September 2004

Environmental Technology Verification Report

RESPONSE BIOMEDICAL CORP.
RAMP® ANTHRAX, BOTULINUM TOXIN, AND RICIN IMMUNOASSAY TEST CARTRIDGES

Prepared by

Battelle
The Business of Innovation

Battelle

Under a cooperative agreement with

EPA **U.S. Environmental Protection Agency**

September 2004

Environmental Technology Verification Report

ETV Advanced Monitoring Systems Center

Response Biomedical Corp.
RAMP®
Anthrax, Botulinum Toxin, and Ricin Immunoassay Test Cartridges

by
Ryan James
Amy Dindal
Zachary Willenberg
Karen Riggs

Battelle
Columbus, Ohio 43201

Notice

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

Foreword

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

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

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

Acknowledgments

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

Contents

Page

Notice ii

Foreword iii

Acknowledgments iv

List of Abbreviations vii

1 Background 1

2 Technology Description 2

3 Test Design and Procedures 4
- 3.1 Introduction 4
- 3.2 Test Samples 5
  - 3.2.1 Performance Test Samples 7
  - 3.2.2 Drinking Water Samples 8
  - 3.2.3 Quality Control Samples 9
- 3.3 Test Procedure 9
  - 3.3.1 Laboratory Testing 9
  - 3.3.2 Non-Laboratory Testing 9
  - 3.3.3 Drinking Water Characterization 10

4 Quality Assurance/Quality Control 12
- 4.1 Sample Chain-of-Custody Procedures 12
- 4.2 Equipment/Calibration 12
- 4.3 Characterization of Contaminant Stock Solutions 12
  - 4.3.1 Characterization of Botulinum Toxin and Ricin 12
  - 4.3.2 Characterization of Anthrax Spores 13
  - 4.3.3 Anthrax Enumeration Data 14
- 4.4 Technical Systems Audit 16
- 4.5 Audit of Data Quality 18
- 4.6 QA/QC Reporting 18
- 4.7 Data Review 18

5 Statistical Methods and Reported Parameters 20
- 5.1 Qualitative Contaminant Presence/Absence 20
- 5.2 False Positive/Negative Responses 20
- 5.3 Consistency 20
- 5.4 Lowest Detectable Concentration 20
- 5.5 Other Performance Factors 21

6 Test Results 22
6.1 Qualitative Contaminant Presence/Absence 22
6.1.1 Anthrax 22
6.1.2 Botulinum Toxin 25
6.1.3 Ricin 26
6.2 False Positive/Negative Responses 27
6.2.1 Interferent PT Samples 27
6.2.2 DW Samples 28
6.2.3 Cross-Reactivity PT Samples 29
6.3 Consistency 30
6.4 Lowest Detectable Concentration 30
6.5 Other Performance Factors 31

7 Performance Summary 32

8 References 37

Appendix Other Evaluations

Figures

Figure 2-1. Response Biomedical Corp. RAMP® Immunoassay Test Cartridge and Reader . . 2

Tables

Table 3-1. Lethal Dose and Source of Contaminants 5
Table 3-2. Performance Test Samples 6
Table 3-3. Drinking Water Samples 7
Table 3-4. ATEL Water Quality Characterization of the Drinking Water Samples 11
Table 4-1. Characterization Information for Battelle Preparation of Anthrax Spores 13
Table 4-2. Anthrax Enumeration Data for PT Samples 15
Table 4-3. Anthrax Enumeration Results for Fortified Interferent and Drinking Water Sample 17
Table 4-4. Summary of Data Recording Process 19
Table 6-1a. Anthrax Contaminant-Only PT Sample Results 23
Table 6-1b. Botulinum Toxin Contaminant-Only PT Sample Results 26
Table 6-1c. Ricin Contaminant-Only PT Sample Results 26
Table 6-2. Interferent PT Sample Results 27
Table 6-3. DW Sample Results 29
Table 6-4. Potentially Cross-Reactive PT Sample Results 30
Table 7-1. Anthrax Summary Table 32
Table 7-2. Botulinum Toxin Summary Table 34
Table 7-3. Ricin Summary Table 36

