplate readers and spectrophotometers used to read the technology results, those activities will follow directions provided by the technology vendor.

B6 CALIBRATION/VERIFICATION OF TEST PROCEDURES

The GC-MS used for reference analyses will be calibrated as appropriate and as stated in the SOP for each participating laboratory responsible for reference analysis. All plate readers and spectrophotometers used to read the microplates and tubes from the different ELISA test kits will be calibrated as appropriate and as specified in the vendor’s instructions. Plate readers and spectrophotometers will be recalibrated as needed and as appropriate according to the manufacturer’s instructions. All calibrations will be documented as appropriate by the technical staff.
B7 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

In general, this verification test relies on the materials and equipment provided by the vendors. Battelle, EPA NRMRL, and the participating laboratories will provide the following equipment and materials needed for the preparation of the performance test, environmental and QC samples:

- DI water
- various laboratory supplies necessary for accurate preparation of the test samples and subsequent dilutions (i.e., volumetric pipets; pipet bulbs; Eppendorf micro pipette tips, or equivalent; volumetric flasks; disposable pipets; balances; etc.)
- reference standards with a known level of purity for target analytes - NIST traceable, or equivalent if available
- carboy and glass sample containers
- equipment and consumables for the GC-MS
- personal protective equipment
- equipment and supplies necessary for shipping samples
- SPE columns and glass fiber filters

All materials, supplies, and consumables will be ordered by EPA NRMRL with the exception of performance evaluation materials which will be ordered by Battelle or NRMRL. Where possible, EPA NRMRL will rely on material and consumable sources that have been used previously without problems. Battelle will also rely on previous experience or recommendations from EPA advisors, stakeholders, test collaborators, subcontractors, or technology vendors.

Upon receipt of any supplies or consumables, EPA NRMRL will visually inspect and ensure that the materials received are those that were ordered and that there are no visual signs of damage that could compromise the suitability of the materials. If damaged or inappropriate goods are received, they will be returned or disposed of and arrangements will be made to receive replacement materials. Certificates of analysis (COA) or other documentation of analytical purity will be checked for all reagents and standards to ensure suitability for the verification test and a copy will be stored with the test files. Where possible, reagents (i.e., HPLC grade water, sulfuric acid, etc.) used to prepare standards will be checked for background contamination prior to their
use. Unsuitable materials will be returned or disposed of and arrangements for the receipt of replacement materials will be made.

B8 NON-DIRECT MEASUREMENTS

Non-direct measurements will not be used during this verification test.

B9 DATA MANAGEMENT

Various types of data will be acquired and recorded electronically or manually during the verification test. Table 4 summarizes the types of data to be recorded. All maintenance activities, repairs, calibrations, and operator observations relevant to the technology operation will be documented by technical staff in LRBs or on data sheets. Results from the reference methods, including raw data, analyses, and final results, will be compiled by EPA NRMRL and forwarded to Battelle.

Each data package received from individual participating laboratories will be validated by the designated project principal investigators (PIs). The PIs for this project include Eric Kleiner (PI) and Marc Mills (co-PI) from U.S. EPA NRMRL in Cincinnati, OH, and Jennifer Gunderson (co-PI) and Dave Russell (co-PI) from U.S. EPA Region 3 in Fort Meade, MD. Data are assessed for completeness and compliance to determine the adequacy of the data to meet the project DQOs. During data validation, the laboratory performance is assessed against prescriptive requirements and subjective requirements. Evaluation of laboratory performance against prescriptive requirements is assessed through the acceptability of QC sample results that are independent of sample matrix (method blanks, laboratory control samples, and reference tests). An assessment of the subjective requirements involves identification of potential matrix effects, and consists of an evaluation of the analytical results and the results of analytical triplicates and matrix spike samples. The QA review report will include a narrative that details QC exceedances and discusses potential data quality impacts. Data qualification “flags” will be applied by the laboratory for data that do not meet quality criteria. Data validation results that meet subjective requirements will be presented in the verification report and statement.

