

**Environmental Technology
Verification Program**
Advanced Monitoring
Systems Center

Test/QA Plan for
Verification of
Immunoassay Test Kits

ET ✓ ET ✓ ET ✓

TEST/QA PLAN

for

Verification of Immunoassay Test Kits

January 2004

Prepared by

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ETV ADVANCED MONITORING SYSTEMS CENTER

Test/QA Plan for Verification of
Immunoassay Test Kits

Version 1

January 2004

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1.0 INTRODUCTION

1.1 Test Objective

This test/quality assurance (QA) plan provides procedures for a performance verification test of immunoassay test kits for the analysis of biological toxins and agents in drinking and distilled water, under a specific set of test conditions. The verification test will be conducted under the auspices of the U.S. Environmental Protection Agency (EPA) through the Environmental Technology Verification (ETV) Program. 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. The objective of this verification test of immunoassay test kits is to evaluate their ability to rapidly detect specific biological toxins and agents that are particularly toxic to humans and their susceptibility to interferents in several drinking water matrices.

1.2 Test Description

The verification test will be performed by Battelle, which is managing the ETV Advanced Monitoring Systems (AMS) Center 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. In performing the verification test, Battelle will follow the procedures specified in this test/QA plan and will comply with the data quality requirements in the “Quality Management Plan for the ETV Advanced Monitoring Systems Center” (AMS QMP)¹.

Immunoassay test kits are based on immunoassay methods, where specific antibodies are used to detect and measure the contaminants of interest. As seen in Equation 1, immunoassay test kits rely on the reaction of a contaminant or antigen (Ag) with a selective antibody (Ab) to give a product that can be measured (AbAg).



Many types of labels including enzymes, fluorescence, phosphorescence, electro-chemiluminescence, and chemiluminescence have been used for quantifying the assay. The sample concentration can be either directly or inversely proportional to color intensity, depending on the technology. Results from these methods can be qualitative or quantitative. While there are several types of immunoassays, enzyme-linked immunoabsorbent assays are most common.

The test will determine the accuracy and precision of immunoassay test kits in detecting selected biological toxins and agents in American Society of Testing and Materials (ASTM) Type II deionized (DI) water, in the presence of possible interferents added to ASTM Type II DI water, and in drinking water obtained from a variety of geographically dispersed U.S. water utilities that use various water treatment processes. Qualitative characteristics of each immunoassay test kit, such as ease of use, sample throughput, and cost, will also be assessed and reported. While most of the testing will occur in a laboratory, the immunoassay test kits that are designed for field use will be tested outside of the laboratory. To evaluate the applicability of these immunoassay test kits for use by non-technically trained individuals outside the laboratory, a non-technical operator without experience in using the kits will perform the tests with little training and with no assistance from the experienced operator.

1.3 Organization and Responsibility

The verification test will be performed by Battelle, with the participation of the vendors who will be having the performance of their immunoassay test kits verified. The testing will occur at Battelle's West Jefferson, Ohio, laboratories and at a non-laboratory (i.e., field) location in the Columbus, Ohio, area. 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.

1.3.1 Battelle

Dr. Ryan James is the AMS Center Verification Test Coordinator. In this role, Dr. James will have overall responsibility for ensuring that the technical, schedule, and cost goals established for the verification test are met. Specifically, he will

- Assemble a team of qualified technical staff to conduct the verification test.
- Direct the team performing the verification test in accordance with the test/QA plan.
- Ensure that all quality procedures specified in the test/QA plan and in the AMS QMP are followed.

