September 2004

Environmental Technology Verification Report

ADVNT BIOTECHNOLOGIES
BADD™ ANTHRAX, BOTULINUM TOXIN, AND RICIN IMMUNOASSAY TEST STRIPS

Prepared by
Battelle

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ETV ETV ETV

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ETV Advanced Monitoring Systems Center

ADVNT Biotechnologies
BADD™
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.

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List of Abbreviations

AMS	Advanced Monitoring Systems
ATEL	Aqua Tech Environmental Laboratories, Inc.
BADD™	Biowarfare Agent Detection Device
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
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 ADVNT Biotechnologies Biowarfare Agent Detection Devices (BADD™) 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 ADVNT Biotechnologies BADD™. BADD™ test strips (Figure 2-1) are self-contained, qualitative assays for screening environmental samples for the presence of anthrax, botulinum toxin, and ricin. These test strips work on similar principles, but each is single use and can detect only one contaminant. The following is a description of the BADD™ system based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The BADD™ test strips are stored in resealable packages, which include all the items necessary to analyze each sample. Each individually packaged test includes approximately 250 microliters (μL) of buffer in a small plastic screw-top vial, a sample collection swab, a bulb syringe, the test strip (within its own sealed package), and step-by-step instructions. This package is approximately 5 inches (12.7 centimeters) by 6 inches (15.2 centimeters) and weighs only a few ounces. The vendor suggests that the resealable package be used as a sealed waste receptacle for all testing materials.

Figure 2-1. ADVNT BADD™ Immunoassay Test Strips

The testing procedure involves dipping the dry collection swab into a solution suspected of containing anthrax, botulinum toxin, or ricin, followed by eluting (extracting) the collected sample into a collection tube containing a sample diluent. After the sample is collected, it is transferred onto the BADD™ test strip where dye-labeled antibodies detect trace amounts of the contaminant collected by the swab, as indicated by the presence of two bands in the test result window. After 15 minutes, the results are read visually.

BADD™ test strips are sold in boxes of 10 for approximately $250 per box.

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 contaminants of interest. The contaminants, or antigens, react with a selective antibody to produce a result that is indicated by a color change. For the BADD™ test strips, the presence of contaminants is indicated by the appearance of a colored line within approximately 20 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 BADD™ test strips were subjected to various concentrations of anthrax spores, botulinum toxin, 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 concentration, and the source. The BADD™ 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) and magnesium (Mg) and calcium (Ca) in DI water both with and without the addition of the contaminants using the BADD™ test strips. In addition, specificity was evaluated by exposing the BADD™ 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)	1×10^6 spores/mL	200 spores/mL[1]	Battelle and U.S. Army Dugway Proving Ground
Botulinum toxin Types A and B	0.4 mg/L	0.3 mg/L[2]	Metabiologics, Inc. (Madison, Wisconsin)
Ricinus communis Agglutinin II (ricin)	0.4 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 BADD™ 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 BADD™ 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 BADD™ 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.

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	0.5 to 25 mg/L
	Botulinum toxin Type B	0.3 to 1,000 mg/L
	Ricin	0.4 to 2,000 mg/L
Interferent	Contaminants in 46 mg/L Ca and 18 mg/L Mg	Anthrax - 10^7 spores/mL Botulinum toxin (Type B) - 4 mg/L Ricin - 5 mg/L
	Contaminants in 230 mg/L Ca and 90 mg/L Mg	Anthrax - 10^7 and 10^8 spores/mL Botulinum toxin (Type A) - 5 mg/L Botulinum toxin (Type B) - 4 mg/L Ricin - 5 mg/L
	Contaminants in 0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Anthrax - 10^7 spores/mL Botulinum toxin (Type B) - 4 mg/L Ricin - 5 mg/L
	Contaminants in 2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Anthrax - 10^7 and 10^8 spores/mL Botulinum toxin (Type A) - 5 mg/L Botulinum toxin (Type B) - 4 mg/L Ricin - 5 mg/L
Potentially Cross-reactive	*Bacillus thuringiensis* (anthrax analogue)	10^6 spores/mL
	Lipopolysaccharide (botulinum toxin analogue)	5 mg/L
	Lectin from soybean (ricin analogue)	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.

