December 2004

Environmental Technology Verification Report

INVITROGEN CORPORATION PATHALERT™ DETECTION KITS FOR THE DETECTION OF *FRANCISELLA TULARENSIS*, *YERSINIA PESTIS*, AND *BACILLUS ANTHRACIS*

Prepared by
Battelle

Under a cooperative agreement with

U.S. Environmental Protection Agency

December 2004

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ETV Advanced Monitoring Systems Center

Invitrogen Corporation PathAlert™ Detection Kits for the detection of *Francisella tularensis,* *Yersinia pestis,* and *Bacillus anthracis*

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Notice

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

Foreword

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

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

Effective verifications of monitoring technologies are needed to assess environmental quality and to supply cost and performance data to select the most appropriate technology for that assessment. Under a cooperative agreement, Battelle has received EPA 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, thanks go to the Metropolitan Water District of Southern California for concentrating each drinking water sample. We would also like to thank Myriam Medina-Vera, U.S. Environmental Protection Agency National Exposure Research Laboratory; Jorge Santo Domingo, National Risk Management Research Laboratory; Kerri Alderisio, New York City Department of Environmental Protection; Ricardo DeLeon, Metropolitan Water District of Southern California; and Stanley States, Pittsburgh Water and Sewer Authority, for their careful review of the test/quality assurance plan and this verification report.

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List of Abbreviations

AMS	Advanced Monitoring Systems
ASTM	American Society of Testing and Materials
ATEL	AquaTech Environmental Laboratories, Inc.
ATCC	American Type Culture Collection
BSL	Biosafety Level
Ca	calcium
cfu	colony forming unit
cm	centimeter
DI	deionized water
DNA	deoxyribonucleic acid
DW	drinking water
EPA	U.S. Environmental Protection Agency
EPC	external positive control
ETV	Environmental Technology Verification
ID	identification
IPC	internal positive control
L	liter
LOD	limit of detection
MB	method blank
Mg	magnesium
mg	milligram
mL	milliliter
MWD	Metropolitan Water District
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PT	performance test
QA	quality assurance
QC	quality control
QMP	Quality Management Plan
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 Invitrogen Corporation's PathAlert™ Detection Kits for the detection of *Francisella tularensis* (*F. tularensis*), *Yersinia pestis* (*Y. pestis*), and *Bacillus anthracis* (*B. anthracis*).

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 PathAlert™ Detection Kit. The following is a description of the PathAlert™ Detection Kit based on information provided by the vendor. The information provided below was not subjected to verification in this test.

The PathAlert™ Detection Kit is a multiplex polymerase chain reaction (PCR) reagent system capable of detecting *F. tularensis*, *Y. pestis*, *B. anthracis*, or smallpox in individual assays. The PathAlert™ Detection Kit comprises an optimized PCR SuperMix specific to the pathogen of interest, as well as an external positive control (EPC) template for system validation. The kit includes Taq polymerase, pre-complexed with antibodies to maintain "hot start" PCR (for specificity and sensitivity); uracil deoxyribonucleic acid (DNA) glycosylase and deoxyuridane triphosphate to eliminate post-PCR cross-contamination; and an internal positive control (IPC) to identify potential PCR inhibition from sample contaminants or environmental samples. Included in the kit is an EPC that has been engineered to produce different amplicon sizes than either the IPC or the pathogen-specific loci. As a result, pathogen-specific results can be read with minimal interference if contamination by the external control should occur.

Figure 2-1. Invitrogen Corporation's PathAlert™ Detection Kit

PathAlert™ Detection Kit is an endpoint assay; post-amplification products may be analyzed using any platform capable of distinguishing amplicon size, such as the Agilent Bioanalyzer 2100, Agilent ALP (high throughput), transgenomic WAVE high-performance liquid chromatography, gel electrophoresis, and Caliper AMS 90. The Agilent Bioanalyzer 2100 and the Agilent ALP are recommended by Invitrogen Corporation for use with the PathAlert™ Detection Kit because all field testing to date has been performed with these systems, and Agilent and Invitrogen Corporation have established a co-marketing relationship for the complete system. The cost of each PathAlert™ Detection Kit ranges from $12 to $16 per assay.

Chapter 3
Test Design and Procedures

3.1 Introduction

The purpose of this verification test of rapid PCR technologies was to evaluate the ability of these technologies to detect the presence of specific bacteria in water and to determine the technologies' performance when specific interferents were added to pure water and when interferents were inherently present in several drinking water matrices. The technologies for this verification test operate based on the PCR process, which involves enzyme-mediated reactions that allow for target DNA (from the bacteria of interest) replication and amplification through a series of temperature cycles. Before the target DNA can be amplified, however, it must first be extracted from the bacteria and then purified.

Because rapid PCR technologies are anticipated to serve mostly as screening tools in water monitoring scenarios, providing rapid results as to whether or not a pathogen or biological agent is present in the water, this verification test involved only qualitative results. This verification test of the PathAlert™ Detection Kit was conducted according to procedures specified in the *Test/QA Plan for Verification of Rapid PCR Technologies*.[1] The performance of the PathAlert™ Detection Kit was verified in terms of the following parameters:

- Accuracy
- Specificity
- False positive/negative responses
- Precision
- Interferences
- Other performance factors.

The performance of the PathAlert™ Detection Kit was verified by challenging it with various concentration levels of *F. tularensis* LVS [American Type Culture Collection (ATCC) #29684], *Y. pestis* CO92, and *B. anthracis* Ames strain in American Society of Testing and Materials (ASTM) Type II deionized (DI) water; ASTM Type II DI water spiked with various interferents; and concentrated drinking water (DW) samples obtained from four water utilities from different geographical locations in the United States. Each source of DW represented a unique water treatment process. In addition, the interferent and DW samples were analyzed without adding any contaminant to evaluate the potential for false positive results. The kit was only tested for one bacteria at a time.

Contaminant concentrations included the infective/lethal dose concentrations given in Table 3-1 for each contaminant and approximately 2, 5, 10, and 50 times the vendor-reported method limit of detection (LOD) for each technology. The infective/lethal dose of each contaminant was determined by calculating the concentration at which ingestion of 250 milliliters (mL) of water is likely to cause the death of a 70-kilogram (approximately 154 pounds) person based on human LD_{50} or ID_{50} data.[2] The results from quadruplicate analysis of the contaminant performance test (PT) samples and comparison with the known sample compositions provided information on the accuracy and precision of the PathAlert™ Detection Kit. The interferent PT samples contained humic and fulvic acids at two concentrations, both spiked and unspiked with contaminants. Each was analyzed in quadruplicate and provided information on potential matrix interferences.

For the purposes of this test, 1×10^4 colony forming units (cfu)/mL were used to calculate the concentration levels of *F. tularensis* and *B. anthracis* spiked into the PT and DW samples; 100 cfu/mL were used to calculate levels of *Y. pestis* spiked in the PT and DW samples. These vendor-provided concentration levels were anticipated to be the levels for the entire experimental process at which quantifiably reproducible positive results could be obtained from a raw water sample. These concentration levels are referred to as the "method LOD" for a particular assay. The method LOD incorporates the sensitivities and uncertainties of not only the PathAlert™ Detection Kit, but also the DNA purification step; and, as such, it is an experimental detection limit rather than an instrument or reagent-specific detection limit. As mentioned previously, the method LOD provided by the vendor was used specifically as a guideline in calculating sample concentration ranges for use with the PathAlert™ Detection Kit and all other components used in this verification test to analyze a sample, and it should be noted that Invitrogen Corporation does not claim this to be the true LOD of the PathAlert™ Detection Kit alone. The vendor claims the absolute LOD (the least amount of target DNA that would generate a positive result) for the PathAlert™ Detection Kit alone is as low as 1 to 10 copies of DNA, depending on the assay. This information was not verified in this test.

The verification test was conducted at Battelle's Medical Research and Evaluation Facility in West Jefferson, Ohio, from June 9, 2004, through June 30, 2004. Aqua Tech Environmental Laboratories, Inc. (ATEL) of Marion, Ohio, performed physicochemical characterization for each DW sample, including turbidity, dissolved and total organic carbon, specific conductivity, alkalinity, pH, magnesium (Mg), calcium (Ca), hardness, total organic halides, trihalomethanes, and haloacetic acids. Battelle cultured the bacteria, provided the stock solutions of each bacteria used in this test, and then confirmed the presence and quantity of *F. tularensis*, *Y. pestis*, and *B. anthracis* bacteria in the stock solutions using plate enumeration. The stock solutions of *F. tularensis,* and *Y. pestis* were stored frozen as 1 mL aliquots. The *B. anthracis* stock solutions were refrigerated as 1 mL aliquots. A new 1 mL vial of stock solution was thawed and used for each day of testing. All test samples were prepared from the stock solutions on the day of analysis. All purified DNA was used the same day it was extracted and purified. Each set of replicates for a sample came from the same batch of purified DNA.