In addition, any calculations performed by technical staff will be spot-checked by the Battelle Verification Test Coordinator to ensure that calculations are performed correctly.
Calculations to be checked include any statistical calculations described in this test/QA plan. The data obtained from this verification test will be compiled and reported independently for each technology. Results for technologies from different vendors will not be compared with each other, per ETV program guidelines.

Among the QA activities conducted by Battelle Quality Manager will be a technical systems audit (TSA) and an audit of data quality (ADQ). The TSA will involve Battelle QA staff observing the testing and compliance to the TQAP. The ADQ will consist of a review by the Battelle Quality Manager (or his designee) of at least 10% of the test data. During the course of any such audit, the Battelle Quality Manager will inform the Battelle Verification Test Coordinator of any findings and any need for immediate corrective action. Once the assessment reports have been prepared for each audit, the Verification Test Coordinator will ensure that a response is provided for each adverse finding or potential problem, and will implement any necessary follow-up corrective action. The Battelle Quality Manager will ensure that follow-up corrective action has been taken.
SECTION C
ASSESSMENT AND OVERSIGHT

C1 ASSESSMENTS AND RESPONSE ACTIONS

Every effort will be made in this verification test to anticipate and resolve potential problems before the quality of performance is compromised. One of the major objectives of this test/QA plan is to establish mechanisms necessary to ensure this. Internal quality control measures described in this test/QA plan, which is peer reviewed by a panel of outside experts, implemented by the technical staff and monitored by the Verification Test Coordinator and EPA NRMRL coordinator, will give information on data quality on a day-to-day basis. The responsibility for interpreting the results of these checks and resolving any potential problems resides with the EPA NRMRL Coordinator, Eric Kleiner. Technical staff have the responsibility to identify problems that could affect data quality or the ability to use the data. Any problems that are identified will be reported to the EPA NRMRL Coordinator, who will work with the Battelle Verification Test Coordinator and/or the Battelle Quality Manager to resolve any issues. Action will be taken to control the problem, identify a solution to the problem, and minimize losses and correct data, where possible. Independent of any EPA QA activities, Battelle will be responsible for ensuring that the following audits are conducted as part of this verification test.

C1.1 Performance Evaluation Audits

A Performance Evaluation (PE) audit will be conducted to assess the quality of the reference method measurements (GC-MS analyses) made in this verification test. The reference method PE audit will be performed by supplying an independent, NIST-traceable solid standard, if available, or at a minimum a second standard solution of E2, EE2, and NP prepared from a different source other than that used in verification testing. If a different source is unavailable for these compounds, then standards of E2, EE2, and NP may be prepared from the same source as that used during testing. In this case, standards will be prepared either at Battelle Columbus laboratories and shipped to NRMRL for distribution or by NRMRL using different concentrations than those used in the regular reference samples. The PE audit samples will be
analyzed in the same manner as all other samples and the analytical results for the PE audit samples will be compared to the nominal concentration. The target criterion for this PE audit is agreement of the analytical result within 30% of the expected concentration. If the PE audit result does not meet the target criterion, the PE audit will be repeated. If the outlying results persist, the source of error will be investigated and corrective action taken as necessary until successful PE audit results are obtained. This audit will be performed once during testing, and will be the responsibility of the Verification Test Coordinator or designee.

C1.2 Technical Systems Audits

The Battelle Quality Manager or designee will perform a TSA at least twice during this verification test. TSAs will be conducted at random points in the study. Because the round robin study is taking place in multiple laboratories across the country, Battelle’s Quality Manager will only visit two laboratories for in-person TSAs. Based on the EDCs being analyzed at each site, Battelle will conduct TSAs at the Cincinnati, OH and Fort Meade, MD facilities. Both laboratories participating in the round robin study in Cincinnati, OH will be audited. Other TSAs that may be performed by the Battelle Quality Manager designee (such as a quality manager from other participating laboratories) will be coordinated and monitored by the Battelle Quality Manager, with all TSA findings being reported to the Battelle Quality Manager.

