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United States
Environmental Protection
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Development and Verification of Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocols for *Bacillus anthracis* – For application to air filters, water and surface samples

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Office of Research and Development
**National Homeland Security Research Center

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The work presented in this report was performed within a Quality Assurance Project Plan agreed upon by the U.S. Environmental Protection Agency and LLNL.

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Foreword

The mission of the U.S. Environmental Protection Agency (EPA) is to protect human health and to safeguard the natural environment – the air, water, and land upon which life depends. The series of 2001 terrorist attacks, including the anthrax bioterrorism incidents that resulted in human casualties and public facility closures, prompted enhanced and expanded national safeguards. Presidential directives identified EPA as the primary federal agency responsible for the protection and decontamination of indoor-outdoor structures and water infrastructure vulnerable to chemical, biological, or radiological (CBR) terror attacks. EPA's mission, to protect human health and the environment, was thereby expanded to address critical needs related to homeland security.

The National Homeland Security Research Center (NHSRC) within the Office of Research and Development (ORD) is EPA's hub for providing expertise on CBR agents, and for conducting and reporting research to meet its homeland security mission needs. A focus of NHSRC's research is to support the Environmental Response Laboratory Network (ERLN), a nationwide association of federal, state, local, and commercial environmental laboratories, established by EPA. The ERLN can be deployed in response to a large-scale environmental disaster to provide consistent analytical capabilities, to offer increased capacity, and to produce quality data in a systematic and coordinated manner. Preparedness against potential indoor or outdoor wide-area anthrax attacks is currently the highest priority for the ERLN. To this end, NHSRC has developed and verified the Rapid Viability PCR (RV-PCR) method for detection of live anthrax spores in environmental samples.

This report provides a detailed account of the development and verification of the RV-PCR method for detection of live spores of *Bacillus anthracis* Ames in environmental sample matrices such as the BioWatch air filters, surface sampling wipes, and water.

NHSRC has made this publication available to assist in preparing for and recovering from disasters involving anthrax spores contamination. This work specifically represents a very important step in NHSRC's support for the ERLN. It is also key to the Agency's commitment to fulfill its homeland security mission and its overall mission to protect human health and the environment.

Jonathan Herrmann, Director
National Homeland Security Research Center
Office of Research and Development
U.S. Environmental Protection Agency

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List of Acronyms

ABI:	Applied BioSystems, Inc.
ATCC:	American Type Culture Collection
AZ:	Arizona
Ba:	*Bacillus anthracis*
Bg:	*Bacillus atrophaeus* subsp. *globigii*
BHI:	Brain Heart Infusion
BHQ:	Black Hole Quencher
bp:	Base Pairs
BSC:	Biosafety cabinet
CDC:	Centers for Disease Control and Prevention
CFU:	Colony Forming Unit
CT/Ct:	Cycle Threshold
DHS:	Department of Homeland Security
DNA:	Deoxyribonucleic acid
DTRA:	Defense Threat Reduction Agency
ERLN:	Environmental Response Laboratory Network
EPA:	U. S. Environmental Protection Agency
FAM:	6-carboxy-fluorescein
fg:	femtogram
HPLC:	High Performance Liquid Chromatography
HSARPA:	Homeland Security Advanced Research Project Agency
IBRD:	Interagency Biological Restoration Demonstration
ISO:	International Organization for Standardization
L:	Liter
LLNL:	Lawrence Livermore National Laboratory
LOD:	Limit of detection
LRN:	Laboratory Response Network
Mb:	Megabases
mg:	Milligram
µL:	Microliter
MPN :	Most probable number
NBBT:	Neutralization Butterfield's Buffer
NDT:	Non-detected
ng:	Nanogram
NHSRC:	National Homeland Security Research Center
PCR:	Polymerase Chain Reaction
Pa:	*Pseudomonas aeruginosa*

pg:	Picogram
PPE:	Personal Protection Equipment
PSAA:	Public Safety Actionable Assays Program
PSU:	Portable Sampling Unit
QAPP:	Quality Assurance Project Plan
q-PCR:	Quantitative PCR
RNA:	Ribonucleic acid
rpm:	Rotations per minute
RV:	Rapid Viability
RV-PCR:	Rapid Viability- Polymerase Chain Reaction
SOP:	Standard Operating Procedure
T0:	Time 0, prior to incubation
T9:	After 9 hr of incubation
T16:	After 16 hr of incubation
TE:	Tris-ethylene diamine tetra acetic acid
UNG:	Uracil-N-Glycosilase
UV:	Ultra Violet
WA:	Work Assignment

Trademarked Products

Trademark	Holder	Location
ABI GOLD™	Life Technologies	Carlsbad, CA
AB Applied BioSystems™	Life Technologies	Carlsbad, CA
ATCC™	American Type Culture Collection	Manassas, VA
AmpliTaq™	ABI	Carlsbad, CA
Cole Parmer®	Cole Parmer	Vernon Hills, IL
Epicentre®	Biotechnologies Inc.	Madison, WI
Excel®	Microsoft Corp.	Redmond, WA
GenBank®	U.S. Department of Health and Human Services	Bethesda, MD
Invitrogen®	Life Technologies	Carlsbad, CA
Life Technologies™	Life Technologies	Carlsbad, CA
MagneSil® Blood Genomic	Promega	Madison, WI
Microsoft®	Microsoft Corp.	Redmond, WA
Millipore®	Millipore Corp.	Billerica, MA
MyCycler™	Bio-Rad Inc.	Hercules, CA
Nanodrop™	Thermo Scientific	Wilmington, DE
PicoGreen®	Life Technologies	Carlsbad, CA
Quant-iT™	Life Technologies	Carlsbad, CA
Qubit®	Life Technologies	Carlsbad, CA
Qubit™	Life Technologies	Carlsbad, CA
TaqMan™	Life Technologies	Carlsbad, CA

Executive Summary

There has been a critical need for the sophisticated analytical tools necessary to rapidly detect and identify, or rule out, live *Bacillus anthracis*, the microbial agent for anthrax, during a bioterrorism event. Under an interagency agreement between the U.S. Environmental Protection Agency (EPA) and the U.S. Department of Energy, and funding by EPA's National Homeland Security Research Center (NHSRC), a cooperative project was undertaken to provide these tools. The Lawrence Livermore National Laboratory (LLNL) of the Department of Energy and the National Homeland Security Research Center spearheaded this project to develop the rapid viability polymerase chain reaction (RV-PCR) method for the detection and identification of viable *Bacillus anthracis* spores in environmental samples.

The RV-PCR method is aimed at serving the needs of the Environmental Response Laboratory Network (ERLN). The ERLN was established by EPA's Office of Emergency Management to analyze environmental samples during any intentional or natural contamination event of national significance in order to both assess the extent of contamination and evaluate decontamination efficacy. The network, established to respond to biological attacks in addition to chemical and radiological attacks, would respond to any biological attack by conducting sample analysis to determine if facilities and areas have been restored to ambient conditions. Validated, rapid viability test protocols are needed as part of the ERLN capabilities to ensure public safety and to help mitigate impacts due to facility closures following a biological agent release. This critical need for rapid analytical results was highlighted during the response to the 2001 anthrax attacks in which the clearance sampling and analysis required prior to facilities re-opening took an excessive amount of time.

The focus of this effort was to develop real time PCR assays for virulent *Bacillus anthracis*. In addition, the work was to develop and verify manual and semi-automated RV-PCR methods for detection and identification of viable *Bacillus anthracis* Ames spores in air filters, wipes, and water. Challenges administered during testing included high populations of non-target micro-organisms such as *Bacillus atrophaeus* subspecies *globigii* and *Pseudomonas aeruginosa*, high populations of heat-killed *Bacillus anthracis* spores, and high loadings of debris. Criteria for assessment of the PCR methodology included limits of detection, accuracy with plating, absence of PCR and growth inhibition, and turn-around time for results.

Signatures for *Bacillus anthracis* were ranked and selected using *in silico* analysis for signature specificity against all available sequences in GenBank® (U.S. National Institutes of Health's genetic sequence database), virulence gene association, availability of prior assay screening data and amplicon characteristics. Signatures from multiple sources were considered, including the Department of Homeland Security Public Safety Actionable Assays Program, the Homeland Security Advanced Research Project Agency program, and EPA's NHSRC. Published assays as well as new signatures developed for this project were also evaluated. The output of this analysis was a computational prediction of virulent *Bacillus anthracis* strain detection for 44 candidate assays ranked for predicted selectivity, amplicon size, and gene target. Ten assays (3 for the chromosome, 4 for the pXO1 plasmid and 3 for the pXO2 plasmid) were selected based on the *in silico* analysis and then optimized for real-time PCR. Three assays (1 for the chromosome, 1 for the pXO1 plasmid and 1 for the pXO2 plasmid) were selected for RV-PCR based on sensitivity, selectivity and robustness in the presence of growth medium and cell debris.

Rapid viability protocols, initially developed for the Interagency Biological Restoration Demonstration Program and funded under the Department of Homeland Security and the Department of Defense's Defense Threat Reduction Agency, were leveraged and optimized for *Bacillus anthracis* Ames using the selected assays. The method endpoint was shortened from its original 16 hr to 9 hr, by performing a magnetic bead-based DNA extraction and purification procedure prior to PCR analysis. Using this improved method, the total analysis time from start to finish for a batch of 24 samples is reduced from 24 hr to 15 hr. For each subsequent batch of 24 samples, only 3-4 additional hours are required. Therefore, with adequate resources, it is possible to analyze hundreds of samples per day.

Single laboratory verification of both manual and semi-automated versions of this optimized method showed limits of detection at the level of 10 spores per sample, both with and without debris, for all three sample types (clean laboratory water samples had a volume of 20 mL, wipes were 2 x 2" squares of rayon/polyester

gauze, and air filters were 47 mm diameter discs of hydrophobic polytetrafluoroethylene membranes). Live *Bacillus anthracis* Ames spores were consistently detected at the 10 spore level for both manual and semi-automated methods in heat-killed *Bacillus anthracis* spore backgrounds of 10^6 colony forming units per sample, and combined non-target backgrounds of 10^3 live *Bacillus atrophaeus* subspecies *globigii* and 10^6 live *Pseudomonas aeruginosa*. Follow on work will further explore the relationship between limit of detection and incubation time for clean wipe samples. These experiments will be used to evaluate whether the method endpoint could be further reduced when relatively clean samples such as wipes collected from clean indoor locations are processed.

1.0 Introduction

To protect human health and ensure that the environment and facilities are restored to ambient conditions following a biothreat agent release, the U.S. Environmental Protection Agency (EPA) needs rapid viability testing methods to evaluate contaminants. In the event of a biothreat agent release, hundreds to thousands of samples of diverse types (aerosol, surface and environmental) could need to be rapidly processed in order to both characterize the extent of contamination and determine the efficacy of remediation activities. Decision-makers could also need rapid results for re-mobilizing disinfection equipment in the case of incomplete decontamination and for re-opening facilities and areas based on results from clearance sampling. Current viability test methods are too labor- and time-intensive to be able to meet the need for rapid analysis. Typically, with current methods, only 30-40 samples are processed each day and the confirmed results are obtained days later.

The EPA's Office of Emergency Management within the Office of Solid, Waste, and Emergency Response has established a network of laboratories to analyze environmental samples, the Environmental Response Laboratory Network (ERLN). The network was created to respond to biological, chemical and radiological attacks. The network would respond to a biological attack by conducting sample analysis to determine if facilities and areas have been restored to ambient conditions. Validated, rapid viability test protocols are needed as part of the ERLN capabilities to ensure public safety and to help mitigate impacts that are due to facility closures following a biothreat agent release. This critical need was highlighted during the response to the 2001 anthrax attacks in which clearance sampling and analysis required excessive time prior to facilities re-opening. Therefore, to establish a research project in direct support of the ERLN, the EPA Office of Research and Development's National Homeland Security Research Center (NHSRC) initiated and entered into an Interagency Agreement with the Lawrence Livermore National Laboratory (LLNL) of the Department of Energy (DOE). Under that agreement, the LLNL was entrusted to develop and verify a rapid viability test method for anthrax spores in various environmental sample matrices. The NHSRC technical lead for this project was Dr. Sanjiv R. Shah.

LLNL has leveraged the useful features of real-time polymerase chain reaction (PCR) and expanded its capabilities by conducting PCR analysis pre- and post-incubation and using the change in the PCR cycle threshold (Ct) value as an indicator for the presence of viable spores or cells. The approach referred to as "rapid viability" (RV)-PCR uses high throughput sample processing via commercial automation in combination with 96-well real-time PCR to analyze hundreds of surface samples per day (the advantages of the RV-PCR method over the standard plating method are summarized in Table 1). RV-PCR protocols allow detection of low numbers of viable spores in the presence of environmental backgrounds and high populations of non-target microorganisms or dead target spores. Under joint Department of Homeland Security (DHS) and the Department of Defense's Defense Threat Reduction Agency (DTRA) funding, hundreds of samples spiked with *Bacillus anthracis* (Ba) surrogates were processed using RV-PCR protocols and demonstrating high throughput analysis and similar detection limits and accuracy as traditional viability analysis.[1,2]

Table 1. Comparison of Rapid Viability-Polymerase Chain Reaction and Plating Methods

Plating Method (Gold Standard)	RV-PCR Method
Direct method (visible colonies)	Indirect method (Ct values)
Quantitative technique down to 100 cells/mL	LOD ≤ 10 cells/mL; method is quantitative when combined with MPN
Separate PCR confirmation is needed	PCR confirmation is built in the method
Live non-target backgrounds may interfere with plate counts	Live non-target backgrounds do not interfere with PCR
Method is labor intensive (manual) and low throughput (30-40 samples/lab/day)	Method can be automated and high throughput (100s of samples/lab/day)
Method has logistic burdens (large lab space required to accommodate many BSCs and incubators)	~ 96 filter cups can be incubated in one standard shaker incubator and processed with one robot
Time for results ~ 18 hr without confirmation (confirmatory tests require additional time and labor and may take days)	Time for results < 15 hr including confirmation

Acronyms: BSC, biosafety cabinet; Ct, cycle threshold; LOD, limit of detection; MPN, most probable number; PCR, polymerase chain reaction; RV, rapid viability

The focus of the NHSRC-funded effort was to develop real time PCR assays for virulent Ba and to develop and verify manual and semi-automated RV-PCR methods for virulent Ba Ames spores in water, wipes and air filters. (These sample types were primarily chosen based on their diversity (surface, air and water samples) and on the availability of starting protocols developed for the Interagency Biological Restoration Demonstration (IBRD) program under joint DHS-DTRA funding.) Challenges administered during testing included high populations of non-target micro-organisms such as *Bacillus atrophaeus* subsp. *globigii* (Bg) and *Pseudomonas aeruginosa* (Pa) to evaluate the selectivity of the method, high populations of heat-killed Ba spores to evaluate decontamination scenarios, and high loadings of debris to evaluate any PCR and/or growth inhibition from environmental chemical and biological materials. Criteria for assessment of PCR methodology included limits of detection, accuracy with plating, absence of PCR and growth inhibition, and turn-around time for results. The current effort only developed and verified the RV-PCR method for qualitative analysis of samples.

It should be noted that the work presented in this report did not focus on sampling methods and that an exhaustive list of potential growth inhibitors and/or PCR inhibitors was not assessed. Although dirt was added to wipes, no actual surface wiping was performed to test interferences from chemicals and/or debris from sampled surfaces. Although air filter samples were randomly collected from both subway and outdoor locations, seasonal variations such as high versus low pollen levels or high versus low air pollution levels were not systematically tested. Although water samples were spiked with humic acid and ferrous sulfate, which are known PCR inhibitors, no environmental water samples were tested in this study. Finally, any interference of the decontamination method (fumigation, foam) with the RV-PCR method will need to be tested. Typical effects of decontamination are delayed germination and growth and PCR inhibition. Such experiments were outside the scope of this study since virulent Ba was used.

2.0
Quality Assurance Project Plan

A quality assurance project plan (QAPP) was approved by EPA before the experimental work started.[3] All work reported in this report was performed in accordance with the QAPP.

Compliance with Centers for Disease Control (CDC) and DOE safety and security policies was checked by the project principal investigator in monthly laboratory inspections. Quarterly laboratory inspections were also conducted by the LLNL responsible official and LLNL DOE representative. These quarterly inspections included a review of laboratory cleanliness; the certification of laboratory equipment including biosafety cabinet, robotic enclosure and autoclave; a review of the waste handling; an inventory of select agents; and a review of personnel training and vaccination. EPA program managers also toured our laboratory on multiple occasions to ensure that work conducted met quality assurance metrics. The ABI (Applied BioSystems™) cycler was calibrated every 6 months, pipettors were inspected and calibrated by the vendor annually, laboratory swipes were performed twice a year (the second set of swipes were taken and analyzed just before proceeding with single laboratory verification) and were always negative for B. *anthracis*. The refrigerators and freezers used are connected to an alarm system, to ensure storage conditions remain within acceptable ranges. In addition, a temperature recording chart is inserted in the refrigerator where stocks are maintained and checked weekly. Thermometers are also placed in each incubator to provide additional temperature control. During the course of the experiment, *B. anthracis* Ames extracted DNA standards were analyzed on every PCR plate, as described in the Materials and Methods section. Plating of the spiking spore suspension was conducted for each experiment.

Data was reported and discussed with EPA's technical lead monthly. Results obtained on the project were also presented in front of the LLNL directorate review committee, which is an external committee.

3.0
Materials and Methods

Detailed protocols for both manual and semi-automated RV-PCR methods are provided in Appendices A and B, respectively, as well as details of PCR conditions (Appendix F), buffer and media preparation (Appendix E), and consumables information (Appendix G).

Bacterial strain

All work presented in this report, including real-time PCR assay evaluation and RV-PCR method development, was conducted using the *pathogenic Bacillus anthracis* Ames strain. This strain belongs to the LLNL strain collection and has been verified by performing real-time PCR analysis on genomic DNA using primers and probes specific to the Ba chromosome, and pXO1 and pXO2 plasmids. The Ba Ames strain was grown in Brain Heart Infusion (BHI) medium and on BHI agar plates. Spore stocks were stored in a 70% water and 30% ethanol solution at 4 ºC.

B. anthracis Ames spore preparation

Ba was streaked for growth onto BHI agar and incubated overnight at 36°C. The organism was then streaked and incubated a second time for isolation. A 10^8 cells/mL suspension of the 24 hr growth was prepared in phosphate buffer (25mM KH_2PO_4, pH 7.2), plated onto soil extract beef peptone agar and incubated at 36°C until 99% sporulation was achieved. Plates were then scraped and rinsed using sterile water and a cell scraper (the content of each plate was transferred to a 50 mL centrifuge tube in a total of 30 mL of water). The spore preparation was cleaned using vortexing (2 min), centrifugation (4000 rpm for 15 min), removal of the supernatant and addition of sterile water. This cleanup procedure was repeated 4 times. Twenty milliliter of a 1:1 (ethanol:water) solution were then added to the centrifuge tubes, which were vortexed for 2 min to re-suspend the spore pellets. Tubes were then placed on a shaker platform for 1 hr at 80 rpm. After this step, the spore suspension was washed again 7 consecutive times using the vortexing, centrifugation and supernatant exchange technique described above. The suspension titer after these washing steps was 10^9 colony forming units (CFU)/mL, as measured by plating. The fraction of dead spores, measured by microscopy, was < 1%. The final spore re-suspension was performed using a mixture of 70% water and 30% ethanol in order to generate a spore stock for storage at 4ºC.

Sample types used in this study

Three sample types were used in this study, including 2 x 2” wipes (Kendall; catalogue number 8042, 50% rayon and 50% polyester gauze), 47 mm diameter air filters (Millipore, catalogue number FSLW04700, hydrophobic polytetrafluoroethylene membranes) and 20 mL water samples (Milli-Q™-filtered water, pH 7.0.). These sample types were primarily chosen based on their diversity (surface, air and water samples) and on the availability of starting protocols developed for the IBRD Program under joint DHS-DTRA funding.

Preparation of dirty wipes

The well-characterized Arizona Fine Test Dust (Powder Technology Inc., Burnsville, MN) was used for this study. The material consists of Arizona sand including Arizona Road Dust, Arizona Silica, AC Fine and AC Coarse Test Dusts, SAE Fine and Coarse Test Dusts, J726 Test Dusts, ISO (International Organization for Standardization) Ultrafine, ISO Fine, ISO Medium and ISO Coarse Test Dusts, and MIL STD 810 Blowing Dust.[4] Analysis of chemical composition performed by the manufacturer indicates that the material consists of: SiO_2 (68 to 76%), Al_2O_3 (10 to 15%), Fe_2O_3 (2 to 5%), Na_2O (2 to 4%), CaO (2 to 5%), MgO (1 to 2%), TiO_2 (0.5 to 1.0%), and K_2O (2 to 5%). Microbial characterization of the test dust performed by the Centers for Disease Control and Prevention (CDC) found 39 morphologically distinct colony types including *Bacillus cereus*, *Bacillus lichenformis*, *Bacillus mycoides*, *Bacillus endophyticus*, actinomycetes, molds, yeast, micrococcus and streptomyces.[5] A 0.5 mg/mL test dust slurry stock was prepared by weighing 10 g of test dust in a conical tube, adding 20 mL of deionized water, and vortexing at high speed for 20 min. Five hundred microliters of slurry were added to wipes (250 mg of dust) as a challenge.

Preparation of dirty air filters

Air filters collected from portable air sampling units belonging to the BioWatch network (a DHS environmental monitoring program) were used as challenges for this study. Some filters came from outdoor locations such as Houston, Texas, and others came from indoor locations, such as a subway station in Boston, Massachusetts. The goal of this challenge was to assess the presence of growth and/or PCR inhibition from aerosols present in both indoor and outdoor urban

environments (including pollen, dust, dirt and chemical and biological materials).

Preparation of chemically spiked water samples

The water used in this study was filtered through a Milli-Q™ water system (Millipore, MA). Challenge samples were prepared by adding ferrous sulfate and humic acid at levels of 10 mg/L, which are known PCR inhibitors.

Addition of heat-killed Ba Ames spore background

A stock of Ba Ames spores (10^6 CFU/mL confirmed by plate counts) was killed by autoclaving three times at 126 °C and 15 psi for 30 min. Six 100 µL aliquots were cultured on solid BHI medium and incubated for 48 hr to confirm non-viability of the stock. One milliliter of the heat-killed spore stock solution (10^6 CFU/sample) was added to each sample type as a challenge. The goal of this challenge was to simulate a decontamination scenario in which low levels of live spores would need to be detected in a high level of dead spores killed by the decontamination method.

Addition of live non-target background

A stock of *Bacillus atrophaeus* subspecies *globigii* (American Type Culture Collection [ATCC™] No. 9372) was plated to confirm its spore concentration of 10^4 CFU/mL. One hundred microliters (10^3 spore level) were then inoculated on each sample as a live challenge. *Pseudomonas aeruginosa* (ATCC No. 10145) cells were grown overnight in a flask and diluted to a concentration of 10^7 cells/mL using optical density measurements at 620 nm to assess the cell concentration. One hundred microliters (10^6 cell level) of this diluted culture were inoculated on each sample. The final live background for each sample was a combination of 10^3 Bg spores and 10^6 Pa cells. The goal of this challenge was to assess the specificity of the RV-PCR method, as well as any growth issues due to competition for nutrients.

