September 2004

Environmental Technology Verification Report

TETRACORE, INC.

BIOTHREAT ALERT® ANTHRAX, BOTULINUM TOXIN, AND RICIN IMMUNOASSAY TEST STRIPS

Prepared by
Battelle

Battelle
The Business of Innovation

Under a cooperative agreement with

EPA U.S. Environmental Protection Agency

ETV ETV ETV

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Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

Tetracore, Inc.
BioThreat Alert® Anthrax, Botulinum Toxin, and Ricin Immunoassay Test Strips

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Notice

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, has financially supported and collaborated in the extramural program described here. This document has been peer reviewed by the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation by the EPA for use.

Foreword

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the nation's air, water, and land resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development provides data and science support that can be used to solve environmental problems and to build the scientific knowledge base needed to manage our ecological resources wisely, to understand how pollutants affect our health, and to prevent or reduce environmental risks.

The Environmental Technology Verification (ETV) Program has been established by the EPA to verify the performance characteristics of innovative environmental technology across all media and to report this objective information to permitters, buyers, and users of the technology, thus substantially accelerating the entrance of new environmental technologies into the marketplace. Verification organizations oversee and report verification activities based on testing and quality assurance protocols developed with input from major stakeholders and customer groups associated with the technology area. ETV consists of six verification centers. Information about each of these centers can be found on the Internet at http://www.epa.gov/etv/.

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received funding to plan, coordinate, and conduct such verification tests for "Advanced Monitoring Systems for Air, Water, and Soil" and report the results to the community at large. Information concerning this specific environmental technology area can be found on the Internet at http://www.epa.gov/etv/centers/center1.html.

Acknowledgments

The authors wish to acknowledge the support of all those who helped plan and conduct the verification test, analyze the data, and prepare this report. We sincerely appreciate the contribution of drinking water samples from the New York City Department of Environmental Protection (Paul Bennett), the City of Orlando (Terri Slifko), and the Metropolitan Water District of Southern California (Paul Rochelle). Also, thank you to the Metropolitan Water District of Southern California for concentrating each drinking water sample. We would also like to thank Karen Bradham, U.S. EPA National Exposure Research Laboratory (NERL); Steve Allgeier, U.S. EPA Office of Water; Ricardo DeLeon, Metropolitan Water District of Southern California; and Stanley States, Pittsburgh Water and Sewer Authority, for their careful review of the test/QA plan and this verification report. Thanks go to Linda Sheldon, U.S. EPA NERL, for her review of the verification reports and statements.

Contents

Page

Figures

Tables

List of Abbreviations

AMS	Advanced Monitoring Systems
ATEL	Aqua Tech Environmental Laboratories, Inc.
Ca	calcium
CDC	Centers for Disease Control and Prevention
cfu	colony-forming units
COA	certificate of analysis
DI	deionized
DW	drinking water
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
HDPE	high-density polyethylene
L	liter
LOD	limit of detection
MB	method blank
Mg	magnesium
mg/L	milligram per liter
µL	microliter
mL	milliliter
PT	performance test
QA	quality assurance
QC	quality control
QMP	quality management plan
RPD	relative percent difference
SOP	standard operating procedure
TSA	technical systems audit

Chapter 1
Background

The U.S. Environmental Protection Agency (EPA) supports the Environmental Technology Verification (ETV) Program to facilitate the deployment of innovative environmental technologies through performance verification and dissemination of information. The goal of the ETV Program is to further environmental protection by accelerating the acceptance and use of improved and cost-effective technologies. ETV seeks to achieve this goal by providing high-quality, peer-reviewed data on technology performance to those involved in the design, distribution, financing, permitting, purchase, and use of environmental technologies.

ETV works in partnership with recognized testing organizations; with stakeholder groups consisting of buyers, vendor organizations, and permitters; and with the full participation of individual technology developers. The program evaluates the performance of innovative technologies by developing test plans that are responsive to the needs of stakeholders, conducting field or laboratory tests (as appropriate), collecting and analyzing data, and preparing peer-reviewed reports. All evaluations are conducted in accordance with rigorous quality assurance (QA) protocols to ensure that data of known and adequate quality are generated and that the results are defensible.

The EPA's National Exposure Research Laboratory and its verification organization partner, Battelle, operate the Advanced Monitoring Systems (AMS) Center under ETV. The AMS Center recently evaluated the performance of the Tetracore, Inc., BioThreat Alert® anthrax, botulinum toxin, and ricin immunoassay test kits. Immunoassay test kits were identified as a priority technology category for verification through the AMS Center stakeholder process.

Chapter 2
Technology Description

The objective of the ETV AMS Center is to verify the performance characteristics of environmental monitoring technologies for air, water, and soil. This verification report provides results for the verification testing of the Tetracore BioThreat Alert® test strips (Figure 2-1). The following is a description of the Tetracore BioThreat Alert® test strips based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The BioThreat Alert®test strip from Tetracore, Inc., is a lateral flow immunochromatographic device that uses two antibodies in combination to specifically detect target antigen in solution. One of the specific antibodies is labeled with a colloidal gold derivative. Samples applied to the BioThreat Alert® test strips mix with the colloidal gold-labeled antibody and move along the strip membrane by capillary action. The second specific antibody captures the colloidal gold-labeled antibody and bound target. When a sufficient amount of target antigen is present, the colloidal gold label accumulates in the sample ("S") window on the test strip, forming a visible reddish-brown colored line. As an internal control, a second band in the control ("C") window indicates that the test strip functioned properly. Two bands or colored lines (in the "S" and "C" windows) are required for a positive result determination.

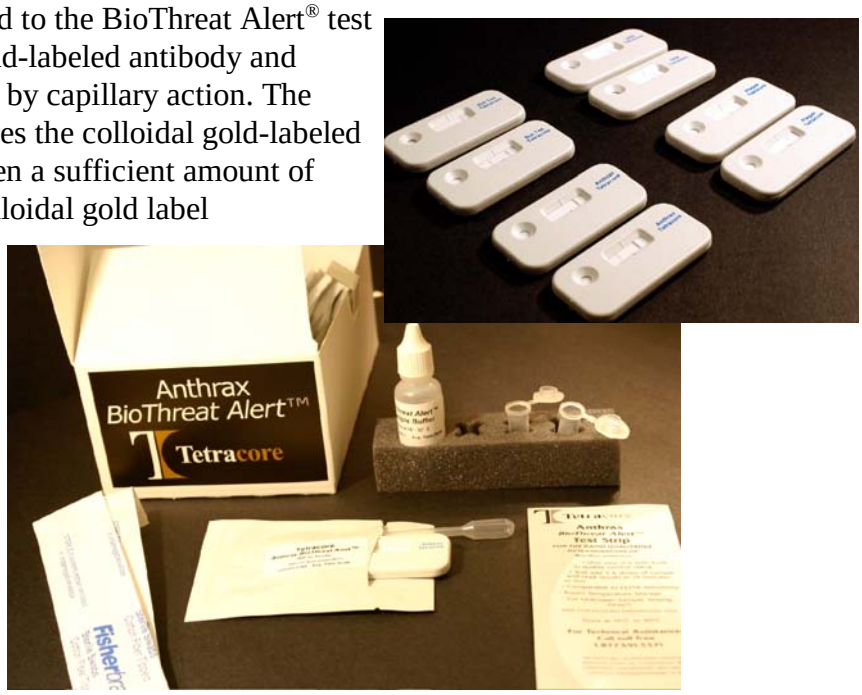

Figure 2-1. Tetracore BioThreat Alert®

Twenty-five individually packaged BioThreat Alert® test strips (including a disposable pipette) are provided in a small box. In addition to the test strips, the box contains several small plastic vials, 25 mL of sample buffer, and step-by-step instructions. To complete a test on a liquid sample, the sample is mixed with the provided buffer, and five or six drops are added to the sample well of the BioThreat Alert® test strip. A positive result is indicated by the appearance of a colored line in the test

window of the test strip and can be read visually or with a reader. During this verification test, a reader was used to make the determination of a positive or negative result.

One kit of 25 strips including sample buffer, instruction brochure, and vials needed for sampling costs approximately $625. The Alexeter strip reader used during this verification test costs approximately $4,000.

