September 2004

Environmental Technology Verification Report

TETRACORE, INC.

ANTHRAX, BOTULINUM TOXIN, AND RICIN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Prepared by
Battelle

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The Business of Innovation

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ETV Advanced Monitoring Systems Center

Tetracore, Inc.
Anthrax, Botulinum Toxin, and Ricin Enzyme-Linked Immunosorbent Assay (ELISA)

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Notice

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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

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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
ELISA	enzyme-linked immunosorbent assay
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 Tetracore, Inc., anthrax, botulinum toxin, and ricin enzyme-linked immunosorbent assays (ELISA). Immunoassay detection technologies 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 ELISA (Figure 2-1), including kits for detecting anthrax, botulinum toxin, and ricin. The following is a description of the Tetracore ELISA based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The antigen-capture Tetracore ELISA detects antigens in samples by capturing them between a sandwich of antibodies. The immunosorbent assay uses immunological reagents to identify antibodies. The Tetracore ELISA can be read qualitatively (visually) and recorded by hand or quantitatively (using a photometer that measures and prints out the optical density of fluid samples in the microplate). Readings were made qualitatively during this verification test.

To perform a test, positive and negative capture antibody reagents are applied to alternating wells of a 96-well plate, where they are passively adsorbed. If the target antigen is present in the sample, it will bind to the reagent. A detector antibody forms the top of the sandwich and binds to any antigen in the sample after it is captured. The conjugate, to which the enzyme is covalently bound, is the third reagent added; and it binds to the detector antibody. The substrate, added after the conjugate, contains 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate), which, in the presence of horseradish peroxidase, changes to a bright green. The amount of color change is directly proportional to the amount of horseradish peroxidase present, which correlates to the amount of antigen (target contaminant) bound in the sandwich. The color change confirms the "capture" of antigen by the antibody reagents. For 48 samples, the process takes approximately 5 hours.

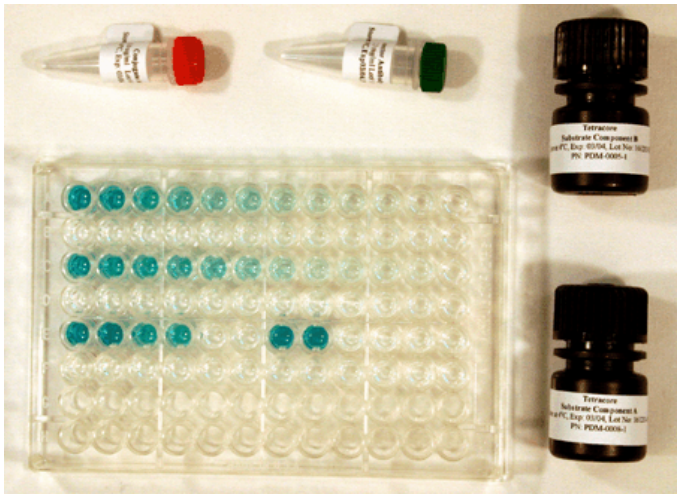

Figure 2-1. Tetracore ELISA

The Tetracore ELISA kit includes two 96-well plates in which the odd-numbered rows had been pre-plated with the antibodies specific for each target contaminant and the even-numbered rows had been pre-plated with antibodies not

specific to the target contaminants. The inclusion of the non-target antibodies provides assurance that a color change is due to an antigen-antibody interaction and is not due to a reaction with one of the other reagents. Also provided are dilution buffer, wash buffer, and the appropriate reagents needed for the analysis. The 96-well microplate is 12.5 centimeters (cm) by 8 cm. One Tetracore ELISA kit (positive and negative coated wells) costs $400.

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 single-use Tetracore ELISA plates detect only one contaminant at a time. For Tetracore ELISA analyses, the presence of contaminants is indicated by a change in color of a solution within a sample well. When a water sample is added, this color change occurs during the last step of a procedure that takes approximately 5 hours. With Tetracore ELISA, usually a large number of samples (i.e., 48 samples) can be analyzed simultaneously. Tetracore ELISA kits can be used in conjunction with an electronic reader to determine the presence or absence of a contaminant or can be read visually. In this verification test, the Tetracore ELISA 96-well plates for anthrax, botulinum toxin, and ricin were read visually, without the aid of a plate reader.