Table 3-1. Infective/Lethal Dose of Target Contaminants

Contaminant	Disease Caused by Contaminant	Infective/Lethal Dose Concentration (cfu/mL)
F. tularensis	Tularemia	4×10^5
Y. pestis	Plague	0.28
B. anthracis	Anthrax	200

3.2 Test Samples

Test samples used in this verification test included PT samples, DW samples, and quality control (QC) samples. Each type of test sample, including QC samples, is described further below.

3.2.1 Performance Test Samples

Table 3-2 lists the PT samples analyzed in this verification test for each bacteria. The bacteria were added individually to each spiked sample. PT samples were prepared in ASTM Type II DI water. The first type of PT sample consisted of ASTM Type II DI water spiked at five concentration levels of each individual contaminant. The contaminant PT sample concentrations ranged from the infective/lethal dose concentration to 50 times the vendor-stated method LOD. The infective/lethal dose concentration was analyzed to document the response of the PathAlert™ Detection Kit at that important concentration level. Four concentration levels at 2, 5, 10, and 50 times the vendor-reported method LOD, in addition to the infective/lethal dose concentration, were analyzed. Each concentration level for the PT samples was analyzed in quadruplicate.

Table 3-2. Performance Test Samples

Type of PT Sample	Sample Characteristics	Approximate Concentrations (cfu/mL)
Contaminant-only	*F. tularensis*	2×10^4 to 5×10^5
	Y. pestis	0.28 to 5×10^3
	B. anthracis	200 to 5×10^5
Interferent	Contaminants in 0.5 milligram per liter (mg/L) humic acid and 0.5 mg/L fulvic acid	*F. tularensis*—1×10^5 *Y. pestis*—1×10^3 *B. anthracis*—1×10^5
	Contaminants in 2.5 mg/L humic acid and 2.5 mg/L fulvic acid	*F. tularensis*—1×10^5 *Y. pestis*—1×10^3 *B. anthracis*—1×10^5

The second type of PT sample was potential interferent samples. Four replicates of each interferent PT sample were analyzed to determine the performance of the PathAlert™ Detection Kit in the presence of humic and fulvic acids. The interferent PT samples contained humic and fulvic acids isolated from Elliot Soil near Joliett, IL, (obtained from the International Humic Substances Society) spiked into ASTM Type II DI water. Each of these interferent mixtures was prepared at two concentration levels. One concentration was near the upper limit of what would be expected in DW (5 mg/L) and one was at a mid-low range of what would be expected (1 mg/L). The 1 mg/L interferent mixture was prepared as 0.5 mg/L humic acid and 0.5 mg/L fulvic acid. Similarly, the 5 mg/L interferent solution was prepared as 2.5 mg/L humic acid and 2.5 mg/L fulvic acid. These interferent levels were confirmed through analysis of aliquots by ATEL. Also, each bacteria was added separately to these samples, along with the potential interferent, at a concentration of 10 times the method LOD and analyzed in quadruplicate.

In all cases, four replicates for each PT sample, DW sample, and QC sample were taken from the extracted and purified product (unspiked) or DNA (spiked) of one sample solution. That is, only one spiked or unspiked sample solution was prepared for each set of replicates and taken through the DNA extraction and purification procedure. Four replicates were then taken from the same purified product or DNA. In an effort to characterize the efficacy of the extraction and purification procedure in the presence of inhibitory substances (humic and fulvic acids), four solutions of humic and fulvic acids at 0.5 mg/L spiked with each contaminant at 10 times the method LOD, were prepared in addition to the samples listed in Table 3-2. Each solution was put through the DNA extraction and purification procedure, and then four replicates from each of the four purified DNA solutions were analyzed using the PathAlert™ Detection Kit.

3.2.2 Drinking Water Samples

Table 3-3 lists the DW samples analyzed for each bacteria in this test. DW samples were collected from four geographically distributed municipal sources (Ohio, California, Florida, and New York) to evaluate the performance of the PathAlert™ Detection Kit with various sample matrices. These samples varied in their source and treatment and disinfection process. All samples had undergone either chlorination or chloramination prior to receipt. Samples were collected from utility systems with the following treatment and source characteristics:

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

All samples were collected in pre-cleaned high density polyethylene containers. After sample collection, to characterize the DW matrix, an aliquot of each DW sample was sent to ATEL to determine the following water quality parameters: turbidity, organic carbon, conductivity, alkalinity, pH, Ca, Mg, hardness, total organic halides, concentration of trihalomethanes, and haloacetic acids. The DW samples were dechlorinated with sodium thiosulfate pentahydrate to prevent the degradation of some of the contaminants by chlorine. Because real-world applications of PCR technologies to screen water samples rely on pre-concentration of the water

sample to be analyzed, approximately 100 L of each of the above sources of DW were dechlorinated and then concentrated through ultrafiltration techniques to a final volume of 250 mL by the Metropolitan Water District (MWD) of Southern California. As shown in Table 3-3, each DW sample was analyzed without adding any contaminant (i.e., unspiked), as well as after fortification with each individual contaminant at a single concentration level (10 times the vendor-stated method LOD).

Table 3-3. Drinking Water Samples

Drinking Water Sample Description			Approximate Contaminant Concentrations (cfu/mL)		
Water Utility	**Water Treatment**	**Source Type**	***F. tularensis***	***Y. pestis***	***B. anthracis***
Columbus, Ohio (OH)	chlorinated filtered	surface	unspiked and 1×10^5	unspiked and 1×10^3	unspiked and 1×10^5
MWD of Southern California (CA)	chloraminated filtered	surface	unspiked and 1×10^5	unspiked and 1×10^3	unspiked and 1×10^5
Orlando, Florida (FL)	chlorinated filtered	ground	unspiked and 1×10^5	unspiked and 1×10^3	unspiked and 1×10^5
New York City, New York (NY)	chlorinated unfiltered	surface	unspiked and 1×10^5	unspiked and 1×10^3	unspiked and 1×10^5

3.2.3 Quality Control Samples

QC samples included method blank (MB) samples consisting of ASTM Type II DI water and positive and negative controls, as provided by the vendor. All of the MB QC samples were exposed to sample preparation and analysis procedures identical to the test samples. External positive and negative controls were prepared and used according to the protocol provided by the vendor. At least one EPC and negative (no-template) control was prepared with each batch of samples placed on the thermal cycler. The MB samples were used to confirm negative responses in the absence of any contaminant and to ensure that no sources of contamination were introduced into handling and analysis procedures. At least 10% of the test samples (eight replicates) for each bacteria were MB samples. The vendor-provided control samples indicated to the technician whether the PathAlert™ Detection Kit was functioning properly. If the controls failed for any reason, that batch of samples would be discarded and the extracts reanalyzed. To the extent practicable, the test samples were analyzed blindly by having the technician label the vials with only a sample number prior to the DNA purification step, so that the samples were tracked through the purification, PCR, and detection steps by only a sample number. Due to special facility use, the identity of the target bacteria was always known by the technician.

3.3 Reference Methods

3.3.1 Plate Enumeration

For all contaminants, plate enumeration was used to quantify bacteria to confirm the concentration of the stock solutions of these contaminants. The Battelle standard operating procedure (SOP) followed was SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique.*

Prior to testing, the *F. tularensis* and *Y. pestis* were grown and then suspended in phosphate buffered saline (PBS). Twenty-five or more individual 1 mL aliquots of stock solution were prepared from each original PBS stock solution. Three 1 mL aliquots were randomly taken for enumeration, while the others were frozen for later use in sample preparation. Each bacteria was enumerated on each of the three selected 1 mL aliquots to confirm the determined concentration.

The *B. anthracis* came from a lot of spores prepared by Battelle and stored in a 1% stock solution of phenol in water. Prior to testing, an aliquot of the *B. anthracis* 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 DI water suspension of spores was then aliquoted into 1-mL portions as with the *F. tularensis* and *Y. pestis*. Because of the known stability of *B. anthracis* spores and based on general facility protocol, the aliquots were refrigerated instead of frozen. An aliquot was enumerated in triplicate prior to testing to confirm the concentration. Another aliquot was enumerated during the verification test to further verify the concentration of *B. anthracis* in the stock solution vials.