Chapter 3
Test Design and Procedures

3.1 Introduction

The objective of this verification test of immunoassay test kits was to evaluate their ability to detect specific biological toxins and agents in water samples and to determine their susceptibility to specific interferents added to pure water and to interferents inherently present in several drinking water (DW) samples. The detection devices are based on immunological interactions, where specific antibodies are used to detect antigens or contaminants of interest. For the BioThreat Alert® test strips, the presence of contaminants is indicated by the appearance of a colored line within 15 minutes of the application of a water sample. The single-use test strips detect only one contaminant at a time.

During this verification test, the BioThreat Alert® test strips were subjected to various concentrations of anthrax spores, botulinum toxin (Types A and B), and ricin in American Society for Testing and Materials Type II deionized (DI) water. Table 3-1 shows the contaminants and information about their detection, including the vendor-stated limit of detection (LOD), the lethal dose concentrations, and the source. The BioThreat Alert® test strips also were used to analyze contaminant-fortified DW samples that were collected from four water utilities that use a variety of treatment methods. The effect of interferents was evaluated by analyzing individual solutions of organic acids (humic and fulvic), magnesium (Mg) and calcium (Ca) in DI water both with and without the addition of the contaminants using the BioThreat Alert® test strips. In addition, specificity was evaluated by exposing the BioThreat Alert® test strips to a potentially cross-reactive compound or spore for each target contaminant.

Table 3-1. Lethal Dose and Source of Contaminants

Contaminant	Vendor-Stated LOD	Lethal Dose Concentration[a]	Source of Contaminant
Bacillus anthracis Ames Strain (anthrax)	1×10^5 spores/mL	200 spores/mL[1]	Battelle and U.S. Army Dugway Proving Ground
Botulinum toxin Types A and B	0.01 mg/L	0.3 mg/L[2]	Metabiologics, Inc. (Madison, Wisconsin)
Ricinus communis Agglutinin II (ricin)	0.035 mg/L	15 mg/L[3]	Vector Laboratories, Inc. (Burlingame, California)

[a] The lethal dose of each contaminant was determined by calculating the concentration at which 250 mL of water would probably cause the death of a 154-pound person based on human mortality data.
mL = milliliter
mg/L = milligrams per liter

The verification test for the BioThreat Alert® test kits was conducted from January 14 through April 23, 2004, according to procedures specified in the *Test/QA Plan for Verification of Immunoassay Test Kits*.[4] This test was conducted at Battelle laboratories in Columbus and West Jefferson, Ohio. Aqua Tech Environmental Laboratories, Inc. (ATEL) of Marion, Ohio, performed physicochemical characterization for each DW sample to determine the following parameters: turbidity; concentration of dissolved and total organic carbon; specific conductivity; alkalinity; concentration of Mg and Ca; pH; hardness; and concentration of total organic halides, trihalomethanes, and haloacetic acids. Battelle confirmed the presence of anthrax spores using plate enumeration.

The BioThreat Alert® test strips were evaluated for the following parameters:

- Qualitative contaminant presence/absence
- False positive/false negative response
  - Interferents
  - DW matrix effects
  - Cross-reactivity
- Consistency
- Lowest detectable concentration
- Other performance factors
  - Field portability
  - Ease of use
  - Sample throughput.

3.2 Test Samples

Tables 3-2 and 3-3 summarize the samples analyzed for each contaminant. The ability of the BioThreat Alert® test strips to individually detect various concentrations of anthrax spores, botulinum toxin, and ricin was evaluated by analyzing performance test (PT) and DW samples. PT samples included DI water fortified with either the target contaminant, an interferent, both, or only a cross-reactive species. DW samples were analyzed using the BioThreat Alert® test strips with and without the addition of each target contaminant. All the samples listed in the test/QA plan were initially analyzed. As discussed below, additional concentration levels and sample types were analyzed to more thoroughly evaluate the performance of the BioThreat Alert® test strips.

Table 3-2. Performance Test Samples

Type of PT Sample	Sample Characteristics	Approximate Concentrations
Contaminant-only	Anthrax spores	200 to 10^{10} spores/mL[(a)]
	Botulinum toxin Type A	0.0 to 0.5 mg/L
	Botulinum toxin Type B	0.01 to 0.5 mg/L
	Ricin	0.035 to 15 mg/L
Interferent	Contaminants in 46 mg/L Ca and 18 mg/L Mg	Anthrax - 10^6 spores/mL Botulinum toxin (Type B only) - 0.1 mg/L Ricin - 0.4 mg/L
	Contaminants in 230 mg/L Ca and 90 mg/L Mg	Anthrax - 10^6 and 10^8 spores/mL Botulinum toxin (Types A and B) - 0.1 mg/L Ricin - 0.4 mg/L
	Contaminants in 0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Anthrax - 10^6 spores/mL Botulinum toxin (Type B only) - 0.1 mg/L Ricin - 0.4 mg/L
	Contaminants in 2.5 mg/L humic acid and 2.5 mg/L fulvic acids	Anthrax - 10^6 and 10^8 spores/mL Botulinum toxin (Types A and B) - 0.1 mg/L Ricin - 0.4 mg/L
Potentially Cross-reactive	*Bacillus thuringiensis* (anthrax analogue)	10^5 spores/mL
	Lipopolysaccharide (botulinum toxin analogue)	0.1 mg/L
	Lectin from soybean (ricin analogue)	0.4 mg/L

[(a)] This concentration range includes all samples analyzed, including spores preserved with and without phenol, spores prepared at Battelle and at Dugway Proving Ground, and vegetative anthrax cells.

Table 3-3. Drinking Water Samples

Drinking Water Sample Description				Approximate Contaminant Concentrations		
Water Utility	**Water Treatment**	**Source Type**	**Conc. / Unconc.**	**Anthrax (spores/mL)**	**Botulinum Toxin (mg/L)**	**Ricin (mg/L)**
Metropolitan Water District of California (CA)	filtered chloraminated	surface	conc.	unspiked 10^6 10^8	unspiked 0.1 (Type B) 0.1 (Type A)	unspiked 0.4
New York City, New York (NY)	unfiltered chlorinated	surface	conc.	unspiked 10^6 10^8	unspiked 0.1 (Type B) 0.1 (Type A)	unspiked 0.4
Metropolitan Water District of California (CA)	filtered chloraminated	surface	unconc.	unspiked 10^6	unspiked 0.1 (Type B)	unspiked 0.4
New York City, New York (NY)	unfiltered chlorinated	surface	unconc.	unspiked 10^6	unspiked 0.1 (Type B)	unspiked 0.4
Columbus, Ohio (OH)	filtered chlorinated	surface	both	unspiked 10^6	unspiked 0.1 (Type B)	unspiked 0.4
Orlando, Florida (FL)	filtered chlorinated	ground	both	unspiked 10^6	unspiked 0.1 (Type B)	unspiked 0.4

3.2.1 Performance Test Samples

The contaminant-only PT samples were prepared in DI water using certified standards of ricin and botulinum toxin. Reference methods were not available for quantitative confirmation of the botulinum toxin and ricin test solutions so certificates of analysis (COA) and QA oversight of solution preparation were used to confirm their concentrations. Anthrax PT samples also were prepared in DI water using anthrax spores prepared and characterized by Battelle using standard methods. All test samples were prepared from the standards or stock solutions on the day of analysis. Spores obtained from Dugway Proving Ground were prepared there and then enumerated by Battelle during this verification test.

Initially, the test/QA plan called for the analysis of PT samples with concentrations including the lethal dose; the vendor-stated LOD; and approximately 5, 10, and 50 times the LOD. These samples were analyzed using the BioThreat Alert® test strips. Preliminary results indicated that anthrax was not detectable; therefore, the original test/QA plan was amended to include the analysis of higher concentration levels of anthrax, as well as anthrax spores that were never preserved in phenol, a second source of anthrax spores, and vegetative anthrax cells. This testing and the subsequent results are fully described in Section 6.1.

The interferent PT samples consisted of samples of humic and fulvic acids isolated from the Elliott River (obtained from the International Humic Substances Society) and Ca and Mg

(prepared from their chlorides), each spiked into DI water at two concentration levels. These solutions were analyzed both with the addition of each target contaminant at one concentration level and without the addition of any target contaminant. To be able to evaluate the susceptibility of the BioThreat Alert® test strip to false negative results due to interferents, the test/QA plan was amended to include the fortification of detectable concentrations of anthrax spores into interferent solutions.