During this verification test, the Tetracore ELISA was 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 concentrations, and the source of the contaminant. The Tetracore ELISA also was 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 with the Tetracore ELISA. In addition, specificity was evaluated by exposing the Tetracore ELISA 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)	2×10^4 spores/mL	200 spores/mL[1]	Battelle and U.S. Army Dugway Proving Ground
Botulinum toxin Types A and B	0.004 mg/L	0.3 mg/L[2]	Metabiologics, Inc. (Madison, Wisconsin)
Ricinus communis Agglutinin II (ricin)	0.0015 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 Tetracore ELISA was conducted 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 physico-chemical 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 Tetracore ELISA 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 Tetracore ELISA 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 Tetracore ELISA with the addition of each target contaminant. All the samples listed in the test/QA plan were initially analyzed. As discussed below, additional concentration levels were analyzed to more thoroughly evaluate the performance of the Tetracore ELISA.

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 Types A and B	0.004 to 0.3 mg/L
	Ricin	0.0015 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) - 0.04 mg/L Ricin - 0.015 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) - 0.04 mg/L Ricin - 0.015 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) - 0.04 mg/L Ricin - 0.015 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) - 0.04 mg/L Ricin - 0.015 mg/L
Potentially Cross-reactive	*Bacillus thuringiensis* (anthrax analogue)	10^4 spores/mL
	Lipopolysaccharide (botulinum toxin analogue)	0.04 mg/L
	Lectin from soybean (ricin analogue)	0.015 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.

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.

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 0.04 (Type B) 0.04 (Type A)	unspiked 0.015
New York City, New York (NY)	unfiltered chlorinated	surface	conc.	unspiked 10^6 10^8	unspiked 0.04 (Type B) 0.04 (Type A)	unspiked 0.015
Metropolitan Water District of California (CA)	filtered chloraminated	surface	unconc.	unspiked 10^6	unspiked 0.04 (Type B)	unspiked 0.015
New York City, New York (NY)	unfiltered chlorinated	surface	unconc.	unspiked 10^6	unspiked 0.04 (Type B)	unspiked 0.015
Columbus, Ohio (OH)	filtered chlorinated	surface	both	unspiked 10^6	unspiked 0.04 (Type B)	unspiked 0.015
Orlando, Florida (FL)	filtered chlorinated	ground	both	unspiked 10^6	unspiked 0.04 (Type B)	unspiked 0.015

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 Tetracore ELISA. 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 a second source of anthrax spores that were never preserved in phenol 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 ELISA test kit 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 plates 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 Tetracore ELISA.

Three replicates of each PT sample were analyzed. A total of 162 PT samples was analyzed by the Tetracore ELISA for this test. The results provided information about how well the Tetracore ELISA detected the presence of each contaminant at several concentration levels, the consistency of the responses, and the susceptibility of the Tetracore ELISA 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 Tetracore ELISA 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. A total of 156 DW samples was analyzed by the Tetracore ELISA for this test.

3.2.3 Quality Control Samples

In addition to the 318 PT and DW samples analyzed, 33 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. With each set of samples, 12 concentration levels (one replicate each) of the target contaminant were analyzed to develop a calibration curve for use if a reader was used. In addition to serving as a calibration curve, those samples served as positive control samples to confirm the function of the Tetracore ELISA plate. Because of this control procedure, 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 testing procedure included the following steps for analyzing liquid samples for the presence of anthrax spores, botulinum toxin, or ricin: (1) 50 µL of dilution buffer were added to all wells that would contain a sample; (2) 50 µL of test sample were added to the dilution buffer, and the plates were incubated for one hour at room temperature; (3) plates were washed three times with 200 µL of Tetracore ELISA wash buffer per well; (4) 100 µL of detector antibody were added to each sample well, and plates were incubated for one hour at room temperature; (5) plates were washed three times with 200 µL of Tetracore ELISA wash buffer per well; (6) 100 µL of conjugate were added to each sample well, and the plates were incubated for one hour at room temperature; (7) the plates were washed three times with 200 µL of Tetracore ELISA wash buffer per well; (8) 100 µL of substrate solution were added to each sample well, and the plates were incubated for 30 minutes at room temperature; and (9) the results were read visually. Wells that changed to a color different than the negative control were recorded as having given positive results. The procedure took approximately 5 hours.