Samples spiking with Ba

Prior to each RV-PCR experiment, the original Ba spore stock (10^8 CFU/mL) was vortexed on a platform vortexer for 20 min. Successive 10-fold dilutions were prepared in phosphate buffer [25 mM KH_2PO_4, pH 7.4], down to 10^2 CFU/mL. Three replicates of the last 2 dilutions were cultured on agar plates following the traditional viability protocol below. Typically, 100 µL of the 10^2 CFU/mL dilution and 50 µL of the 10^3 CFU/mL dilution were plated in triplicate, in order to determine the inoculation levels in terms of CFU/sample; this is why inoculation levels slightly differed from experiment to experiment. Typical error bars on the CFU/sample represent < 5% of the mean value. Water, air filter and wipe samples were inoculated using 100 µL of the 10^2 CFU/mL suspension (10 spore level) and 100 µL of the 10^3 CFU/mL suspension (100 spore level). The targeted levels for this study were the 10 spore level (1 to 99 CFU/sample) and the 100 spore level (100 to 999 CFU/sample). Although the goal was to test the lowest spore numbers for each level, variability with pipetting, vortexing, and surface binding of the spores to stock tubes generated slightly different CFU values for each experiment, which were quantified by systematic plating of the spiking solutions.

Traditional viability

For traditional viability analysis, 2 to 3 successive ten-fold sample dilutions were cultured on BHI agar and incubated overnight at 30 °C. From each dilution, three 100 µL aliquots were plated for spore count accuracy. Colony counts were obtained the next day and corrected for dilution in order to determine the number of viable spores spiked in the samples.

Rapid-Viability PCR

The experimental protocol outline, as well as pictures of the equipment used, are provided in Figure 1 and detailed protocols are provided in Appendices A and B. Briefly, samples were placed in 30 mL conical tubes and spiked with Ba Ames spores as described above. A mesh support was used to maintain wipe and air filter samples in place. Twenty milliliters of extraction buffer (70% of 0.25 mM KH_2PO_4/0.1% Tween 80 [pH 7.2] and 30% ethanol; final pH ~9.5) was added to each tube (for wipes and filters) and the tubes were vortexed for 20 min on a platform vortexer to remove spores from the sample matrix. Thirteen milliliters were then transferred from each sample to a filter cup, and spores in the extraction buffer were collected on a 0.45 µm filter using a vacuum manifold and a vacuum pump. Filters were then washed with 7 mL of filter-sterilized 210 millimolar (mM) KH_2PO_4 buffer (pH 6.0) followed by 3 mL of 25 mM KH_2PO_4 buffer (pH 7.2). Filter cups were then sealed on the bottom, after adding 2.5 mL of BHI growth medium. After mixing, 60 µL aliquots were taken from each filter cup and transferred to a 96-well PCR plate (aliquots taken at time 0; T0). The cups were sealed on the top and incubated for 16 hr at 37 °C and 230 rpm. Another set of 60 µL aliquots were taken after 16 hr of incubation (aliquots taken after 16 hr of incubation; T16). For each set of aliquots, the PCR plate was sealed, centrifuged and placed in a MyCycler™ thermal cycler for 20 min at 95 °C for heat lysis. For samples with high levels of dirt (wipes with Arizona test dust), a 1:10 dilution in PCR-grade water was performed prior to running PCR.

When the RV-PCR protocol was performed manually, all liquid handling was effectuated with serological pipettes or micro-pipettes. In the semi-automated version of the

protocol, with the exception of the initial sample spiking, the Perkin-Elmer Janus robotic platform performed all the liquid handling steps required to implement the RV-PCR method including mixing and transferring buffer from sample extracts to filtration media for spore collection, washes on the filters, and adding growth medium to the filter cups for culturing and sampling cultures for PCR analysis.

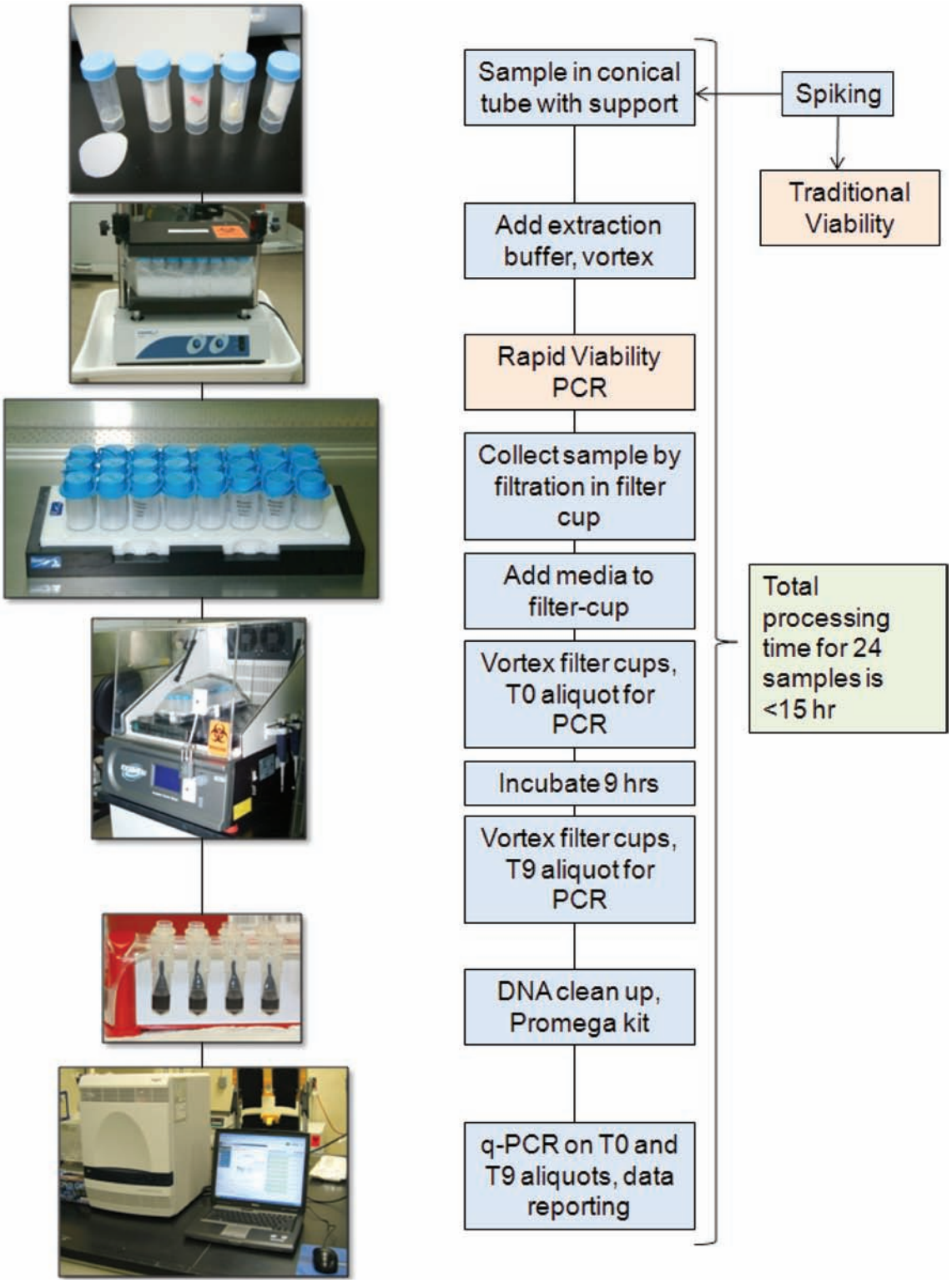

Figure 1. Summary of the optimized RV-PCR protocol steps and pictures of the equipment used to process samples.

Optimized Rapid-Viability PCR

In the optimized protocol, the RV-PCR method was implemented according to the protocol described above, with the exception of the introduction of a 10 min vortexing step of the filter cups prior to aliquoting samples for T0 and T-endpoint (at the end of the incubation period). The incubation time was reduced from 16 hr to 9 hr (T9) and sample aliquots were manually processed using the Promega magnetic bead-based DNA extraction and purification kit (MagneSil® Blood Genomic, Max Yield System; Promega, Catalog number MD1360).[6] Detailed protocols are provided in Appendix A and B. Briefly, 1 mL of each sample was transferred from the filter cup into a 2 mL eppendorf tube, followed by addition of 600 µL of bead mix (combined lysis buffer and bead mix) and 360 µL additional lysis buffer. Sample, buffer and bead mix were mixed by pipetting and tubes were mounted on a tube rack interfacing with a magnet (see Figure 2). Beads with attached DNA were attracted to the magnet and the supernatant was removed by pipetting. An additional lysis with 360 µL of lysis buffer was conducted with mixing by pipetting and removal of the supernatant. Two washes with 360 µL of salt solution were then performed, followed by mixing and removal of the supernatant. Finally, two washes with 360 µL of alcohol wash solution were performed with mixing and supernatant removal. Beads were allowed to air-dry for 2 min, followed by transfer of the tube rack from the magnetic support to a hot plate and heating at 80°C until samples/beads are dry (between 15 and 45 min). DNA elution/concentration was then performed by adding 200 µL of elution buffer while sample tubes remained on the hot plate. The sample with buffer was mixed and transferred to the magnetic support, and the supernatant with eluted DNA was recovered (typically 80 µL). A ten-fold dilution of the eluted sample in PCR-grade water was systematically performed prior to running PCR.

The DNA extraction and purification procedure was performed according to the manufacturer's protocol,[6] with the exception of the first step in which the sample is mixed with bead mix. Since the sample volume used in the RV-PCR method is larger (1 mL) than the sample volume used in the manufacturer's technical bulletin (200 µL), after receiving guidance from the manufacturer, the volume of beads was increased from 130 to 600 µL.

A summary of liquid transfers is provided in Figure 3.

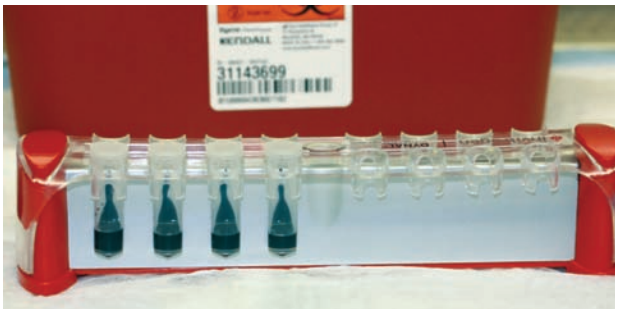

Figure 2. Magnetic rack used to process samples with the Promega DNA extraction and purification kit.

B. anthracis DNA Standards for Real-time PCR

DNA standards were generated for the Ba Ames strain. DNA was extracted from cultured cells using a complete a MasterPure™ Complete DNA and RNA Purification Kit (Epicentre® Biotechnologies Inc.) and DNA concentration was measured with a Qubit™ fluorimeter using the PicoGreen™ assay (Invitrogen™, Quant-iT™ dsDNA HS assay kit for Qubit fluorimeter, Part Number Q32854). Standard concentrations ranging from 1 ng/µL to 1 fg/µL were prepared in PCR-grade water. Seven 10-fold dilutions, ranging from 5 ng per 25-µL PCR to 5 fg per 25-µL PCR, were run with each set of PCR plates. The ROX reference dye contained in the ABI universal mastermix was used to normalize the fluorescent reporter signal.

Real-time PCR

Five microliter sample aliquots were transferred to a 96-well PCR plate with 20 µL of PCR mix. PCR mix was prepared for each primer-probe set according to conditions detailed in Tables 10-11 and in the PCR protocol in Appendix F, using TaqMan™ 2X Universal Master Mix (ABI cat. 4305719). After mixing and centrifugation, PCR was run using the ABI 7500 Fast platform (Applied Biosystems 7500 Fast Real-Time PCR System). Cycle parameters were as follows: 2 min at 50ºC for Uracil-N-glycosilase (UNG) incubation, 10 min at 95ºC for AmpliTaq™ gold activation, followed by 45 amplification cycles (5 s at 95ºC for denaturation and 20 s at 60ºC for annealing/extension). For the assay optimization study, each PCR was run in triplicate. For RV-PCR, each sample was analyzed against each of the 3 primer/probe sets (one PCR was run for each sample against each primer/probe set, 3 replicate samples were analyzed for each set of experimental conditions).

T0 control samples

In the initial part of the work (RV-PCR protocol using a 16 hr incubation step), a 60 µL aliquot was taken out of each filter cup at T0, heat-lysed for 20 min at 95 °C

and analyzed by PCR with the Ba assays to provide T0 background Ct values (these have consistently been >45).

After optimization of the RV-PCR protocol with the addition of the magnetic bead-based DNA extraction and purification, T0 aliquots were handled differently, to match the processing performed at T9 and to also equate the aliquots withdrawn at T0 and T9 (see section 4.3.4, Development of a T0 Control Protocol). In this procedure, three 120 µL aliquots are taken out of filter cups at T0, mixed with 900 µL of BHI and spiked with 500 pg of extracted Bg DNA. Each of the 120 µL aliquot is pipetted out of the filter cup and 100 µL is dispensed into a 2 mL eppendorf tube, to follow BSL3 pipetting guidelines. The T0 samples chosen are 3 replicates spiked at the highest spiking concentration: 10^2 or 10^3 spore level. The three 1 mL samples are then processed following the magnetic bead-based DNA extraction and purification method described above. The extracted samples are then analyzed using the Ba assays in order to determine whether any background is present; a Bg assay is used as a positive control. Ct values at T0 have consistently been >45 for all Ba assays in control experiments.

RV-PCR results interpretation

For the RV-PCR method, the endpoint PCR Ct of ≤ 36 with a ΔCt (Ct[T0]-Ct[T-endpoint]) ≥ 9 were set as cut-off values for a positive detection of viable Ba spores. It should be noted that most laboratories, including the CDC's Laboratory Response Network (LRN), use the PCR Ct value of $\leq$40 as a cut-off value for a positive detection of Ba spores (live or heat-killed). We set a more stringent Ct cut-off value to test the reliability and robustness of the RV-PCR method. Depending upon the end user requirement and the phase of response during an event, a lower ΔCt (Ct[T0]-Ct[T-endpoint]) ≥ 6 (to represent at least a two log difference in DNA concentration), and a higher end point (PCR Ct of $\leq$39) could be set.

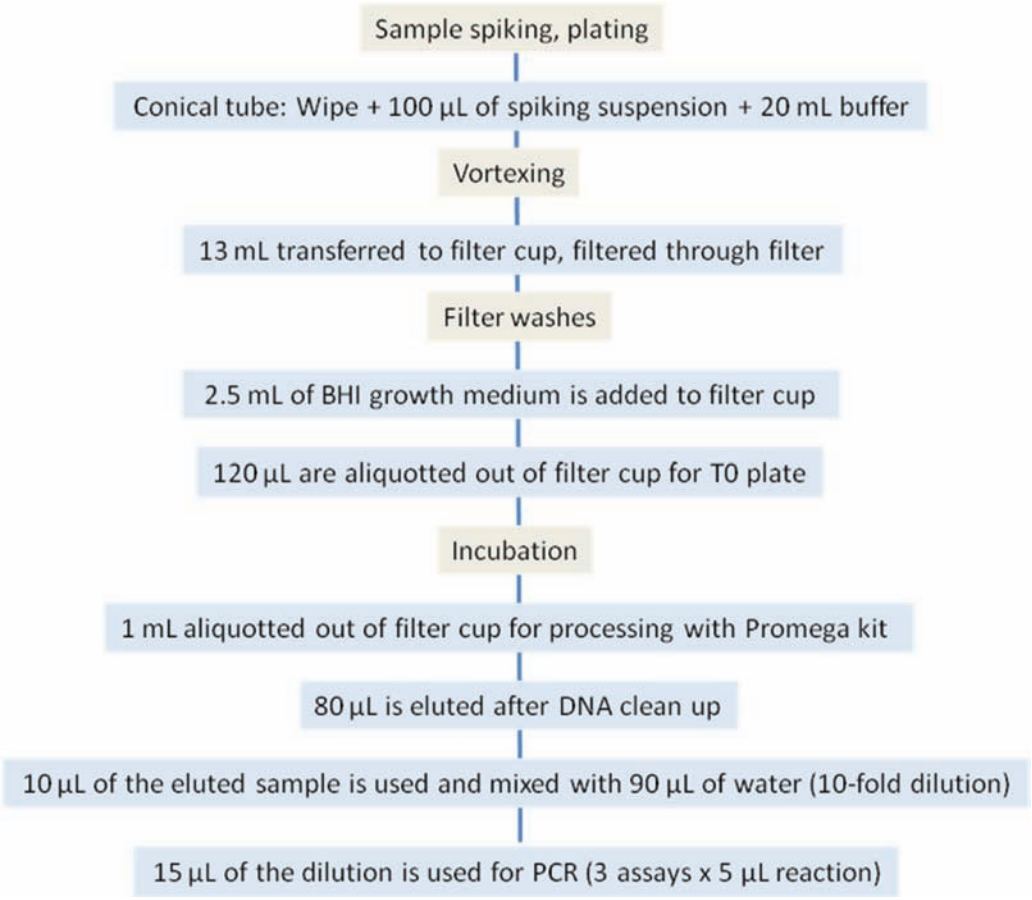

Figure 3. Summary of liquid handling steps performed during the optimized RV-PCR protocol.

Final RV-PCR protocols for real-world sample analysis

Final manual and semi-automated RV-PCR protocols were designed for the analysis of real-world samples. The detailed protocols are located in Appendices A and D.

4.0
Results and Discussion

4.1 Assay Development

4.1.1. In silico Analysis

Signatures for Ba real-time PCR were ranked and selected *in silico* using the following parameters: signature specificity against all available sequences in GenBank® (U.S. National Institutes of Health's genetic sequence database), virulence gene association, availability of prior assay screening data, and amplicon characteristics. Signatures from multiple sources were considered, including the Department of Homeland Security (DHS) Public Safety Actionable Assays Program (PSAA), the Homeland Security Advanced Research Project Agency (HSARPA) Program, and EPA's NHSRC. Published assays, signatures developed at LLNL for other projects, as well as new signatures developed for this project were also evaluated (Table 2).

Erosion/Non-verification analysis provides a prediction of potential cross-reactions and misses as signatures are compared against all available genomic sequence data. The LLNL KPATH microbial database contains all available full-length microbial genomes and is updated weekly. The LLNL erosion analysis software uses algorithms that compare signatures as primer pairs or triplets against all sequence in the large KPATH database and identifies targets with sufficient percent match to each oligomer to represent a potential hybridization. Erosion results include predicted false positives against non-target sequences, and non-verifications which are predicted false negatives against target sequences. True positives are also identified.

Table 2. Summary of All *Bacillus anthracis* Signatures Analyzed *in Silico*

Source	Ba Signatures
HSARPA	18
DHS / PSAA	0
Publication[7]	1
RNA Viability project (LLNL-generated)	17
EPA (Provided by Dr. Sanjiv Shah)	2
EPA (LLNL-generated)	6
Total	**44**

The output of this analysis was a computational prediction of virulent Ba strain detection for 44 candidate assays ranked for predicted selectivity, amplicon size, and gene target. These files are too large to include here in a Microsoft® Office Word document but a summary of signatures selected for this project is presented in Table 3. Ten assays (3 for the chromosome, 4 for the pXO1 plasmid and 3 for the pXO2 plasmid) were selected for optimization based on the *in silico* analysis.

Table 3. List of 10 *Bacillus anthracis* Assays Selected After *in Silico* Analysis

Signature Name	Target	Signature ID	Gene Target
BC1	Chromosome	1776135	hypothetical protein
BC2	Chromosome	1776147	hypothetical protein
BC3	Chromosome	1776201	hypothetical protein
BP11	pXO1 plasmid	1776125	hypothetical protein
BP12	pXO1 plasmid	2186847	hypothetical protein
BP13	pXO1 plasmid	1776115	hypothetical protein
EPA-1	pXO1 plasmid	2212179	hypothetical protein
BP21	pXO2 plasmid	2148341	hypothetical protein
BP22	pXO2 plasmid	2148343	membrane protein, putative
EPA-2	pXO2 plasmid	2212180	capsule biosynthesis protein cap b

4.1.2. Assay Optimization

A dedicated ABI 7500 Fast thermocycler was purchased from Applied BioSystems, Inc. (ABI) with NHSRC funds for this RV-PCR project. PCR for both assay development and Rapid Viability method was always performed using this platform. Selected signatures were ordered from Biosearch Technologies, Inc. (Novato, CA) (all primers and probes were HPLC-purified and probes were modified as 5'-6FAM and 3'-BHQ1). Ba Ames genomic standards were prepared and initially characterized using both Nanodrop™ UV spectrometry and Qubit® fluorimetry with the PicoGreen® double-stranded DNA quantification assay. Initial tests showed

that the concentration of double-stranded Ba Ames DNA measured using the PicoGreen assay was typically 25% of the concentration measured using the Ultra Violet (UV) spectrophotometric method and Nanodrop spectrometer (see Table 4), which was expected since the PicoGreen assay only detects double-stranded DNA. All DNA concentrations were therefore measured using the PicoGreen assay for the entire study.

Table 4. Measurement of *Bacillus anthracis* Ames DNA Concentration by Nanodrop™ UV Spectrometer and Qubit® Fluorimeter based methods

Method	DNA (ng/µL)			
	Replicate 1	Replicate 2	Replicate 3	Average
Nanodrop UV Spectrometer	3.80	3.90	3.90	3.87
Qubit Fluorimeter, PicoGreen Assay	1.24	1.11	1.16	1.17

An initial experiment was conducted to test each primer/probe set with a series of eight 10-fold dilutions of Ba Ames DNA in triplicate, following PCR conditions provided by NHSRC (see Materials and Methods and PCR conditions in Appendix F). This initial experiment showed that the primer/probe sets that had been optimized with this PCR mix and platform (EPA-1 and EPA-2) performed according to specifications, but that other primer/probe sets would need optimization (see Tables 5-7).