The last type of PT sample was a cross-reactivity check sample to determine whether the test strips produce false positive results in response to similar analytes. *Bacillus thuringiensis* (for anthrax), lectin from soybean (for ricin), and lipopolysaccharide (for botulinum toxin) are chemically or biologically similar to the specified targets. Solutions of these were prepared in DI water at concentrations similar to the vendor-stated LOD of the test kits for the specified targets and analyzed using the appropriate BioThreat Alert® test strip.

In most cases, three replicates of each PT sample were analyzed. In some instances, the anthrax test samples were analyzed less than three times, depending on the number of test strips available for the analysis. A total of 186 PT samples was analyzed by the BioThreat Alert® test strips for this test. The results provided information about how well the BioThreat Alert® test strips detected the presence of each contaminant at several concentration levels, the consistency of the responses, and the susceptibility of the BioThreat Alert® test strips to some selected interferents and possibly cross-reactive species.

3.2.2 Drinking Water Samples

Table 3-3 lists the DW samples collected from four geographically distributed municipal sources to evaluate the performance of the BioThreat Alert® with various sample matrices. These samples were unique in terms of their source and treatment and disinfection process. All collected samples were finished DW either ready for the distribution system or from within the distribution system.

Approximately 120 L of each of the DW samples were collected in pre-cleaned high-density polyethylene (HDPE) containers. All but 20 L of the DW samples were shipped to the Metropolitan Water District of Southern California, dechlorinated with sodium thiosulfate, and then concentrated through ultra-filtration techniques to a final volume of 250 mL. This concentration factor was selected because it is the goal of an EPA onsite ultra-filtration method which is currently being developed. The remaining 20 L of each DW sample was shipped to ATEL for water quality analysis. Each DW sample (non-concentrated and concentrated) was analyzed without adding any contaminant, as well as after fortification with individual contaminants at a single concentration level. A total of 156 DW samples was analyzed by the BioThreat Alert® test strips for this test.

3.2.3 Quality Control Samples

In addition to the 342 PT and DW samples analyzed, 41 method blank (MB) samples consisting of DI water also were analyzed to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination were introduced during the analysis procedures.

The Tetracore reader produced an error message if a control line in the result window on the BioThreat Alert® test strips did not appear, according to specifications, during the analysis of each sample. If the Tetracore reader produced an error message instead of a result, that test strip was discarded and a new test strip was used. Such an error message occurred just two times during the verification test. Because of this control feature, other positive control samples were not analyzed.

3.3 Test Procedure

3.3.1 Laboratory Testing

The scope of this verification test required that most of the test samples be analyzed within Battelle laboratories staffed with technicians trained to safely handle anthrax, botulinum toxin, and ricin. Each day, fresh samples were prepared from standards or stock solutions in either DI water, an interferent matrix, or a DW matrix. Each sample was prepared in its own container and labeled only with a sample identification number that also was recorded in a laboratory record book along with details of the sample preparation. Prior to the analysis of each sample, the verification staff recorded the sample identification number on a sample data sheet; then, after the analysis was complete, the result was recorded on the sample data sheet. Three replicates of each test sample were analyzed. The BioThreat Alert® test strip testing procedure included the following steps for analyzing liquid samples for the presence of anthrax spores, botulinum toxin, or ricin: (1) sample buffer was added to the 0.5-mL mark on the sample vial; (2) the liquid sample was added to the buffer until the solution reached the 1-mL mark on the sample vial; (3) the cap was closed, and the vial was shaken vigorously for approximately 10 seconds; (4) the disposable pipette was used to remove liquid from the sample vial; (5) 5 or 6 drops were placed in the sample port of the BioThreat Alert® test strip; and (6) after 15 minutes, the test strip was placed in the reader and the reader's instructions were followed to obtain the result. The reader was operated by turning it on, entering the sample identification, and pressing the "enter" button. The test strip was then taken into the reader, and a positive or negative result was generated within approximately 2 minutes. Each result, along with the time, date, and sample identification was printed using a printer provided by Tetracore.

3.3.2 Non-Laboratory Testing

Because the toxic nature of the contaminants did not permit their use outside special laboratory facilities, MB samples were analyzed at a non-laboratory location to evaluate the BioThreat Alert® test strip performance and ease of use outside of the laboratory. Both a trained technician and a non-technical/untrained, first-time user performed analyses at the non-laboratory location. The purpose of these analyses was to test the performance of the BioThreat Alert® test strips in a non-laboratory setting, not to evaluate thoroughly the effect of changing conditions such as temperature and humidity on the BioThreat Alert® test strips. The non-technical/untrained, first-time user was guided by only the manual or by vendor instructions. The operators for the rest of the verification test had undergraduate degrees in the sciences or equivalent work experience and either participated in a training session provided by the vendor prior to the verification test or were trained by a vendor-trained operator.

3.3.3 Drinking Water Characterization

An aliquot of each DW sample, collected as described in Section 3.2.2, was sent to ATEL prior to concentration to determine the following water quality parameters: turbidity; concentration of dissolved and total organic carbon; conductivity; alkalinity; pH; concentration of Ca and Mg; hardness; and concentration of total organic halides, trihalomethanes, and haloacetic acids.

Table 3-4 lists the methods used to characterize the DW samples, as well as the characterization data from the four water samples collected as part of this verification test. Water samples were collected and water quality parameters were measured by ATEL in January. Samples were then transported and test strips were analyzed from January through March. Because of this, some of the water quality parameters may have changed from the time of analysis by ATEL until testing with the BioThreat Alert® test strips.

Table 3-4. ATEL Water Quality Characterization of Drinking Water Samples

			Sources of Drinking Water Samples			
Parameter	**Unit**	**Method**	**Columbus, Ohio (OH DW)**	**Orlando, Florida (FL DW)**	**New York City, New York (NY DW)**	**MWD, California (CA DW)**
Turbidity	NTU	EPA 180.1[5]	0.2	0.5	1.3	0.1
Dissolved organic carbon	mg/L	SM 5310[6]	2	2	2	2
Total organic carbon	mg/L	SM 5310[6]	2	2	2	2
Specific conductivity	µS/cm^2	SM 2510[6]	357	325	85	740
Alkalinity	mg/L	SM 2320[6]	55	124	4	90
pH		EPA 150.1[7]	7.33	7.93	6.80	7.91
Calcium	mg/L	EPA 200.8[8]	42	41	5.7	35
Magnesium	mg/L	EPA 200.8[8]	5.9	8.4	19	1.5
Hardness	mg/L	EPA 130.2[7]	125	137	28	161
Total organic halides	µg/L	SM 5320[6]	360	370	310	370
Trihalomethanes	µg/L/ analyte	EPA 524.2[9]	26.9	80.9	38.4	79.7
Haloacetic acids	µg/L/ analyte	EPA 552.2[10]	23.2	41.1	40.3	17.6

NTU = nephelometric turbidity unit
MWD = Metropolitan Water District
µS/cm^2 = microSiemens per square centimeter

Chapter 4
Quality Assurance/Quality Control

Quality assurance/quality control (QC) procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[11] and the test/QA plan[4] for this verification test.

4.1 Sample Chain-of-Custody Procedures

Sample custody was documented throughout collection, shipping, and analysis of the samples. Sample chain-of-custody procedures were in accordance with ASAT II-007, *Standard Operating Procedure for Chain of Custody for Dioxin/Furan Analysis*. The chain-of-custody forms summarized the samples collected and analyses requested and were signed by the person relinquishing samples once that person had verified that the custody forms were accurate. The original sample custody forms accompanied the samples; the shipper kept a copy. Upon receipt at the sample destination, sample custody forms were signed by the person receiving the samples once that person had verified that all samples identified on the custody forms were present in the shipping container.