DW samples were analyzed using the BADD™ 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 BADD™ test strips.

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 10^7 10^8	unspiked 4 (Type B) 5 (Type A)	unspiked 5
New York City, New York (NY)	unfiltered chlorinated	surface	conc.	unspiked 10^7 10^8	unspiked 4 (Type B) 5 (Type A)	unspiked 5
Metropolitan Water District of California (CA)	filtered chloraminated	surface	unconc.	unspiked 10^7	unspiked 4	unspiked 5
New York City, New York (NY)	unfiltered chlorinated	surface	unconc.	unspiked 10^7	unspiked 4	unspiked 5
Columbus, Ohio (OH)	filtered chlorinated	surface	both	unspiked 10^7	unspiked 4	unspiked 5
Orlando, Florida (FL)	filtered chlorinated	ground	both	unspiked 10^7	unspiked 4	unspiked 5

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 also were obtained from Dugway Proving Ground and 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 BADD™ test strips. Preliminary results indicated that only the highest concentration PT sample produced positive results for ricin and anthrax, and there were very few positive results for botulinum toxin Type B; therefore, the original test/QA plan was amended to include preparing and analyzing higher concentration samples of anthrax and ricin and testing a preparation of anthrax spores that were never preserved in phenol, a second source of anthrax spores, vegetative anthrax cells, and botulinum toxin Type A. 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 BADD™ test strips to false negative results due to interferents, the test/QA plan was amended to include the fortification of detectable types and concentrations of contaminants 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 BADD™ 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 196 PT samples was analyzed by the BADD™ test strips for this test. The results provided information about how well the BADD™ test strips detected the presence of each contaminant at several concentration levels, the consistency of the responses, and the susceptibility of the BADD™ 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 BADD™ 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 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. During the expanded DW testing for anthrax, a concentration level of approximately 10^8 spores/mL was used to spike the DW samples. A total of 156 DW samples was analyzed by the BADD™ test strips for this test.

3.2.3 Quality Control Samples

In addition to the 352 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. A control line in the result window on the BADD™ test strips appeared during the analysis of each sample that indicated to the operator that the test kit was functioning properly. If this control line did not appear, that test kit would be discarded and a new test strip used. There were no such instances during testing. Because of this control line on the test strips, 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. In most cases, three replicates of each test sample were analyzed. The testing procedure included the following steps for analyzing liquid samples for the presence of anthrax spores, botulinum toxin, or ricin: (1) the test strip was removed from its package; (2) the provided swab was submerged in the test sample (minimum of 2 mL in a small test tube) for approximately 5 seconds to absorb the liquid sample; (3) the swab was transferred into the buffer solution and pressed against the inside of the vial for approximately 10 seconds to squeeze the sample out of the swab and mix the buffer/sample solution; (4) the dropper was used to transfer 5 drops of the buffer/sample solution to the sample well on the test strip (a sixth drop was added to spur the movement of the buffer/sample solution across the adsorbent strip if the solution did not begin moving after 5 drops); and (5) for anthrax, the strip was read after 15 minutes, for botulinum toxin and ricin, after 10 minutes. The appearance of a colored line under the “C” on the test strip indicated that the proper procedure had been followed and the test strip had functioned properly. Because the BADD™ test strips did not require a reader to determine the positive or negative response, the result was read visually. The vendor instructed Battelle to consider the appearance of a colored line under the “T” on the test strip a positive response.

3.3.2 Non-Laboratory Testing

Because the toxic nature of these contaminants did not permit their use outside special laboratory facilities, MB samples were analyzed at a non-laboratory location to evaluate the BADD™ performance and ease of use outside of the laboratory. Both a trained technician and a non-technical/untrained, first-time user performed analyses at a non-laboratory location. The purpose of these analyses was to test the performance of the BADD™ in a non-laboratory setting, not to evaluate thoroughly the effect of changing conditions such as temperature and humidity on the BADD™. Initially, the non-technical/untrained, first-time user was guided only by the manual or by the vendor instructions. If the operators were about to complete the test incorrectly, the Verification Test Coordinator prompted them to re-evaluate the 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 BADD™ test strips.