3.3.2 Drinking Water Analysis

Because most of the contaminants tested can occur naturally in water, and because rapid PCR technologies cannot distinguish between live and dead organisms, each unspiked concentrated DW sample was plate enumerated to verify, to the extent practicable, the presence or absence of the contaminant of interest. The samples were plated onto tryptic soy agar plates with 5% sheep blood and incubated at 30 to 35°C. After 20 hours of incubation, the unspiked OH, CA, and NY DW samples produced lawns of bacteria with a level of contamination estimated to be greater than 1×10^3 cfu/mL. The unspiked FL DW sample showed only 10 to 100 cfu/mL estimated concentration levels after 20 hours. After further incubation, the FL DW sample produced bacteria at a concentration estimated to be greater than 1×10^3 cfu/mL. Each DW sample had at least three distinct types of bacteria growing. Gram stains were performed on any distinct colony types visible in each sample to gain further insight into the colony morphology. For OH and CA DW, three Gram negative bacteria colonies were identified. For NY, four Gram negative colonies were identified; and, for FL, both Gram negative and positive colonies were present.

The CA DW was further evaluated for the presence of *F. tularensis* based on the potential positive results for unspiked CA DW samples during the verification test. An aliquot of the water was plated onto cystine heart agar (*F. tularensis* selective media) and incubated at 30 to

35°C. A single colony type (Gram negative rods) grew on the plates and was subjected to biochemical tests (catalase, oxidase, β-lactamase, and urease) for the presumptive identification of *F. tularensis*. The biochemical test results came back oxidase positive, indicating that the bacteria were not *F. tularensis*. Further identification tests were not conducted on other DW samples because no confirmed positive responses were detected in the remaining unspiked DW samples.

3.4 Test Procedure

3.4.1 Sample Handling

All testing for this verification test was conducted within Battelle laboratories staffed with technicians trained to safely handle *F. tularensis*, *Y. pestis*, and *B. anthracis* bacteria. The technician using the PathAlert™ Detection Kit had prior PCR experience. *F. tularensis* samples were tested in a Biosafety Level 2 (BSL-2) laboratory, *Y. pestis* and *B. anthracis* samples were tested in BSL-3 laboratories. Appropriate safety guidelines associated with each laboratory were followed throughout the verification test. Each day, fresh samples were prepared from a thawed vial of frozen or refrigerated stock solution in either DI water, an interferent matrix, or a DW matrix. Concentration levels for spiked samples at various multiples of the method LOD for the PathAlert™ Detection Kit and associated DNA purification (2, 5, 10, and 50 times the method LOD for PT samples, and 10 times the method LOD for interferent and DW samples) were calculated from the method LOD provided by Invitrogen Corporation. Sample solutions were prepared to these concentrations based on the concentration of the bacteria stock solution, which was determined through triplicate plate enumeration prior to testing. Each sample was prepared in its own container and labeled only with a sample identification (ID) number that also was recorded in a laboratory record book along with details of the sample preparation. Samples were diluted to the appropriate concentration using volumetric pipettes and glassware. Each sample was prepared in 1 mL quantities.

Despite rigorous sample preparation efforts, solutions consisting of low bacterial concentrations, such as the *Y. pestis* infective/lethal dose, may have no DNA present in a given sample or aliquot.[3,4] The rationale for this is based on the Poisson statistical distribution, where there is some probability that a sample taken will contain no particles (i.e., bacteria or target DNA) and thus yield a negative result.[3,4] As a practical example, assume that 1 mL contains exactly five particles (i.e., bacteria or target DNA) of interest. If one takes ten 0.1 mL samples and analyzes them, the maximum number of positives will be five out of the ten samples. From this it follows that there will be at least five negatives. Random variation in the sampling will cause this ratio to change. This verification test was not designed to differentiate between the stochastic nature of the low concentration samples and the capabilities of the assays, but this phenomenon should be noted.

3.4.2 Sample Preparation and Analysis

For this verification test, the following components were used to analyze the samples: Roche High Pure PCR Template Preparation Kit (for DNA extraction and purification); PathAlert™ Detection Kit for *F. tularensis*, *Y. pestis*, *B. anthracis* (each kit included bacteria-specific PathAlert™ PCR SuperMix, PCR-grade water, and PathAlert™ EPC Plasmid); an MJ Research DNA Engine® (PTC-200™) Peltier Thermal Cycler (for performing the PCR) with optical strip tubes; and the Agilent 2100 Bioanalyzer (with a laptop computer, a miniature vortex, the 2100 Bioanalyzer, and a priming station) along with the 2100 Bioanalyzer DNA 500 chips and reagent kit (for detecting the amplified PCR product). The 2100 Bioanalyzer and Bioanalyzer DNA 500 chips are a microfluidics-based detection technology that provides amplicon sizing information, as well as approximate product yields, using lab-on-a-chip technology to provide rapid qualitative and quantitative information. The 2100 Bioanalyzer is approximately 6.5 inches x 16 inches x 11 inches.

Four steps were carried out to test a liquid sample for the presence of *F. tularensis*, *Y. pestis*, and *B. anthracis* bacteria: (1) PCR SuperMix setup, (2) DNA purification, (3) PCR of the DNA, and (4) 2100 Bioanalyzer loading and analysis. To perform these steps, the laboratory work area was separated into three distinct areas: a "clean" area (DNA free), a "medium" area (a moderate amount of DNA present), and a "dirty" area (high sample DNA concentrations). First, in the "clean" area, the PCR SuperMix, part of the PathAlert™ Detection Kit, was added to the PCR tubes. A volume of 12.5 microliters (µL) was added for all controls, as well as the DI water PT samples. For all DW and interferent samples (those samples with inhibitory substances present), 37.5 µL SuperMix was added to the tubes. The negative controls were prepared at this time by adding 12.5 µL of sterile water to the appropriate PCR tubes.

Then, in the "dirty" area, the DNA was isolated and purified from the sample. The entire 1 mL sample was taken through this isolation procedure. The vendor-provided instructions were followed, which were the Roche High Pure Prep Kit instructions for the isolation of bacteria or yeast with the following modifications to accommodate the 1 mL sample: 25 µL of lysozyme was used; the sample was split into two 500 µL aliquots, with each aliquot going through the second incubation steps; 500 µL of Binding Buffer and 100 µL of Protinease K were used prior to the second incubation step; 250 µL of isopropanol were used; and additional initial centrifuging was necessary to pass both 500 µL aliquots through the first filter tube. According to the Roche High Pure Prep Kit instructions, 200 µL of elution buffer were used in the final step.

In the "medium" area, 12.5 µL of purified DNA were added to the appropriate PCR tubes, which already contained the SuperMix. Not all of the purified DNA obtained using the Roche High Pure Prep Kit was used during the verification test. Any unused purified DNA was frozen for possible later use. The EPCs were also prepared at this time. The capped PCR tubes were then loaded onto the thermal cycler, which was pre-programmed by the vendor at the time of training. After the thermal cycler had completed its PCR program run, the amplified product was loaded onto the 2100 Bioanalyzer DNA 500 chip according to the directions provided with the PathAlert™ Detection Kit. Briefly, the chip was inserted into a priming station, and gel-dye mix

was added to the priming well and then pushed throughout the sample wells on the chip using the priming station. Then, the appropriate reagents were added to the remaining wells, and 1 µL amplified DNA was added to each sample well. Each DNA 500 chip held up to 12 samples. In general, at least two positive and two negative control samples were placed on the first 2100 Bioanalyzer chip to be run on a given day to verify the efficacy of the PCR process and the reagents. Before each day's 2100 Bioanalyzer use, a cleaning chip was used. After the chip containing the samples was loaded onto the 2100 Bioanalyzer, the DNA 500 assay was loaded in the 2100 Bioanalyzer software, and the chip run was started. The resulting electropherograms for each sample were analyzed to determine the results for each sample. The bacteria were considered present in the sample if the 2100 Bioanalyzer's electropherogram for a given sample showed three peaks of appropriate amplicon (base pair) size: two for the bacteria being monitored and one for the IPC. The bacteria were considered not present if only the single IPC peak was present. The negative control was considered successful if only the IPC peak was present, and the EPC samples were considered successful if three peaks of the appropriate amplicon size were present. The EPC peaks differ from the sample peaks to help distinguish these samples. If the ladder for a given chip was unsuccessful, the chip was reloaded and run again. The ladder is an external standard for the 2100 Bioanalyzer DNA 500 chip that ensures the proper performance of the chip assay. The technician recorded the sample ID number on a sample data sheet along with the qualitative results (positive or negative) for each sample.

3.4.3 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 used in this verification test. Water samples were collected and water quality parameters were measured by ATEL in January 2004. Some of the water quality parameters may have changed slightly prior to verification testing.