Table 5. Initial Screening of *Bacillus anthracis* Chromosome Assays BC1, BC2 and BC3

DNA (pg)	Average Ct*		
	BC1	BC2	BC3 (selected)
5000	29.1	27.9	23.3
500	32.7	32.4	27.0
50	NDT	42.9	39.8
5	NDT	NDT	41.9
0.5	NDT	NDT	NDT
0.05	NDT	NDT	NDT
0.005	NDT	NDT	NDT

* Average Ct (n=3)
Acronyms: Ct, cycle threshold; n, number of replicates; NDT, no signal detected

Table 6. Initial Screening of *Bacillus anthracis* pXO1 Plasmid Assays BP11, BP12, BP13 and EPA-1

DNA (pg)	Average Ct*			
	BP11	BP12	BP13	EPA-1 (selected)
5000	29.7	28.7	33.8	20.7
500	33.5	33.0	36.3	23.1
50	NDT	32.6	39.7	26.4
5	NDT	NDT	NDT	29.9
0.5	NDT	NDT	NDT	33.4
0.05	NDT	NDT	NDT	37.2
0.005	NDT	NDT	NDT	NDT

* Average Ct (n=3)
Acronyms: Ct, cycle threshold; n, number of replicates; NDT, no signal detected

Table 7. Initial Screening of *Bacillus anthracis* pXO2 Plasmid Assays BP21, BP22, and EPA-2

DNA (pg)	Average Ct*		
	BP21	BP22	EPA-2 (selected)
5000	27.7	24.7	18.5
500	39.1	28.3	21.4
50	40.6	34.8	24.7
5	42.3	41.4	28.4
0.5	44.1	43.7	31.9
0.05	NDT	44.4	35.4
0.005	NDT	NDT	NDT

* Average Ct (n=3)
Acronyms: Ct, cycle threshold; n, number of replicates; NDT, no signal detected

Based on this initial sensitivity screening, EPA-1 and EPA-2 assays were immediately selected for the pXO1 and pXO2 plasmid targets respectively. The BC3 assay was the best performer in the chromosomal assay category in the initial screening and it was therefore selected for optimization. Sequence and amplicon size information for the selected assays are provided in Table 8.

Table 8. Nucleotide Sequences and Amplicon Size for Selected *Bacillus anthracis* RV-PCR Assays

Assay (alias)	LLNL Signature Number	Forward Primer	Reverse Primer	Probe	Amplicon Length (bp)
Chromosome (BC3)	1776201	TTTCGATGATT TGCAATGCC	TCCAAGTTACAG TGTCGGCATATT	ACATCAAGTCAT GGCGTGACTACCCAGACTT	105
pXO1 plasmid (EPA-1)	2212179	GCGGATAGCG GCGGTTA	TCGGTTCGTTAA ATCCAAATGC	ACGACTAAACCG GATATGACATTA AAAGAAGCCCTTAA	101
pXO2 plasmid (EPA-2)	2212180	TGCGCGAATGA TATATTGGTTT	GCTCACCGATAT TAGGACCTTCTTTA	TGACGAGGAG CAACCGATTAAGCGC	77

Acronyms: bp, base pair units; LLNL, Lawrence Livermore National Laboratory

Primer and probe titrations were performed on the BC3 assay in triplicate, using 5 ng and 500 pg of extracted Ba Ames DNA template. The probe concentration was varied between 0.08 and 0.20 µM, while the primer concentration was varied between 0.4 and 1.0 µM per reaction. The conditions chosen were 1.0 µM of primer and 0.08 µM of probe per reaction, since they provided good sensitivity and matched the conditions used for the EPA-1 assay (see highlight in Table 9).

The final PCR conditions for the selected assays are described in detail in Tables 10-12 below.

Reagents:

- Primers and probes
- TaqMan® 2X Universal Master Mix with UNG and AmpliTaq® Gold DNA polymerase (ABI catalogue Number 4305719)
- Molecular Biology grade distilled water, RNase- and DNase-free (Teknova catalogue Number W3350)

Table 9. Optimization of Primer and Probe Concentration for the BC3 Assay

Primer (µM)	Probe (µM)	Average Ct*	
		5 ng DNA	500 pg DNA
0.4	0.08	20.4	23.1
0.4	0.12	20.6	23.2
0.4	0.20	20.7	23.2
0.6	0.08	19.8	22.8
0.6	0.12	19.7	22.7
0.6	0.20	20.0	23.0
0.8	0.08	19.7	22.8
0.8	0.12	19.7	22.6
0.8	0.20	19.9	22.5
1.0	0.08	19.5	22.6
1.0	0.12	19.6	22.7
1.0	0.20	19.6	22.5

* Average Ct (n=3)
Selected PCR conditions are highlighted in blue.
Acronyms: Ct, cycle threshold; n, number of replicate reactions

Table 10. PCR Mix for pXO2 (EPA-2) Primer/Probe Set

Reagent	Volume (µL)	Final Concentration (µM)
TaqMan 2X Universal Master Mix	12.5	1X
Forward primer, 25 µM	0.3	0.3
Reverse primer, 25 µM	0.3	0.3
Probe, 2 µM	1	0.08
Molecular Biology Grade Water	5.9	N/A
Template DNA	5	Variable
TOTAL	25	

Table 11. PCR Mix for Chromosome (BC3) and pXO1 (EPA-1) Primer/Probe Sets

Reagent	Volume (µL)	Final Concentration (µM)
TaqMan 2X Universal Master Mix	12.5	1X
Forward primer, 25 µM	1	1.0
Reverse primer, 25 µM	1	1.0
Probe, 2 µM	1	0.08
Molecular Biology Grade Water	4.5	N/A
Template DNA	5	Variable
TOTAL	25	

Equipment:

- ABI 7500 fast thermocycler
- Optical fast 96-well plates (ABI, cat. Number 4366932)
- Optical adhesive plate covers (ABI, cat. number 4311971)

Table 12. PCR Thermal Cycling Conditions for All 3 Primer/Probe Sets

Steps	UNG incubation	AmpliTaq Gold activation	PCR , 45 cycles	
	Hold*	Hold*	Denatur-ation*	Annealing/extension*
Temper-ature	50ºC	95ºC	95ºC	60ºC
Time	2 min	10 min	5 s	20 s

*Fast Ramp: 3.5oC/s up and 3.5oC/s down

The performance of the three selected assays is summarized in Table 13 and Figure 4 below. After optimization, all three assays enabled detection of less than 10 copies (50 fg) of extracted Ba Ames DNA. The genome copies are based on an estimated genome size of 5.5 Mb. The chromosomal assay provided slightly higher Ct values than the plasmid assays, which is an expected result since more plasmid copies are typically produced.

Table 13. Performance of Optimized BC3, EPA-1, and EPA-2 PCR Assays Tested With *Bacillus anthracis* Ames DNA

DNA (pg)	Genome Copies	Average Ct*		
		BC3	EPA-1	EPA-2
5000	829000	19.4	20.7	18.5
500	82900	22.6	23.1	21.4
50	8290	26.5	26.4	24.7
5	829	30.6	29.9	28.4
0.5	82.9	34.6	33.4	31.9
0.05	8.29	38.5	37.2	35.4

* Average Ct (n = 3)
Acronyms: Ct, cycle threshold; n, number of replicate reactions

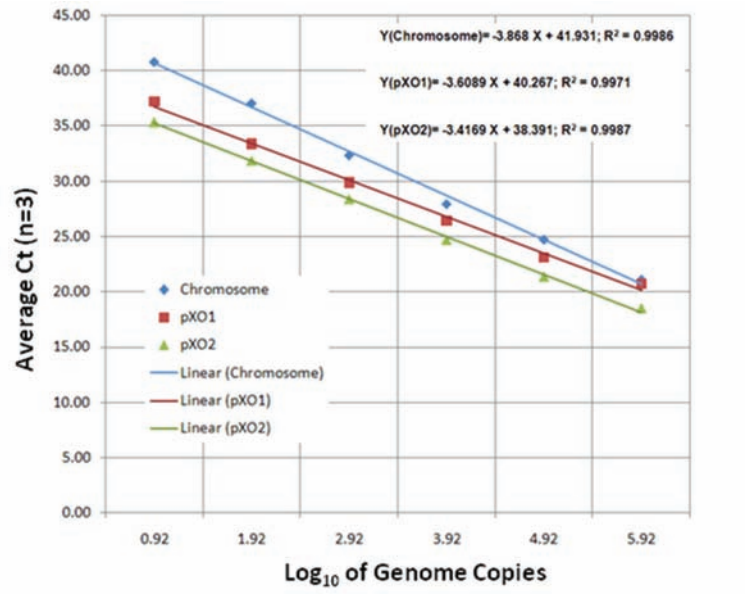

Figure 4. PCR efficiency of selected chromosomal and plasmid *Bacillus anthracis* Ames assays.

Additional experiments were then performed with the assays in order to insure their suitability for Rapid-Viability PCR. Inhibition by the BHI growth medium was tested by comparing the PCR performance of the Ba Ames DNA dilutions in PCR-grade water and BHI medium. Results showed that variations in cycle threshold values induced by the presence of growth medium in the PCR were generally below 1 Ct (see Table 14).

Table 14. Change in average Ct value for *Bacillus anthracis* Ames Assays with BHI Medium in the PCR, Compared to Water

DNA (pg)	Change in Average Ct*		
	EPA-1**	EPA-2**	BC3**
5000	-0.6	0.7	0.5
500	1.1	0.2	-0.1
50	0.2	0.7	-0.9
5	0.8	0.7	-0.5
0.5	0.5	0.5	-0.5
0.05	1.0	-1.3	3.7

* Average Ct (n=3)
** Average Ct (BHI) – Average Ct (water)
Acronyms: pg, picogram

The effect of cell debris on the PCR was also evaluated by comparing Ba Ames DNA dilutions in PCR-grade water and in a lysed culture of Bg (10^9 cells/mL). Results showed that variations in cycle threshold values induced by the presence of cell debris in the PCR were generally below 2 Cts (see Table 15).

Table 15. Change in average Ct value for *Bacillus anthracis* Ames Assays with lysed culture of *Bacillus atrophaeus* (10^9 cells/mL) in the PCR, Compared to Water

DNA (pg)	Change in Average Ct*		
	EPA-1**	EPA-2**	BC3**
5000	-1.1	0.4	1.8
500	-0.7	2.9	0.1
50	-1.2	2.4	-1.4
5	-1.5	2.1	-0.1
0.5	-1.6	2.0	1.3
0.05	-1.0	3.1	1.8

* Average Ct (n=3)
** Average Ct (Bg cell lysate) – Average Ct (water)
Acronyms: pg, picogram

4.1.3. Selectivity Study

Concentrations of extracted DNA templates were measured using the PicoGreen assay, and diluted to appropriate stock concentrations using PCR-grade water. PCR plate lay-outs were prepared to run triplicate reactions for each assay against each target and near-neighbor DNA (two concentrations were run for each template: 500 fg per reaction and 50 fg per reaction for targets and 5 pg and 500 fg per reaction for near neighbors). Three positive controls (extracted Ba Ames DNA) and three negative controls (PCR-grade water) were run for each assay on each PCR plate. Results summarized in Tables 16 and 17 showed that all assays detected Ba targets, as expected, and that no cross

reactivity was observed with near neighbors, with the exception of *Bacillus cereus* 03BB102 for both plasmid assays and *Bacillus cereus* 03BB108 for the pXO2 assay. These interactions of the plasmid assays with *Bacillus cereus* strains were predicted by the *in-silico* analysis.

Table 16. PCR Assay Selectivity Study Using a Panel of *Bacillus anthracis* Strain DNA

Targets	Strain	Average Ct* with indicated PCR assays and DNA concentrations					
		BC3		EPA-1		EPA-2	
		50 fg	500 fg	50 fg	500 fg	50 fg	500 fg
B. anthracis	Turkey 32	36.5 (0.7)	32.7 (0.2)	34.7 (0.4)	31.3 (0.1)	34.0 (0.3)	30.2 (0.1)
B. anthracis	A0149	37.2 (0.6)	32.7 (0.5)	35.6 (0.3)	31.9 (0)	35.8 (0.5)	31.9 (0.3)
B. anthracis	A0248	36.6 (0.7)	32.9 (0.3)	35.6 (0.8)	31.7 (0.2)	35.9 (1.0)	31.5 (0.2)
B. anthracis	V770-NP-1R	36.4 (0.4)	33.0 (0.3)	35.1 (0.8)	31.2 (0.1)	NDT (pXO2-)	NDT (pXO2-)
B. anthracis	Ba1015	38.1 (1.5)	33.7 (0.2)	36.2 (1.0)	32.5 (0.2)	35.5 (0.5)	32.6 (0.2)
B. anthracis	SK-102	37.1 (0.7)	32.9 (0.2)	35.7 (0.2)	32.2 (0.2)	35.0 (0.2)	30.7 (0.4)
B. anthracis	Ba1035	37.4 (1.2)	33.3 (0.5)	35.0 (0.1)	31.4 (0.1)	34.6 (0.7)	30.3 (0.1)
B. anthracis	K3	37.1 (0.3)	33.4 (0.2)	35.5 (0.5)	31.7 (0.1)	35.9 (1.5)	30.7 (0.2)
B. anthracis	Ames	35.9 (0.5)	33.0 (0.3)	35.1 (0.1)	31.4 (0.1)	35.1 (0.9)	31.4 (0.3)
B. anthracis	PAK-1	36.8 (1.7)	33.0 (0.2)	34.8 (0.2)	31.7 (0.1)	34.0 (0.2)	30.6 (0.2)
B. anthracis	RA3	38.4 (0.5)	33.9 (0.3)	35.6 (0.3)	32.5 (0.1)	35.5 (0.8)	31.9 (0.3)
B. anthracis	Vollum 1B	38.8 (0.4)	34.3 (0.3)	37.1 (0.9)	33.3 (0.1)	35.0 (0.2)	31.5 (0.3)
B. anthracis	Sterne	36.7 (0.6)	33.1 (0.1)	35.0 (0.2)	31.5 (0.3)	NDT (pXO2-)	NDT (pXO2-)

* Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; fg, femtogram; n, number of replicates; NDT, no signal detected; PCR, polymerase chain reaction; SD, standard deviation

Table 17. PCR Assay Selectivity Study Using a Panel of Near-Neighbor *Bacillus* species DNA

Near Neighbors	Strain	Average Ct* with indicated PCR assays and DNA concentrations					
		BC3		EPA-1		EPA-2	
		500 fg	5 pg	500 fg	5 pg	500 fg	5 pg
B. cereus	S2-8	NDT	NDT	NDT	NDT	NDT	NDT
B. cereus	3A	NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis	HD1011	NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis	97-27	NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis	HD682	NDT	NDT	NDT	NDT	NDT	NDT
B. cereus	E33L	NDT	NDT	NDT	NDT	NDT	NDT
B. cereus	D17	NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis	HD571	NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis Al Hakam		NDT	NDT	NDT	NDT	NDT	NDT
B. cereus	FM1	NDT	NDT	NDT	NDT	NDT	NDT
B. cereus	03BB102	NDT	NDT	32.4 (0.3)	29.0 (0)	35.0 (0.2)	31.8 (0.3)
B. cereus	03BB108	NDT	NDT	NDT	NDT	37.6 (0.6)	35.2 (0.7)
B. thuringiensis israelensis		NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis kurstaki		NDT	NDT	NDT	NDT	NDT	NDT
B. thuringiensis morrisoni		NDT	NDT	NDT	NDT	NDT	NDT

* Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; fg, femtogram; n, number of replicates; NDT, no signal detected; PCR, polymerase chain reaction; SD, standard deviation

4.2. Select Agent Laboratory Set Up

The CDC Select Agent Program regulates the possession, use, and transfer of biological agents and toxins that could pose a severe threat to public health and safety (select agents). While select agent operations were already approved and ongoing at LLNL, a new laboratory dedicated to the EPA work was set up, and a team with RV-PCR experience was assembled. Any work activity involving select agents requires a permit from CDC which is specific to people, locations, organisms and protocols. Internal LLNL approvals for select agent work were also requested and granted.

Team members applied for and received clearances from the Department of Justice. Training included select agent handling, shipping, receiving, and transferring, as well as a Select Agent Human Reliability Program certification (yearly psychological and medical evaluations, as well as drug testing). All personnel also received the anthrax vaccine.

Dedicated equipment was purchased with NHSRC funds and installed in the dedicated Select Agent laboratory, providing both manual and semi-automated RV-PCR capabilities for Select Agents (see pictures in Figure 5). Instrumentation purchased includes an Applied Biosystems, Inc. ABI 7500 Fast PCR platform, a Beckmann-Coulter (Beckmann-Coulter, Inc., Brea, CA) centrifuge with safety cups, a New-Brunswick shaker incubator (New Brunswick Scientific, Edison, NJ), a VWR refrigerator, a Perkin-Elmer Janus robotic platform for semi-automated liquid handling, and a custom HEPA-filtered enclosure for the Janus built by E-N-G Mobile Systems Inc. The Class II BioSafety Cabinet (BSC), freezer, and standing incubators were provided by LLNL. Both BSC and robot enclosures are certified semi-annually as a best management practice.

4.3. Rapid Viability-Polymerase Chain Reaction (RV-PCR) Method Development

The major test variables for RV-PCR protocol development included inoculum density (10^3 to 10^1 spores per sample), presence/absence of reference background debris, presence/absence of heat-killed target spores, and presence/absence of non-target organisms (including Bg and Pa). Ba spores were spiked directly onto/into wipe, air filter, and water samples rather than collected from surfaces. The determination/evaluation of the efficiency of spore removal from sample matrices was outside the scope of this study.

RV-PCR protocols were evaluated for the following criteria: accuracy with plate counts from spiking solutions, selectivity, sensitivity (< 50 spores per sample), throughput capacity (24-48 samples per day with manual and semi-automated methods), turn-around time for results (< 24 hr), and absence of PCR and/or growth inhibition (a 1:10 dilution of the samples was used as needed to overcome inhibition).

RV-PCR experiments were performed according to the Materials and Methods section and to the detailed protocols provided in Appendices A and B. Experiments consisted of a minimum of three replicates per treatment and routinely used 4 to 6 replicates per treatment. Each replicate sample was analyzed once against each PCR assay (BC3, pXO1, and pXO2). Initial and final cycle thresholds (Ct_0 and Ct_f) from RV-PCR assays were used to determine whether viable spores were present in the sample. Since Ct_0 values were consistently ≥ 45, RV-PCR results during the protocol evaluation and optimization phase were expressed in term of average Ct_f value and corresponding standard deviation for each set of conditions (sample type, spore level, added

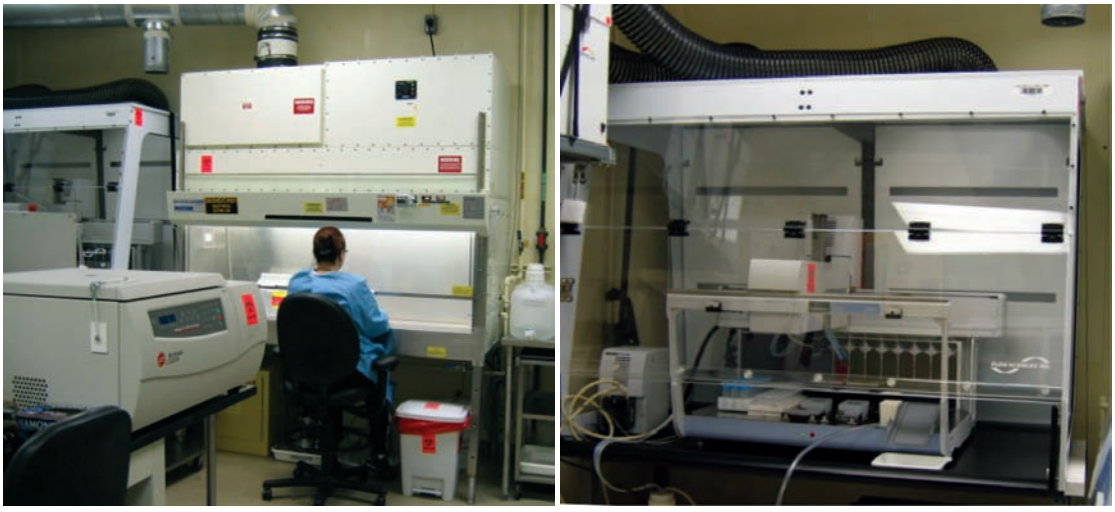

Figure 5. Pictures of the EPA-dedicated Select Agent laboratory at LLNL. On the left is the manual capability (Class II BioSafety Cabinet), and on the right is the automated capability (Janus robotic platform and custom, HEPA-filtered enclosure from E-N-G Mobile Systems Inc.).

background or debris). For the verification phase, Ct_0, Ct_f, and ΔCt were reported for each sample-type analyzed. Traditional viability analysis was used to quantify the level of spores spiked onto/into sample materials in each experiment. Two dilutions were plated and 3 replicate plate counts were used to calculate the standard deviation of the spore level applied, as described in the Materials and Methods.

4.3.1. Development of Manual RV-PCR

Spore levels of 1000, 100 and 10 CFU were spiked on clean wipes in triplicate. Table 18 summarizes the Ct values (average of 3 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 cycles and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for clean wipes with the manual method. Spore levels were determined by plating.

Table 18. Manual RV-PCR at T16 on Clean Wipes Spiked With 3 Spore Levels

CFU/Sample	Average Ct*		
	EPA-1	EPA-2	BC3
22	33.0 (2.0)	26.3 (1.7)	33.4 (3.3)
110	31.1 (2.0)	25.3 (1.0)	33.0 (2.5)
1100	29.9 (1.8)	25.3 (0.8)	31.0 (2.5)

*Average Ct (SD, n = 3)
Acronyms: CFU, colony forming units; Ct, cycle threshold; n, number of replicates; SD, standard deviation

Ultra-fine Arizona test dust was then added to clean wipes at a level of 250 mg per wipe. Spore levels of 100 and 10 CFU were spiked and 8 replicate samples were used for each spore level. Initial PCR results at T16 from experiments performed without diluting the aliquots were 'non-detect' for dirty wipes (spiked with the AZ Test Dust). Ten fold dilutions of all dirty wipes sample aliquots (T16 aliquots) were then prepared in PCR-grade water and positive PCR detection was achieved. PCR inhibition from the Arizona Test Dust is believed to mainly come from metal oxides. Analysis of the chemical composition performed by the manufacturer indicates that the material consists of: SiO_2 (68 to 76%), Al_2O_3 (10 to 15%), Fe_2O_3 (2 to 5%), Na_2O (2 to 4%), CaO (2 to 5%), MgO (1 to 2%), TiO_2 (0.5 to 1.0%), and K_2O (2 to 5%). The fine particles present in this dust also enhance the reactivity of this material by creating a large specific area. Table 19 summarizes the Ct values (average of 8 replicates) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 cycles and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for dirty wipes with the manual method.

Table 19. Manual RV-PCR at T16 on Clean and Dirty Wipes Spiked With 2 Spore Levels

CFU/Sample	Wipe	Average Ct*		
		EPA-1	EPA-2	BC3
12	Clean	29.9 (2.6)	24.8 (2.9)	27.1 (0.6)
	Dirty	34.8 (2.1)	32.9 (1.6)	33.3 (1.7)
110	Clean	22.6 (0.6)	22.6 (1.1)	24.0 (0.4)
	Dirty	27.9 (4.0)	23.7 (1.2)	29.1(2.6)

*Average Ct (SD, n = 3)
*For dirty wipes, a 1:10 dilution of the heat-lysed samples was performed in PCR-grade water prior to running PCR to reduce inhibition from the AZ test dust. Ct values were not corrected from the dilution factor.
Acronyms: CFU, colony forming units; t, cycle threshold; n, number of replicates; SD, standard deviation

Spore levels of 1000, 100 and 10 CFU were spiked on clean filters in triplicate. Table 20 summarizes the Ct values (average of 3 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 cycles and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for clean air filters with the manual method.