List of Abbreviations

AMS	Advanced Monitoring Systems
ATEL	Aqua Tech Environmental Laboratories, Inc.
Ca	calcium
CDC	Centers for Disease Control and Prevention
cfu	colony-forming units
COA	certificate of analysis
DI	deionized
DW	drinking water
EPA	U.S. Environmental Protection Agency
ETV	Environmental Technology Verification
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
RAMP®	Rapid Analyte Measurement Platform
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 Response Biomedical Corp. Rapid Analyte Measurement Platform (RAMP®) anthrax, botulinum toxin, and ricin immunoassay test kits. Immunoassay test kits were identified as a priority technology category for verification through the AMS Center stakeholder process.

Chapter 2
Technology Description

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

RAMP® is a rapid immunochromatographic system for screening environmental samples. The RAMP® system comprises a portable fluorescence reader and RAMP® test cartridges specific for detecting anthrax, botulinum toxin, and ricin. Test cartridges specific for detecting smallpox are also available, but were not tested.

Figure 2-1. Response Biomedical Corp. RAMP® Immunoassay Test Cartridge and Reader

The RAMP® reader is a scanning fluorometer and data analysis system used to measure fluorescence from RAMP® test cartridges. The reader can be operated on built-in battery power or using an alternating current adapter. RAMP® uses an immunochromatographic strip, housed in the disposable test cartridges. Each test cartridge is single-use, disposable, and analyte-specific and is used to detect whether an analyte (e.g., anthrax spores) is present in an aqueous sample.

Twenty-five individually packaged RAMP® test cartridges are provided in a small box. In addition to the test cartridges, the box contains 25 small plastic screw-top vials containing approximately 250 microliters (µL) of buffer, a box of sample collection swabs, a 70-µL micropipette, a lot card for insertion into the reader, a marking pen, and step-by-step instructions. To perform a test on a liquid sample, a small amount (10 µL) of sample is added to the provided buffer, and that solution is mixed and 70 µL of sample is pipetted onto the RAMP® test cartridge. The cartridge is then read using the reader, and a positive or negative result is generated on the reader's display. Each result, along with the time, date, and sample identification is printed using a printer provided by Response Biomedical Corp. The reader is also capable of downloading the results to a computer.

The dimensions of the RAMP® are 10.5 inches wide by 10 inches deep by 6 inches high (27 centimeters wide by 25 centimeters deep by 5 centimeters high), and it weighs 4.6 pounds (2.1 kilograms). A RAMP® system including 25 test cartridges, a reader, a printer, and a carrying case costs approximately $10,000. Regardless of whether the test strips are specific to anthrax, botulinum toxin, ricin, or smallpox, each additional box of 25 test cartridges costs approximately $500.

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. For the RAMP® test cartridges, the contaminants, or antigens, react with a selective antibody to produce a result that is indicated by fluorescence from the test cartridge in less than 15 minutes. The presence of contaminants is indicated by positive or negative reading from the fluorescence reader. The single-use test cartridges detect only one contaminant at a time.

During this verification test, the RAMP® test cartridges 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 concentrations, and the source. The RAMP® test cartridges 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 RAMP® test cartridges. In addition, specificity was evaluated by exposing the RAMP® test cartridges 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)	4×10^5 spores/mL	200 spores/mL[1]	Battelle and U.S. Army Dugway Proving Ground
Botulinum toxin Types A and B	0.5 mg/L	0.3 mg/L[2]	Metabiologics, Inc. (Madison, Wisconsin)
Ricinus communis Agglutinin II (ricin)	1 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 RAMP® test kits 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 RAMP® test cartridges 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 RAMP® test cartridges to individually detect various concentrations of anthrax spores, botulinum toxin, and ricin was evaluated by analyzing performance test (PT) and DW samples. PT samples