4.2 Equipment/Calibration

The BioThreat Alert® test strips and all appropriate reagents and supplies specific for the detection of anthrax, botulinum toxin, and ricin were provided to Battelle by the vendor. These test kits, each containing an internal control line, required no calibration. For DW characterization and confirmation of the possible interferents, analytical equipment was calibrated by ATEL according to the procedures specified in the appropriate standard methods. Pipettes used during the verification test were calibrated according to Battelle Standard Operating Procedure (SOP) VI-025, *Operation, Calibration, and Maintaining Fixed and Adjustable Volume Pipettes.*

4.3 Characterization of Contaminant Stock Solutions

4.3.1 Characterization of Botulinum Toxin and Ricin

Certificates of analysis for botulinum toxin and ricin were provided by the supplier. Because standard reference methods do not exist, the concentration of botulinum toxin and ricin were not

independently confirmed. The COAs stated that the ricin standard (Vector Laboratories, Inc., Burlingame, California) had a concentration of 1,000 mg/L and the botulinum toxin standards (Metabiologics, Inc., Madison, Wisconsin) had concentrations of 2,000 mg/L for Type B and 1,000 mg/L for Type A. Test samples containing these contaminants were prepared by diluting aliquots of these stock solutions with DI water.

4.3.2 Characterization of Anthrax Spores

Multiple sources and forms of the Ames strain of *Bacillus anthracis* (anthrax) were evaluated during this verification test. The primary source was a lot of spores prepared by Battelle and stored in a 1% stock solution of phenol in water as a means to prevent vegetative cell growth. This lot of spores is referred to in this report as Battelle-prepared, phenol-preserved. Prior to testing, an aliquot of the stock solution described above was centrifuged, the phenol/water solution was removed, and the spores were reconstituted with DI water. This process was repeated two times to ensure that the spores were suspended only in DI water. This lot of spores was characterized with an 11-step characterization process prior to use in the verification test. For confidentiality reasons, Table 4-1 gives the outcome of only five of the characterization parameters, as well as the location at which each step was performed. These characterization steps were performed when this lot of spores was prepared in September 2003. It should be noted that, once a stock solution of spores is characterized, less concentrated solutions of spores can be prepared from the stock solution without questioning the integrity of the spores. This lot of spores met all 11 acceptance criteria. Two parts of the characterization process—DNA sequencing and gene identification—were performed by Dr. Alex Hoffmaster at the Epidemiologic Investigations Laboratory, Meningitis and Special Pathogens Branch of the Centers for Disease Control and Prevention (CDC). The CDC analyses confirmed that the spores were Ames strain anthrax spores, and the guinea pig LD_{50} study confirmed their virulence. The stock solution of spores was enumerated after preparation to determine its original concentration. In addition, a vegetative cell analysis showed that the stock solution was 99.94% anthrax spores. Because at least one spore is needed to spur the growth of a colony during an enumeration, the concentrations determined represented a minimum concentration of spores. Care was taken to spread the samples to avoid clumping; but, if clumping occurred, the spore concentrations would only be higher than shown in the data tables.

Table 4-1. Characterization Information for Battelle Preparation of Anthrax Spores

Characterization	Outcome	Analysis Performed By
% vegetative cells	0.06%	Battelle
Viable spore count	5.26×10^9	Battelle
Guinea pig 10 day LD_{50}	10 spores	Battelle
DNA fingerprinting	MLVA Genotype 62	CDC
PA gene sequencing	Protective Antigen Type I	CDC

Another lot of anthrax spores prepared by Battelle was used during the verification test. This lot had been prepared in the same way as the other, but it had never been stored in phenol or any other preservative. The second lot had been subjected only to enumeration to determine the concentration. Test solutions were made from this stock solution to investigate whether the phenol preservation was affecting the sensitivity of the test strips.

Similarly, a lot of anthrax spores from Dugway Proving Ground was obtained and used to investigate the sensitivity of BioThreat Alert® test strips to a different spore preparation (referred to as Dugway-prepared in this report). Again, enumeration was the only characterization step performed on this lot of spores.

A stock solution of vegetative anthrax cells also was prepared and used during this verification test. This solution was prepared by harvesting vegetative cells from an enumeration of the Battelle-prepared, phenol-preserved spores, placing them into solution, and then enumerating to determine the solution's concentration. No further characterization was performed on these vegetative cells. Solutions of these cells were used to determine the sensitivity of the BioThreat Alert® test strips to vegetative cells.

Regardless of the source and type of anthrax stock solution used to make test samples, its concentration was confirmed by a plate enumeration method. This was done within 24 hours of any stock solution being used for test sample preparation and is described in Battelle SOP MREF X-054, *Enumeration of BL-2 and BL-3 Bacteria Samples Via the Spread Plate Technique*. In addition, four times during the verification test the serial dilution method was validated by enumerating the PT samples. For example, for a 10^9 spores/mL sample to be enumerated, the method requires that it be diluted to at least 10^3 spores/mL so 100 µL of sample will provide a countable number of spores on a culture plate. Therefore, if 100 µL of the 10^3 spores/mL solution provided the correct number of spores to the plate, the concentration of every serial dilution made to obtain that concentration was confirmed.

4.3.3 Anthrax Enumeration Data

Table 4-2 gives the results of all plate enumerations performed throughout the verification test on anthrax solutions prepared in DI water. The data from enumerations to validate the serial dilution method are also given in Table 4-2. The expected concentration, as determined from a previous enumeration (if available), the actual concentration, and the relative percent difference between the two are given in the table. Relative percent difference (*RPD*) is determined using the following equation, where *E* is the expected concentration and *A* is the actual concentration as determined by the enumeration.

$$RPD = \frac{|E - A|}{E} \times 100\%$$

For the Battelle-prepared, phenol-preserved spores, only one enumeration resulted in a concentration that was more than 25% different from the expected concentration. The average

Table 4-2. Anthrax Enumeration Data for PT Samples

Spore Solution Description (units)	Date	Expected Concentration	Actual Concentration[(a)]	RPD
Battelle-prepared, phenol-preserved stock solution **(10^8 spores/mL)**	January 28	53	58	9
	January 28	58	53	9
	January 30	53	61	15
	February 2	61	53	14
	February 10	61	82	55
	February 26	82	63	23
	March 1	63	67	5
	March 23	67	57	14
Battelle-prepared, phenol-preserved serial dilution validations **(10^4 spores/mL)**	January 28	10	7.8	22
	January 30	40	32	20
	March 2	10	7.7	24
	March 23	1,000	992	1
Battelle-prepared, non-phenol-preserved **(10^8 spores/mL)**	February 5	Unknown	14	NA
	February 12	14	106	657
Vegetative anthrax **(10^4 cfu/mL)**	March 23	Unknown	26	NA
	March 24	260	350	35
Dugway-prepared **(10^6 spores/mL)**	March 22	Unknown	666	NA
	March 23	0.010	0.0081	19
	March 24	10	8.0	20

[(a)] Each enumeration involved the development of three to five plates. The average, standard deviation, and relative standard deviation for each set of Battelle-prepared, phenol-preserved enumeration data were determined, and the average relative standard deviation of all enumerations was calculated to estimate the variability in the enumeration process, which was 15%.

NA = not applicable.

concentration of the Battelle stock solution was 6×10^9 spores/mL (ranging from 5.3×10^9 to 8.2×10^9 spores). Over the two-month period that the stocks were used and the enumerations performed, the relative standard deviation of the eight results was 15%. The accuracy and precision of these enumerations indicate that the concentration of the spore stock solution was consistent over several months and was usually close to the expected concentration. The serial dilution validation data confirm that the PT samples containing the Battelle-prepared, phenol-preserved spores were prepared accurately at various concentration levels. Also shown in Table 4-2 are the enumerations performed to determine the concentration of the alternate Battelle preparation of spores (Battelle-prepared, non-phenol-preserved), vegetative anthrax cells, and a stock solution of spores obtained from Dugway Proving Ground. Notable among these results was the significant increase in concentration of the alternative Battelle-prepared stock solution from February 5 to February 12, 2004. Because this lot of spores was used only to

determine the effect of phenol preservation on the sensitivity of the BioThreat Alert® test strips, this observation was not fully investigated. For enumerations with unknown expected concentrations, the concentration of that particular solution or the stock from which it had been prepared had not previously been determined.