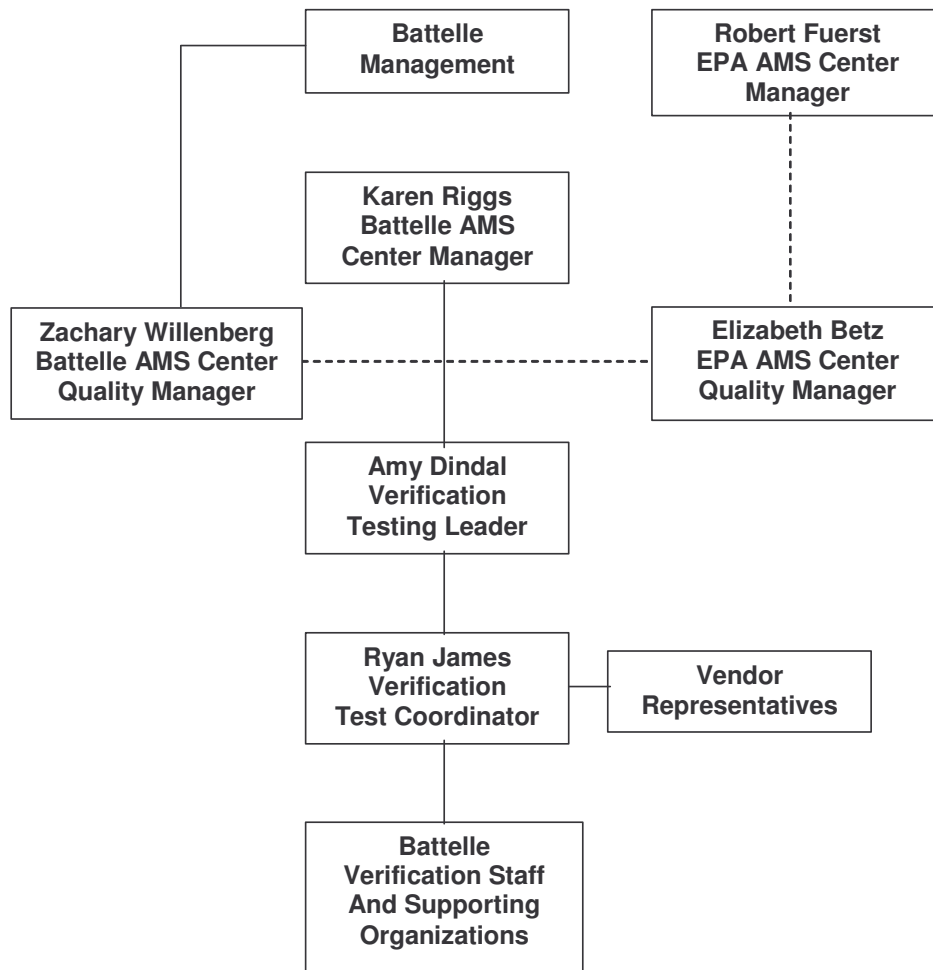


Figure 1. Organization Chart

- Prepare the draft and final test/QA plan, verification reports, and verification statements.
- Revise the draft test/QA plan, verification reports, and verification statements in response to reviewers' comments.
- Respond to any issues raised in assessment reports and audits, including instituting corrective action as necessary.
- Serve as the primary point of contact for vendor representatives.
- Coordinate distribution of the final test/QA plan, verification reports, and statements.

- Establish a budget for the verification test and monitor staff effort to ensure that budget is not exceeded.
- Ensure that confidentiality of vendor information is maintained.

Ms. Amy Dindal is a Verification Testing Leader for the AMS Center. Ms. Dindal will provide technical guidance and oversee the various stages of verification testing. She will

- Support Dr. James in preparing the test/QA plan and organizing the testing.
- Review the draft and final test/QA plan.
- Review the draft and final verification reports and verification statements.

Ms. Karen Riggs is Battelle's manager for the AMS Center. Ms. Riggs 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 vendor confidentiality is maintained.
- Support Dr. James in responding to any issues raised in assessment reports and audits.
- Maintain communication with EPA's technical and quality managers.
- Facilitate a stop work order if Battelle or EPA QA staff discovers adverse findings that will compromise test results.

Battelle Technical Staff will conduct the testing of the immunoassay test kits during the verification test. The responsibilities of the technical staff will be to

- Assist in the collection, receipt, and storage of drinking water samples.
- Prepare drinking water samples, as required for analysis.
- Prepare the stock solutions and the test samples as required.
- Perform the immunoassay test kit analyses by following the vendor's protocol.
- Make qualitative observations about the operation of the immunoassay test kit.

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

Mr. Willenberg will

- Review the draft and final test/QA plan.
- Conduct a technical systems audit once during the verification test, or designate another QA manager to conduct the audit.
- Audit at least 10% of the verification data.
- Prepare and distribute an assessment report for each audit.
- Verify implementation of any necessary corrective action.
- Issue a stop work order if self audits indicate that data quality is being compromised; notify Battelle's AMS Center Manager if a stop work order is issued.
- Provide a summary of the QA/ quality control (QC) activities and results for the verification reports.
- Review the draft and final verification reports and verification statements.
- Assume overall responsibility for ensuring that the test/QA plan is followed.

1.3.2 Vendors

The responsibilities of the vendor representatives are as follows:

- Review the draft test/QA plan.
- Approve the test/QA plan prior to test initiation.
- Provide off-the-shelf immunoassay test kits for analysis of all verification test samples.
- Provide all other equipment needed to complete the immunoassay analyses.
- As desired, provide training to Battelle personnel on operating the immunoassay test kits and associated equipment prior to testing.
- Provide written instructions for operation of test kit.
- Review the draft verification report and statement.

1.3.3 EPA

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 the specific EPA staff are as follows:

Ms. Elizabeth Betz is EPA's AMS Center Quality Manager. For the verification test, Ms. Betz will

- Review the draft test/QA plan.
- Perform at her option one external technical system audit during the verification test.
- Notify the EPA AMS Center Manager of the need for a stop work order if external audit indicates that data quality is being compromised.
- Prepare and distribute an assessment report summarizing results of external audit.
- Review draft verification reports and statements.

Mr. Robert Fuerst is EPA's manager for the AMS Center. Mr. Fuerst will

- Review the draft test/QA plan.
- Approve the final test/QA plan.
- Review the draft verification reports and statements.
- Oversee the EPA review process for the verification reports and statements.
- Coordinate the submission of verification reports and statements for final EPA approval.

1.3.4 Supporting Organizations

When methods are available, the stock solution concentrations of contaminants and interferences will be confirmed. Physico-chemical characterization including turbidity, organic carbon, specific conductivity, alkalinity, pH, hardness, total organic halides, trihalomethanes and haloacetic acids (Table 3) will be performed for all drinking water samples. Battelle will establish a subcontract with Aqua Tech Environmental Laboratories, Inc. (hereafter called subcontract laboratory) to perform the analyses.

The Metropolitan Water District of Southern California will concentrate each drinking water sample by a factor of 400 using ultrafiltration contraction techniques.

2.0 VERIFICATION APPROACH

2.1 Scope of Testing

This test/QA plan specifically addresses verification of immunoassay test kits that provide qualitative or quantitative measurements of ricin, botulinum toxin, and anthrax in drinking water. The contaminants were selected based on the capabilities of the kits being tested. Immunoassay test kits that provide qualitative results only indicate the presence or absence of the contaminants within a specified concentration interval, while immunoassay test kits that provide quantitative results report concentrations using a digital display or an electronic output signal. The vendor will specify in which category their immunoassay test kit should be included and which contaminants the immunoassay test kit is designed to detect. Each technology will be verified only for contaminants for which they are designed to detect. The immunoassay test kits will be verified by subjecting them to various concentration levels of individual contaminants in ASTM Type II DI water, a single concentration of contaminant in the presence of possible interferents (i.e., magnesium and calcium, fulvic and humic acids, biological analogues) spiked into ASTM Type II DI water, and a single concentration of each contaminant spiked into drinking water samples obtained from four water utilities from different geographical locations in the United States. Each source of water will represent a unique water treatment process. Also, both the possible interferent samples and the drinking water matrices will be analyzed without the addition of any contaminant to evaluate the potential for false positive results. The performance of each immunoassay test kit will be evaluated by comparing the immunoassay test kit results with known concentrations of spiked contaminants.