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	1 to 50 mg/L
Interferent	Contaminants in 46 mg/L Ca and 18 mg/L Mg	Anthrax - 10^7 spores/mL Botulinum toxin (Type B) - 5 mg/L Ricin - 10 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) - 5 mg/L Ricin - 10 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) - 5 mg/L Ricin - 10 mg/L
	Contaminants in 2.5 mg/L humic acid and 2.5 mg/L fulvic acids	Anthrax - 10^7 and 10^8 spores/mL Botulinum toxin (Type A) - 5 mg/L Botulinum toxin (Type B) - 5 mg/L Ricin - 10 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)	10 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.

included DI water fortified with either the target contaminant, an interferent, both, or only a cross-reactive species. DW samples were analyzed using the RAMP® test cartridges 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 RAMP® test cartridges.

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 4×10^6 10^9	unspiked 5 (Type B) 5 (Type A)	unspiked 10
New York City, New York (NY)	unfiltered chlorinated	surface	conc.	unspiked 4×10^6 10^9	unspiked 5 (Type B) 5 (Type A)	unspiked 10
Metropolitan Water District of California (CA)	filtered chloraminated	surface	unconc.	unspiked 4×10^6	unspiked 5	unspiked 10
New York City, New York (NY)	unfiltered chlorinated	surface	unconc.	unspiked 4×10^6	unspiked 5	unspiked 10
Columbus, Ohio (OH)	filtered chlorinated	surface	both	unspiked 4×10^6	unspiked 5	unspiked 10
Orlando, Florida (FL)	filtered chlorinated	ground	both	unspiked 4×10^6	unspiked 5	unspiked 10

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 determine their concentrations. Anthrax PT samples also were prepared in DI water using anthrax spores prepared and characterized by Battelle using standard methods. All test samples were prepared from the standards or stock solutions on the day of analysis. Spores obtained from Dugway Proving Ground were prepared there and then enumerated by Battelle during this verification test.

Initially, the test/QA plan called for the analysis of PT samples with concentrations including the lethal dose; the vendor-stated LOD; and approximately 5, 10, and 50 times the LOD. These samples were analyzed using the RAMP® test cartridges. Preliminary results indicated that anthrax and botulinum toxin were not detectable; therefore, the original test/QA plan was amended to include preparing and analyzing higher concentration samples of anthrax and botulinum toxin Type A and testing a preparation of anthrax spores that were never preserved in phenol, a second source of anthrax spores, a sample of anthrax vaccine, 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 RAMP® test cartridges 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 cartridges 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 RAMP® test cartridge.

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 cartridges available for the analysis. A total of 192 PT samples was analyzed by the RAMP® test cartridges for this test. The results provided information about how well the RAMP® test cartridges detected the presence of each contaminant at several concentration levels, the consistency of the responses, and the susceptibility of the RAMP® test cartridges 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 RAMP® test cartridges 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 liters (L) of each of the above 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 water 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 RAMP® test cartridges for this test.

3.2.3 Quality Control Samples

In addition to the 348 PT and DW samples analyzed, 43 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. Each RAMP® test cartridge runs an internal fluorescent control. The RAMP® reader produces an error message if the internal fluorescent control fails. If the RAMP® reader ever produced an error message instead of a result, that test kit would have been discarded and a new test cartridge used. There were no instances of this during ETV testing. Because of this internal fluorescent control, 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. The following procedure was performed to test a liquid sample for the presence of anthrax spores, botulinum toxin, and ricin: (1) 10 μL[1] of test sample were added to the sample buffer vial and mixed; (2) the reader was turned on and a lot card was inserted, its information uploaded, and then it was removed (one time only per manufacturer's lot of test cartridges); (3) user and sample identification were entered into the reader: (4) the test cartridge was removed from its package along with the 70-μL pipette tip (the presence of a pink dot on the inside of the tip was noted); (5) the tip was firmly pressed on the micropipette; (6) the sample was mixed by filling and dispensing the micropipette 10 times (after which it was confirmed that the pink dot in the tip had disappeared); (7) the micropipette was filled and dispensed into the sample well on the test cartridge; and (8) the test cartridge was immediately pressed into the reader until resistance was felt. The reader then automatically performed all time and measurement processes and generated the results in approximately 15 minutes. 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.