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

Parameter	Unit	Method	Sources of Drinking Water Samples			
			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	$\mu S/cm^2$	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
$\mu S/cm^2$ = microSiemen 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 BADD™ 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 supernatant consisting of the phenol/water solution was decanted from the spores, 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 and had not been characterized like the previously described lot. The second lot had been subjected only to enumeration in order 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 BADD™ 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. Cells from an enumeration of the Battelle-prepared, phenol-preserved spores were collected, placed in solution, and then enumerated to determine the concentration. No further characterization was performed on these cells. Solutions of these cells were used to determine the sensitivity of the BADD™ 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 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 (RPD) between the two are given in the table. *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 BADD™, 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 ADVNT had claimed, additional PT 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 BADD™ 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 BADD™ 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 BADD™ performance such as ease of use, field portability, and sample through-put are discussed in Section 6. Also addressed are qualitative observations of the verification staff pertaining to the performance of the BADD™.

Chapter 6
Test Results

6.1 Qualitative Contaminant Presence/Absence

The responses for the BADD™ test strips using the contaminant-only PT samples containing anthrax, botulinum toxin, and ricin are discussed in the following sections. The BADD™ test strips provide indication of only a positive or negative response based on whether or not a red line appears under the "T" (for test) after a liquid test sample is applied to the adsorbent strip. A red line appears under the "C" (for control) after every test sample regardless of whether or not the target contaminant is present.

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 only the 4 x 10^7 concentration level (50 times the vendor-stated LOD) produced detectable results. The Battelle-prepared, phenol-preserved serial dilution validation enumeration on January 30 (1 x 10^5 spores/mL expected) was a part of the serial dilution process to make all five of these PT samples. The results of this enumeration confirms the concentration of spores in these samples. After discussions with ADVNT Biotechnologies, 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 BADD™ to the affected spores.)

2. The sensitivity of the BADD™ 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.

3. The BADD™ test strips are more sensitive to vegetative anthrax cells than spores. (This hypothesis stemmed from the analysis of one sample that was prepared by collecting a single vegetative anthrax colony from an enumeration plate and placing it into DI water and mixing well. This sample produced one out of two positive results using the BADD™ test strips; however, the solution was not enumerated so the concentration was not known.)

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
	8×10^5 spores/mL[c]	Spores	Battelle	Yes	0/3
	3×10^6 spores/mL	Spores	Battelle	Yes	0/3
	8×10^6 spores/mL	Spores	Battelle	Yes	0/3
	4×10^7 spores/mL	Spores	Battelle	Yes	2/3
Investigation of phenol storage of spores	5×10^7 spores/mL	Spores	Battelle	No	2/3
	1×10^9 spores/mL	Spores	Battelle	No	2/2
	8×10^8 spores/mL	Spores	Battelle	Yes	2/2
	1×10^{10} spores/mL	Spores	Battelle	No	2/2
	8×10^9 spores/mL	Spores	Battelle	Yes	2/2
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	0/3
Alternate spore preparation	8×10^7 spores/mL	Spores	Dugway	No	3/3
	8×10^6 spores/mL	Spores	Dugway	No	0/1
Vegetative cell sensitivity	Unknown Conc.	Vegetative	Battelle	NA	1/2
	4×10^6 cfu/mL	Vegetative	Battelle	NA	1/1
	3×10^5 cfu/mL	Vegetative	Battelle	NA	2/3
	3×10^4 cfu/mL	Vegetative	Battelle	NA	0/1

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.

(c) This concentration is very close to the vendor-stated LOD.

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 BADD™ 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 BADD™ test strips; increasing the concentration of spores beyond what was required by the test/QA plan; 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; and testing the response of the test strips to vegetative anthrax cells at various concentrations.