Both the quantitative and qualitative immunoassay test kits are designed to be operated by users with little technical training. Because the verification test involves a multi-step process, both types of immunoassay test kits will probably yield more consistent and reliable results when operated by users familiar with performing these tests. Therefore, for this verification test, technical operators, i.e., operators with a degree in biology or chemistry, that have been trained by the vendor in the operation of the test kits, will analyze all the test samples. However, outside the laboratory, a non-technical operator, i.e., an operator with little or no scientific training and with no assistance or training from the vendor, will also perform the test using only the written instructions or manual provided by the vendor.

A variety of natural and contaminant-fortified water samples (i.e., unspiked and spiked) will be analyzed using the immunoassay test kits. These matrices are examples of drinking water types that could be monitored using immunoassay test kits; however, this is not intended to be an exhaustive study or to represent all possible water types that could be tested.

The immunoassay test kits will be evaluated for the following parameters:

- Accuracy
- Precision
- Linearity
- Method detection limit (MDL)
- Cross reactivity
- Matrix effects
- Occurrence of false positives and false negative results
- Field portability by technical and non-technical operator
- Ease of use by technical and non-technical operator
- Sample throughput.

Qualitative immunoassay test kits will not be evaluated for linearity or MDL because they only provide a positive or negative result relative to a specified concentration level. However, the lowest concentration PT sample to produce consistently positive results will be reported as an approximate detection limit.

2.2 Experimental Design

This verification test will determine the performance capability of the immunoassay test kits to detect individual contaminants in three types of samples—performance test (PT), drinking water (DW), and quality control (QC). PT samples will include all the samples prepared in ASTM Type II DI water. The contaminant PT samples will be fortified with individual contaminants at four concentrations. Concentrations will range from the lethal dose concentration given in Table 1 for each contaminant to approximately 50 times the vendor reported detection limit (LOD) of each technology. The lethal dose of each contaminant will be determined by calculating the concentration at which 250 mL of water would probably cause the death of a 154-pound person based on human LD50 data. The results from triplicate analysis of the contaminant PT samples and comparison with the known concentrations will provide information on accuracy, precision, and linearity of the immunoassay test kits. The MDL of each immunoassay test kit will be determined by analyzing seven replicates of PT sample containing a concentration of each individual contaminant approximately five times greater than the LOD provided by the vendor.

One type of interferent PT samples will consist of calcium and magnesium and humic and

fulvic acid at two concentrations, both spiked and unspiked with each contaminant. Another type of interferent PT sample will test the susceptibility of the immunoassay test kits to producing positive results in response to compounds or spores structurally and functionally similar to the target contaminants. This will be assessed by analyzing samples that contain only the potentially cross reactive compound or spore. These will include: (1) *Bacillus thuringiensis*, a spore-forming bacteria similar to *Bacillus anthracis*, which causes anthrax; (2) lectin from soybean (a lectin similar to ricin); and (2) lipopolysaccharide (an extract from gram negative bacteria, indicative of general groups of organisms found in water that could falsely indicate the presence of botulinum toxin).

Table 1. Lethal Dose of Target Contaminants

Contaminant	Lethal Dose Concentration	Source of Contaminant
<i>Bacillus anthracis</i> (anthrax)	200,000 spores/L	United States Government
Botulinum toxin Complex Type B	0.3 mg/L	Metabiologics, Inc. (Madison, WI)
Ricinus communis Agglutinin II (Ricin)	15 mg/L	Vector Laboratories, Inc. (Burlingame, CA)

DW samples will be collected from four water systems that get water from various sources and employ different treatment processes. The DW samples disinfected by chlorination will be obtained from surface (filtered and unfiltered) and groundwater sources. A DW sample from a surface water source disinfected by chloramination will also be collected. Each of these water samples will be analyzed in triplicate, both unspiked and spiked with each contaminant at a single concentration level approximately 10 times greater than the detection limit of the immunoassay test kit. These same water samples will be concentrated by a factor of 400 at the Metropolitan Water District of Southern California using their ultrafiltration concentration system and analyzed in the manner described above to evaluate the performance of the immunoassay test kits when analyzing in a concentrated drinking water matrix.

Quality control samples will include method blanks consisting of unspiked ASTM Type II DI water and positive controls. Positive control samples will be prepared in ASTM Type II DI water with a known concentration of a toxin specified by the vendor and will be used as a quality check to ensure immunoassay test kit performance.

Performance parameters, such as ease of use and reliability, will be based on documented observations of the operators by the Verification Test Coordinator. Sample throughput will be

estimated based on the time required to analyze a sample set. All immunoassay test kits will be tested in the laboratory; applicable immunoassay test kits will be analyzed for their performance and ease of use outside of the laboratory. Because the contaminants of interest cannot be safely handled outside of the surety laboratory, the samples analyzed in the field will be method blank samples and non-toxic positive control samples if provided by the vendor. Both a technical and a non-technical, first-time user will perform analyses in the field. The only guidance the non-technical, first-time user will receive is from the manual or instructions provided by the vendor.

Given the agent facility restrictions, vendors will not be able to operate their immunoassay test kits during this verification test. Each immunoassay test kit will be operated by a Battelle staff member and tested independently. All technical operators will have a bachelor's degree in the sciences or equivalent work experience. The vendor will train the operators by means of a visit to Battelle or a conference call prior to starting the verification test and then will be asked to sign a consent form stating the names of the Battelle staff they have trained. Each operator will manipulate the water samples and reagents to generate solutions that can be analyzed by the immunoassay test kits. More than one operator may be used by Battelle, but operators will be restricted to only operating immunoassay test kits on which they have been trained by either the vendor or a Battelle staff trained by the vendor.