3.3.2 Non-Laboratory Testing

Because the toxic nature of the contaminants did not permit their use outside special laboratory facilities, MB samples were analyzed at a non-laboratory location to evaluate the RAMP® performance and ease of use outside of the laboratory. Both a trained technician and a non-technical, untrained first-time user performed analyses at the non-laboratory location. The

[1]The instructions provided within the box of test cartridges stated that the sample should be collected by placing a swab in the test sample for 3 seconds, transferring the swab into the sample buffer, and repeating once. At the suggestion of Response Biomedical Corp., a 10-μL pipettor was used to increase the efficiency of our analyses.

purpose of these analyses was to test the performance of the RAMP® in a non-laboratory setting, not to evaluate thoroughly the effect of changing conditions such as temperature and humidity on the RAMP®. Initially, the non-technical, untrained first-time user was guided only 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 cartridges 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 RAMP® test cartridges.

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 RAMP® test cartridges 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 fluorescent control, required no calibration. For DW characterization and confirmation of the possible interferent, 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 of those contaminants. 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 cartridges.

Similarly, a lot of anthrax spores from Dugway Proving Ground was obtained and used to investigate the sensitivity of RAMP® test cartridges 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. Vegetative cells from an enumeration of the Battelle-prepared, phenol-preserved spores were collected and placed in a 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 RAMP® test cartridges to vegetative cells. At the request of Response Biomedical Corp., Battelle obtained an aliquot of anthrax vaccine from Colorado Serum Company, Denver, Colorado, which is Stern *Bacillus anthracis*, to confirm the functionality of their technology. No characterization or enumeration was performed on the vaccine.

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

4.3.3 Anthrax Enumeration Data

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

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

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.

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 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 (not preserved with phenol), 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 RAMP® test cartridges, 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 higher than Response Biomedical Corp. had claimed, additional samples containing higher concentration levels of anthrax were prepared and analyzed. Additional resources were not expended to determine the cross-reactivity of *Bacillus thuringiensis* at comparable concentration levels.

4.4 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) to ensure that the verification test was performed in accordance with the test/QA plan[4] and the AMS Center QMP.[11] As part of the audit, the Battelle Quality Manager reviewed the standards and methods used, compared actual test procedures with those specified in the test/QA plan,[4] and reviewed data acquisition and handling procedures. Observations and findings from this audit were

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

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%.

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 RAMP® test cartridges 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 RAMP® test cartridges 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 cartridge 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 RAMP® test cartridge 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 RAMP®.

Chapter 6
Test Results

6.1 Qualitative Contaminant Presence/Absence

The responses for the RAMP® test cartridges using the contaminant-only PT samples containing anthrax, botulinum toxin, and ricin are discussed in the following sections. The RAMP® test cartridges provide indication of only a positive or negative response based on whether or not the level of fluorescence is above the detectable threshold. Upon sample application, the test cartridge was inserted into the RAMP® reader, which determined conclusively whether the test result was positive or negative. Note that the appendix to this report contains a vendor-supplied reference on the performance of the test cartridges.

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 these 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 RAMP® test cartridges. In addition, the serial dilution validation enumeration on January 30 (4×10^5 spores/mL expected) confirmed that the anthrax solutions can be accurately diluted using standard techniques. After discussions with Response Biomedical Corp., 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 RAMP® test cartridges to the affected spores.)