Table 4-3 gives the enumeration data for all of the interferent PT (shaded) and DW samples that were spiked with anthrax spores. For possible interferent samples and samples prepared in DW, the addition of spores was confirmed by enumeration for at least one sample representing each matrix. The results of the DW samples enumerated in late January and early February indicated that the relative difference between the expected concentration and the actual concentration ranged from 17 to 96%. The larger percent differences for the DW samples as compared with the PT samples were not a surprise, considering that DW is presumably an interferent-prone matrix. These data suggest that spore health is dependent on whether the solution is in DI water or DW. However, the effect of DW on spore health seemed to be less significant when the concentration of spores was higher. For example, in March, when the DW and interferent samples were spiked with higher concentrations of anthrax spores, the difference between the expected concentration and the actual concentration for the interferent samples was between 0 and 21% and for the DW samples between 7 and 55%. Enumerations were performed to characterize the concentration of spores in each sample matrix. For each test matrix, spores were enumerated within a day of testing. In the Chapter 6 tables, the actual concentrations of the test samples have been corrected for the result of the appropriate enumeration for that sample. Because not every test sample was enumerated and some of the test samples were the result of dilutions of enumerated samples, not every actual concentration will be represented directly in Table 4-2 or Table 4-3.

The concentrations of the possible cross-reactive interferents of soybean lectin (analogue of ricin) and lipopolysaccharide (analogue of botulinum toxin) were not confirmed independent of the COA received from the supplier because of the lack of available analytical methodologies for these analytes. Samples containing *Bacillus thuringiensis* (analogue of anthrax) were confirmed by the same enumeration method used for anthrax and were approximately an order of magnitude less than expected because some spores were lost during washing with water. Because the lowest detectable concentration of anthrax was much more concentrated than Tetracore, Inc., had claimed, additional samples containing higher concentration levels of anthrax were prepared and analyzed. Additional resources were not expended to determine the cross-reactivity of *Bacillus thuringiensis* at comparable concentration levels.

4.4 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) to ensure that the verification test was performed in accordance with the test/QA plan[4] and the AMS Center QMP.[11] As part of the audit, the Battelle Quality Manager reviewed the standards and methods used, compared actual test procedures with those specified in the test/QA plan,[4] and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Battelle Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are permanently stored with the Battelle Quality Manager.

Table 4-3. Anthrax Enumeration Results for Fortified Interferent and Drinking Water Samples

Sample Description	Date (2004)	Expected Concentration (10^5 spores/mL)	Actual Concentration[(a)] (10^5 spores/mL)	RPD
Conc. CA DW	January 28	10	0.38	96
Conc. CA DW	January 30	100	8.7	91
Unconc. CA DW	January 30	40	8	80
0.5 mg/L OC	February 2	15	16	9
2.5 mg/L OC	February 3	15	16	9
230 mg/L Ca 90 mg/L Mg	February 3	15	5.6	63
46 mg/L Ca 18 mg/L Mg	February 3	15	8.3	45
Conc. CA DW	February 3	15	6.9	54
Unconc. CA DW	February 3	15	6.5	57
Conc. OH DW	February 3	15	5.7	62
Unconc. OH DW	February 3	15	6.9	54
Conc. NY DW	February 3	15	13	17
Unconc. NY DW	February 3	15	12	21
Conc. FL DW	February 3	15	9.1	39
Unconc. FL DW	February 3	15	7.5	50
Conc. NY DW	March 3	1,000	933	7
Conc. CA DW	March 3	1,000	1,100	10
2.5 mg/L OC	March 3	1,000	993	1
230 mg/L Ca 90 mg/L Mg	March 3	1,000	1,000	0
2.5 mg/L OC	March 23	1,000	962	4
Conc. CA DW	March 23	1,000	448	55
230 mg/L Ca 90 mg/L Mg	March 24	1,000	788	21
Conc. NY DW	March 24	1,000	486	51

OC = Organic carbon (humic and fulvic acids)
Shading on table distinguishes the interferent and cross-reactivity PT samples from the DW samples.
[(a)] The uncertainty of the enumeration technique is approximately 15%.

4.5 Audit of Data Quality

At least 10% of the data acquired during the verification test was audited. Battelle's Quality Manager or designee traced the data from the initial acquisition, through reduction and statistical analysis, to final reporting, to ensure the integrity of the reported results. All calculations performed on the data undergoing the audit were checked.

4.6 QA/QC Reporting

Each internal assessment and audit was documented in accordance with Sections 3.3.4 and 3.3.5 of the QMP for the ETV AMS Center.[11] Once the assessment report was prepared, the Battelle Verification Test Coordinator responded to each potential problem and implemented any necessary follow-up corrective action. The Battelle Quality Manager ensured that follow-up corrective action was taken. The results of the TSA were sent to the EPA.

4.7 Data Review

Records generated in the verification test were reviewed before they were used to calculate, evaluate, or report verification results. Table 4-4 summarizes the types of data recorded. The review was performed by a technical staff member involved in the verification test, but not the staff member who originally generated the record. The person performing the review added his/her initials and the date to a hard copy of the record being reviewed.

Table 4-4. Summary of Data Recording Process

Data to Be Recorded	Responsible Party	Where Recorded	How Often Recorded	Disposition of Data
Dates and times of test events	Battelle	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
Sample collection and preparation information, including chain-of-custody	Battelle	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
Detection device procedures and sample results	Battelle	ETV data sheets	Throughout test duration	Manually incorporated in data spreadsheets
Anthrax enumeration data	Battelle	Enumeration data forms	With every enumeration	Manually incorporated in data spreadsheets
Reference method procedures and sample results	ATEL	Data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets and reported to Battelle

Chapter 5
Statistical Methods and Reported Parameters

The methods presented in this chapter were used to verify the performance parameters listed in Section 3.1. The BioThreat Alert® test strips produce qualitative results; i.e., they indicate only the presence or absence of a contaminant, not a measure of the concentration present. Therefore, the data evaluation methods were used in that context.

5.1 Qualitative Contaminant Presence/Absence

Accuracy was assessed by reporting the number of positive results out of the total number of samples tested for the BioThreat Alert® test strips at each concentration level of contaminant-only PT sample tested for anthrax spores, botulinum toxin, and ricin.

5.2 False Positive/Negative Responses

A false positive response was defined as a positive response when the DI water or DW sample was spiked with a potential interferent, a cross-reactive compound, or not spiked at all. A false negative response was defined as a negative response when any sample was spiked with a contaminant at a concentration greater than the lowest detectable concentration of the test strip for each analyte in DI water. Interferent PT samples, cross-reactivity PT samples, and DW samples were included in the analysis. The number of false positive and negative results is reported.

5.3 Consistency

The reproducibility of the results was assessed by calculating the percentage of individual test samples that produced positive or negative results without variation within replicates.

5.4 Lowest Detectable Concentration

The lowest detectable concentration for each contaminant was determined to be the concentration level at which at least two out of the three replicates generated positive responses. These concentration levels are determined for each target contaminant in solutions of DI water.

5.5 Other Performance Factors

Aspects of the instrument performance such as ease of use, field portability, and sample throughput are discussed in Section 6. Also addressed are qualitative observations of the verification staff pertaining to the performance of the BioThreat Alert® test strips.

Chapter 6
Test Results

6.1 Qualitative Contaminant Presence/Absence

The responses for the BioThreat Alert® test strips using the contaminant-only PT samples containing anthrax, botulinum toxin, and ricin are discussed in the following sections. The BioThreat Alert® test strips provide indication of only a positive or negative response based on whether or not a line appears in the left half (sample) of the absorbent strip window after a liquid test sample is applied. A line appears in the right half (control) after every test sample regardless of whether or not the target contaminant is present. For this verification test, Tetracore, Inc., instructed Battelle to use an electronic reader to determine whether or not that line appeared.

6.1.1 Anthrax

The results obtained for the performance test samples containing anthrax spores are given in Table 6-1a. The first five concentration levels listed were initially analyzed, and the results indicated that none of those samples (up to 50 times the vendor-stated LOD) produced detectable results. The Battelle-prepared, phenol-preserved serial dilution validation enumeration on January 30 (1×10^5 spores/mL expected) was a part of the serial dilution process to make all five of these PT samples, so the results of this enumeration confirm the concentration of spores in these samples. After discussions with Tetracore, Inc., the following speculative explanations for these results were considered:

1. The target proteins on the spore's surface may have been stripped off or chemically altered by phenol in the storage solution. (The absence or alteration of these proteins would probably decrease the sensitivity of the BioThreat Alert® test strips to the affected spores.)

2. The sensitivity of the BioThreat Alert® test strips to anthrax spores is dependent on the method used to prepare the spores; therefore, the spores prepared at Battelle may result in decreased responsiveness compared with spores prepared elsewhere.