The purpose of this audit is to ensure that the verification test is being performed in accordance with the AMS Center QMP, this test/QA plan, any published reference methods, testing protocol, and any Standard Operating Procedures (SOPs) used. In the TSA, the Battelle Quality Manager, or a designee, may review the reference methods used, compare actual test procedures to those specified or referenced in this plan, and review data acquisition and handling procedures. In the TSA, the Battelle Quality Manager or designee will tour the laboratory where verification and reference testing are taking place, inspect sample COC documentation, and review technology-specific record books. He or she will also check standard certifications and technology data acquisition procedures, and may confer with the technology vendors and technical staff. A TSA report will be prepared, including a statement of findings and the actions taken to address any adverse findings. The EPA NRMRL Director of QA will receive a copy of Battelle’s TSA report. At EPA’s discretion, EPA QA staff may also conduct an independent on-
site TSA during the verification test. The TSA findings will be communicated to technical staff at the time of the audit and documented in a TSA report.

C1.3 Data Quality Audits

The Battelle Quality Manager or his designee will audit at least 10% of the verification data acquired in the verification test. The Battelle Quality Manager will trace the data from initial acquisition, through reduction and statistical comparisons, to final reporting. All calculations performed on the data undergoing the audit will be checked.

Each data package received from the laboratory will be validated by the Project PIs to assess compliance with the reference method data quality criteria defined in Table 3. Data are assessed for completeness and compliance to determine the adequacy of the data to meet the project objectives. During data validation, the laboratory performance is assessed against prescriptive requirements and subjective requirements. Evaluation of laboratory performance against prescriptive requirements is assessed through the acceptability of QC sample results that are independent of sample matrix (method blanks, laboratory control samples, and reference tests). An assessment of the subjective requirements involves identification of potential matrix effects, and consists of an evaluation of the analytical results and the results of analytical triplicates and matrix spike samples. The QA review report will include a narrative that details QC exceedances and discusses potential data quality impacts. Data qualification “flags” will be applied by the laboratory for data that do not meet quality criteria.

C1.4 QA/QC Reporting

Each assessment and audit will be documented in accordance with Section 3.3.4 of the AMS Center QMP. The results of the technical systems audit will be submitted to EPA. Assessment reports will include the following:

• Identification of any adverse findings or potential problems
• Response to adverse findings or potential problems
• Recommendations for resolving problems
• Confirmation that solutions have been implemented and are effective
• Citation of any noteworthy practices that may be of use to others.
C2 REPORTS TO MANAGEMENT

The Battelle Quality Manager, during the course of any assessment or audit, will identify to the technical staff performing experimental activities any immediate corrective action that should be taken. If serious quality problems exist, the Battelle Quality Manager is authorized to request that Battelle’s AMS Center Manager issue a stop work order. Once the assessment report has been prepared, the Verification Test Coordinator will ensure that a response is provided for each adverse finding or potential problem and will arrange for implementation of any necessary follow-up corrective action. The Battelle Quality Manager will ensure that follow-up corrective action has been taken. The test/QA plan and final report will be reviewed by the EPA NRMRL Director of QA and EPA AMS Center Project Officer. Upon final review and approval, both documents will then be posted on the ETV website (www.epa.gov/etv).

An effective Quality System requires prompt and thorough correction of non-conformance conditions that can affect quality. Rapid and effective corrective action minimizes the possibility of questionable data or documentation. Corrective action procedures for this project depend on the severity of the nonconformance condition. In cases in which immediate and complete corrective action is implemented by project personnel, the corrective action will be recorded in the appropriate log book. Non-conformance conditions which could have an impact on project data quality must be communicated to the EPA NRMRL Coordinator within 24 hours. These issues will then be relayed to the Battelle Verification Test Coordinator or Battelle Quality Manager. These types of issues require a formal corrective action and root cause analysis. The PIs, EPA QA Officer, or Battelle Quality Manager can require laboratory activities to be limited or discontinued until the corrective action is complete and the non-conformance issue has been eliminated. Laboratory corrective action procedures are defined in each participating laboratory’s QA manual and SOPs. The Quality Assurance Officer at each organization is responsible for verifying that corrective action is implemented according to internal laboratory policies and this test QA/plan. The individual laboratory PIs are responsible for investigating and implementing test-level corrective actions to address errors or deviations in the laboratory.
SECTION D
DATA VALIDATION AND USABILITY