2. The sensitivity of the RAMP® test cartridges 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 RAMP® test cartridges 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 RAMP® test cartridges; 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
	3×10^5 spores/mL[c]	Spores	Battelle	Yes	0/3
	2×10^6 spores/mL	Spores	Battelle	Yes	0/3
	4×10^6 spores/mL	Spores	Battelle	Yes	0/3
	2×10^7 spores/mL	Spores	Battelle	Yes	0/3
Investigation of phenol storage of spores	2×10^7 spores/mL	Spores	Battelle	No	0/3
	1×10^9 spores/mL	Spores	Battelle	No	2/2[d]
	8×10^8 spores/mL	Spores	Battelle	Yes	3/3
	1×10^{10} spores/mL	Spores	Battelle	No	low signal
	8×10^9 spores/mL	Spores	Battelle	Yes	low signal
Sensitivity determination	8×10^8 spores/mL	Spores	Battelle	Yes	3/3
	8×10^7 spores/mL	Spores	Battelle	Yes	0/3
	8×10^6 spores/mL	Spores	Battelle	Yes	0/3
Alternate spore preparation	7×10^8 spores/mL	Spores	Dugway	No	3/3
	8×10^7 spores/mL	Spores	Dugway	No	0/1
	Unknown concentration	Stern vaccine	Colorado Serum	No	4/4
Vegetative cell sensitivity	Unknown Conc.	Vegetative	Battelle	NA	2/2
	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.

[d] The result for one additional replicate was “low signal” error.

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 RAMP® test cartridges. 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 RAMP® test cartridges; increasing the concentration of spores beyond what was required by the test/QA plan; subjecting the test cartridges to Ames strain anthrax spores prepared by Dugway Proving Ground using a preparation method that is different from the one Battelle uses; subjecting the test cartridges to the anthrax vaccine (Stern strain anthrax spores); and testing the response of the test cartridges to vegetative anthrax cells at various concentrations.

To address the possibility that storing spores in phenol affected the sensitivity of the RAMP® test cartridges, a series of samples was prepared and analyzed using one anthrax spore stock solution that had been stored in a phenol solution and one that had not. The data are given in Table 6-1a under "Purpose of Analysis, Investigation of phenol storage of spores." Both solutions had been prepared at Battelle using the same preparation method. The 2×10^7 spores/mL sample made with spores not stored in phenol produced negative results in all three samples, as did the spores stored in phenol. In addition, samples containing concentrations of approximately 10^{10} and 10^9 spores/mL of spores from both phenol and non-phenol stock solutions were analyzed. The 10^{10} spores/mL solutions produced a "low signal" error message for each replicate, a message that indicates that the rate of sample moving across the adsorbent cartridge was not adequate. Response Biomedical Corp. informed Battelle that this error can occur when analyzing highly concentrated solutions of spores because of the increased viscosity of the sample. The approximately 10^9 spores/mL solution made with the Battelle-prepared non-phenol-preserved spores generated two out of three positive results and the Battelle-prepared phenol-preserved spores generated three out of three positive results. These results suggested that the effect of phenol storage was probably inconsequential to the sensitivity of the RAMP® test cartridges 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, and 10^7 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 RAMP® test cartridges to the Battelle-prepared spores. Only the highest concentration level was detectable; therefore, the lowest detectable concentration was approximately 10^9 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 7×10^8 spores/mL, a level similar to that determined for the Battelle-prepared spores. In addition, upon discussions with Response Biomedical Corp., Battelle obtained Stern anthrax vaccine and analyzed it using two separate

lots of the RAMP® test cartridges. The vaccine generated two out of two positive results for two lots of test cartridges (4 out of 4 positive results overall). This indicated that Battelle was operating the test cartridges properly and that the test cartridges were functioning.

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 3×10^5 colony-forming units (cfu)/mL, an order of magnitude lower than the vendor-stated LOD for anthrax spores. While Response Biomedical Corp. has not provided information with regard to the RAMP® test kit's vegetative cell sensitivity, these results suggest that RAMP® test cartridges 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, there were no positive responses. In response to these results, Response Biomedical Corp. informed Battelle that their test cartridges 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 RAMP® test cartridges, 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 RAMP® test cartridges were sensitive to botulinum toxin Type A at approximately 2 mg/L, but were not able to 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
	0.5[b]	B	0/3
	2.5	B	0/3
	5	B	0/3
	25	B	0/3
Expanded testing	0.5	A	0/3
	2	A	2/3
	5	A	2/3
	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. With the exception of the 1 mg/L sample, the vendor-stated LOD, each concentration level analyzed generated 3 out of 3 positive responses.