Table 20. Manual RV-PCR at T16 on Clean Filters Spiked With 3 Spore Levels

CFU/ Sample	Average Ct*		
	EPA-1	EPA-2	BC3
20	28.3 (1.9)	22.2 (4.0)	27.8 (0.5)
120	24.8 (1.2)	21.0 (1.5)	28.2 (0.3)
1000	23.1 (0.5)	20.9 (0.3)	25.2 (0.3)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Spore levels of 1000, 100 and 10 CFU were spiked on dirty filters. Dirty filters used in this experiment were collected from a Portable Sampling Unit (PSU) in

Phoenix, AZ in November 2008. Table 21 summarizes the Ct values (average of 8 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 cycles and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for dirty air filters with the manual method.

Table 21. Manual RV-PCR at T16 on Dirty Air Filters Spiked With 3 Spore Levels

CFU/Sample	Average Ct*		
	EPA-1	EPA-2	BC3
8	32.8 (2.5)	30.4 (4.9)	33.6 (0.9)
116	31.7 (3.5)	26.0 (5.4)	29.5 (2.1)
1100	30.6 (1.8)	22.6 (1.6)	31.8 (1.7)

*Average Ct (SD, n = 8)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Spore levels of 1000, 100 and 10 CFU were spiked in 20 mL clean laboratory water samples (deionized, sterile water) and dirty water samples (10 mg/L humic acid and 10 mg/L ferrous sulfate spiked in deionized, sterile water) in triplicate. Table 22 summarizes the Ct values (average of 3 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 cycles and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for both clean and dirty water with the manual method.

Figure 6 summarizes the data presented in this section for both clean and dirty sample types, with an inoculum level of 10 Ba CFU/sample. Detection limits at the 10 CFU/sample level were demonstrated on both clean and dirty samples (all Ct values were below 35). A 1:10 dilution of the sample was required prior to running PCR on dirty wipes in order to reduce inhibition from the AZ test dust. Other samples did not require any dilution.

Table 22. Manual RV-PCR at T16 on Clean and Dirty Water Spiked With 3 Spore Levels

CFU/ Sample	Water	Average Ct*		
		EPA-1	EPA-2	BC3
78	Clean	27.4 (1.5)	24.1 (1.1)	29.1 (2.0)
	Dirty	28.6 (2.0)	22.1 (3.1)	28.6 (1.4)
780	Clean	26.6 (2.7)	22.6 (2.2)	30.3 (0.2)
	Dirty	27.7 (1.3)	22.0 (1.3)	28.3 (1.4)
7800	Clean	29.5 (3.3)	23.9 (2.4)	30.0 (0.8)
	Dirty	28.7 (1.3)	24.2 (1.0)	29.4 (3.3)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

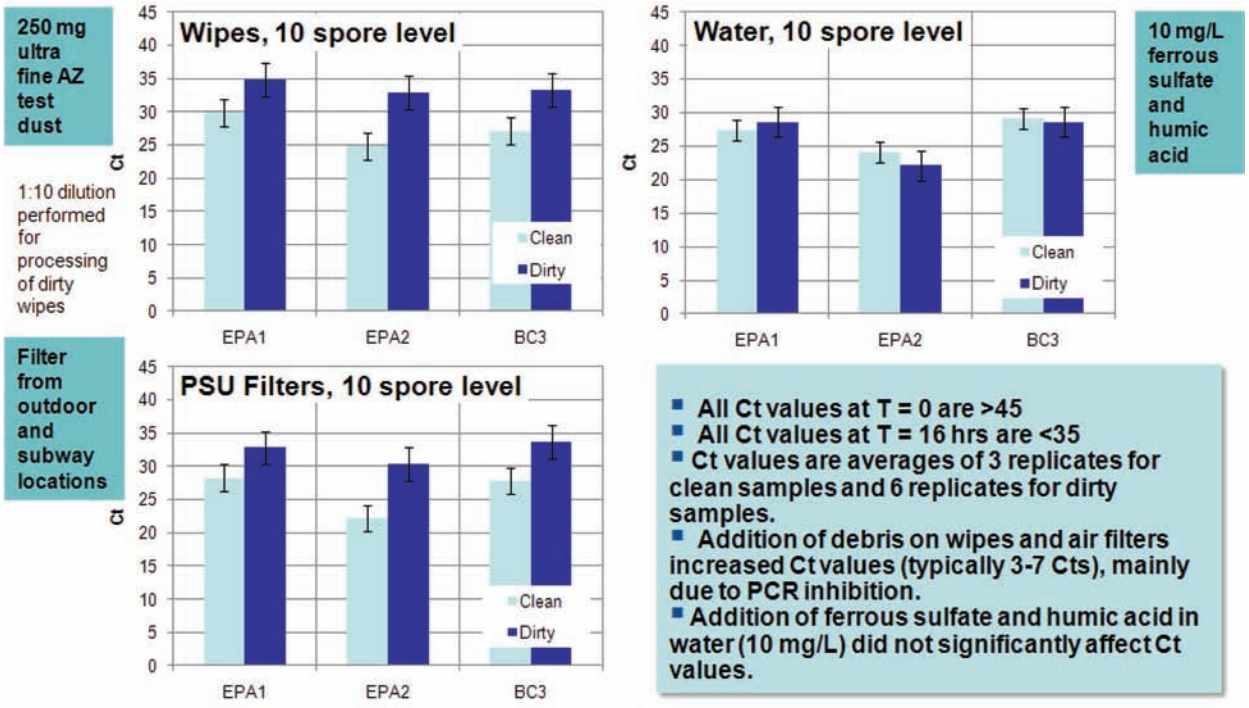

Figure 6. Summary of Ct values obtained with the manual RV-PCR protocol on both clean and dirty sample types (wipes, water and air filters) using a spiking level of 10 *Bacillus anthracis* spores per sample.

Manual RV-PCR experiments to evaluate the influence of high levels of live non-target background on the limit of detection for Ba were started with clean wipes. Spore levels of 100 and 1000 spores per sample were spiked on clean wipes in the presence of live Pa only (10^6 CFU/sample), as well as in the presence of live Bg (10^3 CFU/sample) combined with live Pa (10^6 CFU/sample). Table 23 summarizes the Ct values (average of 5 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level (the Ct at time zero was >45 cycles for all samples).

All Ct values obtained in the presence of live Pa were <36. All Ct values obtained in the presence of both live Pa and Bg were <39 (in all cases, 2 out of 3 assays had Ct values <35), confirming the robustness of the RV-PCR approach in the presence of high levels of non-target organisms (Ct values above 36.0 are highlighted in blue in Table 23). Note that the EPA-2 assay allowed detection of the 100 live Ba Ames spores level in a combined background of up to 10^6 live Pa and 10^5 live Bg (Ct[100 spores, T16]=35.0, Ct [650 spores, T16]=33.4).

Table 23. Manual RV-PCR at T16 on Clean Wipes Spiked With 2 Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
100	10^6/sample Pa	35.8 (3.4)	23.9 (1.7)	34.5 (1.2)
	10^6/sample Pa & 10^3/sample Bg	38.3 (5.6)	25.4 (5.1)	33.3 (3.5)
650	10^6/sample Pa	35.2 (2.1)	26.8 (5.8)	30.8 (2.0)
	10^6/sample Pa & 10^3/sample Bg	37.2 (4.4)	23.4 (2.3)	33.0 (3.8)

*Average Ct (SD, n = 5)
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Spore levels of 10 and 100 CFU were spiked on clean filters in the presence of live Pa only (10^6 CFU/sample), as well as in the presence of live Bg (10^3 CFU/sample) combined with live Pa (10^6 CFU/sample). Table 24 summarizes the Ct values (average of 5 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level (the Ct at time zero was >45 cycles for all samples).

All Ct values obtained in the presence of live Pa were <35. All Ct values obtained in the presence of both live Pa and Bg were <41 (in all cases, 2 out of 3 assays had Ct values <35), confirming the robustness of the RV-PCR approach in the presence of high levels of non-target organisms (Ct values above 36.0 are highlighted in blue in Table 24). Note that the EPA-2 assay allowed detection of the 100 live Ba Ames spores level in a combined background of up to 10^6 live Pa and 10^5 live Bg (Ct[85 spores, T16]=39.0, Ct [330 spores, T16]=34.7).

Table 24. Manual RV-PCR at T16 on Clean Filters Spiked With 2 Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
85	10^6/sample Pa	33.4 (0.2)	30.9 (2.5)	33.6 (2.0)
	10^6/sample Pa & 10^3/sample Bg	33.5 (5.0)	31.3 (5.3)	40.1 (4.2)
330	10^6/sample Pa	31.4 (1.7)	24.4 (1.3)	32.3 (1.1)
	10^6/sample Pa & 10^3/sample Bg	33.8 (3.3)	29.7 (1.0)	38.1 (3.1)

*Average Ct (SD, n = 5)
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Spore inocula at the 100 spore level were spiked in clean laboratory water (filtered and sterilized) in the presence of live Pa only (10^6 CFU/sample), as well as in the presence of live Bg (10^3 CFU/sample) combined with live Pa (10^6 CFU/sample). Table 25 summarizes the Ct values (average of 5 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level (the Ct at time zero was >45 cycles for all samples).

All Ct values obtained in the presence of live Pa were <35. All Ct values obtained in the presence of both live Pa and Bg were <41 (in all cases, 2 out of 3 assays had Ct values <35), confirming the robustness of the RV-PCR approach in the presence of high levels of non-target organisms (Ct values above 36.0 are highlighted in blue in Table 25).

Table 25. Manual RV-PCR at T16 on Clean Water Spiked With 2 Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
123	10^6/sample Pa	27.2 (5.2)	20.6 (1.0)	26.6 (0.2)
	10^6/sample Pa & 10^3/sample Bg	39.0 (1.9)	29.7 (1.9)	40.1 (3.0)
250	10^6/sample Pa	29.6 (2.5)	22.2 (1.4)	27.9 (1.9)
	10^6/sample Pa & 10^3/sample Bg	33.2 (1.5)	29.6 (3.0)	39.9 (4.2)

*Average Ct (SD, n = 5)
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

In order to investigate the effect of high levels of dead Ba Ames on the detection of low levels of the same live organism, an experiment was performed in which two levels of live Ba spores were spiked on clean wipes, air filters and water samples in the presence of 10^6 heat-killed Ba Ames spores/sample. A stock of Ba Ames spores (10^6 CFU/mL confirmed by plate counts) was killed by autoclaving three times at 126 °C for 30 min. Six 100 µL aliquots were then plated on solid BHI medium and no colonies were observed after 48 hr, confirming that all spores were dead. A manual RV-PCR experiment was performed, in which each sample type (wipes, filters, and water) was spiked with 10^6 heat-killed Ba spores and 75 and 400 live Ba spores. Four replicates were generated for each sample type and spore level. Table 26 summarizes the Ct values (average of 4 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

All Ct values obtained in the presence of heat-killed Ba spores were <37 (in all cases, 2 out of 3 assays had Ct values <35), confirming the detection of low levels of live spores in a high background of dead spores (decontamination scenario) (Ct values above 36.0 are highlighted in blue in Table 26).

Background Ct values (average of 4 replicate samples) for 10^6 heat-killed spores/sample only (no live spores) after 16 hr of growth were: 42.89, 44.02, and 40.57 for the BC3, EPA-1, and EPA-2 assays respectively. Such background may originate from DNA leaking from dead cells.

Table 26. Manual RV-PCR at T16 on Clean Wipe, Air Filter and Water Samples Spiked With 2 Live Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Ames Spores

Sample Type	CFU/ Sample	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	75	33.3 (7.2)	20.2 (0.8)	27.8 (1.4)
	400	29.2 (0.5)	20.5 (1.5)	25.5 (1.0)
Air Filters	75	37.0 (7.7)	19.6 (1.2)	26.2 (2.9)
	400	31.3 (6.8)	20.1 (2.4)	27.0 (2.3)
Water	75	29.2 (4.9)	20.6 (0.8)	27.0 (2.2)
	400	29.3 (5.8)	19.7 (1.6)	24.3 (2.1)

*Average Ct (SD, n = 4)
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

4.3.2 Development of Semi-automated RV-PCR

Since no Select Agent work had previously been performed with robotics in our facility, a work observation by the Biosafety Officer and Select Agent Manager was required to authorize the new robotics protocol at LLNL. The observation was recorded in the safety documents and approval to operate the Janus robotic platform with Select Agents was granted. Figure 7 shows the Janus platform and the HEPA-filtered enclosure in the Select Agent laboratory.

The Janus robotic platform was programmed to perform all the liquid handling steps required to implement the RV-PCR method, with the exception of sample spiking. Semi-automated protocols include mixing and transferring buffer from sample extracts to filtration media for spore collection, as well as performing washes on the filters, adding growth medium to filter cups for culturing and sampling cultures for PCR analysis.

Figure 7. Janus robotic platform and custom HEPA-filtered enclosure in the Select Agent laboratory.

Spore levels of 100 and 10 CFU were spiked on all sample types in triplicate. Table 27 summarizes the Ct values (average of 3 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays.

The Ct values at T0 were >45 and Ct values at T16 were <35 for all samples, confirming a detection limit at the 10 spore level for clean sample types with the semi-automated method.

The same experiment was then performed with dirty samples. Dirty wipes were generated by adding 250 mg of ultra-fine AZ test dust to clean wipes, dirty air filters were collected from a subway location (Boston, Massachusetts, 02/2008) and dirty water was generated by spiking clean and sterile laboratory water with humic acid and ferrous sulfate at a level of 10 mg/L. Table 28 summarizes the Ct values (average of 5 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays (Ct values above 36.0 are highlighted in blue).

The Ct values at T0 were >45 and Ct values at T16 were <35 for dirty filter and dirty water samples, confirming a detection limit at the 10 spore level for these sample types in the presence of dirt.

PCR inhibition was observed in the presence of AZ test dust on the wipe samples, similar to what was observed with the manual protocol. Systematic 1:10 dilutions of the dirty wipe samples were therefore prepared and re-analyzed by PCR. With 250 spores per sample, 2 out of 3 assays had Ct values <35. With 36 spores per sample, Ct values were between 36.3 and 37.3 for all 3 assays. Additional experiments were then conducted in order to optimize the protocol (see the Method Optimization section). It was found that the up and down pipetting performed by the Janus platform did not provide enough mixing of the cultured filter cups prior to aliquoting the T16 samples. Vortexing filter cups for 10 min prior to aliquoting the T16 samples generated lower Ct values.

Table 27. Semi-automated RV-PCR at T16 on Clean Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels

Sample Type	CFU/ Sample	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	36	32.0 (1.8)	21.9 (1.2)	24.8 (1.1)
	244	20.1 (0.6)	19.3 (2.1)	26.3 (1.4)
Air Filters	36	29.2 (1.7)	20.7 (1.7)	26.6 (0.1)
	244	22.2 (3.8)	19.7 (2.5)	24.0 (1.2)
Water	36	26.9 (0.7)	20.4 (0.4)	25.0 (0.8)
	244	21.8 (0.4)	21.4 (0.3)	23.8 (0.7)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 28. Semi-automated RV-PCR at T16 on Dirty Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels

Sample Type	CFU/ Sample	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes**	36	37.3 (3.2)	36.3 (4.1)	36.4 (3.6)
	244	38.0 (5.4)	31.1 (5.3)	28.4 (8.2)
Air Filters	36	28.4 (2.2)	21.4 (0.9)	23.3 (1.3)
	244	27.6 (3.6)	20.3 (1.1)	26.2 (3.5)
Water	36	28.0 (3.1)	20.8 (1.6)	25.4 (2.3)
	244	29.2 (4.5)	21.5 (1.5)	25.6 (1.1)

*Average Ct (SD, n = 5)
**A 1:10 dilution of the sample was made prior to running PCR in order to reduce inhibition from the AZ test dust. Ct values were not corrected from this dilution factor.
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 29. Semi-automated RV-PCR at T16 on Clean Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

Sample Type	CFU/ Sample	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	45	31.8 (1.1)	31.1 (1.5)	32.7 (1.0)
	151	27.3 (0.3)	26.3 (0.3)	29.0 (0.6)
Air Filters	45	30.5 (1.7)	29.8 (1.4)	31.1 (1.0)
	151	27.3 (0.3)	27.0 (0.9)	29.9 (1.0)
Water	45	29.2 (0.6)	29.0 (1.8)	31.2 (2.1)
	151	27.1 (1.2)	26.3 (0.6)	29.3 (1.0)

*Average Ct (SD, n = 4)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

In order to investigate the effect of high levels of dead Ba Ames on the detection of low levels of the same live organism, we performed an experiment in which two levels of live Ba spores were spiked on clean wipes, air filters and water samples in the presence of 10^6 heat-killed Ba Ames spores/sample. A stock of Ba Ames spores (10^6 CFU/mL confirmed by plate counts) was killed by autoclaving three times at 126 ºC for 30 min. Six 100 µL aliquots were then plated on solid BHI medium and no colonies were observed after 48 hr, confirming that all spores were dead. A semi-automated RV-PCR experiment was performed, in which each sample type (wipes, filters, and water samples) was spiked with 10^6 heat-killed Ba spores and 45 and 151 live Ba spores. Four replicate samples were generated for each sample type and spore level. Table 29 summarizes the Ct values (average of 4 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

All Ct values obtained in the presence of heat-killed Ba spores were <35, confirming the robustness of the RV-PCR method in the presence of high dead target organisms (decontamination scenario). Baseline Ct values (average of 4 replicate samples) at T0 for 10^6 heat-killed spores/sample only were: 45, 45, and 41.7 for the BC3, EPA-1, and EPA-2 assays, respectively.

Semi-automated RV-PCR in the presence of high levels of live non-target organisms was then conducted. Spore levels of 10 and 100 CFU were spiked on clean samples in the presence of live Pa only (10^6 CFU/sample), as well as in the presence of live Bg (10^3 CFU/sample) combined with live Pa (10^6 CFU/sample). Tables 30-32 summarize the Ct values (average of 4 replicate samples) obtained at the endpoint (T16) for the 3 Ba assays at each spore level.

The Ct values at T0 were >45 for all samples. Ct values obtained in the presence of live Pa were <35. Ct values obtained in the presence of combined Pa and Bg were also <35, confirming the robustness of the RV-PCR method in the presence of high levels of live non-target organisms.

Table 30. Semi-automated RV-PCR at T16 on Clean Wipes Spiked With 2 Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
36	10^6/sample Pa	21.0 (0.3)	20.0 (0.4)	24.1 (2.7)
	10^6/sample Pa & 10^3/sample Bg	23.1 (0.3)	21.4 (0.2)	25.1 (0.6)
274	10^6/sample Pa	20.7 (1.2)	20.0 (0.5)	24.4 (0.5)
	10^6/sample Pa & 10^3/sample Bg	21.0 (0.4)	19.3 (0.4)	23.1 (0.7)

*Average Ct (SD, n = 4)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 31. Semi-automated RV-PCR at T16 on Clean Filters with 2 Live Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
36	10^6/sample Pa	20.9 (1.4)	19.7 (1.2)	23.6 (2.0)
	10^6/sample Pa & 10^3/sample Bg	22.7 (0.8)	21.4 (0.4)	25.3 (0.9)
274	10^6/sample Pa	20.2 (1.1)	19.3 (1.2)	23.2 (1.3)
	10^6/sample Pa & 10^3/sample Bg	20.9 (0.4)	19.2 (0.3)	23.2 (0.4)

*Average Ct (SD, n = 4)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 32. Semi-automated RV-PCR at T16 on Clean Water Spiked With 2 Spore Levels in the Presence of *B. globigii* and *P. aeruginosa* Background

Ba CFU/ Sample	Non-Target Organisms	Average Ct*		
		EPA-1	EPA-2	BC3
36	10^6/sample Pa	21.9 (1.2)	20.7 (0.8)	24.5 (1.2)
	10^6/sample Pa & 10^3/sample Bg	23.4 (0.8)	22.0 (0.6)	25.6 (0.9)
274	10^6/sample Pa	19.8 (0.7)	19.1 (1.1)	26.9 (6.7)
	10^6/sample Pa & 10^3/sample Bg	19.9 (0.1)	18.8 (0.5)	23.0 (0.2)

*Average Ct (SD, n = 4)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Both manual and semi-automated RV-PCR methods were showed to have detection limits at the 10 spore level. Figure 8 provides a direct comparison of Ct values obtained on clean samples using manual and semi-automated RV-PCR methods. The semi-automated protocol was found to provide slightly lower Ct values (up to 4 Cts) than the manual protocol, most probably due to improved pipetting precision and reproducibility. The detection limit was maintained for both manual and semi-automated methods in the presence of challenges including dirt, high levels of heat-killed Ba Ames spores, and high levels of live, combined non-target *Bacillus* species and vegetative cells (although Ct values increased in the presence of challenges, they remained <35 in most cases). As will be seen in the next section, modifications of the protocols were implemented in order to further improve Ct values. While the semi-automated protocol performed well on clean samples, it was found that for dirty wipes, additional mixing of the cultured samples in filter cups by vortexing was required after the incubation step in order to obtain optimal results.

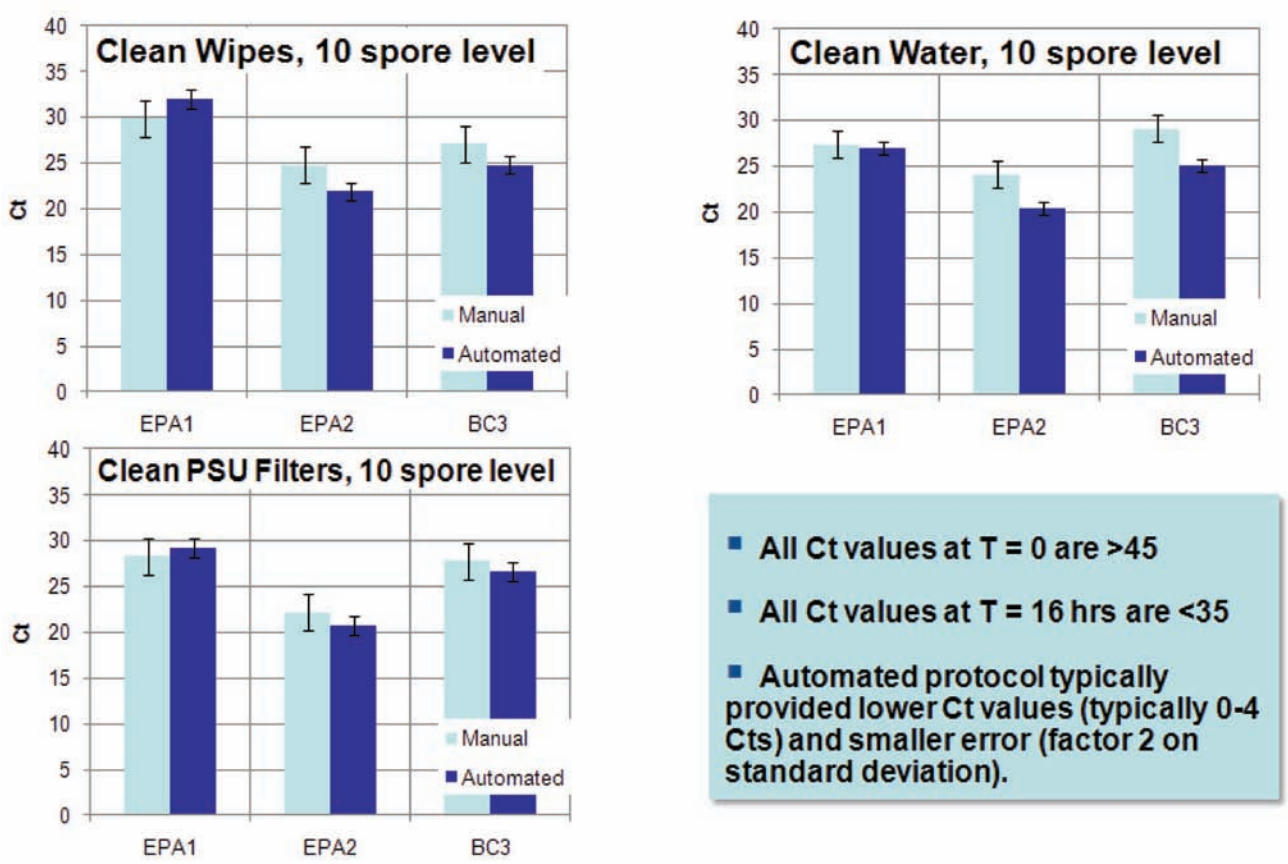

Figure 8. Summary of Ct values obtained with manual and semi-automated RV-PCR protocols on clean samples (wipes, filters and water) spiked at the 10 Ba spore level.