3.3.2 Non-Laboratory Testing

The test/QA plan called for testing the Tetracore ELISA in a non-laboratory setting with both a trained operator and an operator that had not been trained to operate the Tetracore ELISA and had no laboratory experience. This approach was designed to test the performance of the Tetracore ELISA in a non-laboratory setting, not to thoroughly evaluate the effect of changing conditions such as temperature and humidity on the Tetracore ELISA. However, two characteristics of the Tetracore ELISA prompted amending the test/QA plan to omit field portable verification parameters for both the technically trained and untrained operators. First, the Tetracore ELISA requires the use of a multichannel micropipettor to precisely manipulate solutions. Using a multichannel pipettor is a skill that requires training and practice. The objective of this portion of the testing was to evaluate the effectiveness of the Tetracore ELISA as a detection tool for untrained operators. Therefore, the test/QA plan was amended to omit this testing parameter for technologies such as the Tetracore ELISA that cannot be operated without some acquired technical skill. Second, the testing procedure does not change at all when performing tests outside the laboratory. The Tetracore ELISA plates and reagents could be packaged into a shoebox-sized container and transported to a non-laboratory location. In the manner that the Tetracore ELISA was tested, there was no requirement for electrical power. The only items required were a waste container and a flat surface. Because observing the analyses in the laboratory was adequate for determining the feasibility of using the Tetracore ELISA outside the laboratory, the non-laboratory testing including the trained operator was omitted from the test/QA plan.

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 Tetracore ELISA 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 = 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 Tetracore ELISA and all appropriate reagents and supplies specific for the detection of anthrax, botulinum toxin, and ricin were provided to Battelle by the vendor. Because no plate reader was used, the Tetracore ELISA test results were read visually; and, therefore, no calibration was required. 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 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.

Another lot of anthrax spores from Dugway Proving Ground was obtained and used to investigate the sensitivity of Tetracore ELISA to a different spore preparation (referred to as Dugway-prepared in this report). No characterization data except for enumerations were performed on this lot of spores. Vegetative *Bacillus anthracis* was also analyzed during this verification test. Vegetative cells from an enumeration of the Battelle-prepared, phenol-preserved spores were collected, placed in solution, and then enumerated to determine the concentration of vegetative *Bacillus anthracis* in the solution. No further characterization was performed on these vegetative anthrax cells. Solutions of these cells were used to determine the sensitivity of the Tetracore ELISA to vegetative cells.

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

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.

For the Battelle-prepared, phenol-preserved spores, only one enumeration resulted in a concen-

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

tration that was more than 25% different from the expected concentration. The average 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 enumeration performed, the relative standard deviation of the eight results was 15%. The accuracy and

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
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.

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 vegetative anthrax cells and a stock solution of spores obtained from Dugway Proving Ground. 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.

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%.

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. However, samples containing *Bacillus thuringiensis* (analogue of anthrax) were enumerated and were determined to be 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 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.

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 Tetracore ELISA test kits produce qualitative results; i.e., the color change within a sample well on a Tetracore ELISA plate indicates 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 Tetracore ELISA 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 Tetracore ELISA 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 Tetracore ELISA 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 Tetracore ELISA.

Chapter 6
Test Results

6.1 Qualitative Contaminant Presence/Absence

The responses for the Tetracore ELISA using the contaminant-only PT samples containing anthrax, botulinum toxin, and ricin are discussed in the following sections. The Tetracore ELISA provides indication of only a positive or negative response based on whether the color of a solution in a sample well changes to a color different from that of the method blank sample.

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 February 2 confirmed the concentration of the stock solution used to prepare the solutions analyzed by the Tetracore ELISA. In addition, the serial dilution validation enumeration on January 30 (1×10^5 spores/mL expected) confirmed that anthrax solutions can be accurately diluted using standard techniques. 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 Tetracore ELISA to the affected spores.)