2.3 Test Samples

Test samples to be used in this verification test will include PT samples, DW samples, and QC samples. Table 2 lists the number and type of each sample to be analyzed for each contaminant in the verification test. Each type of test sample is described further below.

2.3.1 PT Samples

PT sample types (listed in Table 2) will be prepared from ASTM Type II DI water. The first type of PT sample will consist of ASTM Type II DI water spiked at four concentration levels of each individual contaminant. The PT sample concentrations will range from the lethal dose concentration to 50 times the vendor-stated LOD. The lethal dose concentration will be analyzed to document the response of each technology at that important concentration level. Three concentration levels in addition to the lethal dose concentration will be analyzed by each technology. Those concentration levels will be approximately 5, 10, and 50 times the LOD of each individual technology. The maximum concentrations will be limited by the available standards. These maximum concentrations for each contaminant are as follows: for ricin, 5,000

mg/L, for botulinum toxin, 1,000 mg/L, and for anthrax, 10^9 spores/mL. Three replicates of each PT sample will be analyzed using the immunoassay test kits.

The second type of PT sample will be used to determine the MDL of each quantitative immunoassay test kit. Seven replicates of a contaminant sample prepared to be approximately five times greater than the LOD will be analyzed and the precision of the results used for the MDL determination. The third type of PT sample will be potential interferent samples. One sample will contain calcium and magnesium from carbonates and the second humic and fulvic acids isolated from the Suwanee River (obtained from the International Humic Substances Society) spiked into ASTM Type II DI water. Each of these contaminant mixtures will be prepared at two different concentration levels. One concentration will be near the upper limit of what would be expected in drinking water and one concentration at a mid-low range of what would be expected. Three replicates of each interferent PT sample will be analyzed to determine each immunoassay test kit's susceptibility to commonly found interferences in DW. Also, each contaminant will be added to these samples along with the potential interferents, at a concentration of 10 times the LOD and analyzed in triplicate.

The fourth type of PT sample will be a cross-reactivity check sample. *Bacillus thuringiensis* (for anthrax), lectin from soybean (for ricin), and lipopolysaccharide (for botulinum toxin), all biologically similar to the specified targets, will be prepared in ASTM Type II DI water at concentrations equivalent to 10 times the LOD of the test kits. Three replicates of each cross-reactivity check sample will be analyzed using the immunoassay test kits to determine if there are false positive results in response to these similar bacteria.

2.3.2 Drinking Water Samples

Drinking water samples will be collected from four geographically distributed municipal sources to evaluate the performance of the immunoassay test kits with various sample matrices. These samples will vary in their source and treatment and disinfection process. All samples will have undergone either chlorination or chloramination disinfection prior to receipt. Samples will be collected from water utility systems with the following treatment and source characteristics:

- Chlorinated filtered surface water source
- Chlorinated unfiltered surface water source
- Chlorinated groundwater source
- Chloraminated filtered surface water

All samples will be collected in pre-cleaned high density polyethylene (HDPE) containers. After sample collection, to characterize the DW matrix, an aliquot of each DW sample will be sent to a subcontract laboratory to determine the following water quality parameters: concentration of trihalomethanes, haloacetic acids, total organic halides, calcium, magnesium, pH, conductivity, alkalinity, turbidity, organic carbon, and hardness. Because chlorine can degrade some of the contaminants, the DW samples will be dechlorinated with sodium thiosulfate pentahydrate prior to use. To evaluate the effect of a concentrated DW matrix on the immunoassay test kits performance, approximately 100 L of each of the above sources of DW will be dechlorinated and then concentrated through ultrafiltration techniques to a final volume of 250 mL. As shown in Table 2, each DW sample (non-concentrated and concentrated) will be analyzed without adding any contaminant, as well as after fortification with each individual contaminant at a single concentration level (10 times greater than the vendor-stated LOD).

Table 2. Summary of Test Samples for Immunoassay Test Kit Verification

Performance Test	Performance Factor	Sample Description	Reps
ASTM Type II DI Water	Accuracy, Precision, Linearity ^(a)	Contaminant PT sample @ Lethal Dose	3
		Contaminant PT sample @ 5 times the LOD ^(b)	3
		Contaminant PT sample @ 10 times the LOD	3
		Contaminant PT sample @ 50 times the LOD	3
	Method Detection Limit ^(a)	Contaminant MDL sample @ 5 times the LOD	7
	Interferent	Calcium and Magnesium @ 50 mg/L	3
		Calcium and magnesium @ 50 mg/L + contaminant @ 10 times the LOD	3
		Calcium and magnesium @ 250 mg/L	3
		Calcium and magnesium @ 250 mg/L + contaminant @ 10 times the LOD	3
		Fulvic and humic acids @ a total concentration of 1 mg/L	3
		Fulvic and humic acids @ a total concentration of 1 mg/L + contaminant @ 10 times the LOD	3
		Fulvic and humic acids @ 5 mg/L	3
		Fulvic and humic acids @ 5 mg/L + contaminant @ 10 times the LOD	3
	Cross Reactivity	Bacillus thuringiensis (anthrax) Lectin from soybean (ricin) Lipopolysaccharide (botulinum toxin)	3

	Ease of Use, Field Portability	Technical operator analysis of method blank	3
		Non-technical first-time user analysis of method blank	3
Drinking Water	Performance Factor	Sample Description	Reps
Filtered chlorinated surface water	Accuracy, Precision, Linearity, Matrix Effects, False Positives and False Negatives	Unspiked	3
		Spiked with contaminant @ 10 times the LOD	3
		Concentrated unspiked	3
		Concentrated and spiked with contaminant @ 10 times the LOD	3
Unfiltered chlorinated surface water		Unspiked	3
		Spiked with contaminant @ 10 times the LOD	3
		Concentrated unspiked	3
		Concentrated and spiked with contaminant @ 10 times the LOD	3
Filtered chlorinated surface water		Unspiked	3
		Spiked with contaminant @ 10 times the LOD	3
		Concentrated unspiked	3
		Concentrated and spiked with contaminant @ 10 times the LOD	3
Filtered chloraminated surface water	Unspiked	3	
	Spiked with contaminant @ 10 times the LOD	3	
	Concentrated unspiked	3	
	Concentrated and spiked with contaminant @ 10 times the LOD	3	
Quality Control	Performance Factor	Sample description	Reps
Method Blank	Quality Check	DI water - 10% of all samples	10
Positive Control	Quality Check	Contaminant in DI water - 5% of all samples	5
Approximate total number of samples per contaminant			115

(a) Not determined for qualitative immunoassay test kits.