To address the possibility that storing spores in phenol affected the sensitivity of the BADD™, 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 Analyses, Investigation of phenol storage of spores." Both solutions had been prepared at Battelle using the same preparation method. The 5×10^7 spores/mL sample made with spores not stored in phenol produced positive results in two out of three samples, as did the 4×10^7 spores/mL sample made with spores that had been 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. All four samples were detectable in each of two replicates analyzed. These results suggested that the effect of phenol storage was probably inconsequential to the sensitivity of the BADD™ 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 BADD™ 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 by tenfold factors several times. 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 10^8 spores/mL, a level similar to that determined for the Battelle-prepared spores.

The third explanation of the results 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 10^5 colony-forming units (cfu)/mL, an order of magnitude lower than what the vendor claimed to be able to attain for anthrax spores. While

ADVNT Biotechnologies has not provided information with regard to the BADD™ test kit's vegetative cell sensitivity, these results suggest that BADD™ test strips are much more sensitive to vegetative cells than to spores.

6.1.2 Botulinum Toxin

The results obtained for the PT samples containing botulinum toxin are given in Table 6-1b. Upon analyzing the first five concentration levels listed in the test/QA plan, the only positive results were produced from one replicate out of three at the 2 and 4 mg/L concentration levels, with no positive responses at concentrations above or below those levels. In response to these results, ADVNT Biotechnologies informed us that their test strips were only sensitive to botulinum toxin Type A. Since botulinum toxin Type B was described for use in the test/QA plan, samples were not initially analyzed using botulinum toxin Type A. To more completely verify the BADD™ test strips, a limited amount of expanded testing was conducted by analyzing four PT samples containing a range of concentration levels (0.5, 2, 5, and 25 mg/L) of botulinum toxin Type A and two higher concentration levels (200 and 1,000 mg/L) of botulinum toxin Type B. The results showed that the BADD™ test strips were sensitive to botulinum toxin Type A at approximately 5 mg/L, but were not able to reproducibly detect botulinum toxin Type B at concentration levels up to 1,000 mg/L.

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

Purpose of Analysis	Concentration (mg/L)	Type of Botulinum Toxin	Positive Results Out of Total Replicates
Original test/QA plan PT samples	0.3[a]	B	0/3
	0.4[b]	B	0/3
	2	B	1/3
	4	B	1/3
	20	B	0/3
Expanded testing	0.5	A	1/3
	2	A	0/3
	5	A	3/3
	25	A	3/3
	200	B	0/3
	1,000	B	0/3

[a] Lethal dose concentration.

[b] Vendor-stated LOD.

6.1.3 Ricin

The results obtained for the PT samples containing ricin are given in Table 6-1c. Upon analyzing the first five concentration levels listed, the only positive results were produced at the 20 mg/L concentration level. Two additional concentration levels (200 and 2,000 mg/L) were analyzed to better define the performance of BADD™ for the detection of ricin. Positive results were obtained at each of these additional concentration levels.

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

Purpose of Analysis	Concentration (mg/L)	Positive Results Out of Total Replicates
Original test/QA plan PT samples	0.4[a]	0/3
	2	0/3
	5	0/3
	15[b]	0/3
	20	3/3
Expanded testing	200	3/3
	2,000	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 BADD™ 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 and botulinum toxin,

Table 6-2. Interferent PT Sample Results

Interferent Sample	Positive Results Out of Total Replicates							
	Anthrax (spores/mL)			Botulinum Toxin (mg/L)			Ricin (mg/L)	
	Blank	1×10^7 [a]	1×10^8	Blank	Type B 4	Type A 5	Blank	5
46 mg/L Ca 18 mg/L Mg	0/3	0/3[b] 6×10^6	NA	0/3	0/3	NA	0/3	0/3
230 mg/L Ca 90 mg/L Mg	0/3	0/3[b] 4×10^6	3/3[b] 1×10^8	0/3	0/3	3/3	0/3	0/3
0.5 mg/L humic and fulvic acids	0/3	0/3[b] 1×10^7	NA	0/3	0/3	NA	0/3	0/3
2.5 mg/L humic and fulvic acids	0/3	0/3[b] 1×10^7	3/3[b] 1×10^8	0/3	0/3	3/3	0/3	0/3

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

expanded testing included additional interferent PT samples (a higher concentration in the case of anthrax and a different type in the case of botulinum toxin). No expanded testing involving interferent PT samples was done for the ricin test strips.