Table 6-1a. Anthrax Contaminant-Only PT Sample Results

Purpose of Analysis	Actual Fortified Concentration[a]	Anthrax Description	Prep Location	Phenol-Preserved	Positive Results Out of Total Replicates
Original test/QA plan PT samples	200 spores/mL[b]	Spores	Battelle	Yes	0/3
	8×10^4 spores/mL	Spores	Battelle	Yes	0/3
	4×10^5 spores/mL	Spores	Battelle	Yes	0/3
	8×10^5 spores/mL	Spores	Battelle	Yes	0/3
	4×10^6 spores/mL	Spores	Battelle	Yes	0/3
Investigation of phenol storage of spores	5×10^6 spores/mL	Spores	Battelle	No	0/3
	1×10^9 spores/mL	Spores	Battelle	No	0/3
	8×10^8 spores/mL	Spores	Battelle	Yes	3/3
	1×10^{10} spores/mL	Spores	Battelle	No	3/3
	8×10^9 spores/mL	Spores	Battelle	Yes	3/3
Sensitivity determination	8×10^8 spores/mL	Spores	Battelle	Yes	3/3
	8×10^7 spores/mL	Spores	Battelle	Yes	3/3
	8×10^6 spores/mL	Spores	Battelle	Yes	0/3
	8×10^5 spores/mL	Spores	Battelle	Yes	0/1
Alternate spore preparation	7×10^8 spores/mL	Spores	Dugway	No	2/2
	8×10^7 spores/mL	Spores	Dugway	No	0/1
Vegetative cell sensitivity	Unknown Conc.	Vegetative	Battelle	NA	2/2
	4×10^6 cfu/mL	Vegetative	Battelle	NA	1/1
	3×10^5 cfu/mL	Vegetative	Battelle	NA	1/1
	3×10^4 cfu/mL	Vegetative	Battelle	NA	2/3
	3×10^3 cfu/mL	Vegetative	Battelle	NA	0/1

Vendor-stated LOD was 1×10^5 spores/mL.

NA = not applicable. Vegetative cells were not prepared from any stock solution; they were grown and placed in solution.

[a] Actual concentrations were corrected for the enumeration of the stock solution from which each sample was prepared. The uncertainty of the enumeration technique is approximately 15%.

[b] Lethal dose concentration.

Additional testing beyond that described in the test/QA plan was performed to explore these possible explanations and to gain more information about the performance of the BioThreat Alert® test strips. It included evaluating whether Battelle's storage of the stock solution of anthrax spores in a 1% solution of phenol had any impact on the performance of the BioThreat Alert® test strips; increasing the concentration of spores beyond what was required by the test/QA plan; and subjecting the test strips to Ames strain anthrax spores prepared by Dugway Proving Ground using a preparation method that is different from the one Battelle uses.

To address the possibility that storing spores in phenol affected the sensitivity of the BioThreat Alert® test strips, a series of samples was prepared and analyzed using one anthrax spore stock solution that had been stored in a phenol solution and one that had not. The data are given in Table 6-1a under "Purpose of Analysis, Investigation of phenol storage of spores." Both solutions had been prepared at Battelle using the same preparation method. The 5×10^6 spores/mL sample made with spores not stored in phenol was not detectable, as was the case for the 4×10^6 spores/mL solution made from a stock that was stored in phenol. In addition, samples containing concentrations of approximately 10^{10} and 10^9 spores/mL of spores from both phenol and non-phenol stock solutions were analyzed. The approximately 10^9 spore/mL solutions were not detectable, but the 10^{10} spore/mL solutions were detectable. These results suggested that the effect of phenol storage was probably inconsequential to the sensitivity of the BioThreat Alert® test strips to anthrax spores.

The second explanation of the results at the first five concentration levels was investigated by preparing and analyzing samples containing approximately 10^9, 10^8, 10^7, and 10^6 spores/mL from the original stock solution that had been stored in phenol, but washed with water prior to testing. Since phenol storage apparently did not affect the sensitivity of the technologies to spores, this series of samples was analyzed to determine the approximate sensitivity of the BioThreat Alert® test strips to the Battelle-prepared spores. Only the two highest concentration levels were detectable; therefore, the lowest detectable concentration was approximately 10^8 spores/mL. Solutions of spores that were prepared at Dugway Proving Ground and received at Battelle in 2001 were then analyzed. Since 2001, the Dugway stock solution had been refrigerated as a solution of spores in spent media. The solution was washed in DI water as described for the phenol storage solution above and diluted to make several solutions with concentrations separated by factors of ten. Both the stock solution concentration and the dilution methodology were confirmed by plate enumeration as shown in Table 4-2. These samples were analyzed one concentration level at a time by decreasing concentration to determine the approximate sensitivity to these spores. Three replicate analyses were performed on the lowest detectable individual replicate. When determined in this manner, the lowest detectable concentration of Dugway spores was approximately 10^9 spores/mL, a concentration higher than the lowest detectable concentration of the Battelle-prepared spores.

Tetracore informed Battelle that the BioThreat Alert® test strips are more sensitive to vegetative anthrax than spores. This was investigated by preparing a solution of vegetative cells as described above. This solution was diluted by a factor of 10 four times, and then the stock and two diluted samples were enumerated to determine the concentration of vegetative cells in each sample. These samples were analyzed one concentration level at a time by decreasing concentration to determine the approximate sensitivity to these vegetative cells. The lowest

detectable concentration of vegetative cells was 3×10^4 colony-forming units (cfu)/mL, approximately an order of magnitude lower than Tetracore claimed to be able to attain for anthrax spores.

6.1.2 Botulinum Toxin

The results obtained for the PT samples containing botulinum toxin Types A and B are given in Table 6-1b. The results showed that the BioThreat Alert® test strips were reproducibly sensitive to botulinum toxin Type A at 0.01 mg/L and Type B at 0.05 mg/L.

Table 6-1b. Botulinum Toxin Contaminant-Only PT Sample Results

Purpose of Analysis	Concentration (mg/L)	Positive Results Out of Total Replicates (Type A)	Positive Results Out of Total Replicates (Type B)
Botulinum toxin PT samples	0.01[a]	3/3	1/3
	0.05	3/3	3/3
	0.1	3/3	3/3
	0.3[b]	NA[c]	3/3
	0.5	3/3	3/3

[a] Vendor-stated LOD for botulinum toxin.
[b] Lethal dose concentration.
[c] This concentration level was not analyzed using Type A botulinum toxin.

6.1.3 Ricin

The results obtained for the PT samples containing ricin are given in Table 6-1c. When analyzing concentrations ranging from 0.035 to 15 mg/L, all replicate samples generated positive results.

Table 6-1c. Ricin Contaminant-Only PT Sample Results

Purpose of Analysis	Concentration (mg/L)	Positive Results Out of Total Replicates
Ricin PT samples	0.035[a]	3/3
	0.2	3/3
	0.4	3/3
	2	3/3
	15[b]	3/3

[a] Vendor-stated LOD.
[b] Lethal dose concentration.

6.2 False Positive/Negative Responses

Three types of samples were analyzed to evaluate the susceptibility of BioThreat Alert® test strips to false positive and negative results. These included interferent PT samples, made up of DI water fortified with Ca and Mg and samples fortified with humic and fulvic acids with and without the addition of target contaminants; cross-reactivity PT samples, made up of DI water fortified with a contaminant similar biologically or chemically with each specific target contaminant; and DW samples both concentrated and unconcentrated and both with and without the addition of target contaminants. A false positive result was defined as a positive result in the absence of the target contaminant, and a false negative result was defined as a negative result from a sample containing detectable levels of each target contaminant.

6.2.1 Interferent PT Samples

The results from the interferent PT samples are given in Table 6-2. For test strips specific to each contaminant, the number of positive results out of the number of replicates is given for PT samples containing only the possible interferents and those possible interferents in the presence of the listed concentration of target contaminant. For anthrax, expanded testing included additional interferent PT samples with a higher concentration of anthrax. Results for botulinum toxin Types A and B and ricin are also presented.