2. The sensitivity of the Tetracore ELISA 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
	2×10^4 spores/mL[c]	Spores	Battelle	Yes	0/3
	8×10^4 spores/mL	Spores	Battelle	Yes	0/3
	2×10^5 spores/mL	Spores	Battelle	Yes	0/3
	8×10^5 spores/mL	Spores	Battelle	Yes	0/3
Expanded 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	3/3
Alternate spore preparation	8×10^6 spores/mL	Spores	Dugway	No	0/3
	8×10^5 spores/mL	Spores	Dugway	No	0/3
	8×10^4 spores/mL	Spores	Dugway	No	0/3
	8×10^3 spores/mL	Spores	Dugway	No	0/3
Vegetative cell sensitivity	3×10^5 cfu/mL	Vegetative	Battelle	NA	3/3
	3×10^4 cfu/mL	Vegetative	Battelle	NA	3/3
	3×10^3 cfu/mL	Vegetative	Battelle	NA	0/3
	3×10^2 cfu/mL	Vegetative	Battelle	NA	0/3

[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] Vendor-stated LOD.
NA = not applicable. Vegetative cells were not prepared from any stock solution; they were grown and placed in solution.

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 Tetracore ELISA. 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 Tetracore ELISA, increasing the concentration of spores beyond what was required by the test/QA plan, and subjecting the plates 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 Tetracore ELISA, as well as the possibility that the Tetracore ELISA could not detect the spores prepared by Battelle as readily as the spores prepared elsewhere, a series of samples was prepared using one anthrax spore stock solution that had been stored in a phenol solution and one that had not. Using the Battelle preparation, samples containing approximately 10^9, 10^8, and 10^7 spores/mL were prepared and analyzed. All three concentration levels were detectable, whereas 10^6 spores/mL had been shown to be not detectable during prior testing. Therefore, the lowest detectable concentration for Battelle-prepared spores was approximately 10^7 spores/mL. 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 to prepare solutions with various concentration levels (10^7, 10^6, 10^5, and 10^4 spores/mL). Both the stock solution concentration and the dilution methodology were confirmed by plate enumeration as shown in Table 4-2. These samples were analyzed to determine the approximate sensitivity to these spores. None of these concentration levels were detectable.

Tetracore informed Battelle that its ELISA is more sensitive to vegetative anthrax than spores. To evaluate this premise, a solution of vegetative cells was prepared by collecting a single vegetative anthrax colony from an enumeration plate, placing it into DI water, and mixing it well. This solution was diluted by a factor of 10 four times, and then the stock solution and two diluted samples were enumerated to determine the concentration of vegetative cells in each sample. These samples were analyzed to determine the approximate sensitivity for these vegetative cells. The lowest detectable concentration of vegetative cells was 3×10^4 colony-forming units (cfu)/mL, approximately the concentration of the vendor-stated LOD 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 Tetracore ELISA was reproducibly sensitive to botulinum toxin Type A at concentrations as low as 0.02 mg/L. However, for botulinum toxin Type B, the results were very inconsistent. The lowest concentrated sample (0.004 mg/L) generated 2 out of 3 positive results, and the next two higher concentration levels (0.02 and 0.04 mg/L) resulted in only 1 positive result out of 6 total replicates. The 0.2-mg/L samples generated 3 out of 3 detectable results, and the 0.3-mg/L sample generated 1 out of 3 detectable results. These results are especially puzzling because the Tetracore ELISA was able to reproducibly detect 0.04 mg/L of botulinum toxin Type B when it was spiked into possible interfering matrices, as well as DW. There is no certain explanation for the inconsistent botulinum toxin Type B PT sample results.

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	0.004[a]	0/3	2/3
	0.02	3/3	0/3
	0.04	3/3	1/3
	0.2	3/3	3/3
	0.3[b]	NA[c]	1/3

[a] Vendor-stated LOD.
[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. With the exception of the 0.0015 mg/L sample (which generated all negative results), all replicate samples analyzed 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	0.0015[a]	0/3
	0.0075	3/3
	0.015	3/3
	0.075	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 Tetracore ELISA 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 Tetracore ELISA plates coated with antibodies 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 presented.

For anthrax, no false positive results were generated from the interferent samples that did not contain anthrax spores (blanks). The two spiked Ca and Mg interferent samples contained 6 and 8×10^5 spores/mL and were falsely negative with respect to the vendor-stated LOD. However, because these concentrations were very similar to the concentration of the PT samples that were also determined to be not detectable, it is not clear if the negative results were due to the interferents. For both spiked humic and fulvic acid interferent samples containing 2×10^6 spores/mL, a concentration that had previously been shown not to be detectable in the PT samples, the results were positive. There is not a clear explanation for these positive results. Expanded testing was performed to evaluate the performance of the Tetracore ELISA when analyzing higher concentrations of anthrax spores. During that testing, 1×10^8 spores/mL, a detectable concentration in DI water, were spiked into two interferent solutions. In Ca and Mg, as well as in humic and fulvic acids, none of the three replicates had false negative results.