4.3.3. Method Optimization

4.3.3.1. Improvement of Sample Mixing after Incubation

After noticing high Ct values in the presence of debris with the semi-automated protocol, vortexing of the filter cups at T16 was evaluated to improve sample mixing before aliquoting after overnight growth. Results obtained without any vortexing were compared to results obtained after implementing 10 min of vortexing of the filter cups prior to aliquoting the sample after incubation. The data, summarized in Table 33, showed a reduction of both Ct values (2-4 Ct values) and standard deviations for detection using 36 spores when the vortexing step was implemented (see Figure 9).

Table 33. Semi-automated RV-PCR at T16 on Clean Wipe, Air Filter and Water Samples Spiked with 36 *Bacillus anthracis* Ames Spores

Sample Type	Protocol	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	Vortexing	32.0 (1.8)	21.9 (1.2)	24.8 (1.1)
	No Vortexing	35.3 (4.6)	23.2 (0.5)	31.5 (1.7)
Air Filters	Vortexing	29.2 (1.7)	20.7 (1.7)	26.6 (0.1)
	No Vortexing	33.9 (2.8)	23.1 (0.4)	30.9 (0.4)
Water	Vortexing	26.9 (0.8)	20.4 (0.4)	25.0 (0.8)
	No Vortexing	30.8 (0.9)	22.2 (1.4)	28.0 (1.6)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates

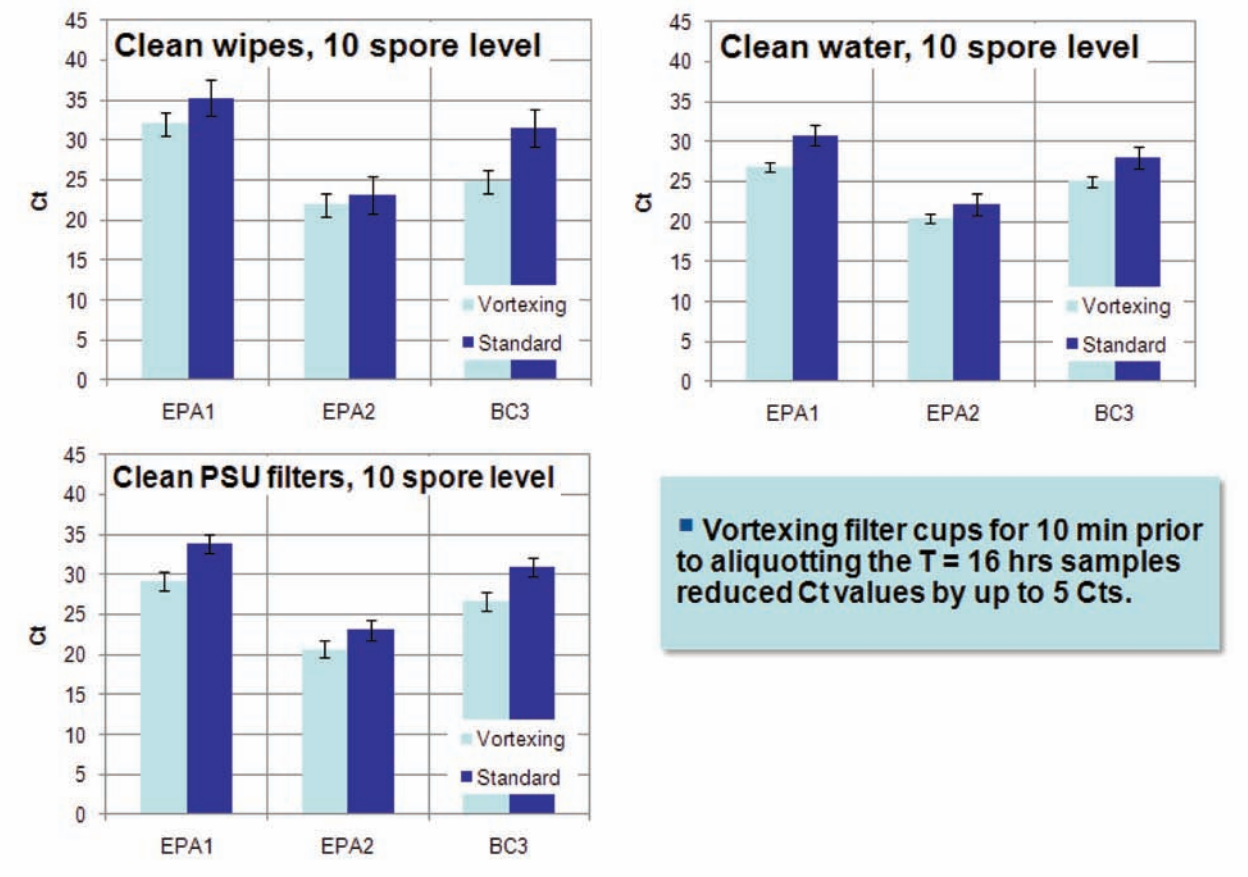

Figure 9. Summary of Ct values obtained with the semi-automated RV-PCR protocol on clean samples with and without vortexing filter cups prior to aliquoting sample for PCR. Samples were spiked at the 10 spore level.

4.3.3.2. Shortening of the RV-PCR Method Endpoint for More Rapid Detection of Bacillus anthracis Spores

A manual RV-PCR experiment was conducted with clean wipe, filter and water samples spiked at the 10 and 100 Ba spore levels in order to assess whether live Ba spores could be detected after 9 hr of incubation (four replicate samples were processed for each set of conditions). In this experiment, the only changes made in the protocol were the reduction of the incubation time from 16 to 9 hrs, and the addition of 10 min of vortexing of the filter cups prior to aliquoting the samples.

Results obtained after heat lysis at T9 are summarized in Table 34 below (no dilution of the samples was performed) (Ct values above 36.0 are highlighted in blue). At a level of 10 CFU/sample, the BC3 and EPA-1 assays did not provide any measurable Ct values and the EPA-2 assay provided Ct values above 34. At a level of 100 CFU/sample, the BC3 assay did not typically generate measurable Ct values and the EPA-1 and EPA-2 assays provided Ct values typically above 30, with high standard deviations. It was therefore determined that an additional DNA extraction step was required in order to generate reliable Ct values for low spore levels at T9.

Table 34. Manual RV-PCR at T9 on Heat-Lysed Extracts of Clean Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels

Sample Type	CFU/ Sample**	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	28	NDT	NDT	NDT
	272	36.7 (2.4)	30.9 (2.1)	NDT
Air Filters	28	NDT	34.8 (3.1)	NDT
	272	37.1 (3.6)	31.4 (3.5)	NDT
Water	28	NDT	34.3 (3.4)	NDT
	272	38.6 (3.0)	33.7 (3.3)	39.6 (4.2)

*Average Ct (SD, n = 4)
**Samples were lysed with heat lysis and analyzed with PCR (no dilution was performed)
Ct values above 36.0 are highlighted in blue.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

Two manual RV-PCR experiments (one performed on clean wipes, filters and water, and the other performed on dirty wipes, filters and water) were then performed (the method is described in the Materials and Method section and a detailed protocol is provided in Appendix A) in order to demonstrate the potential of the introduction of a DNA extraction and purification step to shorten the method endpoint without compromising the limit of detection. Two spore levels were tested (10 CFU/sample and 100 CFU/sample) and 4 replicate samples were processed for each set of conditions. Dirty wipes were generated by adding 250 mg of ultra-fine AZ test dust to clean wipes dirty air filters were collected from a subway location (Boston, Massachusetts, 02/2008) and dirty water was generated by spiking clean and sterile laboratory water with humic acid and ferrous sulfate at a level of 10 mg/L.

In these experiments, the manual RV-PCR method was used according to the regular protocol, with the exception of the introduction of a 10 min vortexing step of the filter cups prior to aliquoting samples for T0 and T-endpoint. The incubation time was reduced from 16 hr to 9 hr and sample aliquots were manually processed using the Promega DNA extraction and purification kit. Since the protocol starts from a 1 mL sample and the final elution is performed using a 200 µL volume, a 5-fold concentration of the DNA is achieved. In addition, a cleaner DNA sample is expected for PCR analysis since growth medium and debris are removed.

Tables 35 and 36 summarize the results obtained with clean and dirty sample types respectively. Both data sets showed that levels down to 10 spores per sample were detected after 9 hr of incubation using the new protocol, even in the presence of debris. The total manual processing time from start to finish for 24 samples was <15 hr (25 min vortexing, 1.5 hr for set up of filter cups, 9 hr of incubation, 2 hr for DNA extraction and purification, and 1.5 hr for PCR). A 1:10 dilution of the DNA extracted and purified from the samples was necessary to obtain consistent Ct values in the presence of ultra-fine Arizona test dust, but all Ct values were <35 without any correction from the dilution factor.

Table 35. Manual RV-PCR at T9 on Clean Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels and Processed with the DNA Extraction and Purification Protocol

Sample Type	CFU/ Sample**	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes	40	22.7 (0.5)	20.6 (0.3)	23.7 (0.1)
	130	20.1 (1.1)	20.5 (0.2)	21.2 (1.0)
Air Filters	40	24.0 (0.7)	21.5 (0.7)	24.7 (0.9)
	130	21.7 (1.2)	19.7 (0.7)	22.7 (0.8)
Water	40	23.7 (0.6)	21.5 (0.6)	24.3 (0.5)
	130	23.2 (1.9)	21.3 (2.0)	24.2 (1.9)

*Average Ct (SD, n = 4)
**Samples were processed using the DNA extraction and purification protocol
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 36. Manual RV-PCR at T9 on Dirty Wipe, Air Filter and Water Samples Spiked With 2 Spore Levels and Processed with the DNA Extraction and Purification Protocol

Sample Type	CFU/ Sample**	Average Ct*		
		EPA-1	EPA-2	BC3
Wipes***	40	33.8 (2.3)	31.4 (1.5)	35.1 (2.9)
	130	27.6 (0.8)	25.4 (0.5)	29.4 (0.8)
Air Filters	40	24.1 (1.3)	22.2 (1.7)	25.5 (1.8)
	130	21.4 (2.6)	20.1 (1.4)	23.0 (1.4)
Water	40	23.9 (1.5)	21.5 (1.2)	24.7 (1.4)
	130	22.2 (1.9)	20.3 (1.7)	23.1 (1.6)

*Average Ct (SD, n = 4)
**Samples were processed using the DNA extraction and purification protocol.
***A 1:10 dilution of the sample was prepared prior to running PCR in order to reduce inhibition from the AZ test dust. Ct values were not corrected from this dilution factor.
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

An additional experiment with wipe samples was performed to assess whether the incubation time could be further reduced to 8 hr and whether the improved RV-PCR method would work in all the verification conditions (clean samples, dirty samples, clean samples with heat-killed Ba background, and clean samples with live non-target background). Wipe samples were spiked at the 10 live Ba Ames spore level and six replicate samples were analyzed for each set of conditions. Samples were processed using the semi-automated RV-PCR protocol with 10 min of vortexing of the filter cups prior to aliquoting samples. The incubation time was reduced to 8 hr and samples were cleaned using the Promega DNA extraction and purification protocol. At T0, a 60 µL aliquot was taken out of each filter cup in order to perform a heat lysis control. At T8, a 60 µL aliquot was taken out of each filter cup to perform heat lysis, and a 1 mL aliquot was taken for DNA extraction and purification.

As shown in Table 37, heat lysis at T0 did not generate any measurable Ct values, confirming the absence of Ba DNA background. Various protocols were compared for the analysis of the T8 samples. PCR was performed after heat lysis, after heat lysis and a 1:10 dilution, after DNA extraction and purification, and after DNA extraction and purification followed by 1:10 dilution. Results for all 4 analysis methods are presented in Table 38. Heat lysis did not generate Ct values below 37 after 8 hr of incubation. The addition of a 1:10 dilution often led to measurable Ct values, but these were typically in the high 30s. Processing of the samples using the Promega DNA extraction and purification kit did not consistently lead to measurable Ct values at T8, while the addition of a 1:10 dilution after the Promega extraction and purification did. This experiment showed the necessity to perform the Promega DNA extraction and purification protocol to generate reliable data with the shortened incubation time and the necessity to perform a 1:10 dilution to reduce PCR inhibition coming from dirt and debris, chemical residues from wipe samples, and/or residual chemicals from the magnetic bead-based DNA extraction and purification protocol, including alcohol.

The data summarized in Table 38 also showed that the 10 spore level could be detected in all cases but that Ct values were generally closer to 35 (35.7 for the chromosomal assay in the presence of AZ test dust, after 1:10 dilution). In light of these results, it was decided to use a 9 hr incubation time as the end point for the single laboratory method verification. A flow chart of the optimized protocol used for the verification phase of the project is provided in Figure 10.

Table 37. Manual RV-PCR After Heat Lysis at T0 on Wipes Spiked with 28 *Bacillus anthracis* Spores

Sample Treatment	Average Ct*		
	EPA-1	EPA-2	BC3
Clean	NDT	NDT	NDT
Dirty (250 mg AZ test dust)	NDT	NDT	NDT
10^6/sample heat-killed Ba Ames	NDT	NDT	NDT
10^6/sample Pa & 10^3/sample Bg	NDT	NDT	NDT

*Average Ct (SD, n = 6)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

Table 38. Manual RV-PCR at T8 on Wipes Spiked With 28 *Bacillus anthracis* Spores

T8 PCR condition	Assay	Average Ct With:*			
		Clean Sample	250 mg AZ test dust	10^6/sample heat-killed Ba Ames	10^6/sample Pa & 10^3/sample Bg
Heat Lysis	EPA1	NDT	NDT	NDT	41.5 (1.5)
Heat Lysis + 1:10 Dilution		34.8 (1.3)	38.6 (1.8)	34.1 (0.8)	33.8 (0.6)
Promega kit		NDT	NDT	35.6 (3.9)	36.0 (2.3)
Promega kit + 1:10 dilution		33.1 (0.8)	33.9 (2.2)	31.3 (1.4)	31.1 (1.7)
Heat Lysis	EPA2	40.0 (3.6)	NDT	37.3 (1.0)	37.8 (3.0)
Heat Lysis + 1:10 Dilution		32.8 (1.2)	37.3 (1.4)	32.3 (0.4)	31.5 (1.4)
Promega kit		NDT	NDT	28.4 (1.8)	28.9 (2.4)
Promega kit + 1:10 dilution		31.9 (1.4)	31.8 (2.2)	29.8 (1.4)	29.1 (1.8)
Heat Lysis	BC3	NDT	NDT	NDT	43.1 (0.4)
Heat Lysis + 1:10 Dilution		37.9 (1.5)	43.3 (2.0)	36.5 (1.0)	35.7 (1.2)
Promega kit		NDT	NDT	35.1 (1.9)	36.0 (2.8)
Promega kit + 1:10 dilution		33.1 (1.9)	35.7 (3.3)	32.9 (2.2)	32.3 (2.0)

* Average Ct (SD, n = 6)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

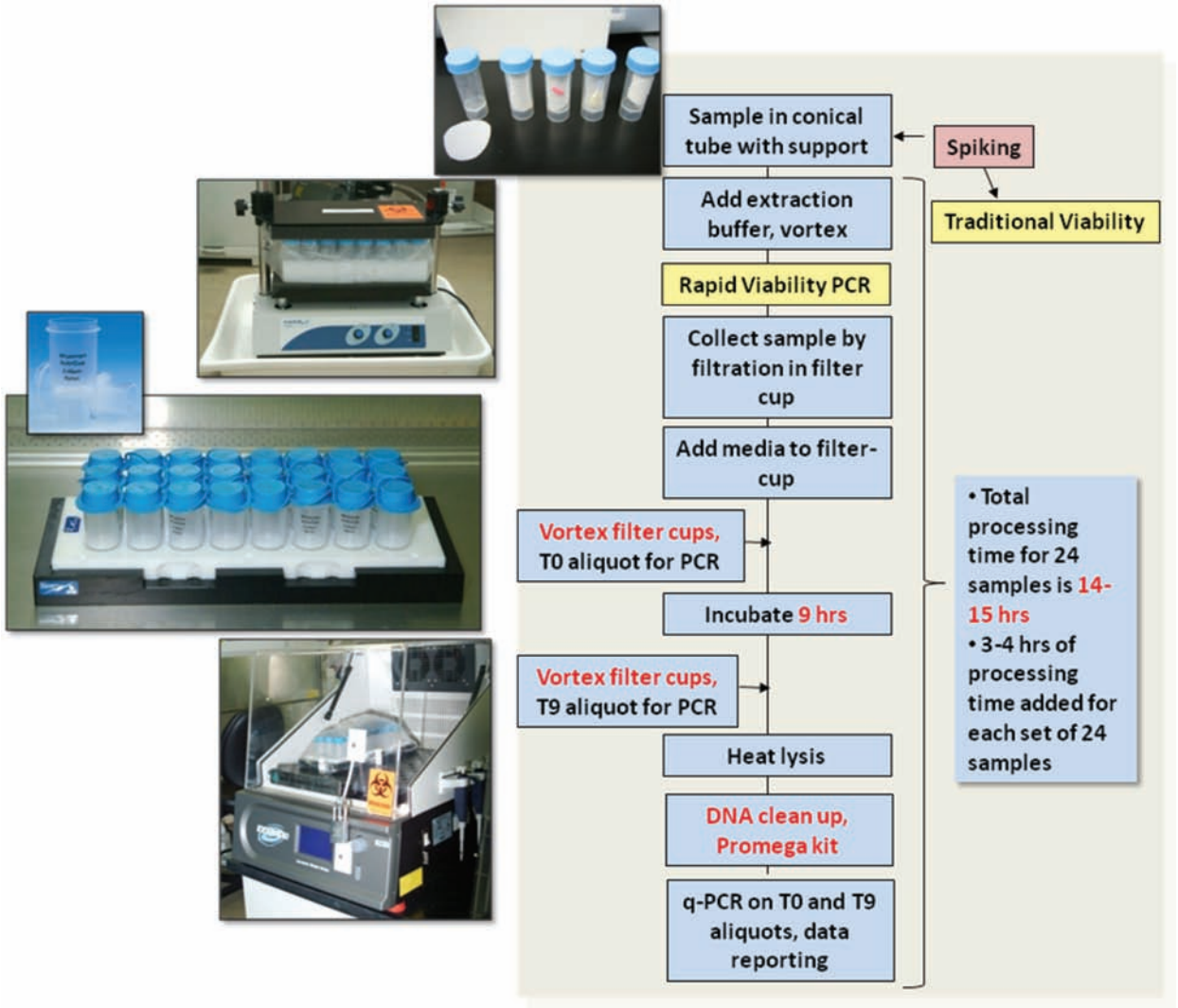

Figure 10. Optimized RV-PCR method overview. Items in red indicate optimization steps.

4.3.4. Development of a T0 Control Protocol

After adding the new DNA extraction and purification step to the RV-PCR protocol, a new control had to be implemented at T0. A series of experiments were performed in order to: 1) assess whether any detectable Ba DNA was present at T0 when analyzing T0 samples with the new DNA extraction and purification protocol, and 2) determine the most effective way to implement a T0 control, the challenge being that a 1 mL aliquot cannot be withdrawn from the filter cup at T0 when the total volume of growth medium is 2.5 mL without significantly affecting the spore growth and the limit of detection at the endpoint.

The first T0 control experiment consisted in processing 1 mL T0 aliquots using the Promega DNA extraction and purification protocol. The goal was to determine if any detectable Ba DNA was present at T0. Clean wipes were spiked with increasing levels of Ba Ames spores ranging from 10 CFU/sample up to 10^6 CFU/sample. A set of clean wipes was also spiked at the 10^6 heat-killed Ba Ames spore level and another set was spiked at the 100 live Ba Ames spore level in a background of 10^6 heat-killed Ba Ames spores. Three replicates were processed for each set of experimental conditions using the RV-PCR protocol. One hundred microliters of samples spiked at the 10^3 spore level were plated at T0 (plate counts in 100 µL were: 41, 28 and 26 colonies) and T9 (plate counts in 100 µL were too numerous to count on all 3 plates) as a control. Sixty microliter aliquots were removed out of each filter cup in order to perform heat lysis at T0. One milliliter aliquots were then removed out of each filter cup at T0 and processed by heat lysis or using the Promega DNA extraction and purification protocol (undiluted and 10-fold diluted samples were analyzed by PCR using all 3 Ba assays).

Neither the heat lysis nor the DNA extraction and purification method (undiluted or diluted) generated any measurable Ct values at T0. The only exceptions were a few high Ct values (1 out of 3 replicates), bolded in Tables 39 and 40, which correspond to a spiking level of 10^6 live spores per sample. No T0 Ct values were measured on samples spiked with 10^6 heat-killed Ba Ames spores.

These results confirm that neither heat lysis nor the Promega DNA extraction and purification protocol lyse Ba spores at T0 and that background at T0 does not affect experiments conducted with spiking levels below 10^5 CFU/sample. It should be noted that no background was measured on the samples spiked with heat-killed spores, which indicates that spore killing by autoclaving did not induce significant DNA leakage from the Ba spores.

Finally, these results confirm the cleanliness of the spore preparation used in this study. For samples spiked at the 10^6 spore level, each 5 µL PCR would contain ~ 6500 spores, assuming 100% recovery. At this level, heat lysis did not provide any measurable Ct, and DNA extraction and purification provided Ct values around 40.