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

Parameter	Unit	Method	Sources of Drinking Water Samples			
			Columbus, Ohio (OH DW)	MWD, California (CA DW)	Orlando, Florida (FL DW)	New York City, New York (NY DW)
Turbidity	NTU	EPA 180.1[5]	0.2	0.1	0.5	1.3
Dissolved organic carbon	mg/L	SM 5310[6]	1.9	2.3	1.7	1.5
Total organic carbon	mg/L	SM 5310[6]	1.6	2.1	1.8	2.1
Specific conductivity	microSiemens	SM 2510[6]	357	740	325	85
Alkalinity	mg/L	SM 2320[6]	55	90	124	4
pH		EPA 150.1[7]	7.33	7.91	7.93	6.80
Ca	mg/L	EPA 200.8[8]	42	35	41	5.7
Mg	mg/L	EPA 200.8[8]	5.9	1.5	8.4	19
Hardness	mg/L	EPA 130.2[7]	125	161	137	28
Total organic halides	µg/L	SM 5320[6]	360	370	370	310
Trihalomethanes	µg/L/analyte	EPA 524.2[9]	26.9	79.7	80.9	38.4
Haloacetic acids	µg/L/analyte	EPA 552.2[10]	23.2	17.6	41.1	40.3

NTU = nephelometric turbidity unit
µg = microgram

Chapter 4
Quality Assurance/Quality Control

Quality assurance/quality control procedures were performed in accordance with the quality management plan (QMP) for the AMS Center[11] and the test/QA plan for this verification test.[1]

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 generally those provided in the guidelines in 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 PathAlert™ Detection Kit, Agilent 2100 Bioanalyzer, and all associated reagents and supplies specific for the detection of *F. tularensis*, *Y. pestis*, and *B. anthracis* were provided to Battelle by the vendor. This system required no calibration. The performance of the system was monitored through ladders, EPC, IPC, and negative controls. 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 SOP VI-025, *Operation, Calibration, and Maintaining Fixed and Adjustable Volume Pipettes.*

4.3 Characterization of Contaminant Stock Solutions

F. tularensis, *Y. pestis*, and *B. anthracis* were grown and prepared by Battelle. All bacteria were plate enumerated in triplicate for confirmation of the concentration of the 1 mL aliquot stock solutions. Prior to enumeration, the *B. anthracis*, originally stored as a 1% stock solution of

phenol in water, was aliquoted and washed twice with DI water and resuspended in only DI water for analysis.

The lot of *B. anthracis* spores used for this verification test was previously characterized in September 2003 by Battelle and the Centers for Disease Control and Prevention. This characterization involved 11 criteria, including the percent of vegetative cells present, the viable spore count, the guinea pig 10-day LD_{50}, as well as DNA fingerprinting and gene sequencing. This lot of spores met all 11 acceptance criteria, proving that they were viable and of the specified strain (Ames). The vegetative cell count indicated that the stock solution of spores was 99.94% pure spores, with only 0.06% of the solution containing vegetative cells.

The Battelle SOP No. MREF X-054, *Standard Operating Procedure (SOP) for the Enumeration of BSL-2 and BSL-3 Bacteria Samples Via the Spread Plate Technique*, was followed for the plate enumeration of *F. tularensis, Y. pestis*, and *B. anthracis*. The results of the plate enumerations for each bacteria are presented in Table 4-1. For all bacteria, the plate enumeration was conducted prior to testing. Because the *B. anthracis* stock solution aliquots were stored at 2 to 8°C, another 1 mL aliquot stock solution vial was enumerated during testing activities to further confirm the concentration of the aliquots. The average of triplicate enumerations for each bacteria was used to calculate and prepare all spiked sample solutions. The percent difference between the concentration of the initial preparation of *B. anthracis* spores and the second analysis of these spores during testing was 23%. Because this difference falls within the bounds of expected plate enumeration errors and is close to the standard deviations found for the plate enumerations of other bacteria used in this verification test, the concentration determined from the initial set of plate enumerations on the *B. anthracis* spores was used in calculating solution concentrations.

Table 4-1. *F. tularensis, Y. pestis*, *and B. anthracis* Triplicate Plate Enumeration Data

Bacteria	Plate 1 Concentration (cfu/mL)	Plate 2 Concentration (cfu/mL)	Plate 3 Concentration (cfu/mL)	Average (cfu/mL)	Relative Standard Deviation
F. tularensis	1.0×10^9	1.1×10^9	1.2×10^9	1.1×10^9	9%
Y. pestis	5.8×10^7	6.5×10^7	5.0×10^7	5.8×10^7	13%
B. anthracis (initial prep)	8.7×10^7	8.1×10^7	7.8×10^7	8.2×10^7	6%
B. anthracis (second analysis)	5.7×10^7	5.7×10^7	7.6×10^7	6.3×10^7	17%

4.4 Quality Control Samples

MB samples consisting of ASTM Type II DI water, and EPC and negative control samples, as provided in the PathAlert™ Detection Kit, were analyzed to help identify potential cross-contamination issues as well as verify that the PCR process was functioning properly. IPCs were part of each sample that was analyzed and provided further checks on the performance of the PathAlert™ Detection Kit, especially in identifying the presence of potential inhibitory substances. EPC and negative control samples were run with each set of samples placed on the thermal cycler. Eight MB replicates were analyzed over the course of the verification test for each bacteria. Ladder samples, an external standard for the 2100 Bioanalyzer DNA 500 chip, were analyzed with each chip to ensure the proper performance of the 2100 Bioanalyzer.

Each set of eight MB sample replicates for each bacteria returned negative results. IPC peaks were present in all contaminant-only PT samples and interferent and DW samples using 37.5 µL of SuperMix for the PCR. In those interferent and DW samples containing 12.5 µL of SuperMix, the IPC peak was often suppressed, indicating the presence of inhibitors in the sample (see section 6.5.1 for further details). For all three bacteria tested, no EPC or negative controls failed. In two separate analysis events, the ladder on the DNA 500 chip failed because of improper loading onto the chip. DNA 500 chips had to be reloaded and rerun on the 2100 Bioanalyzer before results could be obtained for the samples on the failed chips.

4.5 Audits

4.5.1 Technical Systems Audit

The Battelle Quality Manager conducted a technical systems audit (TSA) on June 11, 2004, to ensure that the verification test was performed in accordance with the test/QA plan[(1)] 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 to those specified in the test/QA plan, and reviewed data acquisition and handling procedures. Observations and findings from this audit were documented and submitted to the Verification Test Coordinator for response. No findings were documented that required any significant action. The records concerning the TSA are stored for at least seven years with the Battelle Quality Manager.

4.5.2 Audit of Data Quality

At least 10% of the data acquired during the verification test was audited. Battelle's Quality Manager 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 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 Verification Test Coordinator ensured that a response was provided for each adverse finding or 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 these records were used to calculate, evaluate, or report verification results. Table 4-2 summarizes the types of data recorded. The review was performed by a Battelle technical staff member involved in the verification test, but not the staff member that 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 4-2. Data Recording Process

Data to Be Recorded	Where Recorded	How Often Recorded	Disposition of Data[a]
Dates and times of test events	ETV data sheets	Start/end of test and at each change of a test parameter	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
Sample collection and preparation information, including chain-of-custody	ETV data sheets and chain-of-custody forms	At time of sample collection and preparation	Used to organize/check test results; manually incorporated in data spreadsheets as necessary
PathAlert™ Detection Kit procedures and sample results	ETV data sheets and data acquisition system	Throughout test duration	Manually incorporated in data spreadsheets
Enumeration data	Enumeration data forms and ETV data sheets	With every enumeration	Used to organize/check test results
Reference method procedures and sample results	Data acquisition system, as appropriate	Throughout sample analysis process	Transferred to spreadsheets

[a] All activities subsequent to data recording were carried out by Battelle, except for the reference method analyses (DW characterization), which were carried out by ATEL.

Chapter 5
Data Analysis

The PathAlert™ Detection Kit was evaluated for qualitative results (i.e., positive/negative responses to samples) based on the expected application of rapid PCR technologies as rapid screening tools. All data analyses were based on these qualitative results. QC and MB samples were not included in any of the analyses.

5.1 Accuracy

Accuracy was assessed by evaluating how often the PathAlert™ Detection Kit results were positive in the presence of a concentration of contaminant above the method LOD. Contaminant-only PT samples were used for this analysis. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples above the method LOD.

5.2 Specificity

The ability of the PathAlert™ Detection Kit to provide a negative response when the contaminant was absent was assessed. The specificity rate was determined by dividing the number of negative responses by the total number of unspiked samples.

5.3 False Positive/Negative Responses

A false positive response was defined as a detectable or positive PathAlert™ Detection Kit response when the ASTM Type II DI water (including interferent samples) or DW samples were not spiked. A false positive rate was reported as the frequency of false positive results out of the total number of unspiked samples.

A false negative response was defined as a non-detectable response or negative response when the sample was spiked with a contaminant at a concentration greater than the method LOD. Spiked PT (contaminant and interferent) samples and spiked DW samples were included in the analysis. A false negative rate was evaluated as the frequency of false negative results out of the total number of spiked samples for a particular contaminant.

5.4 Precision

The precision of the four replicates of each sample set were assessed. Responses were considered consistent if all four replicates gave the same result. The precision of the PathAlert™ Detection Kit was assessed by calculating the overall number of consistent responses for all the sample sets.