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**	**2×10^{6}[a]**	**1×10^{8}**	**Blank**	**Type B 0.04**	**Type A 0.04**	**Blank**	**0.015**
46 mg/L Ca 18 mg/L Mg	0/3	0/3 8×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
230 mg/L Ca 90 mg/L Mg	0/3	0/3 6×10^{5}[b]	3/3 8×10^{7}[b]	0/3	3/3	3/3	0/3	3/3
0.5 mg/L humic and fulvic acid	0/3	3/3 2×10^{6}[b]	NA	0/3	0/3	NA	0/3	3/3
2.5 mg/L humic and fulvic acid	0/3	3/3 2×10^{6}[b]	3/3 1×10^{8}[b]	0/3	3/3	1/3	0/3	3/3

NA = not applicable. Sample not analyzed during expanded testing of anthrax or testing of botulinum toxin Type A.
[a] Expected concentration.
[b] Actual concentration.

For botulinum toxin, no false positive results were generated from the interferent samples that did not contain botulinum toxin. When botulinum toxin Type B was added to the interferent samples at 0.04 mg/L, all the results were positive except for the 0.5 mg/L humic and fulvic acid sample. This apparent interferent was unexpected because the higher concentration of humic and fulvic acid did not cause false negative results. Two interferent samples were fortified with botulinum toxin Type A. The Ca and Mg sample generated three out of three positive results (no false negatives), but the humic and fulvic acid sample generated only one out of three positive results (two false negatives). For ricin, there were no false positive or false negative results with respect to the interferent samples.

6.2.2 DW Samples

The results from the DW samples are given in Table 6-3. For Tetracore ELISA plates coated with antibodies 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.

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**	**2×10^{6}[a]**	**1×10^{8}**	**Blank**	**Type B 0.04**	**Type A 0.04**	**Blank**	**0.015**
Unconcentrated CA DW	0/3	0/3 7×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated CA DW	0/3	0/3 7×10^{5}[b]	3/3 5×10^{7}[b]	0/3	3/3	3/3	0/3	3/3
Unconcentrated FL DW	0/3	0/3 8×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated FL DW	0/3	0/3 9×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Unconcentrated NY DW	0/3	0/3 1×10^{6}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated NY DW	0/3	0/3 1×10^{6}[b]	3/3 5×10^{7}[b]	0/3	3/3	3/3	0/3	3/3
Unconcentrated OH DW	0/3	0/3 7×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3
Concentrated OH DW	0/3	0/3 6×10^{5}[b]	NA	0/3	3/3	NA	0/3	3/3

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

For anthrax, there were no false positive results for the unspiked DW samples. Also, the DW samples initially spiked with an expected concentration of 2×10^6 spores, but with actual concentrations of between 7×10^5 and 2×10^6 spores/mL, generated false negative results with respect to the vendor-stated LOD. As had been observed for the humic and fulvic acid interferent samples, the negative results cannot necessarily be attributed to the DW matrix. The DW samples spiked with approximately 1×10^8 spores/mL generated no false negative results.

For botulinum toxin, there were no false positive results for the unspiked DW samples or false negative results for either the Type B or Type A botulinum toxin. In 24 out of 24 Type B replicate samples spiked with 0.04 mg/L botulinum toxin Type B, all the results were positive, again contrasting with the PT sample results, where 0.04 mg/L was only detected in 1 out of 3 replicates. Similar results were generated for ricin. No false positives were observed for the unspiked DW samples, and no false negative results were observed for the 0.015 mg/L spiked DW samples.

6.2.3 Cross-Reactivity PT Samples

The results from the cross-reactivity PT samples are given in Table 6-4. For Tetracore ELISA plates coated with antibodies 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 of the target contaminant. The number of positive results out of the number of replicates is given for each sample. All 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 (1×10^4 spores/mL)[(a)]	0/3		
Lipopolysaccharide (0.04 mg/L)		0/3	
Lectin from soybean (0.015 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 Tetracore ELISA analysis of anthrax spores and ricin, the results were 100% consistent. The Tetracore ELISA plates coated with antibodies specific for ricin generated results for 30 sample sets of three replicates, and all of them produced results that were either all positive or all negative. Similarly, for anthrax, all 47 sets were entirely positive or negative. The botulinum toxin results were not as consistent as the anthrax and ricin results. The PT samples containing botulinum toxin Type B generated 3 out of 5 sample sets in which the results were not consistent.