Table 39. Manual RV-PCR at T0 on Wipes Processed with DNA Extraction and Purification of on 1 mL Aliquots

CFU/Sample	Average Ct*		
	BC3	EPA-1	EPA-2
10 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^2 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^3 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^4 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^5 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, **43.2**
10^6 live Ba Ames	NDT, NDT, NDT	NDT, NDT, **42.8**	NDT, NDT, **38.0**
10^6 heat-killed Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT

* Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

Table 40. Manual RV-PCR on Wipes Processed with DNA Extraction and Purification on 1 mL Aliquot at T0 Followed by 1:10 Dilution of the Extracted Samples

CFU/Sample	Average Ct*		
	BC3	EPA-1	EPA-2
10 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^2 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^3 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^4 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^5 live Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^6 live Ba Ames	NDT, NDT, **43.6**	NDT, NDT, **38.9**	NDT, NDT, **42.4**
10^6 heat-killed Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	NDT, NDT, NDT	NDT, NDT, NDT	NDT, NDT, NDT

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

A second control experiment was performed in which the goal was to equate sample volumes and concentrations at T0 and T9 (see Table 41 for details). Clean wipes were spiked with increasing levels of Ba Ames spores ranging from 10 CFU/sample up to 10^6 CFU/sample. A set of clean wipes was also spiked with 10^6 heat-killed Ba Ames spores and another set was spiked with 100 live Ba Ames spores in a background of 10^6 heat-killed Ba Ames spores. Three replicates were processed for each set of experimental conditions using the RV-PCR protocol. One hundred microliters of samples spiked at the 10^3 spore level were plated from filter cups at T0 (plate counts in 100 µL were 53, 52 and 33 colonies) and T9 (plate counts in 100 µL were too numerous to count on all 3 plates) as a control. Sixty-microliter aliquots were removed out of each filter cup at both T0 and T9 in order to perform heat lysis. Another 60 µL aliquot was removed out of each filter cup at T0 in order to perform a modified version of the Promega DNA extraction and purification protocol in which 60 µL of the sample are mixed with 940 µL of TE buffer prior to processing. One-milliliter aliquots were also removed out of each filter cup at T9 and analyzed using the Promega DNA extraction and purification protocol. Bg carrier DNA was added in the samples processed with the Promega DNA extraction and purification protocol in order to provide a positive control. The presence of Bg DNA was evaluated with a Bg assay available at LLNL.

Table 41. Experiment Design for Aliquot Equivalency between T0 and T9 Samples

T0	T0, carrier DNA concentration (Bg)	T9	T9 carrier DNA concentration (Bg)
60 µL sample + 940 µL TE **(Total sample volume is 1 mL)**	30 pg/mL	**1 mL sample**	500 pg/mL
DNA Extraction and Purification	150 pg/mL (5X concentration by DNA extraction and purification)	DNA extraction and purification	2500 pg/mL (5X concentration by DNA extraction and purification
No dilution	150 pg / mL	**1:17 dilution**	150 pg / mL (1:17 dilution)
PCR (Results in Table 42)	0.750 pg / rxn (5 µL sample) (Results in Table 42)	PCR (Results in Table 45)	0.750 pg / rxn (5 µL sample) (Results in Table 45)

Acronyms: µL, microliter; pg, picogram; rxn, reaction

Heat lysis analysis at T0 did not provide any measurable Ct values for any assay on any sample.

DNA extraction and purification with the Promega protocol at T0 did not provide any measurable Ct values for any assay on any sample (Table 42).

Table 42. Manual RV-PCR on Wipes Processed with DNA Extraction and Purification at T0 (Table 41 Column 1)

CFU/Sample	Average Ct*			
	BC3	EPA-1	EPA-2	Bg
10 live Ba Ames	NDT	NDT	NDT	NDT
10^2 live Ba Ames	NDT	NDT	NDT	NDT
10^3 live Ba Ames	NDT	NDT	NDT	NDT
10^4 live Ba Ames	NDT	NDT	NDT	NDT
10^5 live Ba Ames	NDT	NDT	NDT	NDT
10^6 live Ba Ames	NDT	NDT	NDT	37.9 (2.0)
10^6 heat-killed Ba Ames	NDT	NDT	NDT	37.3 (0.6)
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	NDT	NDT	NDT	36.9 (0.4)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

Heat lysis analysis at T9 did not allow the detection of the 10 Ba spore level but generated measurable Ct values for spore levels above 100 CFU/sample (Table 43).

Table 43. Manual RV-PCR at T9 on Wipes with Heat Lysis

CFU/Sample	Average Ct*		
	BC3	EPA-1	EPA-2
10 live Ba Ames	NDT	NDT	35.6 (1.0)
10^2 live Ba Ames	37.4 (1.5)	35.1 (1.7)	29.2 (1.4)
10^3 live Ba Ames	31.9 (1.4)	30.8 (0.5)	28.2 (1.6)
10^4 live Ba Ames	29.3 (1.2)	28.5 (1.7)	24.5 (1.3)
10^5 live Ba Ames	28.6 (0.8)	28.0 (2.2)	23.7 (0.7)
10^6 live Ba Ames	25.9 (1.5)	24.6 (0.7)	22.4 (2.7)
10^6 heat-killed Ba Ames	NDT	NDT	NDT
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	36.1 (0.3)	33.8 (1.8)	29.4 (1.0)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

DNA extraction and purification results at T9 are summarized in Table 44 (1:10 dilution performed to insure reliable Ct values) and Table 45 (1:17 dilution performed to equate volumes and concentrations between T0 and T9 aliquots and demonstrate sufficient ΔCt to detect the presence of viable spores at T9). All samples were detected using both 1:10 and 1:17 dilutions at T9. Note that although the Ct values qualitatively decrease with increasing concentrations of Ba spiked in the samples, the Ct values are not separated by a full log from one spore level to the other. However, overall results are not affected because of an optimum ΔCt (Ct change from T0 to T9 at all spore levels).

Table 44. Manual RV-PCR at T9 on Wipes Processed with DNA Extraction and Purification Followed by 1:10 Dilution of Extracted Samples

CFU/Sample	Average Ct*			
	BC3	EPA-1	EPA-2	Bg
10 live Ba Ames	32.7 (1.0)	29.6 (1.1)	29.9 (1.2)	31.4 (1.3)
10^2 live Ba Ames	32.2 (2.0)	28.5 (1.2)	27.1 (0.8)	30.7 (0.8)
10^3 live Ba Ames	31.3 (1.0)	27.1 (1.1)	24.6 (0.7)	31.2 (1.6)
10^4 live Ba Ames	29.7 (0.2)	26.2 (0.5)	24.7 (0.9)	30.5 (0.6)
10^5 live Ba Ames	28.2 (0.3)	23.7 (0.4)	22.4 (0.4)	30.9 (1.4)
10^6 live Ba Ames	27.3 (0.9)	23.4 (0.3)	21.9 (0.5)	30.3 (2.6)
10^6 heat-killed Ba Ames	NDT	39.0 (1.1)	38.7 (0.9)	32.2 (0.9)
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	33.9 (1.5)	30.0 (1.2)	28.7 (0.9)	31.0 (0.7)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; NDT, no Ct detected

Table 45. Manual RV-PCR at T9 on 1:17 Diluted Purified DNA from Wipes (Table 41 Column 3)

CFU/Sample	Average Ct*			
	BC3	EPA-1	EPA-2	Bg
10 live Ba Ames	36.6 (3.4)	32.9 (2.4)	30.2 (1.6)	32.3 (2.1)
10^2 live Ba Ames	35.7 (2.9)	31.4 (3.9)	28.8 (2.5)	32.8 (2.7)
10^3 live Ba Ames	34.8 (2.6)	31.4 (2.3)	26.0 (2.6)	32.5 (2.5)
10^4 live Ba Ames	30.9 (0.4)	27.5 (0.4)	24.8 (1.1)	30.6 (0.3)
10^5 live Ba Ames	31.0 (2.8)	26.7 (2.5)	23.8 (1.5)	30.8 (2.4)
10^6 live Ba Ames	30.2 (2.4)	26.2 (2.5)	23.5 (1.5)	30.9 (2.8)
10^6 heat-killed Ba Ames	43.5 (1.5)	40.0 (1.2)	36.1 (1.2)	32.3 (0.1)
10^2 live Ba Ames + 10^6 heat-killed Ba Ames	35.6 (2.5)	32.6 (1.6)	29.7 (1.2)	31.9 (1.2)

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

The Bg carrier DNA control at T0 was not consistently detected while consistent Ct values were measured on the T9 samples. Table 46 shows the sensitivity of the Bg assay with extracted Bg DNA, which is typically 5 fg per reaction. Based on these results, an additional control experiment was performed in which TE and BHI samples were spiked with a fixed concentration of Ba spores and increasing concentrations of Bg DNA (see Table 47).

Table 46. Ct Values Obtained with Extracted *B. globigii* DNA Dilution Series

Bg DNA (pg)	Average Ct*
5000	15.8 (0.1)
500	17.2 (0.1)
50	20.3 (0.1)
5	23.8 (0.2)
0.5	27.1 (0.1)
0.05	31.5 (0.3)
0.005	35.6 (0.3)
0	NDT

*Average Ct (SD, n = 3)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; NDT, no Ct detected

A final T0 control experiment was then performed in order to define the concentration of Bg carrier DNA to spike in the T0 samples as a positive control. In this experiment, 2 sets of twelve 900 µL samples were prepared (1 set with 900 µL of TE and 1 set with 900 µL of BHI). To these samples, 100 µL of Ba Ames spore suspension at 10^4 CFU/mL were added, bringing the final spore concentration to the 10^3 spore level in each sample (the highest spiking concentration used in this study). Samples were then spiked with various concentrations of extracted Bg DNA in triplicate (final Bg DNA concentrations of 50, 250, 500 and 750 pg/mL were used). All twenty four 1 mL samples were then processed with the Promega DNA extraction and purification protocol and analyzed for the presence of Bg DNA by PCR.

Results presented in Table 47 clearly show that TE interferes with the binding chemistry of the Promega DNA extraction and purification protocol since no Ct values were detected. Using similar concentrations of Bg DNA in BHI samples, Bg was reliably detected for spiking concentrations above 500 pg/mL.

Table 47. Ct values for 1 mL Samples Spiked with 10^3 *Bacillus anthracis* CFU/mL and *B. globigii* DNA, and Processed with DNA Extraction and Purification

Sample Matrix	Final Concentration of Bg DNA (pg/mL)	Bg Assay*
TE	50	NDT
TE	250	NDT
TE	500	NDT
TE	750	NDT
BHI	50	NDT
BHI	250	30.8 (0.6)**
BHI	500	30.4 (1.0)
BHI	750	29.5 (1.0)

*Average Ct (SD, n = 3)
**Ct values for 3 replicates were: 30.3, NDT, 31.2
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units; pg, picogram; NDT, no Ct detected

Based on the results of these T0 control experiments, final manual and semi-automated RV-PCR protocols were designed for the analysis of real-world samples. The detailed protocols in Appendices C and D and an overall summary are provided in Table 48.

Table 48. Summary of Aliquot Equivalency between T0 Controls and T9 Samples for the Processing of Real-World Samples

T0	T9
100 µL sample + 900 µL BHI **(Total sample volume is 1 mL)**	**1 mL sample**
DNA extraction and purification	DNA extraction and purification
No dilution	**1:10 dilution**
PCR	PCR

Acronyms: µL, microliter

4.4. Single Laboratory Method Verification

Based on the optimization experiments presented above, a decision was made to proceed with the optimized RV-PCR method (10 min of vortexing of the filter cups prior to aliquoting the sample and 9 hr of incubation followed by magnetic bead-based DNA extraction and purification/extraction and purification prior to running PCR) for the method verification phase of the project. Both manual and semi-automated methods were verified with a total of 192 wipe, air filter and water samples spiked with Ba Ames spores. Dirt (wipes containing 250 mg of Arizona test dust, dirty BioWatch filters and chemically spiked water), high levels of heat-killed Ba spores and high levels of non-target cells were also evaluated. Four replicates of each sample were analyzed. For each replicate sample, PCR was run against each of the three assays (one PCR per sample and per assay). All experiments were conducted according to manual and semi-automated RV-PCR protocols detailed in Appendices A and B.

4.4.1. Verification of the Manual RV-PCR Method

Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore levels and four replicates were analyzed for each sample. Results are presented in Tables 49-51 below.

The Ct values at T9 obtained during the verification of the manual method for clean samples were ≤ 35.0 for all samples, confirming the 10 spore detection level with the manual RV-PCR method.

Table 49. Average Ct Values for Manual RV-PCR at T9 on Clean Wipes with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
31	T0	45	18.3	45	18.0	45	20.4
	T9	26.7 (2.5)		27.0 (2.7)		24.6 (3.0)	
272	T0	45	24.2	45	24.9	45	22.2
	T9	20.8 (1.5)		20.1 (0.6)		22.8 (0.6)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 50. Average Ct Values for Manual RV-PCR at T9 on Clean Filters with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
31	T0	45	14.5	45	14.0	45	17.6
	T9	30.5 (2.4)		31.0 (2.4)		27.4 (2.1)	
272	T0	45	23.2	45	24.9	45	21.4
	T9	21.8 (1.1)		20.1 (0.5)		23.6 (0.8)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 51. Average Ct Values for Manual RV-PCR at T9 on Clean Water with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
31	T0	45	11.3	45	12.6	45	13.8
	T9	33.7 (1.8)		32.4 (1.5)		31.2 (2.1)	
272	T0	45	23.7	45	26.1	45	21.6
	T9	21.3 (0.7)		18.9 (1.1)		23.4 (1.0)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Dirty wipes (250 mg of Arizona test dust), dirty air filters (BioWatch air filters collected in Houston, Texas on 02/20/08) and dirty water (10 mg/L humic acid and 10 mg/L ferrous sulfate) samples were spiked at the 10 and 100 live Ba Ames spore level and four replicates were analyzed for each sample. Results are presented in Tables 52-54 below.

The Ct values at T9 obtained during the manual method verification on dirty samples were ≤ 36.0 for all samples, confirming the 10 spore detection level in the presence of dirt with the manual RV-PCR method.

Table 52. Average Ct Values for Manual RV-PCR at T9 on Dirty Wipes with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
49	T0	45	10.4	45	11.8	45	9.4
	T9	34.6 (0.4)		33.2 (1.3)		35.6 (1.8)	
301	T0	45	13.6	45	17.2	45	10.8
	T9	31.4 (1.8)		27.8 (1.7		34.2 (2.1)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 53. Average Ct Values for Manual RV-PCR at T9 on Dirty Filters with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
49	T0	45	10.4	45	11.5	45	9.0
	T9	34.6 (2.8)		33.5 (1.7)		36.0 (1.3)	
301	T0	45	13.3	45	16.6	45	11.0
	T9	31.7 (0.9)		28.4 (1.5)		34.0 (1.0)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 54. Average Ct Values for Manual RV-PCR at T9 on Dirty Water with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
49	T0	45	11.2	45	13.4	45	10.1
	T9	33.8 (0.5)		31.6 (0.4)		34.9 (1.0)	
301	T0	45	14.1	45	15.1	45	10.9
	T9	30.9 (0.9)		29.9 (1.4)		34.1 (1.2)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore level in the presence of 10^6 heat-killed Ba Ames spores/sample. Four replicates were analyzed for each sample. Results are presented in Tables 55-57 below.

The Ct values at T9 obtained during the manual method verification in the presence of heat-killed Ba Ames spores were ≤ 35.0 for all samples, confirming the 10 spore detection level in the presence of a high level of dead Ba Ames spores with the manual RV-PCR method.

Table 55. Average Ct Values for Manual RV-PCR at T9 on Clean Wipes with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
14	T0	45	15.5	45	18.9	45	12.2
	T9	29.5 (1.1)		26.1 (0.7)		32.8 (0.7)	
143	T0	45	16.2	45	18.9	45	11.9
	T9	28.8 (0.4)		26.1 (0.2)		33.1 (0.5)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 56. Average Ct Values for Manual RV-PCR at T9 on Clean Filters with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
14	T0	45	13.7	45	16.8	45	10.1
	T9	31.3 (2.2)		28.2 (2.4)		34.9 (0.4)	
143	T0	45	17.9	45	21.2	45	14.2
	T9	27.1 (1.9)		23.8 (0.6)		30.8 (1.2)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 57. Average Ct Values for Manual RV-PCR at T9 on Clean Water with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
14	T0	45	11.4	45	16.3	45	10.1
	T9	33.6 (1.6)		28.7 (1.0)		34.9 (1.5)	
143	T0	45	13.6	45	18.2	45	10.2
	T9	31.4 (1.8)		26.8 (1.0)		34.8 (0.9)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Manual RV-PCR method verification with clean samples and a high live non-target background was then performed. Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore level in the presence of 10^6 live Pa CFU/sample and 10^3 live Bg CFU/sample. Four replicates were analyzed for each sample. Results are presented in Tables 58-60 below.

The Ct values at T9 obtained during the manual method verification in the presence of live non-target background were ≤ 36.0 for all samples, confirming the 10 spore detection level in the presence of high levels of live non-target organisms with the manual RV-PCR method.

Table 58. Average Ct Values for Manual RV-PCR at T9 on Clean Wipes with 2 Spore Levels in the Presence of live *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
33	T0	45	13.4	45	19.6	45	11.5
	T9	31.6 (2.7)		25.4 (0.8)		33.5 (1.6)	
186	T0	45	15.3	45	23.8	45	14.7
	T9	29.7 (3.0)		21.2 (0.9)		30.3 (3.1)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 59. Average Ct Values for Manual RV-PCR at T9 on Clean Filters with 2 Spore Levels in the Presence of live *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
33	T0	45	12.5	45	18.7	45	11.2
	T9	32.5 (1.8)		26.3 (0.6)		33.8 (3.8)	
186	T0	45	14.9	45	21.6	45	13.0
	T9	30.1 (3.5)		23.4 (2.0)		32.0 (3.0)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 60. Average Ct Values for Manual RV-PCR at T9 on Clean Water with 2 Spore Levels in the Presence of live *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
33	T0	45	10.7	45	15.1	45	9.7
	T9	34.3 (4.5)		29.9 (0.6)		35.3 (3.8)	
186	T0	45	13.7	45	18.3	45	15.6
	T9	31.3 (3.4)		26.7 (2.1)		29.4 (1.7)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

4.4.2. Verification of the Semi-Automated RV-PCR Method

Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore levels and four replicates were analyzed for each sample. Results are presented in Tables 61-63 below.

The Ct values at T9 obtained during the semi-automated method verification on clean samples were ≤ 35.0 for all samples, confirming the 10 spore detection level with the semi-automated RV-PCR method.

Table 61. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Wipes with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
26	T0	45	16.1	45	18.5	45	13.1
	T9	28.9 (1.1)		26.5 (0.3)		31.9 (1.0)	
203	T0	45	20.0	45	21.7	45	16.9
	T9	25.0 (1.0)		23.3 (1.6)		28.1 (2.6)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 62. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Filters with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
26	T0	45	14.2	45	16.3	45	11.8
	T9	30.8 (2.1)		28.7 (2.2)		33.2 (3.5)	
203	T0	45	18.6	45	19.7	45	11.2
	T9	26.4 (2.9)		25.3 (2.6)		33.8 (1.7)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 63. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Water with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
26	T0	45	16.0	45	17.4	45	11.8
	T9	29.0 (1.9)		27.6 (2.0)		33.2 (2.0)	
203	T0	45	18.2	45	19.1	45	13.6
	T9	26.8 (1.3)		25.9 (1.8)		31.4 (2.2)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

A replicate manifold of clean samples was spiked at the 10 and 100 live Ba spore levels, in order to plate samples at T0 and T9. This manifold was processed in a similar manner as the previous manifold. Filter cups were vortexed for 10 min at T0 and a 100 µL aliquot was plated out of each filter cups (24 plates). After 9 hr of incubation, filter cups were vortexed for 10 min and serial dilutions were prepared in BHI and plated in triplicate for each sample (216 plates). Results are provided in Table 64.

No live organism was detected by plating at T0 for the 10 spore level (10 spores spiked in the sample become 2.6 CFU/mL in the filter cup assuming 100% recovery, which then becomes 0.26 spores/100 µL for plating). For the 100 spore level (100 spores spiked in the sample become 26 CFU/mL in the filter cup assuming 100% recovery which then becomes 2.6 spores/100 µL for plating), colonies were detected most of the time (the number of colonies per plate was below 10, as expected). These results exemplify the advantage of the RV-PCR method over the plating method for the detection of low levels of live Ba spores. After 9 hr of incubation in filter cups, the cell concentration was 10^6 cells/filter cup when starting with a spiking level of 10 CFU/sample, and 10^7 cells/filter cup when starting with a spiking level of 100 CFU/sample. Such growth corresponds to a doubling time in the order of 30 min and a germination time in the 30-60 min range, which suggests optimal growth conditions for Ba in filter cups and confirms that the Ct values recorded at T9 originate from viable spores.

Table 64. Spore Recovery Experiment from Filter Cup Aliquots at T0 and T9

Sample Type	Ba Ames Spore Level	Plate Counts		
		CFU spiked/sample	CFU/filter cup T0*	CFU/filter cup T9**
Clean Filter	10	29	0	1,720,000
		29	0	1,020,000
		29	0	1,560,000
		29	0	1,340,000
Clean Water	10	29	0	1,000,000
		29	0	840,000
		29	0	820,000
		29	0	840,000
Clean Wipe	10	29	0	1,760,000
		29	0	1,940,000
		29	0	1,080,000
		29	20	2,020,000
Clean Filter	100	262	120	12,600,000
		262	60	11,200,000
		262	0	10,200,000
		262	20	7,200,000
Clean Water	100	262	40	8,000,000
		262	20	7,400,000
		262	0	7,800,000
		262	20	5,800,000
Clean Wipe	100	262	20	28,400,000
		262	60	28,800,000
		262	0	24,600,000
		262	0	21,200,000

*One 100 µL aliquot was plated out of each filter cup at T0. Number of spores in filter cup = number of colonies x 10 x 2.
**Serial 10-fold dilutions were prepared out of each filter cup and plated in triplicate. The third, fourth and fifth dilutions were plated for the samples spiked at the 10 spore level. The fourth, fifth and sixth dilutions were plated for the samples spiked at the 100 spore level. Values presented in the table are corrected from dilution. Each value is the average of three replicate plates.
Acronyms: CFU, colony forming unit

Dirty wipes (250 mg of Arizona test dust), air filters (BioWatch air filters collected in Houston, Texas on 02/20/08) and water (10 mg/L humic acid and 10 mg/L ferrous sulfate) samples were spiked at the 10 and 100 live Ba Ames spore level and four replicates were analyzed for each sample. Results are presented in Tables 65-67 below.

The Ct values at T9 obtained during the semi-automated method verification on dirty samples were ≤ 35.0 for all samples, confirming the 10 spore detection level in the presence of dirt with the semi-automated RV-PCR method.