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	1[a]	0/3
	5	3/3
	15[b]	3/3
	20	3/3
	50	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 RAMP® test cartridges 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 cartridges 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, 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 cartridges.

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**	**4×10^{6}[a]**	**1×10^{9}[a]**	**Blank**	**Type B 5**	**Type A 5**	**Blank**	**10**
46 mg/L Ca 18 mg/L Mg	0/3	0/3 2×10^{6}[b]	NA	0/3	0/3	NA	0/3	3/3
230 mg/L Ca 90 mg/L Mg	0/3	0/3 2×10^{6}[b]	3/3 1×10^{9}[b]	0/3	0/3	3/3	0/3	3/3
0.5 mg/L humic and fulvic acid	0/3	0/3 4×10^{6} [b]	NA	0/3	0/3	NA	0/3	3/3
2.5 mg/L humic and fulvic acid	0/3	0/3 4×10^{6}[b]	3/3 1×10^{9}[b]	0/3	0/3	2/3	0/3	3/3

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

When interferent solutions not fortified with target contaminants were analyzed, no false positive results occurred for the test cartridges specific for any of the three target contaminants. The lack of detectable results at 4×10^6 spores/mL for anthrax and 5 mg/L for botulinum toxin Type B 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 cartridges 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 anthrax spores, there were no false negative responses for the expanded testing. For botulinum toxin Type A, there was one false negative response in the 2.5 mg/L humic and fulvic acid sample. 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 cartridges 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 cartridges.

Table 6-3 shows that there were no false positive results for the test cartridges specific for any of the three target contaminants when the unspiked DW samples were analyzed. The second column of results under anthrax and botulinum toxin and the only results under ricin show false negative responses with respect to the vendor-stated LOD (not as defined in Section 5.2). But for the reasons detailed in the previous section, many of the negative results in Table 6-3 cannot be attributed to the presence of the DW matrix. No further testing was completed for ricin, but for anthrax spores and botulinum toxin, expanded testing for the spiked DW samples was done as for the spiked interferent testing (Section 6.2.1). Only two DW samples, concentrated CA and concentrated NY DW, were analyzed during the expanded testing of anthrax and botulinum toxin samples. No false negative responses resulted from this expanded testing; in both cases the analyses resulted in three out of three positive results.

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

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

6.2.3 Cross-Reactivity PT Samples

The results from the cross-reactivity PT samples are given in Table 6-4. For test cartridges 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. There were not any false positive results for this evaluation. Each sample generated 3 out of 3 negative responses. For *Bacillus thuringiensis*, the concentration analyzed was significantly less than the lowest detectable concentration of anthrax; therefore, the results may not indicate a lack of cross-reactivity.

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)		0/3	
Lectin from soybean (10 mg/L)			0/3

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

6.3 Consistency

For the anthrax testing, at times the number of replicate analyses was reduced to conserve time or available supplies. However, the available replicate data for anthrax suggests that performance of the test cartridges was very consistent. Including all the sample sets analyzed for anthrax, only one sample set generated results that were not either all negative or all positive, for a consistency of 96%.

For botulinum toxin, the 2- and 5-mg/L samples of botulinum toxin Type A produced positive results in two out of three replicates, while the 25 mg/L botulinum toxin Type A samples produced three out of three positive results and the 0.5 mg/L Type A botulinum toxin generated three out of three negative results. These results may suggest a correlation between consistency and concentration. The rest of the botulinum toxin sample sets, with the exception of the 2.5 mg/L humic and fulvic acid interferent sample spiked with 5 mg/L botulinum toxin Type A (two out of three positive), produced either all positive or all negative results. For botulinum toxin, the consistency was 95%. For ricin, the results were consistent 100% of the time for all the sample matrices. Either all replicates within a sample were positive or all were negative. Overall, 95% of all the results were obtained in sets of two or three replicates in which all the individual replicates had the same result, whether positive or negative.