All four botulinum toxin Type A PT sample sets, however, generated consistent results. The botulinum toxin Type B interferent and DW samples were 100% consistent over 16 sample sets. Overall, 98% of the Tetracore ELISA samples analyzed generated results in sets of 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 spores, that concentration was 8×10^6 spores/mL (for Battelle-prepared spores) and 3×10^4 cfu/mL for the vegetative anthrax cells. The spores from the Dugway preparation were not detectable at levels up to 8×10^6 spores/mL. For botulinum toxin Type A, the lowest detectable concentration was 0.02 mg/L; but, for Type B, it was less clear because of the inconsistent results for the PT samples. The PT sample results indicated that the lowest detectable concentration was 0.004 mg/L, but some concentrations above that were not detectable. However, 0.04 mg/L was consistently detectable in the interferent and DW samples, so the LOD is at least 0.04 mg/L. For ricin, the lowest detectable concentration was 0.0075 mg/L.

6.5 Other Performance Factors

Battelle technicians, who had been trained by Tetracore to perform testing using the Tetracore ELISA, performed all of the required laboratory testing. The technicians had no problem performing the tests as they were trained. In addition to the antibody-coated Tetracore ELISA plates and other reagents, a multichannel pipette, disposable tips, and plastic troughs for filling the multichannel pipette with solution were required for testing. Because the Tetracore ELISA plates were made from transparent plastic, a color change in a sample well could be observed visually against a white background, such as a piece of paper. At times it was difficult to determine whether a sample was different from the negative control sample. A plate reader would eliminate that judgment call by the operator.

The performance of the Tetracore ELISA was not demonstrated outside the laboratory because the functioning of the tools/equipment required for the Tetracore ELISA is independent of location. Also, the procedure for laboratory analysis would not change if testing was moved from the laboratory. No carrying case is provided, but a small box could easily be used for transporting the supplies needed. The main requirement for a non-laboratory setting would be a well-lighted, flat work space. Depending on the conditions, it may be beneficial to cover the ELISA plates during incubation to avoid contamination.

Similarly, testing using an untrained operator was not performed because of the background knowledge necessary to understand the detailed procedures for performing the testing and to correctly use a multichannel pipettor. The operators with a technical background could analyze 48 samples on a single plate in approximately five hours.

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	3/3
			8×10^5 spores/mL	0/3
		Vegetative cells	3×10^5 cfu/mL	3/3
			3×10^4 cfu/mL	3/3
			3×10^3 cfu/mL	0/3
			3×10^3 cfu/mL	0/3
		Dugway-prepared spores	8×10^6 spores/mL	0/3
			8×10^5 spores/mL	0/3
			8×10^4 spores/mL	0/3
			8×10^3 spores/mL	0/3
	Interferent PT samples	230 mg/L Ca 90 mg/L Mg	8×10^7 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
		Humic acid and fulvic acid	2×10^6 spores/mL[b]	6/6
		Ca and Mg	2×10^6 spores/mL	0/6
	DW samples	Concentrated CA	5×10^7 spores/mL[b]	3/3
		Concentrated NY	5×10^7 spores/mL[b]	3/3
		Unconcentrated DW	2×10^6 spores/mL	0/24
	Cross-reactivity	1×10^4 spores/mL *Bacillus thuringiensis*	unspiked	0/3
False positives		No false positives resulted from the analysis of the interferent, DW, or cross-reactivity samples. However, two humic and fulvic acid samples, spiked at concentrations below what was detectable in DI water, 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.		