(b) LOD in all cases is limit of detection provided by the vendor for their immunoassay test kit.

2.3.3 QC Samples

QC samples will include method blank (MB) samples consisting of ASTM Type II DI water, and positive control (PC) samples, consisting of ASTM Type II DI water fortified with a known contaminant at a concentration and type selected by the vendor. All of the QC samples will

be prepared from ASTM Type II DI water and will be exposed to identical sample preparation and analysis procedures as the test samples. The MB samples will be used to ensure that no sources of contamination are introduced in the sample handling and analysis procedures. At least 10% of the test samples will be MB samples. The PC samples will indicate to the operator whether the immunoassay test kit is functioning properly. If the immunoassay test kit is qualitative in nature, a positive result will be adequate for the operator to be assured of the proper functioning of the immunoassay test kit. If the immunoassay test kit is quantitative in nature, the vendor will provide acceptable control limits for results in which no corrective action will have to be taken. If the results are outside these control limits, the vendor will be contacted to find out if corrective action is necessary. PC samples will account for approximately 5% of all the test samples. The test samples will be analyzed blindly by the technical operator.

2.4 Reference Method

Standard laboratory reference methods do not exist for ricin or botulinum toxin. To ensure the accuracy of sample concentrations of ricin and botulinum toxin, QA oversight of the solution preparation in the form of audits, and purity tracking of standards will be performed. Section 6.0 describes the QA/QC procedures for this test. For anthrax, a plate enumeration technique of quantifying bacteria will be followed to confirm the concentration of the stock solution of these contaminants on a daily basis. The Battelle standard operating procedure (SOP) to be followed is SOP No: MERF X-054⁴ "Enumeration of BL-2 and BL-3 Bacteria Samples Via the Spread Plate Technique."

3.0 MATERIALS AND EQUIPMENT

In general, this verification test will rely on immunoassay test kit materials and equipment provided by the vendors. Battelle will provide the following equipment and materials for the collection, preparation, storage, and shipment of test samples.

3.1 Testing Supplies

The following supplies will be needed throughout testing for sample collection and preparation of the DW and QC samples:

- ASTM Type II DI water
- Various laboratory supplies necessary for accurate preparation of the test samples (i.e., volumetric pipettes, pipet bulbs, Eppendorf micro pipettes/pipette tips, volumetric flasks, disposable pipettes, etc.)
- 25-L HDPE containers for sample collection and shipment
- Various smaller sizes of pre-cleaned HDPE and glass containers for sample aliquot storage
- Standards of contaminant and interferents with a known level of purity (NIST traceable or equivalent)
- Sodium thiosulfate pentahydrate
- n,n-diethyl-p-phenylenediamine (DPD) tablet
- Personal protective equipment.

3.2 Field Analysis Supplies

For the analysis of the method blank samples in the field, Battelle will provide only the water used for analysis. The operators will depend on only supplies provided by the vendor to analyze the samples.

3.3 Special Facilities

All of the contaminants to be evaluated in this verification test (botulinum toxin, ricin, and anthrax) can only be handled in laboratories that are specially designed and certified for the use of chemical and biological agents and by operators who are trained in their use. Battelle's Medical Research and Evaluation Facility (MREF), which is a Department of Defense laboratory-scale facility conducting research with chemical and biological agents, will provide the facilities and

staff for verification testing of the specified contaminants. The MREF is licensed to ship, receive, and handle select agents, as defined by the Centers for Disease Control and Prevention (CDC)⁵. The facility maintains state-of-the-art equipment and professional and technical staffing expertise to safely conduct testing and evaluation of hazardous chemical and biological materials.

The MREF and its personnel have the demonstrated capability for storing and safely handling botulinum toxin, ricin, and anthrax. Biological agent use will be according to the CDC Select Agents Program (32 CFR 626 and 627)⁵ administered through the Biological Defense Research Program and the Battelle MREF Facility Safety Plan.

4.0 PROCEDURES

4.1 Test Sample Collection, Preparation, and Storage

Stock solutions of each contaminant and interferent will be prepared in ASTM Type II DI water from certified standards. The concentration of the anthrax stock solutions will be confirmed daily using plate enumeration method. The ricin and botulinum toxin solutions will be prepared daily.

PT and QC samples will be prepared in ASTM Type II DI water using the aforementioned stock solutions. Aliquots of each stock solution will be diluted to the appropriate concentration using volumetric pipets and glassware. The DW samples will be collected as described in Section 2.3.2. Because free chlorine will degrade many of the contaminants and interferences during storage, the sample will be immediately dechlorinated with sodium thiosulfate pentahydrate (or other dechlorination reagents as per vendor protocol). The dechlorination of the DW will be qualitatively confirmed by adding a diethyl-p-phenylene diamine (DPD) tablet to an aliquot of DW. If the water does not turn pink, the dechlorination process will be determined to be successful. If the water does turn pink, an additional dechlorinating reagent will be added and the dechlorination confirmation procedure will be repeated. Once dechlorination is confirmed, 100 L of each DW sample will be concentrated as described previously, and approximately 25 L will remain unconcentrated. The dechlorinated concentrated and native DW samples will be analyzed unspiked and spiked. Aliquots of each stock solution will be diluted to the appropriate concentration using volumetric pipets and glassware and with each DW sample as the dilution solvent. All spiked DW samples will be analyzed on the same day as they are prepared.