5.5 Interferences

The potential effect of the DW matrix on the PathAlert™ Detection Kit performance was evaluated qualitatively by comparing the results for the spiked and unspiked DW samples to those for the PT samples. Similarly, the potential effect of interferent PT samples containing fulvic and humic acids at two levels, both spiked and not spiked with bacteria, were evaluated.

5.6 Other Performance Factors

Aspects of the PathAlert™ Detection Kit performance such as ease of use and sample throughput are discussed in Section 6. Also addressed are qualitative observations of the verification staff pertaining to the performance of the PathAlert™ Detection Kit.

Chapter 6
Test Results

The results for the PathAlert™ Detection Kit were evaluated based on the responses provided by the 2100 Bioanalyzer electropherogram output. An example positive electropherogram for *B. anthracis* is presented in Figure 6-1. The electropherogram displays the fluorescence intensity (fluorescence units) versus migration time (seconds) for each sample component, which is displayed as a peak in the electropherogram. Only qualitative (positive/negative) responses were recorded for each sample. To determine the results of each sample, peak sizes were monitored in the electropherogram display. In Figure 6-1, peak sizes are shown above each peak. Approximate amplicon sizes were listed on each bacteria-specific PathAlert™ Detection Kit (consisting of reagents necessary for the PCR of each sample) for each expected peak in a positive sample. These were used as guidelines in identifying positive responses. The threshold for the peak height was set to 10 fluorescence units, and the peak filter width was set to 1 second, at the direction of the vendor. Peaks that met these criteria, as well as other criteria left at the default settings, were automatically picked and integrated by the 2100 Bioanalyzer software. The bacteria were considered present in the sample if the electropherogram for a given sample showed three peaks of appropriate amplicon (base pair) size that appeared at approximately the same time throughout the samples: two for the bacteria being monitored and one for the IPC. The bacteria were considered not present, and thus a negative response was recorded, if the only peak present was for the IPC. The results for a sample were considered inconclusive if the IPC and only one bacteria peak were present in the electropherogram results. In a real-world scenario, samples with inconclusive results would likely be rerun, as well as subjected to a battery of other tests to confirm the presence or absence of the bacteria of interest.

Negative controls and EPCs were monitored with each day's sample set. No controls failed throughout the testing process. The ladder well results for a given 2100 Bioanalyzer DNA 500 chip were also monitored to ensure the integrity of the chip analysis. The ladder is an external standard for the 2100 Bioanalyzer DNA 500 chip that ensures that each chip is properly working. If the ladder failed or was unsuccessful, no results were recorded for that chip, and the samples were loaded onto another chip and run again.

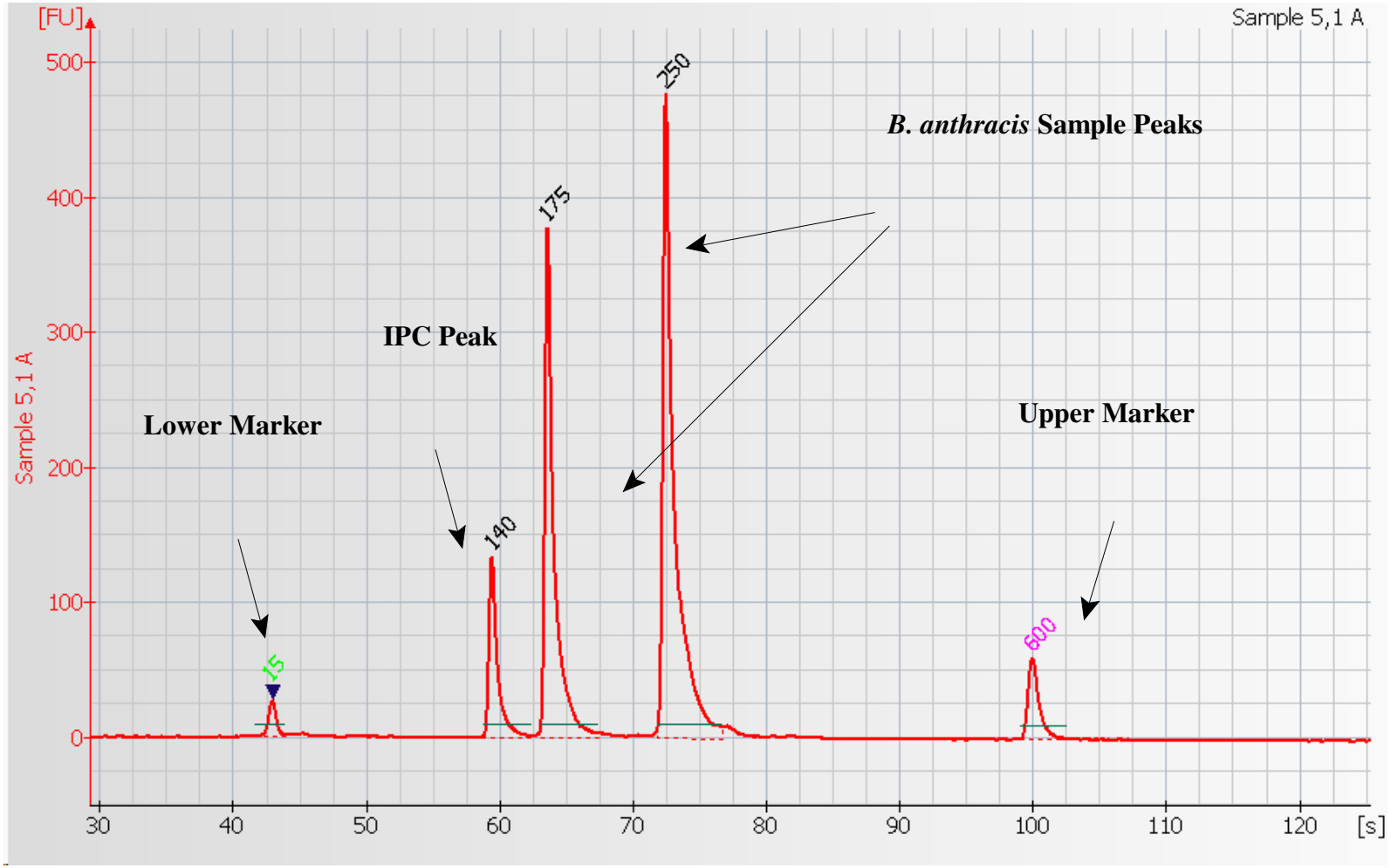

Figure 6-1. Positive Electropherogram for *B. anthracis*

6.1 Accuracy

The results for the PathAlert™ Detection Kit using the contaminant-only PT samples containing *F. tularensis, Y. pestis*, and *B. anthracis* are discussed in this section. The infective/lethal dose samples for each bacteria were included in the contaminant-only PT samples. In the case of *Y. pestis* and *B. anthracis*, the infective doses (see Table 3-1) were below the vendor-stated method LOD. The results for each bacteria at the infective/lethal dose are presented in the following tables, but those for *Y. pestis*, and *B. anthracis* were not included in the overall accuracy calculations for those bacteria.

6.1.1 F. tularensis

The results obtained for the PT samples containing *F. tularensis* are given in Table 6-1a. All concentration levels analyzed generated 4 out of 4 positive responses. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT samples. This resulted in 100% agreement for the overall accuracy of the PathAlert™ Detection Kit in detecting *F. tularensis*.

Table 6-1a. *F. tularensis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
PT samples	4×10^{5}[b]	4/4
	2×10^{4}	4/4
	5×10^{4}	4/4
	1×10^{5}	4/4
	5×10^{5}	4/4
Overall accuracy		**100% (20/20)**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose.

6.1.2 Y. pestis

The results obtained for the PT samples containing *Y. pestis* are given in Table 6-1b. All samples with concentration levels above 0.28 cfu/mL generated 4 out of 4 positive responses. The infective/lethal dose of *Y. pestis* (0.28 cfu/mL) was below the method LOD for this bacteria and produced no positive responses in four replicates. Only the IPC was present in the electropherogram for these samples. An overall percent agreement was determined by dividing the number of positive responses by the overall number of analyses of contaminant-only PT

samples above the method LOD. This resulted in 100% agreement for the overall accuracy of the PathAlert™ Detection Kit in detecting *Y. pestis* above the method LOD.

Table 6-1b. *Y. pestis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
PT samples	0.28[b]	0/4
	2×10^2	4/4
	5×10^2	4/4
	1×10^3	4/4
	5×10^3	4/4
Overall accuracy		**100% (16/16)[c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below the method LOD for *Y. pestis*.
[c] Excludes infective/lethal dose concentration, which was below the method LOD.