Parameter	Sample Information
False negatives	No false negative results were generated for the analysis of interferent or DW samples spiked with detectable levels of anthrax. Tetracore ELISA was not able to detect anthrax at the vendor-stated LOD, but was able to at much higher concentrations. All of the unconcentrated DW and six Ca and Mg samples were spiked at concentrations less than detectable by the test strips and, therefore, were, as expected, negative.
Consistency	100% (47 out of 47) 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	8×10^6 spores/mL - Battelle prep (vendor-stated LOD: 2×10^4 spores/mL); 3×10^4 cfu/mL - vegetative anthrax (no vendor-stated LOD); the Dugway preparation of spores was not detectable at concentrations up to 8×10^6 spores/mL
Other performance factors	A technically trained operator easily performed the Tetracore ELISA analysis. Untrained, non-technical, first-time users would not likely be able to perform the testing because of the need to use a multichannel pipettor, prepare solutions, and read a technical operating procedure. The Tetracore ELISA could be used outside the laboratory without a problem. At times it was difficult to determine whether the color of the sample had changed; no reader was used. Sample throughput was 48 samples in 5 hours.

(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	0.004	0/3
			0.02	3/3
			0.04	3/3
			0.2	3/3
		Type B	0.004	2/3
			0.02	0/3
			0.04	1/3
			0.2	3/3
			0.3	1/3
	Interferent PT samples	Ca and Mg	0.04	3/3 Type A 6/6 Type B
		Humic acid and fulvic acid	0.04	1/3 Type A 3/6 Type B
	DW samples	Concentrated DW	0.04	6/6 Type A 12/12 Type B
		Unconcentrated DW	0.04	6/6 Type A 12/12 Type B
	Cross-reactivity	0.04 mg/L Lipopolysaccharide	unspiked	0/3
False positives		There were no false positive results for the interferent, DW, or cross-reactivity samples.		
False negatives		Two out of three results were false negative when 0.04 mg/L botulinum toxin Type A was spiked into 2.5 mg/L humic and fulvic acids, and three out of three were false negatives when botulinum toxin Type B was spiked into 0.5 mg/L humic and fulvic acids. There were no false negatives for the spiked DW samples.		
Consistency		With the exception of 2.5 mg/L humic and fulvic acids spiked with 0.04 mg/L botulinum toxin Type A (1 out of 3 positive), results generated for botulinum toxin Type A were 100% consistent. The DW and interferent samples spiked with botulinum toxin Type B were equally consistent, but the contaminant PT samples containing botulinum toxin Type B generated consistent results in just 2 out of 5 sample sets. Overall, 98% of the results were from sample sets that were either all positive or all negative.		
Lowest detectable concentration		0.02 mg/L (Type A); not clear for Type B because of sporadic results. (vendor-stated LOD for botulinum toxin [non-specific]: 0.004 mg/L)		
Other performance factors		A technically trained operator easily performed the Tetracore ELISA analysis. Untrained, non-technical, first-time users would not likely be able to perform the testing because of the need to use a multichannel pipettor, prepare solutions, and read a technical operating procedure. The Tetracore ELISA could be used outside the laboratory without a problem. At times it was difficult to determine whether the color of the sample had changed; no reader was used. Sample throughput was 48 samples in 5 hours.		

Table 7-3. Ricin Summary Table

Parameter		Sample Information	Ricin Concentration (mg/L)	Positive Results Out of Total Replicates
Qualitative contaminant positive results	Contaminant-only PT samples	Ricin PT samples	0.0015	0/3
			0.0075	3/3
			0.015	3/3
			0.075	3/3
			15	3/3
	Interferent PT samples	Ca and Mg	0.015	6/6
		Humic acid and fulvic acid	0.015	6/6
	DW samples	Concentrated CW	0.015	12/12
		Unconcentrated DW	0.015	12/12
	Cross-reactivity	0.015 mg/L Lectin from soybean	unspiked	0/3
False positives		No false positive results were generated for ricin in DW or interferent samples.		
False negatives		There were no false negative results for interferent or DW samples spiked with detectable concentrations of ricin.		
Consistency		100% of the results for ricin were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		
Lowest detectable concentration		0.0075 mg/L (vendor-stated LOD: 0.0015 mg/L)		
Other performance factors		A technically trained operator easily performed the Tetracore ELISA analysis. Untrained, non-technical, first-time users would not likely be able to perform the testing because of the need to use a multichannel pipettor, prepare solutions, and read a technical operating procedure. The Tetracore ELISA could be used outside the laboratory without a problem. At times it was difficult to determine whether the color of the sample had changed; no reader was used. Sample throughput was 48 samples in 5 hours.		

Chapter 8
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