Table 65. Average Ct Values for Semi-automated RV-PCR at T9 on Dirty Wipes with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
40	T0	45	12.9	45	16.5	45	14.5
	T9	32.1 (7.7)		28.5 (4.9)		30.5 (1.8)	
327	T0	45	19.0	45	21.5	45	17.4
	T9	26.0 (3.6)		23.5 (3.2)		27.6 (3.3)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 66. Average Ct Values for Semi-automated RV-PCR at T9 on Dirty Filters with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
40	T0	45	12.7	45	17.6	45	10.4
	T9	32.3 (2.3)		30.4 (2.1)		34.6 (3.0)	
327	T0	45	13.1	45	15.2	45	10.2
	T9	31.9 (1.8)		29.8 (1.6)		34.8 (1.4)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 67. Average Ct Values for Semi-automated RV-PCR at T9 on Dirty Water with 2 Spore Levels

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
40	T0	45	15.3	45	16.8	45	13.0
	T9	29.7 (2.3)		28.2 (2.5)		32.0 (2.2)	
327	T0	45	18.2	45	20.1	45	16.0
	T9	26.8 (0.3)		24.9 (0.3)		29.0 (0.9)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore level in the presence of 10^6 heat-killed Ba Ames spores/sample. Four replicates were analyzed for each sample. Results are presented in Tables 68-70 below.

The Ct values at T9 obtained during the semi-automated verification in the presence of heat-killed Ba Ames spores were ≤ 35.0 for all samples, confirming the 10 spore detection level in the presence of a high level of dead Ba Ames spores with the semi-automated RV-PCR method. These results directly relate to real-world situations since the presence of high levels of dead Ba spores will be encountered in post-decontamination samples.

Table 68. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Wipes with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
38	T0	45	13.2	45	15.8	45	12.3
	T9	31.8 (1.2)		29.2 (1.2)		32.7 (1.8)	
256	T0	45	14.7	45	17.0	45	13.0
	T9	30.3 (1.0)		28.0 (1.6)		32.0 (1.8)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 69. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Filters with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
38	T0	45	18.5	45	20.3	45	16.0
	T9	26.5 (1.8)		24.7 (1.5)		29.0 (1.8)	
256	T0	45	19.1	45	21.1	45	16.5
	T9	25.9 (1.4)		23.9 (1.0)		28.5 (1.2)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 70. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Water with 2 Spore Levels in the Presence of Heat-Killed *Bacillus anthracis* Spore Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
38	T0	45	14.7	45	16.9	45	14.4
	T9	30.3 (3.2)		28.1 (3.0)		30.6 (0.7)	
256	T0	45	18.2	45	20.5	45	16.6
	T9	26.8 (1.0)		24.5 (0.6)		28.4 (1.0)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Semi-automated RV-PCR verification with clean samples and a high live non-target background was then performed. Clean wipes, air filters and water samples were spiked at the 10 and 100 live Ba Ames spore level in the presence of 10^6 live Pa CFU/sample and 10^3 live Bg CFU/sample. Four replicates were analyzed for each sample. Results are presented in Tables 71-73 below.

The Ct values at T9 obtained during the semi-automated verification in the presence of live non-target background were ≤ 35.5 for all samples, confirming the 10 spore detection level in the presence of high levels of live non-target organisms with the semi-automated RV-PCR method.

Table 71. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Wipes with 2 Spore Levels in the Presence of *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
21	T0	45	17.8	45	20.2	45	13.7
	T9	27.2 (0.4)		24.8 (0.5)		31.3 (0.7)	
233	T0	45	18.2	45	20.2	45	14.0
	T9	26.8 (0.4)		24.8 (0.6)		31.0 (1.0)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 72. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Filters with 2 Spore Levels in the Presence of *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
21	T0	45	13.4	45	16.5	45	9.8
	T9	31.6 (1.3)		28.5 (0.8)		35.2 (0.5)	
233	T0	45	15.5	45	17.5	45	11.0
	T9	29.5 (1.5)		27.5 (1.6)		34.0 (1.8)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

Table 73. Average Ct Values for Semi-automated RV-PCR at T9 on Clean Water with 2 Spore Levels in the Presence of *P. aeruginosa* and *B. globigii* Background

CFU/Sample	Time Point	Assay					
		EPA-1		EPA-2		BC3	
		Ct*[1]	ΔCt**	Ct*[1]	ΔCt**	Ct*[1]	ΔCt**
21	T0	45	16.5	45	18.4	45	12.2
	T9	28.5 (0.3)		26.6 (0.2)		32.8 (0.3)	
233	T0	45	16.8	45	18.7	45	12.2
	T9	28.2 (0.3)		26.3 (0.3)		32.8 (0.3)	

* Ct = Average Ct (SD, n = 4)
[1] Ct values at T0 were all 'non-detect'. A Ct value of 45 was entered in the table in order to calculate the ΔCt value.
**ΔCt =Ct(T0)-Ct(T9)
Acronyms: Ct, cycle threshold; SD, standard deviation; n, number of replicates; CFU, colony forming units

To summarize Section 4, single laboratory verification of the RV-PCR method was conducted for wipes, air filters and water samples for both manual and semi-automated protocols, using 9 hr of incubation followed by DNA extraction and purification and PCR. Spore levels of 10 and 100 CFU were tested and 4 replicates were processed for each sample type/spore level/challenge combination. Challenges introduced during testing included the addition of dirt or debris, high levels of heat-killed Ba spores and high levels of live non-target organisms. Out of 192 positive samples analyzed during verification, 188 samples presented Ct values < 35 for all 3 assays (chromosome, and pXO1 and pXO2 plasmids). For the 4 samples outside this range, the 2 plasmid assays had Ct values < 35 and the chromosome assay Ct values were 35.6 (manual, with dirt), 36.0 (manual, with dirt), 35.3 (manual, with live background) and 35.2 (semi-automated, with live background) respectively. Detection at 10 spore level was maintained during the verification of both the manual and semi-automated version of the Ba RV-PCR method.

5.0 Conclusions

Single laboratory verification of both manual and semi-automated versions of this optimized method showed limits of detection at the 10 spore level with and without debris for all three sample types. Live Ba Ames spores were consistently detected at the 10 spore level for both manual and semi-automated methods in heat-killed Ba spore backgrounds of 10^6 spores/sample and live, combined non-target backgrounds of 10^3 Bg and 10^6 *Pa*. The method endpoint was shortened from its initial overnight incubation (16 hr) to 9 hr by performing a magnetic bead-based DNA extraction and purification procedure before PCR analysis. Using this method, the total manual processing time from start to finish for 24 samples was reduced to 14-15 hr (3-4 hr of processing time should be added for each set of 24 samples).

Additional work is needed however. This research did not focus on sampling methods and no exhaustive list of potential growth and/or PCR inhibitors was assessed. Although dirt was added to wipes, no actual surface wiping was performed to test interferences from chemicals and/or debris from sampled surfaces. Although air filter samples were randomly collected from subway and outdoor locations, seasonal variations such as high versus low pollen levels or high versus low air pollution levels were not systematically tested. Although water samples were spiked with humic acid and ferrous oxide, which are known PCR inhibitors, no environmental samples were tested in this study. Interference of the decontamination method (fumigation, foam) with the RV-PCR method was not examined although the typical effects of decontamination are delayed germination, growth, and PCR inhibition. Experiments addressing these additional issues were outside the scope of this study since virulent Ba was used.

In addition, the RV-PCR method described here was developed for qualitative and not quantitative analysis. Although the results showed a qualitative decrease in Ct values with increasing spore spiking levels, the Ct difference between two consecutive 10-fold dilutions was typically below the theoretical value in the presence of challenges, which suggests that the lack of quantitation of the method may be tied to the environmental component of the samples (dirt, dead spore background, live non-target background). Follow on work will be performed in FY10 to determine the relationship between limit of detection and incubation time, using clean wipe samples. These experiments will evaluate whether the method endpoint could be further reduced when processing relatively clean samples such as wipes collected from clean indoor locations.

Although the main focus of the NHSRC-funded effort was on delivering verified, rapid viability test protocols to support ERLN capabilities to ensure public safety, other potential applications for the RV-PCR method may lie in surveillance, public health and food safety.

6.0
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7.0
References

1. S. Kane, S. Létant, G. Murphy, T. Alfaro, P. Krauter, R. Mahnke, T. Legler and E. Raber. 2009. Rapid, high-throughput, culture-based PCR methods to analyze samples for viable spores of *Bacillus anthracis* and its surrogates. *J. Microbiol. Methods* 76(3), 278-284.
2. S. Létant, S. Kane, G. Murphy, T. Alfaro, L. Hodges, L. Rose and E. Raber. 2010. Most-probable-number rapid viability PCR method to detect viable spores of *Bacillus anthracis* in swab sample. *J. Microbiol. Methods* 81(2), 200-202.
3. S. Kane, S. Létant, T. Mitchell-Hall, T. Bunt, and E. Raber. 2008. *Quality Assurance Project Plan: Development and Verification of Rapid Viability Polymerase Chain Reaction (RV-PCR) Protocols for Bacillus anthracis – For Application to Air Filter, Water and Surface Samples: for EPA under IAG #DW-89-92261601-0.* LLNL-TM-406527.
4. Materials Safety Data Sheet. Arizona Test Dust, 2006. Powder Technology Inc., 14331 Ewing Avenue S., Burnsville, MN 55306. http://www.powdertechnologyinc.com/products/test-dust/arizona-test-dust.php
5. L. Hodges, L. Rose, H. O'Connell and M. Arduino. National validation study for a swab protocol for the recovery of *Bacillus anthracis* spores from surfaces. Poster session presented at: American Society for Microbiology (ASM) Biodefense and Emerging Diseases Research Conference, February 22-25, 2009, Baltimore, Maryland.
6. Promega Technical Bulletin TB312. *MagneSil(R) blood genomic, max yield system*. 2009. http://www.promega.com/tbs/tb312/tb312.pdf
7. E. Bode, W. Hurtle, and D. Norwood. 2004. Real-Time PCR assay for a unique chromosomal sequence of *Bacillus anthracis*. *J. Clin. Microbiol*. 42(12), 5825–5831.

Appendix A:
Manual RV-PCR Protocol

Qualitative detection of *Bacillus anthracis* (Ba) Ames Spores in wipe, air filter and water samples

See Appendix E, buffers and media; Appendix F, PCR Conditions; and Appendix G, Consumables

Laboratory set-up

- Put PPE (personal protection equipment) on: lab coat, safety glasses, double gloves.
- Prepare fresh bleach solution (1 volume bleach + 9 volumes water). Date and label with initials.
- Clean/bleach BioSafety Cabinet (BSC) and bench surfaces.
- All sample manipulations are performed in the BSC

Place samples in tubes

- Wipes and air filters: Using sterile forceps, transfer sample collection type to 30 mL tube behind internal mesh/ support (1.5 mL of 1X wash buffer is added to each wipe prior to spiking).
- Water samples: No mesh support is needed. Aliquot 20 mL of water directly in 30 mL tube.

Preparation of the spore working stock for sample spiking

Using Biological Safety Level 2 (BSL2) practices and BSL 3 pipetting techniques, start the protocol below.

1. Place original Ba Ames (10^8 CFU/mL; check vial for correct concentration) spore suspension on platform vortexer for 20 min using position 7 (high).
2. Transfer stock to BSC.
3. Aliquot 100 µL of spore suspension into 900 µL of 1X wash buffer (working suspension of 10^7 CFU/mL).
4. Vortex on single tube vortexer for 1 min.
5. Aliquot 500 µL of the working suspension into 4.5 mL of 1X wash buffer (10^6 CFU/mL).
6. Vortex on single tube vortexer for 1 min.
7. Aliquot 3 mL of 10^6 CFU/mL solution to 27 mL of 1X wash buffer (10^5 CFU/mL).
8. Vortex on single tube vortexer for 1 min.
9. Aliquot 1 mL of 10^5 CFU/mL to 9 mL of 1X wash buffer (10^4 CFU/mL).
10. Aliquot 1 mL of 10^4 CFU/mL to 9 mL of 1X wash buffer (10^3 CFU/mL).
11. Aliquot 1 mL of 10^3 CFU/mL to 9 mL of 1X wash buffer (10^2 CFU/mL).
12. Plate on BHI agar plates as follows:

    100 µL 10^2 CFU/mL (3 replicates)

    50 µL 10^3 CFU/mL (3 replicates)
13. Incubate agar plates at 30°C overnight; count plates the next day to determine exact number of spores spiked in the samples.
14. Inoculate sample tubes with 100 µL of 10^2 CFU/mL (10 spore level) and 10^3 CFU/mL (100 spore level); record manifold layout.

Preparation of BioSafety Cabinet for manual RV-PCR

Materials:

- Bleach wipes and daily bleach bottle
- Sharps waste container
- Absorbent pad
- Beaker with top filter-cup caps
- Capping tray with bottom caps
- 2 large biohazard bags + rubber bands
- Allen wrench for manifold
- Serological pipettes and pipettor

Setup:

- Set up manifold: connect vacuum pump, pump in line filter, and waste container with fresh bleach; tape pump exhaust tube to BSC to vent exhaust inside BSC
- Add filter cups to manifold
- Tape filter-cup layout on glass window of the BSC
- Tape PCR plate layout on glass window of the BSC

Preparation of the cart for manual RV-PCR

Materials:

- Gloves
- Bleach wipes
- Waste bags
- Absorbent pads
- Isopropyl alcohol squeeze bottle
- Deionized water squeeze bottle
- Red biohazards bags + rubber bands
- Autoclave tape
- Large photo-tray
- Sharps container
- Zip-lock bag containing: 96-well plate, plate support, foil seals, and plate sealer
- 2 mL eppendorf tubes
- 200 µL pipettor and pipette tips
- Marker

Media and Buffers (See Appendix E):

- Brain Heart Infusion medium
- High salt wash buffer (pH 6.0)
- 1X wash buffer (pH 7.4)

Manual RV-PCR protocol (T0)

1. In the BSC, add 20 mL of extraction buffer to wipe or air filter samples placed in a 30 mL tubes (no addition is needed for water samples).
2. Seal sample rack in large waste bag; bleach exterior of bag with bleach wipe.
3. Vortex samples for 20 min on platform vortexer (outside BSC), position 7.
4. After vortexing samples, transfer sample rack to BSC. Remove tube rack from plastic bag.
5. Turn on vacuum pump at 10 psi.
6. Using BSL3 pipetting technique, transfer sample from 30 mL sample tube to filter-cup using a serological pipette (the total sample volume is 20 mL. Pipette 15 mL and deliver 13 mL to filter-cup). Dispose pipette in waste container. Cap sample tube, bleach the outer surface of sample tube. Re-place tube back on rack. Change gloves.
7. Repeat step 6 for each sample.
8. Complete filtration of liquid through filter cups.
9. Bleach sample tube rack. Double-bag rack. Bleach outer surface of biohazard bag. Change gloves.
10. Transfer double-bagged sample tube rack to refrigerator.
11. Transfer 7 mL of high salt wash buffer (pH 6.0) to each filter-cup using a serological pipette. Use new disposable pipette for each sample cup.
12. Complete filtration of liquid through filter cups.
13. Transfer 3 mL of 1X wash buffer (pH 7.4) to each filter-cup using a serological pipette. Use new disposable pipette for each sample cup.
14. Complete filtration of liquid through filter cups. Change gloves.
15. Stop vacuum pump.
16. Bring 5 mL graduated pipettes, 200 µL pipettor, 200 µL tips, BHI medium, sharps container, centrifuge safety cup, zip-lock bag with PCR plate, plate support, foil seals, and plate sealer to BSC. Set up work space in BSC. Change gloves.
17. Pipette 2.5 mL of Brain Heart Infusion medium into each filter cup using a serological pipette. Use new disposable pipette for each filter cup.
18. Transfer upper part of manifold to capping tray fitted with bottom caps. Press down to cap bottom of filter-cups. Change gloves.
19. **When performing standard RV-PCR protocol (16 hr incubation):**
    - Take 96-well PCR plate and plate support out of zip-lock bag.
    - For each sample: swirl pipette tip gently in filter cup to mix sample, aliquot 60 µL volume out of each cup and transfer 50 µL to the 96-well plate (T0 sample plate) according to plate layout (see example at the end of this protocol).
    - Seal T0 sample plate with foil seal. Change gloves.
    - Place T0 96-well plate in thermocycler for lysis: 20 minutes at 95ºC. Once cycle is completed and sample is back at room temperature, bag 96-well plate in zip-lock bag, bleach the bag.
    - Transfer bagged 96-well plate to freezer and store at -20ºC until qPCR is run (use a photo-tray to transport plate).

    When performing the optimized RV-PCR protocol (9 hr incubation and Promega DNA extraction and purification protocol):
    - For 3 samples (3 replicate samples spiked at a level of 10^2 or 10^3): swirl pipette tip gently in filter cup to mix sample, aliquot 120 µL volume out of each cup using and transfer 100 µL to 2 mL eppendorf tubes.
    - Add 900 µL of BHI to each eppendorf tube.
    - Add 500 pg of extracted Bg DNA to each eppendorf tube.
    - Process 1 mL samples according to Promega MD1630 protocol described below.
20. Cap filter-cups. Double-bag filter-cup manifold. Bleach bag.

21. Transfer bagged filter-cup tray to shaker incubator at 37ºC, speed 230 rpm. **Incubate for 16 hr for the standard RV-PCR protocol or for 9 hr for the optimized RV-PCR protocol.**
22. Follow laboratory cleanup protocol in Appendix E.

Manual RV-PCR protocol (T16 for standard protocol)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cups for 10 min on platform vortexer.
3. Transfer filter-cup manifold to BSC.
4. Using BSL3 pipetting technique, aliquot 60 µL volume out of each cup and transfer 50 µL to 96-well plate using a micropipette (T16 sample plate) according to plate layout.
5. Seal T16 plate with foil seal.
6. Lyse sample plate for 20 minutes at 95ºC on thermocycler.
7. Once lysis is complete and plate is at room temperature, bag 96-well plate in zip-lock bag, bleach the bag.
8. Transfer plate to freezer and store at -20ºC until qPCR is run (use a photo-tray to transport plate).

Manual Promega MD1630 DNA Extraction and Purification Protocol (T9 for optimized protocol)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cups for 10 min on platform vortexer.
3. Transfer filter-cup manifold to BSC.

DNA extraction and purification phase:

4. Aliquot 1000 µL volume out of each filter cup to 2 mL eppendorf tube using a micropipette (T9 samples). Do not use the 1.5 mL eppendorf tubes.
5. 1st lysis: add 600 µL of bead mix (lysis buffer + magnetic beads) to each tube and mix.
6. Add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
7. 2nd lysis: add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
8. 1st salt wash: add 360 µL of salt wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
9. 1st alcohol wash: add 360 µL of alcohol wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
10. Air dry (2 min)
11. Heat dry on heat block at 80°C until samples are dry. Allow all alcohol solution to evaporate since alcohol may interfere with qPCR.

DNA concentration and elution phase:

12. Move sample tubes out of heat block and add 200 µL of elution buffer to each tube. Mix, place on magnet, collect supernatant with micropipette and transfer to clean eppendorf tubes (typically, 80 µL are collected). Visually verify absence of magnetic bead carry over during final transfer. If magnetic bead carry over occurred, place tube back on magnet, collect supernatant, and transfer to clean tube.
13. Store DNA samples at 4 ºC until qPCR is run (use photo-tray to transport sample tubes).
14. Follow laboratory cleanup protocol in Appendix E.

PCR for standard RV-PCR protocol with 16 hr incubation (follow PCR conditions provided in Appendix F) – Perform on T0 and T16 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal and transfer to BSC.
2. Take 96-well sample crude lysate plate out of freezer (use a photo-tray to transport plate), transfer to BSC. Change gloves. Crude lysate plate should be kept on a cool block or at 4 ºC at all times.
3. Using BSL3 centrifugation technique (PCR plates are loaded in safety cups), centrifuge sealed sample plate for 1 min at 2000 rpm.
4. Open crude lysate plate in BSC.
5. Mix samples up and down 10 times and transfer 5 µL from crude lysate plate to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
6. When working with dirty wipes, perform 1:10 dilution of crude lysate:

   Add 90 µL of PCR-grade water to wells of a sterile 96-well bioblock (See Appendix G).

   Mix crude cell lysate up and down 5 times and transfer 10 µL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 µL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
7. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
8. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
9. Run PCR (Please see Appendix F).
10. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
11. Follow laboratory cleanup protocol in Appendix E.

PCR for optimized RV-PCR protocol with 9 hr of incubation and Promega DNA extraction and purification (follow PCR conditions provided in Appendix F) – Perform on T0 and T9 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal, transfer to BSC.
2. If sample tubes were frozen, transfer them to BSC and let them thaw to room temperature.
3. For T0 samples: mix samples up and down 10 times and transfer 5 µL from each tube to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
4. Perform 1:10 dilution of all T9 samples:

   Add 90 µL of PCR-grade water to wells of a sterile 96-well bioblock.

   Mix sample up and down 5 times and transfer 10 µL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 µL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
5. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
6. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
7. Run PCR (Please see Appendix F).
8. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
9. Follow laboratory cleanup protocol in Appendix E.

Example of 24 sample manifold lay-out:

Sample A1	Sample A2	Sample A3
Sample B1	Sample B2	Sample B3
Sample C1	Sample C2	Sample C3
Sample D1	Sample D2	Sample D3
Sample E1	Sample E2	Sample E3
Sample F1	Sample F2	Sample F3
Sample G1	Sample G2	Sample G3
Sample H1	Sample H2	Sample H3

Corresponding PCR lysis plate lay-out:

A1	A2	A3									
B1	B2	B3									
C1	C2	C3									
D1	D2	D3									
E1	E2	E3									
F1	F2	F3									
G1	G2	G3									
H1	H2	H3									

Appendix B:
Semi-automated RV-PCR Protocol

Qualitative detection of *Bacillus anthracis* (Ba) Ames Spores in wipe, air filter and water samples

Laboratory set-up

- Put PPE (personal protective equipment) on: lab coat, safety glasses, double gloves.
- Prepare fresh bleach solution (1 volume bleach + 9 volumes water). Date and label with initials.
- Clean/bleach BioSafety Cabinet (BSC), Janus enclosure and bench surfaces.
- All sample manipulations are performed in the BSC or in the HEPA-filtered Janus enclosure.

Place samples in tubes

- Wipes and air filters: Using sterile forceps, transfer sample collection type to 30 mL tube behind internal mesh/ support (1.5 mL of 1X wash buffer is added to each wipe prior to spiking).
- Water samples: No mesh support is needed. Aliquot 20 mL of water directly in 30 mL tube.

Preparation of the spore working stock for sample spiking

Using Biological Safety Level 2 (BSL2) practices and BSL3 pipetting techniques, start the following protocol:

1. Place original Ba Ames (10^8 CFU/mL; check vial for correct concentration) spore suspension on platform vortexer for 20 min using position 7 (high).
2. Transfer stock to BSC
3. Aliquot 100 µL of spore suspension into 900 µL of 1X wash buffer (working suspension of 10^7 CFU/mL)
4. Vortex on single tube vortexer for 1 min
5. Aliquot 500 µL of the working suspension into 4.5 mL of 1X wash buffer (10^6 CFU/mL)
6. Vortex on single tube vortexer for 1 min
7. Aliquot 3 mL of 10^6 CFU/mL solution to 27 mL of 1X wash buffer (10^5 CFU/mL)
8. Vortex on single tube vortexer for 1 min
9. Aliquot 1 mL of 10^5 CFU/mL to 9 mL of 1X wash buffer (10^4 CFU/mL)
10. Aliquot 1 mL of 10^4 CFU/mL to 9 mL of 1X wash buffer (10^3 CFU/mL)
11. Aliquot 1 mL of 10^3 CFU/mL to 9 mL of 1X wash buffer (10^2 CFU/mL)
12. Plate on BHI agar plates as follow:

    100 µL 10^2 CFU/mL (3 replicates)

    50 µL 10^3 CFU/mL (3 replicates)
13. Incubate agar plates at 30°C overnight; count plates the next day to determine exact number of spores spiked in the samples.
14. Inoculate sample tubes with 100 µL of 10^2 CFU/mL (10 spore level) and 10^3 CFU/mL (100 spore level); record manifold layout.