D1 DATA REVIEW, VALIDATION, AND VERIFICATION REQUIREMENTS

The key data review requirements for the verification test are stated in Section B10 of this test/QA plan. In general, the data review for each data package received from individual participating laboratories will be validated by the designated laboratory PI before being sent to NRMRL or the Verification Test Coordinator. This process will serve both as the data review and the data verification, and will ensure that data have been recorded, transmitted, and processed properly. Furthermore, this process will ensure that the ELSIA test kit data and the reference method data are collected under appropriate testing conditions and that the reference method data meet the reference method specifications.

The critical measurements for the reference laboratories listed in Table 3 will be used to validate the data quality. The QA audits described within Section C of this document, including the data quality audit, are designed to validate the data quality.

D2 VALIDATION AND VERIFICATION METHODS

Data verification is conducted as part of the data review, as described in Section B10 for this test/QA plan. Each data package received from individual participating laboratories will be validated by the designated laboratory PIs. A visual inspection of handwritten data will be conducted to ensure that all entries were properly recorded or transcribed, and that any erroneous entries were properly noted (i.e., single line through the entry with an error code and the initials of the recorder and date of entry). Data manually incorporated into spreadsheets for use in calculations will be checked against handwritten data to ensure that transcription errors have not occurred. All calculations will be reviewed to ensure the accuracy and the appropriateness of the calculations. Calculations performed manually will be reviewed and repeated using a handheld calculator or commercial software (e.g., Excel). Calculations performed using standard commercial office software (e.g., Excel) will be reviewed by inspecting the equations used in calculations and verifying selected calculations by handheld calculator. Calculations performed
using specialized commercial software (i.e., for analytical instrumentation) will be reviewed by inspection and, when feasible, verified by handheld calculator, or standard commercial office software.

To ensure that the data generated from this test meet the goals of the test, a number of data validation procedures will be performed. Section C of this test/QA plan provides a description of the validation safeguards employed for this verification test. Data validation and verification efforts include the completion of QC activities and the performance of TSA audits as described in Section C. Data failing to meet the criteria in Table 3 will be flagged in the data set and not used for evaluation of the technologies, unless these deviations are accompanied by descriptions of their potential impacts on the data quality.

A data quality audit or review will be conducted by the Battelle Quality Manager or designated individual laboratory PIs to ensure that data review, verification, and validation procedures were completed, and to assure the overall data quality.

D3 RECONCILIATION WITH USER REQUIREMENTS

The purpose of a verification test performed following this test/QA plan is to evaluate the performance of commercial ELISA test kit technologies which screen for endocrine disruptors in water. This evaluation will include comparisons of the results from each technology to results from standard reference techniques. To meet the requirements of the user community, the data obtained in such a verification test will include thorough documentation of the technology’s performance during the verification test. The data review, verification, and validation procedures described above will assure that verification test data meet these requirements, are accurately presented in the verification reports generated from the test, and that data not meeting these requirements are appropriately flagged and discussed in the verification reports. Additionally, all data generated using the reference method, which are used to evaluate technology results during the verification test, should meet the QA requirements of any applicable standard operating procedures or instrumentation instruction manuals.