4.2 Sample Identification

Aliquots to be analyzed by each immunoassay test kit will be drawn from the PT, QC or DW samples and placed in sample containers with unique identification (ID) numbers. A master log of the samples and sample ID numbers for each immunoassay test kit will be maintained by Battelle. The ID number, date, person collecting, sample location, and time of collection will be recorded on a chain-of-custody form for all field samples.

4.3 Sample Analysis

4.3.1 Drinking Water Characterization

Table 3 lists the methods to be used to characterize the DW samples collected from the various water sources by the subcontract laboratory. The suppliers of the DW samples will also be asked to provide water quality characteristics data that are typical for their water source. If the results from characterization analysis are different from suppliers data, the variance will be noted by the Verification Test Coordinator. Information produced on physio-chemical parameters of drinking water by the subcontract laboratory for this test will be utilized for verifying performance of the immunoassay test kits.

Table 3. Physio-Chemical Characterization of Drinking Water^a

Parameter	Method	Method Detection Limits ^(b)
Turbidity	EPA 180.1 ⁶	0.067 ntu
Organic carbon	SM 5310 ⁷	0.7 mg/L
Specific conductivity	SM 2510 ⁷	2 µmho
Alkalinity	SM 2320 ⁷	2 mg/L
pH	EPA 150.1 ⁸	NA
Hardness	EPA 130.2 ⁸	5 mg/L
Total organic halides	SM 5320 ⁷	5 µg/L
Trihalomethanes	EPA 524.2 ⁹	0.5 µg/L/analyte
Haloacetic acids	EPA 552.2 ¹⁰	1.0 µg/L/analyte

(a) Physio-chemical DW characterization to be performed by the subcontract laboratory

(b) Method detection limits based on standard methods.

4.3.2 Stock Solution Confirmatory Methodologies

Botulinum toxin and ricin and their cross reactivity analogues do not have methods available to confirm their concentrations. QA procedures will ensure accurate sample preparation. The concentration of anthrax and its corresponding cross-reactivity analogue will be confirmed by a plate enumeration method following Battelle SOP No.: MERF X-054. For the interferent samples, the concentration of calcium and magnesium will be confirmed by EPA Method 200.8¹¹ and the concentration of humic and fulvic acid will be confirmed by Standard Method 5310⁷ for total and dissolved organic carbon.

4.4 Immunoassay Test Kits

Each vendor will provide immunoassay test kits, consumables like reagents and other necessary equipment (e.g. vortexer, spectrophotometer) for the analysis of all samples. The full set of samples listed in Table 2 unless otherwise noted will be analyzed by each immunoassay test kit for each applicable contaminant. The analyses will be performed according to the vendor's recommended procedures as described in the standard written instructions or manual provided with the immunoassay test kits. Calibration and maintenance of the immunoassay test kits will be performed as specified in the written instructions or manual.

Immunoassay test kit results will be recorded manually by the technical and non-technical operator on data sheets designed specifically for this verification test. In addition to the immunoassay test kit results, the data sheets will include records of the time required for sample analysis and technical and non-technical operator observations concerning the use of the immunoassay test kit (e.g., ease of use, maintenance, etc.).

4.5 Schedule

The verification test described here will take place during approximately three weeks in January 2004 at Battelle's laboratories in West Jefferson, Ohio, and at a nearby non-laboratory location. It will be necessary for participating vendors to provide their immunoassay test kits to Battelle prior to testing so staff may become familiar with operating the immunoassay test kits before testing begins. Vendor staff are requested to provide training in operating the immunoassay test kits either in person or by teleconference. Unused immunoassay test kits and associated equipment (but not consumables) will be returned to the vendors at the completion of report writing. As appropriate, immunoassay test kits will be decontaminated by washing with an aqueous solution of bleach before being returned to the vendor.

5.0 DATA HANDLING AND REPORTING

5.1 Data Acquisition and Review

Various types of data will be acquired and recorded electronically or manually by Battelle during this verification test. Table 4 summarizes the type of data to be recorded. All data and observations will be documented by Battelle staff on data sheets or in laboratory record books. Results from the laboratory reference instruments will be compiled in electronic format.

Records received by or generated by Battelle staff in the verification test will be reviewed by a more Battelle senior staff member within two weeks of receipt or generation, respectively, before the records are used to calculate, evaluate, or report verification results. This review will be performed by a Battelle technical staff member involved in the verification test, but not the staff member that originally received or generated the record. The review will be documented by the person performing the review by adding his/her initials and date to a hard copy of the record being reviewed. This hard copy will then be returned to the Battelle staff member who will be storing the record. In addition, data calculations performed by Battelle will be spot-checked by Battelle technical staff 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 immunoassay test kit. Results for immunoassay test kits from different vendors will not be compared with each other.

Table 4. Summary of Data Recording Process

Data to Be Recorded	Where Recorded	How Often Recorded	Disposition of Data^(a)
Dates and times of test events	ETV data sheets	Start/end of test, and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Calibration information and results for physico-chemical parameters (temperature, salinity, pH, conductivity, etc.)	ETV data sheets	Prior to sample preparation	Manually incorporated in data spreadsheets as necessary
Sample collection and preparation information, including chain-of-custody	ETV data sheets and chain-of-custody forms	At time of sample collection and preparation	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Immunoassay test kit procedures and sample results	ETV data sheets	Throughout test duration	Manually incorporated in data spreadsheets
Reference method procedures and sample results	ETV data sheets, or data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets

(a) All activities subsequent to data recording are carried out by Battelle.

5.2 Data Analysis

5.2.1 Accuracy

For immunoassay test kits that provide quantitative results, PT and DW sample accuracy will be assessed relative to the contaminant spike level. The results for each set of analyses averaged, and the accuracy will be expressed in terms of a percent recovery (R) as calculated from Equation 2:

$$R = \bar{C} / C_R \times 100 \quad (2)$$

where \bar{C} is the average concentration measured by the immunoassay test kit and C_R is the spike

level for the PT or DW sample.