6.4 Lowest Detectable Concentration

The lowest detectable concentration of each target contaminant was defined as the lowest concentration of contaminant-only PT sample to have at least two out of three positive results. For anthrax, that concentration was determined to be 8×10^8 spores/mL (Battelle-prepared), 7×10^8 spores/mL (Dugway-prepared), and 3×10^5 cfu/mL (vegetative cells); for botulinum toxin Type A, 2 mg/L; and for ricin, 5 mg/L. The RAMP® test cartridges could not detect botulinum toxin Type B.

6.5 Other Performance Factors

Battelle technicians, who had been trained by Response Biomedical Corp. to perform testing using the RAMP® test cartridges, performed all of the testing in a laboratory setting. The technicians had no problem performing the tests as they were trained. The RAMP® test cartridges require the use of a reader contained in a rugged carrying case that weighs approximately 20 pounds and is about the size of a medium-sized suitcase. To test the ability of the RAMP® test cartridges to be used outside a laboratory environment and by a non-trained user, both a trained operator and an operator without any training in the sciences or in the operation of the RAMP® test cartridges were given a liquid sample (DI water) and told to analyze the sample three times. For the samples analyzed in a non-laboratory setting, the reader was powered by batteries. Initially, the non-technical operator was guided only by the instructions provided with each box of test cartridges. However, if the operator was about to complete the test incorrectly, the Verification Test Coordinator prompted the operator to reevaluate the instructions. Initially, the non-technical operator was confused by what the micropipette was and how to use it, but figured it out without any assistance. During the first sample analysis, the Verification Test Coordinator stopped the non-technical operator before attempting to perform the mixing step after applying the sample to the sample well. The instructions seemed to be clear, but the operator apparently misunderstood. When directed back to the instructions, the operator then understood the proper technique and was able to perform each step, including the operation of the reader, successfully. The non-technical operator repeated the sample analysis two additional times. On the second attempt, the reader prompted the operator to repeat the test because it took too long to insert the cartridge into the reader after sample application. The operator completed the final two samples successfully with no assistance. The experienced operator analyzed this sample in the correct way on the first attempt.

Over 400 RAMP® test cartridges were tested during the verification test. In nine instances, the result instead of positive or negative was “low signal.” This error message occurred when insufficient sample was transferred to the test cartridge. However, eight out of nine instances occurred when analyzing highly concentrated anthrax solutions when we were very confident of the testing procedure. Upon discussions with Response Biomedical Corp., we were informed that “low signal” result can also be generated from highly concentrated spore solutions because of the increased viscosity of the sample. According to Response Biomedical Corp., this precaution is in place to stop the over-sampling of powdered samples. Sample throughput was 4 samples per hour.