This test/QA plan and any resulting ETV verification report(s) generated following procedures described in this test/QA plan will be subjected to review by participating technology vendors, ETV AMS center staff, test collaborators, EPA, and external expert peer reviewers.
These reviews will assure that this test/QA plan, verification test(s) of ELISA test kits, and the resulting report(s) meet the needs of potential users and regulators. The final report(s) will be submitted to EPA in 508 compliant adobe portable document format (pdf) and subsequently posted on the ETV website.
SECTION E
REFERENCES

E1 REFERENCES


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

4. Analysis of Nonylphenol (NP), Nonylphenol Monoethoxylate (NP1EO), Nonylphenol Diethoxylate (NP2EO), Bisphenol A and 4-(Tert-Octyl) Phenol (Octylphenol) in Water by Selected Ion Monitoring Gas Chromatography/Mass Spectrometry (GC/MS-SIM) with Large Volume Injection, CRL SOP Number GCMS004-013106 for APs, 01/31/06.
Appendix A

Cleaning and Silanization Protocol for Glassware
### Cleaning and Silanization Protocol for Glassware

<table>
<thead>
<tr>
<th>Procedures</th>
<th>Used Vials and Beakers</th>
<th>New Ordered Vials, beakers and Volumetric Flasks</th>
<th>Used Volumetric Flasks and Pipets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clean</strong></td>
<td>Put small items in a .5 or 1 L beaker. Soak in hot water with *detergent and sonicate for 15 minutes. Rinse with hot water four times and then rinse with D.I. Water four times.</td>
<td>NA</td>
<td>Soak in Hot water with *detergent for one hour and rinsed with hot water six times and D.I. water four times, individually.</td>
</tr>
<tr>
<td><strong>Oven</strong></td>
<td>Cover items with watch glass or aluminum foil and dry at 105 °C in the oven.</td>
<td>NA</td>
<td>Dry at 105 °C in the oven wrapped or capped with aluminum foil.</td>
</tr>
<tr>
<td><strong>Solvent Rinse</strong></td>
<td>Methanol rinse four times and let them dry at 105 °C in the oven.</td>
<td>Subsequently rinse with DCM, acetone, and methanol (one time for each solvent), and dry the in the hood loosely covered, finish drying at 105 °C in the oven.</td>
<td>Subsequently rinse with DCM, acetone, and methanol (3 times for each solvent) and air out in the hood, then dry them in a oven at 105 °C.</td>
</tr>
<tr>
<td><strong>Muffle Furnace</strong></td>
<td>Bake at 550 °C for at lest 6 hours. Then cooling down for 30 minutes.</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Deactivation</strong></td>
<td>Use a clean beaker or other container to soak vials with silanizing reagent (Sylon CT) for two or more minutes (with a glass cover). Shake it several times and make sure there are no bubbles in the vials. Decant and store the reagent in a clean bottle for re-use if the reagent stays clean and without water. Rinse the items three times with toluene, then four times with methanol (until the rinsings are neutral). Let sit in the hood for one hour and emit the solvent, then dry it at 105 °C in the oven for 15 to 30 minutes.</td>
<td>Use the procedure at left for new vials and small beakers, and use the procedure at right for volumetric flasks and bigger beakers.</td>
<td>Pour the silanizing reagent (Sylon CT) into the flask, roll that glass to make sure all surfaces are contacted or fill and let sit 1-2 minutes. Drain the reagent into a clean bottle for reuse. Rinse three times with toluene and then four times with methanol (until the rinsings are neutral). Put the flask in the hood for 15 minutes and emit the solvent, then dry it at 105 °C in the oven.</td>
</tr>
</tbody>
</table>

* Detergent such as powdered Alconox, Deto-Tet, Luminox or citrojet can be used. Detergents containing alkylphenolic compounds must not be used.
Appendix B

Procedure for Preparation of 20L Glass Carboys
Preparation of 20L Glass Carboys

- Rinse out carboy with water.

- Place Teflon stopper inside the carboy.

- Flush carboy with hot water for 15 minutes. Insert tubing from hot water outlet into carboy neck to base of carboy so that the water swishes up the side of the carboy. Close off carboy outlet valve with finger until hot water reaches neck of carboy. Release finger. Adjust flow of water so that influent equals outflow from carboy. Maintain this equilibrium for 15 minutes. Drain all hot water from carboy.