When interferent solutions not fortified with target contaminants were analyzed, no false positive results occurred for the test strips specific for any of the three target contaminants. The lack of detectable results at 1×10^7 spores/mL for anthrax, 4 mg/L for botulinum toxin Type B, and 5 mg/L for ricin indicated false negative responses with respect to the vendor-stated LOD; however, because those tested concentration levels for anthrax were not detectable when analyzed in DI water and the test strips were not sensitive to botulinum toxin Type B (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 and botulinum toxin Type A. For both anthrax spores and botulinum toxin, there were no false negative responses for the expanded testing. The lower concentration interferent matrix was not analyzed during the expanded testing of anthrax and botulinum toxin 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 and botulinum toxin, expanded testing included additional DW samples (a higher concentration in the case of anthrax and a different type in the case of botulinum toxin) fortified with those two target contaminants. No expanded testing involving DW samples was performed for the ricin test strips.

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**	**1×10^7 [a]**	**1×10^8**	**Blank**	**Type B 4**	**Type A 5**	**Blank**	**5**
Unconcentrated CA DW	0/3	0/3 4×10^6 [b]	NA	0/3	0/3	NA	0/3	0/3
Concentrated CA DW	0/3	0/3 4×10^6 [b]	3/3 1×10^8 [b]	0/3	1/3	3/3	0/3	0/3
Unconcentrated FL DW	0/3	0/3 5×10^6 [b]	NA	0/3	1/3	NA	0/3	0/3
Concentrated FL DW	0/3	0/3 6×10^6 [b]	NA	0/3	0/3	NA	0/3	0/3
Unconcentrated NY DW	0/3	0/3 8×10^6 [b]	NA	0/3	0/3	NA	0/3	0/3
Concentrated NY DW	0/3	0/3 9×10^6 [b]	2/3 1×10^8 [b]	0/3	0/3	3/3	0/3	0/3
Unconcentrated OH DW	0/3	0/3 5×10^6 [b]	NA	0/3	0/3	NA	0/3	0/3
Concentrated OH DW	0/3	0/3 4×10^6 [b]	NA	0/3	0/3	NA	0/3	0/3

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

Table 6-3 shows that there were no false positive results for the test strips specific for any of the three target contaminants when the unspiked DW samples were analyzed. Two false positives occurred when the BADD™ test strips were used to analyze DW fortified with botulinum toxin Type B, which, according to the vendor and as noted in Section 6.1.2, the test strips cannot detect. These occurred in one unconcentrated FL DW sample and one concentrated CA DW sample, each generating one out of three positive results. The second column of results under anthrax and botulinum toxin and all results under ricin are false negative responses with respect to the vendor-stated LOD; but for the reasons detailed in Section 6.2.1, the negative responses may not be the result of the DW matrix. Only two DW samples, concentrated CA and concentrated NY DW, were analyzed during the expanded testing of anthrax and botulinum toxin samples. For anthrax spores, one out of three samples of 1×10^8 spores/mL in concentrated DW from NY produced a false negative result; for botulinum toxin Type A, there were no false negative responses.

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 (5×10^5 spores/mL)[(a)]	0/3		
Lipopolysaccharide (5 mg/L)		1/3	
Lectin from soybean (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 the consistency of the positive (or negative) results depended on how close the concentration level was to the lowest detectable concentration for the spores being analyzed. The contaminant PT sample data using the Battelle-prepared spores showed that the lowest detectable concentration for the BADD™ was between 10^7 and 10^8 spores/mL. At approximately 10^7 spores/mL (8×10^6 spores/mL of the original test/QA plan PT samples and of the sensitivity determination samples), there were no positive responses out of six replicates; at concentrations above 10^8 spores/mL, there were 100% positive responses (14 out of 14 replicates). When analyzing two samples containing 5 × 10^7 spores/mL, the results in both cases were two positive responses out of three replicates.