For qualitative results, accuracy will be assessed by evaluating how often the immunoassay test kit result is positive in the presence of a concentration above the LOD. An overall percent agreement will be determined by dividing the number of positive responses to the overall number of analyses.

5.2.2 Precision

For immunoassay test kits that provide quantitative results, the standard deviation (S) of the results for the replicate samples will be calculated for each sample using Equation 3:

$$S = \left[\frac{1}{n-1} \sum_{k=1}^n (C_k - \bar{C})^2 \right]^{1/2} \quad (3)$$

where n is the number of replicate samples, C_k is the concentration measured for the k^{th} sample, and \bar{C} is the average concentration of the replicate samples. The precision for each sample will be reported in terms of the relative standard deviation (RSD) as calculated using Equation 4:

$$RSD = \frac{S}{\bar{C}} \times 100 \quad (4)$$

For immunoassay test kits that provide qualitative results, precision will be assessed by calculating the percentage of consistent responses.

5.2.3 Linearity

For immunoassay test kits that provide quantitative results, linearity will be assessed by performing a linear regression with the spiked contaminant concentration as the independent variable, and the individual immunoassay test kit result as the dependent variable. Individual replicate results for the four PT samples for each contaminant will be used in the linear regression. Linearity will be expressed in terms of the slope, intercept, and correlation coefficient (r). Linearity will not be determined for qualitative immunoassay test kits.

5.2.4 Method Detection Limit

The MDL for each quantitative immunoassay test kit will be assessed using results from seven replicate analyses of a sample spiked at a level of approximately five times the vendor-stated immunoassay test kit detection limit. The standard deviation of the seven replicate samples will be calculated using Equation 3. The MDL will be calculated using Equation 5:

$$MDL = t \times S \quad (5)$$

where t is the Student's t value for a 99% confidence level and S is the standard deviation of the seven replicate samples. An MDL will not be determined for qualitative immunoassay test kits, but the lowest concentration PT sample to consistently exhibit positive results will be evaluated and reported.

5.2.5 Cross Reactivity

Cross reactivity of the immunoassay test kits to the biological agent analogues will be assessed by evaluating the results for samples that contain only the analogues, but not the biological agents themselves. The percent of samples that indicate a positive response for the biological agent when only the analogue is present will be reported. Cross reactivity will be determined for qualitative and quantitative test kits that detect biological agents.

5.2.6 Matrix Interferences

The potential effect of the DW matrix on the immunoassay test kit performance will be evaluated qualitatively by comparing the results for the spiked and unspiked DW samples to those for the PT samples. For the quantitative tests, the accuracy and precision of the results will be compared. For the qualitative tests, the results indicating the correct or incorrect reporting of the presence of a contaminant will be evaluated.

5.2.7 False Positive/False Negative Responses

For both quantitative and qualitative immunoassay test kits, a false positive response is defined as a detectable or positive immunoassay test kit response when the ASTM DI water or

drinking water sample is spiked with a potential interferent or cross reactive compound or not spiked at all. A false negative response is defined as a non-detectable response or negative response when the sample was spiked with a contaminant at a concentration greater than the LOD. Reagent blanks, PT (contaminant, interferent, and cross-reactivity) samples, and DW samples will be included in the analysis.

5.3 Reporting

The data obtained in the verification test will be compiled separately for each immunoassay test kit, and the data evaluation methods described in Section 5.2 will be applied to each data set without comparison to any other immunoassay test kit. At no time will data for immunoassay test kits from different vendors be intercompared or ranked. Following completion of the data evaluation, a draft verification report will be prepared for each immunoassay test kit. The verification report will describe the verification test procedures and document the results. Each draft verification report will be submitted to the corresponding vendor for review and comment. Each draft report will be revised in response to the comments provided by the vendor. The revised reports will be submitted for external peer review, revised again to address the peer review comments, and submitted to EPA for final approval.

A verification statement will also be prepared for each immunoassay test kit. The verification statement is a two- to four page summary of the immunoassay test kit, test procedures, and results. The verification statement will follow the same review and revision process as the verification reports. Upon final approval by EPA, each verification statement will be signed by a senior Battelle manager and an EPA laboratory director. Final verification reports and statements are expected to be posted on the ETV website (<http://www.epa.gov/etv>), and original signed verification statements will be provided to the vendor.

6.0 QUALITY ASSURANCE /QUALITY CONTROL

The QA/QC activities associated with this verification test will focus primarily on sample preparation and handling, data recording and analysis, and reference laboratory analysis. An independent audit covering each of these areas will be performed by the Battelle Quality Manager to ensure the quality of the verification test.

6.1 Sample Chain-of-Custody Procedures

Sample custody will be documented throughout collection, shipping, and analysis of the samples from the water utility to Battelle laboratories. Similar documentation will be recorded for shipping and analysis of samples to the subcontract laboratories. Sample chain-of-custody procedures will be in accordance with the Battelle SOP D/F ASAT II-007¹², *Sample Receipt, Custody, and Handling*. The chain-of-custody form summarizes the samples collected and analyses requested. The chain-of-custody form will track sample release from the field to the Battelle laboratory, and from the Battelle laboratory to the subcontract laboratory. Each chain-of-custody form will be signed by the person relinquishing samples once that person has verified that the chain-of-custody form is accurate. The original sample chain-of-custody forms accompany the samples; the shipper will keep a copy. Upon receipt at the sample destination, chain-of-custody forms will be signed by the person receiving the samples once that person has verified that all samples identified on the chain-of-custody forms are present in the shipping container. Any discrepancies will be noted on the form and the sample receiver will immediately contact the Verification Test Coordinator to report missing, broken, or compromised samples.