Chapter 7
Performance Summary

Table 7-1. Anthrax Summary Table

Parameter		Sample Information	Actual Fortified Anthrax Concentration[a]	Positive Results Out of Total Replicates
Qualitative contaminant results	Contaminant-only PT samples	Battelle-prepared, phenol-preserved spores	8×10^8 spores/mL	3/3
			8×10^7 spores/mL	0/3
			8×10^6 spores/mL	0/3
			3×10^5 spores/mL	0/3
		Vegetative cells	3×10^5cfu/mL	2/3
			3×10^4 cfu/mL	0/1
		Dugway-prepared spores	7×10^8 spores/mL	3/3
			8×10^7 spores/mL	0/1
	Interferent PT samples	230 mg/L Ca 90 mg/L Mg	1×10^9 spores/mL[b]	3/3
		2.5 mg/L humic acid 2.5 mg/L fulvic acid	1×10^9 spores/mL[b]	3/3
	DW samples	Concentrated CA	5×10^8 spores/mL[b]	3/3
		Concentrated NY	5×10^8 spores/mL[b]	3/3
		Unconcentrated DW	4×10^6 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		No false negative results were generated from the analysis of the interferent and DW samples spiked with detectable levels of anthrax spores; the RAMP® test cartridges were not able to detect anthrax spores at the vendor-stated limit of detection (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		96% 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	8×10^8 spores/mL - Battelle prep ; 7×10^8 spores/mL - Dugway prep (vendor-stated LOD: 4×10^5 spores/mL); 3×10^5 cfu/mL - vegetative anthrax (no vendor-stated LOD)
Other performance factors	All components for testing were provided in a box of 25 test cartridges; the required cartridge reader was operated using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test cartridges used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; "low signal" resulted from highly concentrated anthrax solutions; and sample throughput was 4 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	0/3
			2	2/3
			5	2/3
			25	3/3
		Type B	0.3	0/3
			0.5	0/3
			2.5	0/3
			5	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)]	2/3
	DW samples	Concentrated CA	5[(a)]	3/3
		Concentrated NY	5[(a)]	3/3
		Unconcentrated DW	5[(b)]	0/24
	Cross-reactivity	5 mg/L Lipopolysaccharide	unspiked	0/3
False positives		No false positives resulted from the analysis of the interferent, DW, or cross-reactivity samples.		
False negatives		One false negative replicate resulted from the analysis of the 2.5 mg/L humic and fulvic acid interferent samples spiked with a detectable level of Type A botulinum toxin; in addition, the RAMP® test cartridges were not able to detect Type B botulinum toxin spiked into DW at 5 mg/L or in DI water at concentrations up to 1,000 mg/L.		
Consistency		95% of the results were obtained in replicate sets in which all the individual replicates had the same result, whether positive or negative.		

Table 7-2. Botulinum Toxin Summary Table (continued)

Parameter	Sample Information
Lowest detectable concentration	2 mg/L (Type A), Type B was not detectable up to concentrations of 1,000 mg/L. (vendor-stated LOD for botulinum toxin [non-specific]: 0.5 mg/L)
Other performance factors	All components for testing were provided in a box of 25 test cartridges; the required cartridge reader was operated using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test cartridges used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; and sample throughput was 4 samples per hour.

[(a)] Type A botulinum toxin.
[(b)] Type B botulinum toxin.

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 in DI water	1	0/3
			5	3/3
			15	3/3
			20	3/3
			50	3/3
	Interferent PT Samples	Ca and Mg	10	6/6
		Fulvic and humic acid	10	6/6
	DW Samples	Concentrated DW	10	12/12
		Unconcentrated DW	10	12/12
	Cross-reactivity	10 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		No false negative results were generated by analyzing DW and interferent samples spiked with detectable levels of ricin.		
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		5 mg/L (vendor-stated LOD: 1 mg/L)		
Other performance factors		All components for testing were provided in a box of 25 test cartridges; the required cartridge reader was operated using electricity or batteries, was easy to operate, and was contained in a rugged carrying case; test cartridges used easily inside and outside a laboratory with trained operator; non-technical operator needed minor direction from a trained operator; and sample throughput was 4 samples per hour.		

Chapter 8
References

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

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

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

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

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

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

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

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

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

10. U.S. EPA Method 552.2, "Haloacetic Acids and Dalapon by Liquid-Liquid Extraction, Derivatization and GC with Electron Capture Detector," *Methods for the Determination of Organic Compounds in Drinking Water—Supplement III,* EPA/600/R-95/131, August 1995.

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

Appendix
Other Evaluations

It is the belief of Response Biomedical Corp. that the anthrax data generated during this verification test is discrepant from that of data generated by other groups. For more information, please review the following publication, which can be obtained from Response Biomedical Corp.:

"Evaluation of a Rapid Immunoassay System for the Detection of *Bacillus anthracis* Spores," K. Heroux and P. Anderson; U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, Maryland.