Table 6-2. Interferent PT Sample Results

	Positive Results Out of Total Replicates							
	Anthrax (spores/mL)			Botulinum Toxin (mg/L)			Ricin (mg/L)	
Interferent Sample	Blank	1×10^{6}[a]	1×10^{8}[a]	Blank	Type B 0.1	Type A 0.1	Blank	0.4
46 mg/L Ca 18 mg/L Mg	0/3	0/3 5×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
230 mg/L Ca 90 mg/L Mg	0/3	0/3 5×10^{5}[b]	0/3 1×10^{8}[b]	0/3	3/3	3/3	0/3	3/3
0.5 mg/L humic and fulvic acid	0/3	0/3 1×10^{6}[b]	NA	0/3	3/3	NA	0/3	3/3
2.5 mg/L humic and fulvic acid	0/3	0/3 1×10^{6}[b]	3/3 1×10^{8}[b]	1/3	3/3	3/3	0/3	3/3

NA = not applicable. Sample not analyzed during expanded testing.
[a] Expected concentration.
[b] Actual concentration.

One replicate of the botulinum toxin Type B test strips subjected to 2.5 mg/L humic and fulvic acids generated a false positive result. With that exception, when the unspiked interferent solutions were analyzed, there were no false positive results for the test strips specific for any of

the three target contaminants. The lack of detectable results for several DW samples spiked at 1×10^6 spores/mL for anthrax indicated false negative responses with respect to the vendor-stated LOD; however, because those tested concentration levels were not detectable when analyzed in DI water (see Section 6.1.1), the lack of sensitivity within this testing scenario cannot be attributed to the presence of the possible interferents. Expanded testing was performed by analyzing samples prepared using concentration levels of anthrax detectable when prepared in DI water only. Of these samples, three out of three replicates of 230-mg/L Ca and 90-mg/L Mg fortified with 1×10^8 spores/mL anthrax generated false negative responses. For botulinum toxin and ricin, no false negative results were generated from interferent solutions spiked with target contaminants. The lower concentration interferent matrix was not analyzed during the expanded testing of anthrax samples or the botulinum toxin Type A samples.

6.2.2 DW Samples

The results from the DW samples are given in Table 6-3. For test strips specific to each contaminant, the number of positive results out of the number of replicates is given for the DW samples containing no target contaminants and also the DW samples in the presence of the listed concentration of each target contaminant. For anthrax, expanded testing included additional DW samples containing a higher concentration of anthrax. Results for botulinum toxin Types A and B and ricin are also given.

There were several false positive results for the test strips when used to analyze the unspiked DW samples. For the anthrax test strips, one out of three replicates was falsely positive when used to analyze FL and concentrated NY DW. For the botulinum toxin test strips, one out of three replicates was falsely positive when subjected to concentrated OH DW; for the ricin test strips, two out of three replicates generated positive results when used to analyze the concentrated FL DW.

The second column of results under anthrax shows false negative responses with respect to the vendor-stated LOD (not as defined in Section 5.2). But, because those tested concentration levels were not detectable when analyzed in DI water (see Section 6.1.1), the negative results cannot necessarily be attributed to the presence of the DW matrix. For anthrax spores, expanded testing was performed using a concentration level of anthrax that was detectable when prepared in DI water only. Only two DW samples, concentrated CA and concentrated NY DW, were analyzed during the expanded testing of anthrax samples or the botulinum toxin Type A samples. For anthrax, two out of three samples of 1×10^8 spores/mL in concentrated DW from NY and one out of three samples of the same concentration in concentrated CA DW produced false negative results; for botulinum toxin, there were no false negative responses; and for ricin, one replicate out of three was falsely negative when 0.4 mg/L of ricin was spiked into NY DW.

Table 6-3. DW Sample Results

	Positive Results Out of Total Replicates							
	Anthrax (spores/mL)			Botulinum Toxin (mg/L)			Ricin (mg/L)	
DW Sample	Blank	1×10^{6}[a]	1×10^{8}[a]	Blank	Type B 0.1	Type A 0.1	Blank	0.4
Unconcentrated CA DW	0/3	0/3 2×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated CA DW	0/3	0/3 4×10^{4}[b]	2/3 1×10^{8}[b]	0/3	3/3	3/3	0/3	3/3
Unconcentrated FL DW	1/3	0/3 5×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated FL DW	0/3	0/3 6×10^{5}[b]	NA	0/3	3/3	NA	2/3	3/3
Unconcentrated NY DW	0/3	0/3 8×10^{5}[b]	NA	0/3	3/3	NA	0/3	2/3
Concentrated NY DW	1/3	0/3 8×10^{5}[b]	1/3 1×10^{8}[b]	0/3	3/3	3/3	0/3	3/3
Unconcentrated OH DW	0/3	0/3 5×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated OH DW	0/3	0/3 4×10^{5}[b]	NA	1/3	3/3	NA	0/3	3/3

[a] Expected concentration.
[b] Actual concentration.
NA = not applicable. Sample not analyzed during expanded testing.

6.2.3 Cross-Reactivity PT Samples

The results from the cross-reactivity PT samples are given in Table 6-4. For test strips specific to each target contaminant, a PT sample fortified with a spore or chemical similar to each target contaminant was analyzed in the absence of any target contaminant. The number of positive results out of the number of replicates is given for each sample. The only false positive result in this evaluation of cross-reactivity was for lipopolysaccharide, a compound chemically similar to botulinum toxin. The rest of the results were correctly reported as negative.

Table 6-4. Potentially Cross-Reactive PT Sample Results

	Positive Results Out of Total Replicates		
	Anthrax	Botulinum Toxin	Ricin
Bacillus thuringiensis (1×10^5 spores/mL)[(a)]	0/3		
Lipopolysaccharide (0.1 mg/L)		1/3	
Lectin from soybean (0.4 mg/L)			0/3

[(a)] Concentration was determined after the fact to be below the lowest detectable concentration. Therefore, the non-detectable results may not indicate a lack of cross-reactivity.

6.3 Consistency

For the anthrax testing, at times the number of replicate analyses was reduced to conserve time or available supplies. However, the available replicate data for anthrax suggests that performance of the test strips was reproducible for the contaminant and interferent PT samples since 96% of the test strips generated results that were either all negative or all positive. However, the results were less consistent for DW samples (unspiked or spiked with the target contaminant), where 22% of the sets generated mixed results. For the botulinum toxin test strips, there was only one mixed result for the contaminant PT samples, one for the interferent PT samples, and one for the DW samples (overall 92% consistency for botulinum toxin). They were generated from the lowest concentration of botulinum toxin Type B sample, the unspiked 2.5 mg/L humic and fulvic acid sample, and the unspiked concentrated OH DW sample, respectively. For ricin, the results were consistent 100% of the time for the contaminant and interferent PT samples. For the ricin DW samples, two sample sets, the unspiked concentrated FL DW, and the spiked NY DW generated mixed results. Overall, 95% of all the contaminant and interferent PT sample results and 87% of all of the DW results were obtained in sets of two or three in which all the individual replicates had the same result, whether positive or negative.

6.4 Lowest Detectable Concentration

The lowest detectable concentration of each target contaminant was defined as the lowest concentration of contaminant-only PT sample to have at least two out of three positive results. For anthrax, that concentration was 8×10^7 spores/mL (Battelle spores), 7×10^8 spores/mL (Dugway spores), and 3×10^4 cfu/mL (vegetative cells); for botulinum toxin, 0.01 mg/L (Type A); 0.05 mg/L (Type B); and for ricin, 0.035 mg/L.

6.5 Other Performance Factors

Battelle technicians, who had been trained by Tetracore to perform testing using the BioThreat Alert® test strips, performed all of the required laboratory testing. The technicians had no problem performing the tests as they were trained. The BioThreat Alert® test strips do not require the use of a reader because the zone of the test strip that changes color is exposed and therefore the results can be read visually. However, because Tetracore recommended using the reader to improve the sensitivity of the test strips and remove human bias from the results, a reader was used to determine positive or negative results during the verification test. The reader comes in a rugged carrying case that weighs approximately 20 pounds and is about the size of a medium-sized suitcase.

To test the ability of the BioThreat Alert® test strips to be used outside a laboratory environment and by a non-trained user, both a trained operator and person without any training in the sciences or in the operation of the BioThreat Alert® test strips were given a liquid sample (DI water) and told to analyze the sample three times. The non-technical person was guided only by the instructions provided with each test strip. The experienced operator analyzed this sample in the correct way. The non-technical operator followed the instructions properly and tested all three samples without error. Each of the six DI water samples correctly produced negative results. The Verification Test Coordinator observed both operators and made this assessment.