For botulinum toxin, the 5- and 25-mg/L samples of botulinum toxin Type A produced positive results in three out of three replicates, while the rest of the samples produced negative results except for three individual replicates at separate concentration levels. Two of these replicates were generated from botulinum toxin Type B, which was not detectable, and one was from a concentration of botulinum toxin Type A that was below the lowest detectable concentration (false positive results). For ricin, the results were 100% consistent for all the samples analyzed. Either all replicates within a sample were positive or all were negative. Overall, 90% of the anthrax results were obtained in sets of two or three in which all the individual replicates had the same result, whether positive or negative. For botulinum toxin, this statistic was 84%; for ricin, it was 100%.

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 4 × 10^7 spores/mL (Battelle-prepared), 8 × 10^7 spores/mL (Dugway-prepared) and 3 × 10^5 cfu/mL (vegetative cells); for botulinum toxin Type A, 5 mg/L; and for ricin, 20 mg/L.

6.5 Other Performance Factors

Battelle technicians, who had been trained by ADVNT Biotechnologies to operate the BADD™, performed all of the testing in a laboratory setting. The technicians had no problem performing the tests as they were trained. To test the ability of the BADD™ test strips to be used outside a laboratory environment and by a non-trained user, both a trained operator and a person without any training in the sciences or in the operation of the BADD™ were given a liquid sample (DI water) and told to analyze the sample three times. Initially, the non-technical person was guided only by the instructions provided with each test strip. However, if they were about to complete the test incorrectly, the Verification Test Coordinator prompted them to re-evaluate the instructions. The experienced operator analyzed the sample in the correct way. The non-technical operator followed the instructions properly until it was time to drop the sample onto the test strip. The operator had to be stopped from dropping the sample onto the rectangular adsorbent rather than the round sample well. A reason for this may have been that the operator had been using the side of the instruction insert that did not have a diagram of the test strip. The Verification Test Coordinator directed the operator to the instructions with the diagram, and the operator dispensed the sample in the correct place. However, the operator initially held the dropper too close to the sample well, creating many bubbles during dispensing. The Verification Test Coordinator emphasized that the operator was to apply the sample dropwise, and the operator corrected the technique. After those two simple directions, the non-technical operator tested two additional replicates flawlessly. More than 400 BADD™ test strips were used during the verification test, all except one functioned properly. In that instance, upon application of the sample, the sample did not move across the adsorbent strip. That test strip was discarded and the analysis was repeated.

As described in Section 3.3.1, using the BADD™ test strips required the visual determination of the result. The appearance of even a faint line with a red color under the "T" on the test strip indicated a positive result. For many of the results that were determined to be positive, the lines indicating that result were very faint. For anthrax, 38 results were determined to be positive; and, of those, the technician performing the tests described 17, or 44%, of those results to be "faintly" or "extremely faintly" positive. The technician, who has normal vision, made this comment when the indicator line was present, but not obvious at a glance (i.e., the strip had to be studied closely to determine whether or not the line had appeared). For botulinum toxin, 29% of the 24 positive results were described that way; for ricin, all 9 of the positive responses were described in that manner. The verification staff was able to test 20 to 30 samples per hour using the BADD™ test strips.