6.1.3 B. anthracis

The results obtained for the PT samples containing *B. anthracis* are given in Table 6-1c. All samples with concentration levels above the vendor-stated method LOD generated 4 out of 4 positive responses. The infective/lethal dose of *B. anthracis* was below the method LOD for this bacteria, but produced one positive response in four replicates. For the remaining three replicates of the infective dose sample, the IPC and one bacteria peak were present in the electropherogram. This indicated inconclusive results for those replicates (i.e., the sample could be declared neither positive nor negative). In a screening scenario, inconclusive results would lead to further testing of the sample, but this was beyond the scope of this test. The overall accuracy of the PathAlert™ Detection Kit in detecting *B. anthracis* above the method LOD was 100%.

Table 6-1c. *B. anthracis* Contaminant-Only PT Sample Results

Sample Type	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
PT samples	200[b]	1/4[c]
	2×10^4	4/4
	5×10^4	4/4
	1×10^5	4/4
	5×10^5	4/4
Overall accuracy		**100% (16/16)[d]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).
[b] Infective/lethal dose—below the method LOD for *B. anthracis*.
[c] Three replicates had an IPC and one *B. anthracis* peak in the electropherogram. This indicated an inconclusive result (neither positive or negative) for each replicate.
[d] Excludes infective/lethal dose concentration, which was below the method LOD.

6.2 Specificity

Specificity assesses the PathAlert™ Detection Kit's ability to provide a negative response when the contaminant was absent. The results from all unspiked interferent PT samples and unspiked DW samples are presented in this section. Negative results out of total replicates are presented in each table.

6.2.1 F. tularensis

The results obtained for *F. tularensis* for the unspiked interferent and DW samples are given in Table 6-2a. All unspiked interferent PT samples showed negative responses. All OH, FL, and NY unspiked DW samples showed negative responses also, indicating that the bacteria were not present in these samples, as would be expected. For the unspiked CA DW samples, the IPC and one bacteria peak were present in the electropherogram (with a baseline correction) for one replicate. This indicated inconclusive results for this replicate (i.e., the sample could be declared neither positive nor negative). The CA DW was further analyzed to determine the presence (or absence) of *F. tularensis* naturally in the water (see Section 3.3.2). *F. tularensis* could not be identified in the sample.

An overall specificity rate was determined by dividing the number of negative responses by the overall number of analyses of unspiked samples. This resulted in 96% agreement for the overall specificity of the PathAlert™ Detection Kit for *F. tularensis*.

Table 6-2a. *F. tularensis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4
DW samples	OH DW, unspiked	4/4
	CA DW, unspiked	3/4[(a)]
	FL DW, unspiked	4/4
	NY DW, unspiked	4/4
Overall specificity		**96% (23/24)[(a)]**

[(a)] One sample had an IPC and one bacteria peak in the electropherogram. This indicated an inconclusive result.

6.2.2 Y. pestis

The results obtained for unspiked interferent PT and DW samples using the *Y. pestis* PathAlert™ Detection Kit are given in Table 6-2b. All unspiked interferent PT samples showed negative responses. All OH, CA, and NY unspiked DW samples showed negative responses also, indicating that the bacteria were not present in these samples, as would be expected. For the unspiked FL DW samples, the IPC and one bacteria peak were present in the electropherogram (with a baseline correction) for one replicate. This indicated inconclusive results for this replicate (i.e., the sample could be declared neither positive nor negative).

An overall specificity rate was determined by dividing the number of negative responses by the overall number of analyses of unspiked samples. This resulted in 96% agreement for the overall specificity of the PathAlert™ Detection Kit for *Y. pestis*.

Table 6-2b. *Y. pestis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	4/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	4/4
DW samples	OH DW, unspiked	4/4
	CA DW, unspiked	4/4
	FL DW, unspiked	3/4[(a)]
	NY DW, unspiked	4/4
Overall specificity		**96% (23/24)[(a)]**

[(a)] One sample had an IPC and one bacteria peak in the electropherogram. This indicated an inconclusive result.

6.2.3 B. anthracis

The results obtained using *B. anthracis* reagents for the analysis of unspiked interferent and DW samples are given in Table 6-2c. All unspiked interferent PT samples and unspiked DW samples showed negative responses for all of the replicates. The overall specificity rate of the PathAlert™ Detection Kit for *B. anthracis* was 100%.

Table 6-2c. *B. anthracis* Specificity Results

Sample Type	Sample	Negative Results Out of Total Replicates
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid, unspiked	3/3[(a)]
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid, unspiked	3/3[(a)]
DW samples	OH DW, unspiked	4/4
	CA DW, unspiked	4/4
	FL DW, unspiked	4/4
	NY DW, unspiked	4/4
Overall specificity		**100% (22/22)**

[(a)] These samples had to be rerun because of suspected sample preparation problems. Samples were run in triplicate because of limited supplies. Only the rerun results are presented.

6.3 False Positive/Negative Responses

Contaminant-only PT samples, interferent PT samples, and DW samples were evaluated to determine false positive and false negative results for the PathAlert™ Detection Kit. Included in the calculations were the 16 additional interferent samples (0.5 mg/L each humic and fulvic acids) tested to determine the effects of the DNA extraction and isolation on the results. A false positive response was defined as a positive result when bacteria were not spiked into the sample. A false negative response was defined as a negative result when the sample was spiked with a contaminant at a concentration greater than the method LOD for that bacteria.

It should be noted that false positive responses cannot be absolutely confirmed as false because there is a possibility of cross-contamination. All appropriate steps were taken throughout the verification test to avoid this issue by using three work areas ("clean," "medium," and "dirty"), by following daily cleanup procedures, and by loading only one set of samples into the PCR tubes at a time (thus only the optical tubes for one set of replicates were uncapped at a time). However, cross-contamination is always a possibility in any PCR process.[12] No appropriate reference method was available to cross-check the amplified PCR product to confirm the PathAlert™ Detection Kit responses. When sample preparation error was suspected (e.g., a sample appeared to be unspiked when it should have been spiked or spiked when it should have been blank), the sample was reevaluated. If sample preparation errors or cross-contamination were suspected after reanalysis, only the results of the reruns were presented.

6.3.1 F. tularensis

Table 6-3a presents the false positive/negative results for *F. tularensis*. The number of positive samples out of the total replicates analyzed is presented in the table. No false positive or false negative samples were found in any of the sample matrices. One replicate for unspiked CA DW did show one bacteria peak along with the IPC peak in the electropherogram results. Because neither *F. tularensis* peaks was apparent in the electropherogram, the result was determined to be inconclusive.

6.3.2 Y. pestis

Table 6-3b presents the false positive/negative results for *Y. pestis*. The number of positive samples out of the total replicates analyzed is presented in the table. As with *F. tularensis*, no false positive or false negative samples were found in any of the sample matrices. One replicate for unspiked FL DW did show one of the two *Y. pestis* peaks along with the IPC peak in the electropherogram results. Because neither *Y. pestis* peak was apparent in the electropherogram, the result was determined to be inconclusive.

6.3.3 B. anthracis

Table 6-3c presents the false positive/negative results for *B. anthracis*. The number of positive samples out of the total replicates analyzed is presented in the table. No false positive or false negative samples were found in any of the sample matrices.

Table 6-3a. *F. tularensis* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-only PT samples	DI water	4×10^{5}[b]	4/4
	DI water	2×10^{4}	4/4
	DI water	5×10^{4}	4/4
	DI water	1×10^{5}	4/4
	DI water	5×10^{5}	4/4
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^{5}	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^{5}	4/4
DW samples	OH DW	Blank	0/4
	OH DW	1×10^{5}	4/4
	CA DW	Blank	0/4[c]
	CA DW	1×10^{5}	4/4
	FL DW	Blank	0/4
	FL DW	1×10^{5}	4/4
	NY DW	Blank	0/4
	NY DW	1×10^{5}	4/4
False positive rate			**0/24[c]**
False negative rate			**0/60**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] Infective/lethal dose.

[c] One unspiked CA DW replicate had an IPC and one *F. tularensis* peak in the electropherogram. This indicated an inconclusive result.

Table 6-3b. *Y. pestis* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-only PT samples	DI water	2×10^2	4/4
	DI water	5×10^2	4/4
	DI water	1×10^3	4/4
	DI water	5×10^3	4/4
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/4
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^3	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/4
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^3	4/4
DW samples	OH DW	Blank	0/4
	OH DW	1×10^3	4/4
	CA DW	Blank	0/4
	CA DW	1×10^3	4/4
	FL DW	Blank	0/4[b]
	FL DW	1×10^3	4/4
	NY DW	Blank	0/4
	NY DW	1×10^3	4/4
False positive rate			**0/24[b]**
False negative rate			**0/56[c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] One unspiked FL DW had an IPC and one *Y. pestis* peak in the electropherogram. This indicated an inconclusive result.