- Repeat above procedure with house distilled water.

- Remove teflon stopper from carboy. Place in carboy spigot.

- Rinse carboy 3x with ~250-300 ml Reagent Grade Alcohol.

- Remove teflon stopper. Rinse with alcohol. Place in beaker covered with aluminum foil. Do not muffle stopper.

- Cover neck opening and outlet of carboy with alcohol rinsed aluminum foil. Label and date carboy.

To Muffle: Remove tape from carboy. Place in muffle oven. After oven reaches 400°, muffle for one hour. Oven door may be opened when temperature reaches 100°. Remove carboy when cool, label, date and initial. Leave foil on carboy until ready to use.
Appendix C

Extractions for EE2 from Water Sample for ELISA for hormone samples
Extractions of EE2 from Water Sample for ELISA

1. Filter the raw water samples with glass fiber filter (pore size: 1 micrometer)
   - If the pH of the filtrate ranges out of 5-8, adjust it to 5-8 with 1M acetic acid buffer (pH=5.0).

2. Nexus SPE cartridge is preconditioned with 5 mL dichloromethane, and then 5 mL methanol followed by 10mL water on the SPE manifold under vacuum with flow control (ca.10 mL/min).
   - For solid phase extraction (SPE) cartridge, Nexus (Varian), 500 mg of styrene divinyl benzene sorbent, 6 mL reservoir is used.

3. The sample is applied to the cartridge via a Teflon tube and adaptor under the same flow rate of ca. 10 mL/min.

4. The cartridge is rinsed with 5mL water, and then 5mL water/methanol (50/50 % v/v).

5. After drying the cartridge under vacuum for 2 min, EE2 is eluted with 6mL of dichloromethane in the glass vial with a flow rate of 3 to 5 mL/min.

6. The extract is evaporated under a gentle nitrogen stream at 40 to 50°C. Then, the vial is capped and stored at 4°C until the assay is performed.

7. When the sample is analyzed with ELISA, 100% methanol is added to the vial and vigorously mixed with vortex mixer, and then followed by distilled water to give a 10% aqueous methanol solution.
   - For example, 100 micro L of methanol is added to the vial and vigorously mixed with vortex mixer, and then followed by 900 micro L of water to give a 10% aqueous methanol solution of 1 mL volume.
   - If there remains undissolved residue, add 100% DMSO and MeOH to adjust the ration at 1%DMSO and 10%MeOH solution. The solvent for standards also need to adjusted at 1%DMSO and 10%MeOH in this case.
Appendix D

Flowchart: AP ELISA for NP samples
Flowchart: AP ELISA
(Analysis of Alkylphenol with the new AP ELISA)

NEXUS SPE Cleanup & Enrichment

Evaporation

ELISA

*NEXUS Cartridge Precondition*
- Dichloromethane: 10mL (up to 20mL/min)
- Methanol: 5mL (up to 20mL/min)
- Distilled water: 5mL (up to 20mL/min)

*Sample Loading*
- Filtered sample: 1L (up to 10mL/min)

*Washing*
- Distilled water: 5mL (up to 20mL/min)
- Distilled water/Methanol=1:1: 5mL (up to 20mL/min)

*Drying*
- Vacuuming: 45 minute

*Elution*
- Dichloromethane: 6mL (3-5mL/min)

**CAUTION:** The evaporation has to be done under 30 degree Celsius or lower temperature.

*ELISA Sample Preparation*
- To dissolve the residue to 1%DMSO and 10%methanol solutions

NEXUS SPE Cartridge

Producer: VARIAN
PART#: 1210-3102
ABS ELUT-NEXUS, 200MG 6ML, 30/PK

Please use the NEXUS Cartridge made of glass (not plastic), if it is available in the US.
Producer: GL Science Ltd. (Tokyo, Japan)
PART#: 5010-26021
GL-Pak GLASS SPE NEXUS 200mg/6ml 20/PK