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 positive results	Contaminant-only PT samples	Battelle-prepared, phenol-preserved spores	8×10^8 spores/mL	3/3
			8×10^7 spores/mL	3/3
			4×10^7 spores/mL	2/3
			8×10^6 spores/mL	0/3
			8×10^5 spores/mL	0/3
		Vegetative cells	4×10^6 cfu/mL	1/1
			3×10^5 cfu/mL	2/3
			3×10^4 cfu/mL	0/1
		Dugway-prepared spores	8×10^7 spores/mL	3/3
			8×10^6 spores/mL	0/1
	Interferent PT samples	230 mg/L Ca 90 mg/L Mg	1×10^8 spores/mL[b]	3/3
		2.5 mg/L humic acid 2.5 mg/L fulvic acid	1×10^8 spores/mL[b]	3/3
	DW samples	Concentrated CA	1×10^8 spores/mL[b]	3/3
		Concentrated NY	1×10^8 spores/mL[b]	2/3
		Unconcentrated DW	1×10^7 spores/mL[b]	0/24
	Cross-reactivity	5×10^5 spores/mL *Bacillus thuringiensis*	unspiked	0/3
False positives		No false positives resulted from the analysis of the interferent, DW, or cross-reactivity samples. *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		One false negative replicate resulted from the analysis of the interferent and DW samples spiked with detectable levels of anthrax spores (concentrated NY DW); the BADD™ test strips were not able to detect anthrax spores at the vendor-stated LOD, but they were able to detect much higher concentration levels. All of the unconcentrated DW samples were spiked at concentrations less than detectable by the test strips and, therefore, were, as expected, negative.		
Consistency		90% of the results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		

Table 7-1. Anthrax Summary Table (continued)

Parameter	Sample Information
Lowest detectable concentration	4×10^7 spores/mL - Battelle prep; 8×10^7 spores/mL - Dugway prep (vendor-stated limit of detection [LOD]: 1×10^6 spores/mL); 3×10^5 cfu/mL - vegetative anthrax (no vendor-stated LOD)
Other performance factors	All components for testing were provided in a resealable package weighing just a few ounces; strips used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; indicator line color change for the anthrax test strips was very faint 44% of the time, increasing the likelihood of false negative results; and sample throughput was 20 to 30 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	0.5	1/3
			2	0/3
			5	3/3
			25	3/3
		Type B	0.3	0/3
			0.4	0/3
			2	1/3
			4	1/3
			20	0/3
			200	0/3
			1,000	0/3
	Interferent PT samples	230 mg/L Ca 90 mg/L Mg	5[a]	3/3
		2.5 mg/L humic acid 2.5 mg/L fulvic acid	5[a]	3/3
	DW samples	Concentrated CA	5[a]	3/3
		Concentrated NY	5[a]	3/3
		Unconcentrated DW	4[b]	2/24
	Cross-reactivity	5mg/L Lipopolysaccharide	unspiked	1/3
False positives		No false positives resulted from the analysis of the interferent or unspiked DW samples. There was one false positive replicate out of three when lipopolysaccharide was analyzed as a possible cross-reactive compound.		
False negatives		No false negatives resulted from the analysis of the interferent and DW samples spiked with detectable levels of Type A botulinum toxin; however, the BADD™ test strips were not able to reproducibly detect Type B botulinum toxin when spiked into DW or interferent samples at 4 mg/L or DI water up to 1,000 mg/L.		
Consistency		84% 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		5 mg/L (Type A), Type B was not reproducibly detectable. (vendor-stated LOD for botulinum toxin [non-specific]: 0.4 mg/L)		
Other performance factors		All necessary components for testing were provided in a resealable package weighing just a few ounces; strips used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; indicator line color change for the botulinum toxin test strips was very faint 29% of the time, increasing the likelihood of false negative results; and sample throughput was 20 to 30 samples per hour.		

[a] Type A botulinum toxin.

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		0.4	0/3
			2	0/3
			5	0/3
			15	0/3
			20	3/3
			200	3/3
			2,000	3/3
	Interferent PT and DW Samples		5	0/36
	Cross-reactivity	4 mg/L Lectin from soybean	unspiked	0/3
False positives		No false positives resulted from the analysis of the interferent, DW, or cross-reactivity samples.		
False negatives		Ricin was not reproducibly detectable when spiked into DW and interferent samples at 5 mg/L. No expanded testing was done involving the interferent or DW samples.		
Consistency		100% 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		20 mg/L (vendor-stated LOD: 0.4 mg/L)		
Other performance factors		All necessary components for testing were provided in a resealable package weighing just a few ounces; strips used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; indicator line color change for the ricin test strips was very faint for every positive response, increasing the likelihood of false negative results; and sample throughput was 20 to 30 samples per hour.		

Chapter 8
References

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