6.2 Test Kit Calibration

Prior to analysis, the verification test coordinator will identify test kits that require calibration. All test kits will be calibrated according to vendor directed procedures. Other equipment provided by the vendor will be calibrated based on vendor requirements.

6.3 Equipment Calibration

For physio-chemical characterization and confirmation of interferences, analytical equipment will be calibrated by the subcontract laboratory according to the procedure specified in the standard method. Pipettes will be calibrated according to the procedure outlined in Battelle SOP No: VI-025¹³. Pipettes will be calibrated semiannually and the calibration service will

provide a calibration certificate.

6.4 QC of Stock Solutions Preparation and DW Characterization

In lieu of analytical methods for ricin and botulinum toxin, Battelle QA staff will provide oversight of the stock solution preparation for these contaminants. The concentration of the stock solutions of anthrax and their corresponding analogues will be confirmed by the plate enumeration method; additionally, each DW sample will undergo analysis by this method to confirm the absence of these bacteria. Method blanks and control spikes will be analyzed by the subcontract laboratory performing confirmation analysis on the calcium, magnesium, and humic and fulvic acid samples. Method blanks and control spikes will be analyzed with every batch of samples processed. Method blank and control spikes of interferences will be analyzed in accordance with standard methods and QC limits specified therein.

6.5 Audits

6.5.1 Technical Systems Audit

The Battelle Quality Manager will conduct a technical systems audit at least once during the course of the verification test. The purpose of this audit is to ensure that the verification test is being performed in accordance with this test/QA plan and the AMS QMP¹, and that all procedures described in this test/QA plan are being followed. This audit will review the standards and methods used, compare actual test procedures to those specified in this test/QA plan, and review data acquisition and handling procedures. An independent technical systems audit may also be performed by EPA Quality Management staff during the verification test at EPA's discretion.

Before using an outside laboratory to perform stock solution confirmation analyses, the Battelle Quality Manager will conduct an audit of the laboratory's quality documents. If there are areas of concern with the quality documents, the commercial laboratory will be notified, and if they are willing to adapt their procedures, the laboratory will still be used. If not, another laboratory will be selected.

6.5.2 Data Quality Audit

At least 10% percent of the data acquired during the verification test will be audited during the verification test. Battelle's Quality Manager will trace the data from its 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 will be checked.

6.6 QA/QC Reporting

Each assessment and audit will be documented in accordance with Section 3.3.4 of the AMS QMP¹. 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.

6.7 Corrective Action

During the course of any assessment or audit, the Battelle Quality Manager will inform the technical staff of any immediate corrective action that should be taken. If serious quality problems exist, the Battelle Quality Manager is authorized to stop work. 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 implement any necessary follow-up corrective action. The Battelle Quality Manager will ensure that follow-up corrective action has been taken.

7.0 HEALTH AND SAFETY

7.1 Standard/Test Sample Preparation

All handling of solid and highly concentrated aqueous solutions of contaminants and possible interferences will be done inside of a laboratory hood with hood sash set to the lowest height that still allows for safe manipulation of materials. The following guidelines should be adhered to:

- Personal protective equipment shall include safety glasses with side shields, a laboratory coat and nitrile lab gloves. Gloves shall be immediately changed if they become contaminated.
- All contaminated waste shall be handled as hazardous waste and sent out through Battelle Waste Operations.

7.2 Handling During Verification Testing

Laboratory and field handling of any solutions used during the verification test will be accomplished by taking the following precautions:

- All containers shall be stored and transported in double containment.
- Safety goggles, nitrile gloves with long cuffs, and a chemical resistant disposable lab coat shall be worn when handling either chemical. Gloves shall be immediately changed if they become contaminated.

7.3 Testing of Ricin, Botulinum, and Anthrax

Verification of these contaminants will be done following the safety procedures required at the MREF facility as noted in Section 3.3.

8.0 REFERENCES

1. Quality Management Plan (QMP) for the ETV Advanced Monitoring Systems Center, Version 4.0. EPA Environmental Technology Verification Program, prepared by Battelle, Columbus, Ohio, December, 2002.
2. Environmental Technology Verification Program Quality Management Plan, December 2002 (EPA/600/R-03/021).
3. Secondary Drinking Water Regulations: Guidance for Nuisance Chemicals, U.S. EPA Document, EPA-810-K-92-001, July 1992.
4. Battelle MREF X-054. Enumeration of BL-2 and BL-3 Bacterial Samples via the Spread Plate Technique. July 2003.
5. Code of Federal Regulations, Title 32, Chapter 5, Part 626, Biological Defense Safety Program, Technical Safety Requirements and Part 627, Biological Defense Safety Program, Technical Safety Requirements.
6. EPA Method 180.1. Turbidity (Nephelometric), Methods for the Determination of Inorganic Substances in Environmental Samples EPA-600-R-93-100. 1993.
7. American Public Health Association, et al. Standard Methods for Examination of Water and Wastewater. 19th Edition. 1997. Washington D.C.
8. EPA 600/4-79-020. Methods for Chemical Analysis of Water and Wastes. March 1983.
9. EPA/600/R-95-131. EPA Method 524.2. Purgeable Organic Compounds by Capillary Column GC/Mass Spectrometry. Methods for Determination of Organic Compounds in Drinking Water, Supplement III. August 1995.
10. EPA/600/R/R-95-131. EPA Method 552.2. Haloacetic Acids and Dalapon by Liquid-Liquid Extraction, Derivatization and GC with Electron Capture Detector. Methods for the Determination of Organic Compounds in Drinking Water, Supplement III. August 1995.

11. EPA Method 200.8. Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma Mass Spectrometry, Revision 5. 1994.
12. Battelle SOP D/F ASAT II-007. Sample Receipt, Custody and Handling. October 2000.
13. Battelle SOP VI-025. Operation, Calibration, and Maintenance of Fixed and Adjustable Volume Pipettes. January, 2003.