[c] The infective/lethal dose for *Y. pestis* was below the method LOD and thus was not included in this calculation.

Table 6-3c. *B. anthracis* False Positive/Negative Results

Sample Type	Sample	Concentration[a] (cfu/mL)	Positive Results Out of Total Replicates
Contaminant-only PT samples	DI water	2×10^4	4/4
	DI water	5×10^4	4/4
	DI water	1×10^5	4/4
	DI water	5×10^5	4/4
Interferent PT samples	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	Blank	0/3[b]
	0.5 mg/L humic acid and 0.5 mg/L fulvic acid	1×10^5	20/20
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	Blank	0/3[b]
	2.5 mg/L humic acid and 2.5 mg/L fulvic acid	1×10^5	4/4
DW samples	OH DW	Blank	0/4
	OH DW	1×10^5	4/4
	CA DW	Blank	0/4
	CA DW	1×10^5	4/4
	FL DW	Blank	0/4
	FL DW	1×10^5	4/4
	NY DW	Blank	0/4
	NY DW	1×10^5	4/4
False positive rate			**0/22**
False negative rate			**0/56[c]**

[a] Sample solutions were prepared at 2, 5, 10, and 50 times the vendor-stated method LOD from stock solutions based on the enumeration data (see Table 4-1).

[b] These samples had to be rerun because of suspected cross-contamination problems. Samples were run in triplicate because of limited supplies. Only the rerun results are presented.

[c] The infective/lethal dose for *B. anthracis* was below the method LOD and thus was not included in this calculation.

6.4 Precision

The performance of the PathAlert™ Detection Kit *F. tularensis* assay within sample sets of four replicates was consistent. Only one set of replicates, that for unspiked CA DW, was inconsistent, with one of the replicates showing inconclusive results, while the other samples were negative. All other samples showed the same results within a set of replicates. Thus, for *F. tularensis*, one of the 21 sets of replicates that was analyzed was determined to be inconsistent, indicating that 95% of the sample sets showed consistent results among the replicates.

The results for *Y. pestis* were similar to those for *F. tularensis*, with only one set of replicate sample sets showing inconsistent results. One of the four quadruplicate samples for unspiked FL DW showed inconclusive results (only one of the two *Y. pestis* peaks was apparent on the electropherogram, along with the IPC), while the other three samples were all negative. All of the remaining 20 sets of replicate samples analyzed for *Y. pestis* by the PathAlert™ Detection Kit showed the same results within the sample set. Thus, as with *F. tularensis*, 95% of the sample sets showed consistent results among the replicates.

As with *F. tularensis* and *Y. pestis*, 95% of the sample sets for *B. anthracis* (20/21) showed consistent results among the replicates. For this bacteria, the one sample set with inconsistent results was the infective dose PT sample. In this set of replicates, three of the four samples had inconclusive results, while the fourth sample was positive for *B. anthracis*. The infective dose of *B. anthracis* was below the method LOD for this bacteria, so the discrepancy between replicate samples likely has more to do with this fact than the actual precision of the PathAlert™ Detection Kit.

6.5 Interferences

6.5.1 Interferent PT Samples

In the 0.5 mg/L and 2.5 mg/L fulvic and humic acids solution, both spiked with the bacteria of interest and unspiked, the PathAlert™ Detection Kit provided expected results for *F. tularensis*, *Y. pestis*, and *B. anthracis*. In the absence of the bacteria, the samples tested negative; in the presence of the bacteria, the samples tested positive. For the interferent PT samples for *F. tularensis*, *Y. pestis*, and *B. anthracis,* 37.5 µL of the PathAlert™ Detection Kit SuperMix were used for the PCR process. In the case of *F. tularensis*, the first bacteria tested, the spiked interferent PT samples were also analyzed using 12.5 µL of SuperMix in addition to the interferent PT samples analyzed using 37.5 µL of SuperMix. This was done to verify that fulvic and humic acids were acting as potential inhibitors in the PCR process. The presence of an inhibitory substance is signified by the suppression of the IPC peak in the electropherogram results for the sample. For the 0.5 mg/L each humic and fulvic acids solution spiked with 1×10^5 cfu/mL of *F. tularensis*, the IPC peak was suppressed in two of the replicate samples, with one of those replicates showing only one sample peak in the electropherogram. The remaining two replicates indicated positive results, with the IPC clearly present. For the 2.5 mg/L each humic and fulvic acids solution spiked with 1×10^5 cfu/mL of *F. tularensis,* the IPC was suppressed in

three of the four replicate samples, though both sample peaks were apparent in those three samples. The remaining replicate sample was positive, with the IPC and two peaks indicative of *F. tularensis* present in the electropherogram. The suppression of the IPC peaks indicated that the humic and fulvic acids were acting as inhibitory substances in the spiked PT interferent samples. After discussions with Invitrogen Corporation, and in the interest of time, all remaining interferent PT samples for *Y. pestis* and *B. anthracis* were analyzed using only 37.5 µL of SuperMix to overcome the inhibitory actions of the humic and fulvic acids.

As discussed in Section 3.2.1, four solutions of fulvic and humic acids at 0.5 mg/L each, spiked with each contaminant at 1×10^3 and 1×10^5 cfu/mL (depending on the bacteria), were prepared in addition to the initial 0.5 mg/L and 2.5 mg/L fulvic and humic acid solutions. Each solution was put through the DNA extraction and isolation procedure, and then four replicates from each of the four purified DNA solutions were analyzed using the PathAlert™ Detection Kit. These samples were included in the verification test in an effort to evaluate the efficacy of the DNA extraction and isolation procedure in the presence of inhibitory substances. These samples also contribute to the precision evaluations of the PathAlert™ Detection Kit. For *F. tularensis*, *Y. pestis*, and *B. anthracis*, all of the samples tested resulted in positive responses. Thus, 20 out of the 20 spiked 0.5 mg/L humic and fulvic acid samples tested resulted in positive responses.

6.5.2 Drinking Water Samples

The PathAlert™ Detection Kit DW sample results for *F. tularensis*, *Y. pestis*, and *B. anthracis* are presented in Tables 6-3a, 6-3b, and 6-3c, respectively. In general, the PathAlert™ Detection Kit showed positive results for each set of replicates for the spiked DW samples and negative results for each set of replicates for the unspiked DW samples, with two exceptions. For the detection of *F. tularensis* in unspiked CA DW, one of the four replicates had an inconclusive result, where one of the two *F. tularensis* peaks was apparent on the electropherogram, along with the IPC. Similarly, one of the replicates for the detection of *Y. pestis* in unspiked FL DW had an inconclusive result. Analysis of the DW samples did not indicate the presence of *F. tularensis* in the CA DW and could not confirm the presence of *Y. pestis* in the FL DW. The possibility of cross-contamination causing the inconclusive results for these DW samples cannot be ruled out.

As with the interferent PT samples, DW samples spiked with *F. tularensis* were also analyzed using both 12.5 µL and 37.5 µL of SuperMix. Because of time constraints and the amount of SuperMix readily available, this was only done for spiked CA, FL, and NY DW. For two of the spiked FL DW replicates, there was a baseline shift and few identifiable peaks in the electropherogram. After a baseline correction was performed, all *F. tularensis* and IPC peaks were present in the electropherogram. It is not clear whether the baseline rise/shift is attributable to the amount of SuperMix in the sample. The remaining two replicates were positive, with all appropriate peaks present in the electropherogram. For spiked NY DW analyzed using 12.5 µL of SuperMix, one replicate required baseline correction to best view the electropherogram. Upon correction, the sample response was positive. The other three replicates all had suppressed IPC peaks, indicative of the presence of inhibitory substances. Two of the three samples were inconclusive, showing only one *F. tularensis* sample peak, while both *F. tularensis* bacteria

peaks were present in the third replicates, with only the IPC not found. For spiked CA DW samples, all replicate results were positive. In the case of one replicate, all peak sizes were slightly smaller than the other three samples. As with the interferent PT samples, all remaining DW samples for *Y. pestis* and *B. anthracis* were analyzed using only 37.5 µL of SuperMix to overcome the potential inhibitory actions of the DW matrix.

The contaminant-only PT samples spiked at 1×10^5 cfu/mL with *F. tularensis* and *B. anthracis* and 1×10^3 cfu/mL with *Y. pestis*, the level at which the DW samples were spiked, showed consistent positive responses across all bacteria. The interferent PT samples at both 0.5 mg/L and 2.5 mg/L humic and fulvic acids also spiked at 1×10^5 and 1×10^3 cfu/mL showed consistent positive responses for all replicates across all bacteria using 37.5 µL of SuperMix. The IPC peak was present in all of the aforementioned interferent PT samples. The consistency of responses in these PT samples, as well as other contaminant-only PT samples above the method LOD, would seem to indicate that the DW matrices used in this test do not have inhibitory effects on the PCR process for the PathAlert™ Detection Kit using 37.5 µL of SuperMix.