Over 400 BioThreat Alert® test strips were tested during the verification test; all except five functioned properly. In two of those instances, the identification microchip inside the test strip was not recognized by the reader, so the type of test (ricin) had to be manually selected using the reader's arrow buttons, and the test strip was then read by the reader. Additionally, the following three problems caused a test strip to be discarded and the sample re-analyzed using a new test strip: (1) lack of sample flow after the application of the required 6 drops of sample; (2) after a sample had flowed across the strip, no control line was visible; and (3) the reader generated a "control line out of range" that results when the reader does not detect an adequately intense control line. Overall, 99% of the test strips functioned properly. The verification staff were able to test approximately 20 samples per hour.

Chapter 7
Performance Summary

Table 7-1. Anthrax Summary Table

Parameter		Sample Information	Actual Fortified Anthrax Concentration[a]	Positive Results Out of Total Replicates
Qualitative contaminant results	Contaminant-only PT samples	Battelle-prepared, phenol-preserved spores	8×10^8 spores/mL	3/3
			8×10^7 spores/mL	3/3
			8×10^6 spores/mL	0/3
			8×10^5 spores/mL	0/1
		Vegetative cells	4×10^6 cfu/mL	1/1
			3×10^5 cfu/mL	1/1
			3×10^4 cfu/mL	2/3
			3×10^3 cfu/mL	0/1
		Dugway-prepared spores	7×10^8 spores/mL	2/2
			8×10^7 spores/mL	0/1
	Interferent PT samples	230 mg/L Ca and 90 mg/L Mg	1×10^8 spores/mL[b]	0/3
		2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^8 spores/mL[b]	3/3
	DW samples	Concentrated CA	1×10^8 spores/mL[b]	2/3
		Concentrated NY	1×10^8 spores/mL[b]	1/3
		Unconcentrated DW	1×10^6 spores/mL	0/24
	Cross-reactivity	1×10^5 spores/mL *Bacillus thuringiensis*	unspiked	0/3
False positives		Two false positives resulted from the analysis of the DW samples. One out of three replicates for each of the FL DW and concentrated NY DW falsely generated positive results. *Bacillus thuringiensis* was prepared at concentrations much lower than the lowest detectable concentration of *Bacillus anthracis.* Therefore, negative results with these samples do not necessarily indicate a lack of cross-reactivity.		
False negatives		None of three results was positive for the 230-mg/L Ca and 90-mg/L Mg spiked with a detectable concentration of anthrax. In addition, one and two false negative results were generated for the concentrated CA and concentrated NY DW samples, respectively. BioThreat Alert® test strips were not able to detect anthrax spores at the vendor-stated LOD. All of the unconcentrated DW samples were spiked at concentrations less than detectable by the test strips and, therefore, were, as expected, negative.		
Consistency		96% (25 of 26 replicates) of the contaminant and interferent PT sample results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative. This was the case for 78% of the DW samples.		

Table 7-1. Anthrax Summary Table (continued)

Parameter	Sample Information
Lowest detectable concentration	8×10^7 spores/mL - Battelle prep; 7×10^8 spores/mL - Dugway prep (vendor-stated LOD: 1×10^5 spores/mL); 3×10^4 cfu/mL - vegetative anthrax (no vendor-stated LOD)
Other performance factors	All components for testing were provided in a box of 25 test strips; the strip reader used during the verification test was powered using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test strips and reader were used easily inside and outside a laboratory with trained operator; non-technical operator performed tests as well as a trained operator; and sample throughput was 20 samples per hour.

(a) The uncertainty of the enumeration technique was approximately 15%.
(b) Battelle-prepared, phenol-preserved spores.

Table 7-2. Botulinum Toxin Summary Table

Parameter		Sample Information	Botulinum Toxin Concentration (mg/L)	Positive Results Out of Total Replicates
Qualitative contaminant positive results	Contaminant-only PT samples	Type A	0.01	3/3
			0.05	3/3
			0.1	3/3
			0.5	3/3
		Type B	0.01	1/3
			0.05	3/3
			0.1	3/3
			0.3	3/3
			0.5	3/3
	Interferent PT samples	Ca and Mg	0.1	3/3 Type A 6/6 Type B
		Humic acid and fulvic acid	0.1	3/3 Type A 6/6 Type B
	DW samples	Concentrated DW	0.1	6/6 Type A 12/12 Type B
		Unconcentrated DW	0.1	12/12 Type B
	Cross-reactivity	0.1 mg/L Lipopolysaccharide	unspiked	1/3
False positives		There was one false positive replicate out of three for the unspiked 2.5-mg/L humic and fulvic acid interferent PT sample; the unspiked concentrated OH DW sample and the lipopolysaccharide each generated one false positive result out of three replicates.		
False negatives		No false negatives resulted from the analysis of the interferent and DW samples spiked with detectable levels of Types A and B botulinum toxin.		
Consistency		92% of the results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		
Lowest detectable concentration		0.01 mg/L (Type A); 0.05 mg/L (Type B) (vendor-stated LOD for both Types A and B: 0.01 mg/L)		
Other performance factors		All components for testing were provided in a box of 25 test strips; the strip reader used during the verification test was powered using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test strips and reader were used easily inside and outside a laboratory with trained operator; non-technical operator performed tests as well as a trained operator; and sample throughput was 20 samples per hour.		

Table 7-3. Ricin Summary Table

Parameter			Ricin Concentration (mg/L)	Positive Results Out of Total Replicates
Qualitative contaminant positive results	Contaminant-only PT samples		0.035	3/3
			0.2	3/3
			0.4	3/3
			2	3/3
			15	3/3
	Interferent PT samples	Ca and Mg	0.4	6/6
		Humic acid and fulvic acid	0.4	6/6
	DW samples	Concentrated DW	0.4	12/12
		Unconcentrated DW	0.4	11/12
	Cross-reactivity	0.4 mg/L Lectin from soybean	unspiked	0/3
False positives		The unspiked concentrated FL DW generated two false positives out of three. All other DW and cross-reactivity samples resulted in correctly negative responses.		
False negatives		There was one false negative out of three for the NY DW sample spiked with a detectable concentration of ricin. The other spiked interferent and DW samples were correctly determined to be positive.		
Consistency		100% of the contaminant and interferent PT results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative. That was the case 88% (14 out of 16) of the time for the DW samples.		
Lowest detectable concentration		0.035 mg/L (Vendor-stated LOD: 0.035 mg/L)		
Other performance factors		All components for testing were provided in a box of 25 test strips; the strip reader used during the verification test was powered using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test strips and reader were used easily inside and outside a laboratory with trained operator; non-technical operator performed tests as well as a trained operator; and sample throughput was 20 samples per hour.		

Chapter 8
References

1. Personal communication with Dick Burrows, U.S. Army Center for Health Promotion and Preventive Medicine.

2. U.S. EPA threat prioritization study provided by Steve Allgeier, U.S. EPA Office of Water.

3. Center for Defense Information Fact Sheet: Ricin, www.cdi.org/terrorism;ricin-pr.cfm.

4. *Test/QA Plan for Verification of Immunoassay Test Kits,* Battelle, Columbus, Ohio, January 2004.

5. U.S. EPA Method 180.1, "Turbidity (Nephelometric)," *Methods for the Determination of Inorganic Substances in Environmental Samples,* EPA/600/R-93/100, August 1993.

6. American Public Health Association, et al. S*tandard Methods for the Examination of Water and Wastewater*. 19th Edition, Washington, D.C., 1997.

7. U.S. EPA, *Methods for Chemical Analysis of Water and Wastes,* EPA/600/4-79/020, March 1983.

8. U.S. EPA Method 200.8, "Determination of Trace Elements in Waters and Wastes by Inductively-Coupled Plasma Mass Spectrometry," in *Methods for the Determination of Organic Compounds in Drinking Water,* Supplement I, EPA/600/R-94/111, October 1994.

9. U.S. EPA Method 524.2, "Permeable Organic Compounds by Capillary Column GC/Mass Spectrometry," *Methods for the Determination of Organic Compounds in Drinking Water—Supplement III,* EPA/600/R-95/131, August 1995.

10. U.S. 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*, EPA/600/R-95/131, August 1995.

11. Quality Management Plan (QMP) for the ETV Advanced Monitoring Systems Center, Version 5.0, U.S. EPA Environmental Technology Verification Program, Battelle, Columbus, Ohio, March 2004.