6.6 Other Performance Factors

The PathAlert™ Detection Kit was operated by the same Battelle technician throughout the verification test. This technician had prior PCR experience and was trained by Invitrogen and Agilent in operating the PathAlert™ Detection Kit and 2100 Bioanalyzer, respectively, before testing began. This training included the use of the Roche High Pure Prep Kit, the PathAlert™ Detection Kit, the operation of the thermal cycler used for testing, and the use of the 2100 Bioanalyzer. The Battelle technician was familiar with general DNA extraction and isolation techniques, PCR plating techniques, and general thermal cycler operation, as well as general PCR theory prior to training. The overall operation of the PathAlert™ Detection Kit was straightforward, and the experienced technician found the kit easy to use and had no major difficulties using the reagents. The need to use only the SuperMix for the PCR setup added to the ease of operation of the kit, since all of the necessary components for the PCR process were contained in one solution, instead of two or more. Though the DNA extraction procedure was straightforward, many steps were involved in the process. The operation of the 2100 Bioanalyzer was straightforward, though some degree of laboratory skill was required to properly load the chips without bubbles. Training on the 2100 Bioanalyzer software was very helpful in understanding how to interpret the results because an understanding of the software and the expected peak sizes was necessary for the data interpretations.

All testing was performed in a laboratory setting because the PathAlert™ Detection Kit is not field portable. Three distinct and separate testing areas were required in each laboratory to operate the PathAlert™ Detection Kit: “clean” or DNA-free, “medium” or moderate amount of DNA, and “dirty” or high DNA concentration. The PathAlert™ Detection Kit PCR reagents had to be stored at -20°C and thawed before use. The SuperMix was aliquoted into smaller portions to avoid thawing and refreezing the entire allotment of SuperMix each day. The PCR reactions had to be assembled on ice, and the plated SuperMix had to be incubated on ice or placed in the refrigerator until the sample DNA could be added to it.

F. tularensis samples were tested in a BSL-2 laboratory, while *Y. pestis* and *B. anthracis* were tested in a BSL-3 laboratory. Because live bacteria were being handled, special safety requirements and protocols had to be implemented in both the BSL-2 and BSL-3 laboratories. Some of these requirements impacted the analysis time for the PathAlert™ Detection Kit and are inherently present in any throughput estimations for this verification test. Thus, performance factors mentioned also incorporate the safety and facility requirements necessary for this test.

The PathAlert™ Detection Kit was used to test 92 or more sample replicates (including MBs) for each bacteria. Dispensing the SuperMix into the PCR tubes took approximately 15 minutes for each set of samples analyzed on a given day. Loading the sample DNA into the PCR tubes after purification took between 15 and 30 minutes, depending on the number of samples being analyzed. A maximum 36 replicates (nine sample solutions) plus controls were analyzed on a given day. Most sample sets averaged between five and eight sample solutions. On average, the DNA extraction and isolation step for between five and nine solutions took approximately 2.5 hours. The completion of the thermal cycle program to amplify the sample DNA took approximately 1.5 hours. Loading and analyzing each 2100 Bioanalyzer chip took approximately 45 minutes. The verification staff analyzed on average three DNA 500 chips a day, in some instances up to 4 chips a day. This equates to approximately 36 sample replicates a day for three chips and 48 sample replicates a day for four chips, including controls. The PathAlert™ Detection Kits can perform up to 320 assays per kit using 12.5 µL of SuperMix per reaction.

Chapter 7
Performance Summary

The PathAlert™ Detection Kit results for this verification test for samples containing *F. tularensis*, *Y. pestis,* and *B. anthracis* are presented in Tables 7-1 through 7-3. The results for each bacteria assay are presented in a separate table. Qualitative responses for each set of sample replicates as well as accuracy, specificity, false positives and negatives, and precision are presented in each table. A summary of the other performance factors associated with the PathAlert™ Detection Kit is presented at the end of this chapter. These performance factors apply to each kit across all bacteria.

Table 7-1. *F. tularensis* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	4×10^5 cfu/mL[a]	4/4
			2×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
			1×10^5 cfu/mL	4/4
			5×10^5 cfu/mL	4/4
	Interferent PT samples[b]	Humic and fulvic acids	1×10^5 cfu/mL	24/24
	DW samples[b]	Concentrated DW	1×10^5 cfu/mL	16/16
Accuracy		100% (20 out of 20) of the contaminant-only PT samples above the method LOD were positive.		
Specificity		96% (23 out of 24) of the unspiked interferent and DW samples were negative. One unspiked CA DW replicate returned an inconclusive result.[c]		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples. One inconclusive result was obtained for CA DW.[c]		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with *F. tularensis* above the method LOD.		
Precision		95% (20 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[c]		

[a] Infective/lethal dose.

[b] Interferent PT and DW sample results reflect the use of 37.5 µL of SuperMix. Some samples were analyzed using 12.5 µL of SuperMix, and many of the interferent PT and DW samples showed suppressed IPC peaks, indicating the presence of inhibitory substances.

[c] One unspiked CA DW replicate had an IPC and one bacteria peak in the electropherogram. This indicated an inconclusive result and would require reanalysis in a real-world scenario. The remaining three replicates were negative.

Table 7-2. *Y. pestis* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	0.28 cfu/mL[(a)]	0/4[(a)]
			2×10^2 cfu/mL	4/4
			5×10^2 cfu/mL	4/4
			1×10^3 cfu/mL	4/4
			5×10^3 cfu/mL	4/4
	Interferent PT samples[(b)]	Humic and fulvic acids	1×10^3 cfu/mL	24/24
	DW samples[(b)]	Concentrated DW	1×10^3 cfu/mL	16/16
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the method LOD were positive.		
Specificity		96% (23 out of 24) of the unspiked interferent and DW samples were negative. One unspiked FL DW replicate returned an inconclusive result.[(c)]		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples. One inconclusive result was obtained for FL DW.[(c)]		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with *Y. pestis* above the method LOD.		
Precision		95% (20 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[(c)]		

(a) Infective/lethal dose—below the method LOD for *Y. pestis*.

(b) Interferent PT and DW sample results reflect the use of 37.5 µL of SuperMix.

(c) One unspiked FL DW replicate had an IPC and one bacteria peak in the electropherogram. This indicated an inconclusive result and would require reanalysis in a real-world scenario. The remaining three replicates were negative.

Table 7-3. *B. anthracis* Summary Table

Parameter		Sample Information	Concentration	Number Detected/ Number of Samples
Qualitative results	Contaminant-only PT samples	DI water	200 cfu/mL[a]	1/4[b]
			2×10^4 cfu/mL	4/4
			5×10^4 cfu/mL	4/4
			1×10^5 cfu/mL	4/4
			5×10^5 cfu/mL	4/4
	Interferent PT samples [c]	Humic and fulvic acids	1×10^5 cfu/mL	24/24
	DW samples [c]	Concentrated DW	1×10^5 cfu/mL	16/16
Accuracy		100% (16 out of 16) of the contaminant-only PT samples above the method LOD were positive.		
Specificity		100% (22 out of 22) of the unspiked interferent and DW samples were negative.[d]		
False positives		No false positives resulted from the analysis of the unspiked interferent or DW samples.		
False negatives		No false negative results were obtained from the analysis of the interferent and DW samples spiked with *B. anthracis* above the method LOD.		
Precision		95% (20 out of 21) of the sample sets showed consistent results among the individual replicates within that set.[b]		

[a] Infective/lethal dose—below the method LOD for *B. anthracis*.

[b] Three samples in the infective/lethal dose PT sample replicates had an IPC and one *B. anthracis* peak in the electropherogram. This indicated an inconclusive result for each sample.

[c] Interferent PT and DW sample results reflect the use of 37.5 µL of SuperMix.

[d] Three replicates were run for both unspiked fulvic and humic acid samples because of limited supplies for rerun analysis.

Other performance factors: A technician with prior PCR experience operated the PathAlert™ Detection Kit at all times. The kit was straightforward and easy to use. All components necessary for the PCR process (excluding the sample DNA) were contained in one solution, which had to be stored at -20°C. Three separate work areas were needed for testing: a "clean" area free of DNA, a "medium" area with moderate DNA presence, and a "dirty" area, with high DNA presence. SuperMix preparation took approximately 15 minutes, and loading the sample DNA into the PCR tubes took between 15 and 30 minutes for each set of samples. For this verification test, sample throughput (from DNA purification to amplified product detection) was 36 to 48 samples per day. The PathAlert™ Detection Kit cost is around $15 per assay (approximately $15 per sample using 12.5 µL of SuperMix). PathAlert™ Detection Kits can perform up to 320 assays per kit.

Chapter 8
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