Preparation of the Janus Robotic enclosure

Materials:

- Sharps waste container to collect tips
- Beaker with top filter-cup caps
- Capping tray with bottom caps
- 2 large biohazard bags + rubber bands
- Allen wrench for manifold

Setup:

- Bleach wipes and daily bleach bottle
- Set up manifold: connect vacuum pump, pump in line filter, and waste container with fresh bleach; tape pump exhaust tube to Janus enclosure to vent exhaust inside enclosure
- Add filter cups to manifold
- Tape filter-cup layout on glass window of the enclosure
- Tape PCR plate layout on glass window of the enclosure

Preparation of the cart

Materials:

- Gloves
- Bleach wipes
- Waste bags
- Isopropyl alcohol squeeze bottle
- Deionized water squeeze bottle
- Red biohazards bags + rubber bands
- Autoclave tape
- Large photo-tray
- Reservoirs
- Sharps containers
- Robotic tip refills
- Zip-lock bag containing: 96-well plate, plate support, foil seals, and plate sealer
- 2 mL eppendorf tubes
- Pipettor and pipette tips: 200 µL
- Marker

Media and Buffers:

- Brain Heart Infusion medium
- High salt wash buffer (pH 6.0)
- 1X wash buffer (pH 7.4)

Sample preparation in BSC

1. Using BSL3 pipetting technique, add 20 mL of extraction buffer to wipes and air filter samples placed in a 30 mL tubes (not needed for water samples).
2. Seal sample rack in waste plastic bag; bleach exterior of bag with bleach wipes.
3. Vortex samples for 20 min on platform vortexer, position 7 (outside BSC).
4. After vortexing samples, transfer sample rack to Robotic enclosure. Remove tube rack from plastic bag.

Semi-automated RV-PCR (T0)

1. Turn on vacuum pump at 10 psi.
2. Using the Janus robotic system, transfer 13 mL from 30 mL sample tubes to filter-cups. Stop robot, cap sample tubes, bleach the outer surface of sample tubes, bag the tube rack, bleach the bag, and transfer to refrigerator.
3. Use wash solution transfer program 1 to transfer 7 mL of high salt wash buffer (pH 6.0) to each filter-cup.
4. Complete liquid filtration through filter cups.
5. Use wash solution transfer program 2 to transfer 3 mL of 1X wash buffer (pH 7.4) to each filter-cup.
6. Complete liquid filtration through filter cups.
7. Stop vacuum pump.
8. Use media transfer program to transfer 2.5 mL of Brain Heart Infusion medium to each cup. Is there any mixing step here to dislodge the spores from the filter? The vacuum suction may lead to spore getting stuck to the filter in the cup.
9. Transfer upper part of manifold to capping tray fitted with bottom caps. Press down to cap bottom of filter-cups. Bleach bottom part of manifold. Change gloves.
10. **When performing standard RV-PCR protocol (16 hr incubation):**
    - Use PCR aliquot program to transfer 60 µL of sample from each filter cup to a 96-well PCR plate.
    - Seal T0 sample plate with foil seal. Change gloves.
    - Place T0 96-well plate in thermocycler for lysis: 20 minutes at 95ºC. Once cycle is completed and plate is at room temperature, bag 96-well plate in zip-lock bag, bleach the bag.
    - Transfer bagged 96-well plate to freezer and store at -20ºC until qPCR is run (use a photo-tray to transport plate).

    When performing the optimized RV-PCR protocol (9 hr incubation and Promega DNA extraction and purification protocol):
    - Manually mix sample by swirling pipette tip gently in filter cup, aliquot 120 µL volume out of 3 filter cups (3 replicate samples spiked at a level of 10^2 or 10^3 CFU/sample) and transfer 100 µL to 2 mL eppendorf tubes.
    - Add 900 µL of BHI to each tube.
    - Add 500 pg of extracted Bg DNA to each tube.
    - Process 1 mL sample according to Promega MD1630 protocol described below.
11. Cap top of filter-cups. Double-bag filter-cup manifold. Bleach bag.
12. Transfer bagged filter-cup tray to shaker incubator at 37ºC, speed 230 rpm. **Incubate for 16 hr for standard RV-PCR protocol or for 9 hr for optimized RV-PCR protocol with DNA extraction and purification.**
13. Follow laboratory cleanup protocol in Appendix E.

Semi-automated RV-PCR (T16 for standard protocol)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cup manifold for 10 min on platform vortexer.
3. Transfer filter-cup manifold to Janus robotic enclosure. Discard bag and uncap filter cups.
4. Use PCR aliquot program to transfer 60 µL of sample from each filter cup to a 96-well PCR plate (T16 sample plate) according to plate layout. Seal T16 sample plate with foil seal. Change gloves.
5. Lyse plate at 95°C for 20 min on thermocycler.
6. Once lysis is completed and plate is at room temperature, bag 96-well plate in zip-lock bag, bleach the bag, and transfer 96-well crude lysate plate to freezer and store at -20ºC until qPCR is run (use a photo-tray to transport plate).
7. Cap filter cup tray, bag, bleach bag, and transfer to BSC to continue with the Promega DNA extraction and purification Protocol.
8. Follow laboratory cleanup protocol in Appendix E.

Manual Promega MD1630 DNA Extraction and Purification Protocol (T9 for optimized protocol)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cup manifold for 10 min on platform vortexer.
3. Transfer filter-cup manifold to BSC. Discard bag and uncap filter cups.

DNA extraction and purification phase:

4. Aliquot 1000 µL volume out of each filter cup to 2 mL eppendorf tube using a micropipette (T9 samples). Do not use the 1.5 mL eppendorf tubes.
5. 1st lysis: add 600 µL of bead mix (lysis buffer + magnetic beads) to each tube and mix.
6. Add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
7. 2nd lysis: add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
8. 1st salt wash: add 360 µL of salt wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
9. 1st alcohol wash: add 360 µL of alcohol wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
10. Air dry (2 min)
11. Heat dry on heat block at 80°C until samples are dry. Allow all alcohol solution to evaporate since alcohol may interfere with qPCR.

DNA concentration and elution phase:

12. Move sample tubes out of heat block and add 200 µL of elution buffer to each tube. Mix, place on magnet, collect supernatant with micropipette and transfer to clean eppendorf tubes (typically, 80 µL are collected). Visually verify absence of magnetic bead carry over during final transfer. If magnetic bead carry over occurred, place tube back on magnet, collect supernatant, and transfer to clean tube.
13. Store DNA samples at 4 ºC until qPCR is run (use photo-tray to transport sample tubes).
14. Follow laboratory cleanup protocol in Appendix E.

PCR for standard RV-PCR protocol with 16 hr incubation (follow PCR conditions provided in Appendix F) – Perform on T0 and T16 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal, transfer to BSC.
2. Take 96-well sample crude lysate plate out of freezer (use a photo-tray to transport plate), transfer to BSC. Change gloves. Crude lysate plate should be kept on a cool block or at 4ºC at all times.
3. Using BSL3 centrifugation technique (PCR plates are loaded in safety cups), centrifuge sealed sample plate for 1 min at 2000 rpm.
4. Open crude lysate plate in BSC.
5. Mix samples up and down 10 times and transfer 5 µL from lysis plate to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.

   When working with dirty wipes, perform 1:10 dilution of crude cell lysate:

   Add 90 µL of PCR-grade water to wells of a sterile 96-well bioblock.

   Mix crude cell lysate up and down 5 times and transfer 10 µL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 µL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
6. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
7. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.

8. Run PCR (see Appendix F).
9. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
10. Follow laboratory cleanup protocol in Appendix E.

PCR for optimized RV-PCR protocol with 9 hr of incubation and Promega DNA extraction and purification (follow PCR conditions provided in Appendix F) – Perform on T0 and T9 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal, transfer to BSC.
2. If sample tubes were frozen, transfer them to BSC and let them thaw to room temperature.
3. For T0 samples: mix samples up and down 10 times and transfer 5 µL from sample tubes to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
4. Perform 1:10 dilution of T9 samples:

   Add 90 µL of PCR-grade water to wells of a sterile 96-well bioblock.

   Mix sample up and down 5 times and transfer 10 µL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 µL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
5. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
6. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
7. Run PCR (Please see Appendix F).
8. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
9. Follow laboratory cleanup protocol in Appendix E.

Appendix C:
Final Manual RV-PCR Protocol

Qualitative detection of *Bacillus anthracis* (Ba) Ames Spores in wipe, air filter and water samples

Laboratory set-up

- Put PPE (personal protective equipment) on: lab coat, safety glasses, double gloves.
- Prepare fresh bleach solution (1 volume bleach + 9 volumes water). Date and label with initials.
- Clean/bleach BioSafety Cabinet (BSC) and bench surfaces.
- All sample manipulations are performed in the BSC

Place samples in tubes

- Wipes and air filters: Using sterile forceps, transfer sample collection type to 30 mL tube behind internal mesh/ support (1.5 mL of 1X wash buffer is added to each wipe prior to spiking).
- Water samples: No mesh support is needed. Aliquot 20 mL of water directly in 30 mL tube.

Plating

Although no plating is included in the RV-PCR method, it is suggested to preserve samples for any further confirmation using plating and other microbiological culture-based methods.

Preparation of BioSafety Cabinet for manual RV-PCR

Materials:

- Bleach wipes and daily bleach bottle
- Sharps waste container
- Absorbent pad
- Beaker with top filter-cup caps
- Capping tray with bottom caps
- 2 large biohazard bags + rubber bands
- Allen wrench for manifold
- Serological pipettes and pipettor

Set up:

- Set up manifold: connect vacuum pump, pump in line filter, and waste container with fresh bleach; tape pump exhaust tube to BSC to vent exhaust inside BSC
- Add filter cups to manifold
- Tape filter-cup layout on glass window of the BSC

Preparation of the cart for manual RV-PCR

Materials:

- Gloves
- Bleach wipes
- Waste bags
- Absorbent pads
- Isopropyl alcohol squeeze bottle
- Deionized water squeeze bottle
- Red biohazards bags + rubber bands
- Autoclave tape
- Large photo-tray
- Sharps container
- 2 mL eppendorf tubes
- 200 µL pipettor and pipette tips
- Marker

Media and Buffers:

- Brain Heart Infusion medium
- High salt wash buffer (pH 6.0)
- 1X wash buffer (pH 7.4)

Manual RV-PCR protocol (T0)

1. In the BSC, add 20 mL of extraction buffer to wipe or air filter samples placed in a 30 mL tubes (no addition is needed for 20 mL water samples).
2. Seal sample rack in waste bag; bleach exterior of bag with bleach wipe.
3. Vortex samples for 20 min on platform vortexer (outside BSC), position 7.
4. After vortexing samples, transfer sample rack to BSC. Remove tube rack from plastic bag.
5. Turn on vacuum pump at 10 psi.
6. Using BSL3 pipetting technique, transfer sample from 30 mL sample tube to filter-cup using a serological pipette (the total sample volume is 20 mL. Pipette 15 mL and deliver 13 mL to filter-cup). Dispose pipette in waste container. Cap sample tube, bleach the outer surface of sample tube. Re-place tube back on rack. Change gloves.
7. Repeat step 6 for each sample.
8. Complete filtration of liquid through filter cups.
9. Bleach original sample rack. Double-bag rack. Bleach outer surface of biohazard bag. Change gloves.
10. Transfer double-bagged original sample rack to refrigerator.
11. Transfer 7 mL of high salt wash buffer (pH 6.0) to each filter-cup using a serological pipette. Use new disposable pipette for each sample cup.
12. Complete filtration of liquid through filter cups.
13. Transfer 3 mL of 1X wash buffer (pH 7.4) to each filter-cup using a serological pipette. Use new disposable pipette for each sample cup.
14. Complete filtration of liquid through filter cups. Change gloves.
15. Stop vacuum pump.
16. Bring 5 mL graduated pipettes, 200 µL pipettor, 200 µL tips, BHI medium, sharps container, centrifuge safety cup, zip-lock bag with PCR plate, plate support, foil seals, and plate sealer to BSC. Set up work space in BSC. Change gloves.
17. Pipette 2.5 mL of Brain Heart Infusion medium into each filter cup using a serological pipette. Use new disposable pipette for each filter cup.

18. Transfer upper part of manifold to capping tray fitted with bottom caps. Press down to cap bottom of filter-cups. Change gloves.
19. For each sample: swirl pipette tip gently in filter cup to mix sample, aliquot 120 µL volume out of each cup using a micropipette, and transfer 100 µL to 2 mL eppendorf tubes.

    Add 900 µL of BHI to each eppendorf tube.

    Add 500 pg of extracted Bg DNA to each eppendorf tube.

    Process 1 mL samples according to Promega MD1630 protocol described below.
20. Cap filter-cups. Double-bag filter-cup manifold. Bleach bag.
21. Transfer bagged filter-cup tray to shaker incubator at 37ºC, speed 230 rpm. Incubate for 9 hr.
22. Follow laboratory cleanup protocol in Appendix E.

Manual Promega MD1630 DNA Extraction and Purification Protocol (T9)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cups for 10 min on platform vortexer.
3. Transfer filter-cup manifold to BSC.

DNA extraction and purification phase:

4. Aliquot 1000 µL volume out of each filter cup to 2 mL eppendorf tube using a micropipette (T9 samples). Do not use the 1.5 mL eppendorf tubes.
5. 1st lysis: add 600 µL of bead mix (lysis buffer + magnetic beads) to each T0 and T9 tubes and mix.
6. Add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
7. 2nd lysis: add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
8. 1st salt wash: add 360 µL of salt wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
9. 1st alcohol wash: add 360 µL of alcohol wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
10. Air dry (2 min)
11. Heat dry on heat block at 80°C until samples are dry. Allow all alcohol solution to evaporate since alcohol may interfere with qPCR.

DNA concentration and elution phase:

12. Move sample tubes out of heat block and add 200 µL of elution buffer to each tube. Mix, place on magnet, collect supernatant with micropipette and transfer to clean eppendorf tubes (typically, 80 µL are collected). Visually verify absence of magnetic bead carry over during final transfer. If magnetic bead carry over occurred, place tube back on magnet, collect supernatant, and transfer to clean tube.
13. Store DNA samples at 4 ºC until qPCR is run (use photo-tray to transport sample tubes).
14. Follow laboratory cleanup protocol in Appendix E.

PCR (follow PCR conditions provided in Appendix F) – Perform on T0 and T9 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal and transfer to BSC.
2. Transfer sample tubes to BSC.
3. For T0 samples: mix samples up and down 10 times and transfer 5 μL from sample tubes to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
4. Perform 1:10 dilution of T9 samples:

   Add 90 μL of PCR-grade water to wells of a sterile 96-well bioblock.

   Mix sample up and down 5 times and transfer 10 μL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 μL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
5. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
6. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
7. Run PCR (Please see Appendix F).
8. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
9. Follow laboratory cleanup protocol in Appendix E.

Appendix D:
Final Semi-automated RV-PCR Protocol

Qualitative detection of *Bacillus anthracis* (Ba) Ames Spores in wipe, air filter and water samples

Laboratory set-up

- Put PPE (personal protective equipment) on: lab coat, safety glasses, double gloves.
- Prepare fresh bleach solution (1 volume bleach + 9 volumes water). Date and label with initials.
- Clean/bleach BioSafety Cabinet (BSC), Janus enclosure and bench surfaces.
- All sample manipulations are performed in the BSC or in the HEPA-filtered Janus enclosure.

Place samples in tubes

- Wipes and air filters: Using sterile forceps, transfer sample collection type to 30 mL tube behind internal mesh/ support (1.5 mL of 1X wash buffer is added to each wipe prior to spiking).
- Water samples: No mesh support is needed. Aliquot 20 mL of water directly in 30 mL tube.

Plating

Although no plating is included in the RV-PCR method, it is suggested to preserve samples for any further confirmation using plating and other microbiological culture-based methods.

Preparation of the Janus Robotic enclosure

Materials:

- Bleach wipes and daily bleach bottle
- Sharps waste container to collect tips
- Beaker with top filter-cup caps
- Capping tray with bottom caps
- 2 large biohazard bags + rubber bands
- Allen wrench for manifold

Setup:

- Set up manifold: connect vacuum pump, pump in line filter, and waste container with fresh bleach; tape pump exhaust tube to Janus enclosure to vent exhaust inside enclosure
- Add filter cups to manifold
- Tape filter-cup layout on glass window of the enclosure
- Tape PCR plate layout on glass window of the enclosure

Preparation of the cart

Materials:

- Gloves
- Bleach wipes
- Waste bags
- Isopropyl alcohol squeeze bottle
- Deionized water squeeze bottle
- Red biohazards bags + rubber bands
- Autoclave tape
- Large photo-tray
- Reservoirs
- Sharps containers
- Robotic tip refills
- Zip-lock bag containing: 96-well plate, plate support, foil seals, and plate sealer
- 2 mL eppendorf tubes
- Pipettor and pipette tips: 200 µL
- Marker

Media and Buffers:

- Brain Heart Infusion medium
- High salt wash buffer (pH 6.0)
- 1X wash buffer (pH 7.4)

Sample preparation in BSC

1. Using BSL3 pipetting technique, add 20 mL of extraction buffer to wipes and air filter samples placed in a 30 mL tubes (not needed for 20 mL water samples).
2. Seal sample rack in plastic bag; bleach bag.
3. Vortex samples for 20 min on platform vortexer, position 7 (outside BSC).
4. After vortexing samples, transfer sample rack to Robotic enclosure. Remove tube rack from plastic bag.

Semi-automated RV-PCR (T0)

1. Turn on vacuum pump at 10 psi.
2. Using the Janus robotic system, transfer 13 mL from 30 mL sample tubes to filter-cups. Stop robot, cap sample tubes, bleach the outer surface of sample tubes, bag the original sample tube rack, bleach the bag, and transfer to refrigerator.
3. Use wash solution transfer program 1 to transfer 7 mL of high salt wash buffer (pH 6.0) to each filter-cup.
4. Complete liquid filtration through filter cups.
5. Use wash solution transfer program 2 to transfer 3 mL of 1X wash buffer (pH 7.4) to each filter-cup.
6. Complete liquid filtration through filter cups.
7. Stop vacuum pump.
8. Use media transfer program to transfer 2.5 mL of Brain Heart Infusion medium to each cup. Is there any mixing step here to dislodge the spores from the filter? The vacuum suction may lead to spore getting stuck to the filter in the cup.
9. Transfer upper part of manifold to capping tray fitted with bottom caps. Press down to cap bottom of filter-cups. Bleach bottom part of manifold. Change gloves.

10. For each sample: Use PCR aliquot program to transfer 100 µL volume out of each filter cup and transfer 100 µL to 2 mL eppendorf tubes.

    Add 900 µL of BHI to each tube.

    Add 500 pg of extracted Bg DNA to each tube.

    Process 1 mL sample according to Promega MD1630 protocol described below
11. Cap top of filter-cups. Double-bag filter-cup manifold. Bleach bag.
12. Transfer bagged filter-cup tray to shaker incubator at 37ºC, speed 230 rpm. Incubate for 9 hr.
13. Follow laboratory cleanup protocol in Appendix E.

Manual Promega MD1630 DNA Extraction and Purification Protocol (T9)

1. After incubation, take filter-cup manifold out of incubator.
2. Vortex filter cup manifold for 10 min on platform vortexer.
3. Transfer filter-cup manifold to BSC. Discard bag and uncap filter cups.

DNA extraction and purification phase:

4. Aliquot 1000 µL volume out of each filter cup to 2 mL eppendorf tube using a micropipette (T9 samples). Do not use the 1.5 mL eppendorf tubes.
5. 1st lysis: add 600 µL of bead mix (lysis buffer + magnetic beads) to each T0 and T9 tubes and mix.
6. Add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
7. 2nd lysis: add 360 µL of lysis buffer to each tube. Mix, place on magnet, discard supernatant to waste.
8. 1st salt wash: add 360 µL of salt wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
9. 1st alcohol wash: add 360 µL of alcohol wash solution to each tube. Mix, place on magnet, discard supernatant to waste. Repeat one more time.
10. Air dry (2 min)
11. Heat dry on heat block at 80°C until samples are dry. Allow all alcohol solution to evaporate since alcohol may interfere with qPCR.

DNA concentration and elution phase:

12. Move sample tubes out of heat block and add 200 µL of elution buffer to each tube. Mix, place on magnet, collect supernatant with micropipette and transfer to clean eppendorf tubes (typically, 80 µL are collected). Visually verify absence of magnetic bead carry over during final transfer. If magnetic bead carry over occurred, place tube back on magnet, collect supernatant, and transfer to clean tube.
13. Store DNA samples at 4ºC until qPCR is run (use photo-tray to transport sample tubes).
14. Follow laboratory cleanup protocol in Appendix E.

PCR (follow PCR conditions provided in Appendix F) – Perform on T0 and T9 samples

1. Set up PCR plate with PCR mix according to plate layout in PCR-preparation hood, seal and transfer to BSC.
2. Transfer sample tubes to BSC and let them thaw to room temperature.
3. For T0 samples: mix samples up and down 10 times and transfer 5 μL from sample tubes to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
4. Perform 1:10 dilution of T9 samples:

   Add 90 μL of PCR-grade water to wells of a sterile 96-well bioblock.

   Mix sample up and down 5 times and transfer 10 μL to bioblock wells, maintaining the plate layout.

   Mix diluted samples up and down 10 times and transfer 5 μL from bioblock well to the PCR plate (with PCR mix). Seal PCR plate with clear seal. Change gloves.
5. Using BSL3 centrifugation technique, centrifuge sealed PCR plate for 1 min at 2000 rpm.
6. Open safety cup in BSC, place plate on photo-tray, change gloves, transfer PCR plate to ABI thermocycler.
7. Run PCR (Please see Appendix F).
8. After thermal cycling completion, discard sealed PCR plate to waste. Autoclave. PCR plates with amplified product are never to be opened in the laboratory.
9. Follow laboratory cleanup protocol in Appendix E.

Appendix E:
Lab cleanup procedure, buffers and media

Lab Cleanup Protocol

- Dispose of all biological materials in autoclave bags (double bagged)
- Autoclave all waste materials
- Decontaminate counters and all equipment with fresh bleach (1 volume water and 9 volumes commercial bleach), followed by 70% isopropanol, and finally rinse with DI water.

Buffer and media preparation

1 M Sodium Hydroxide Solution (NaOH)

Note: Label all bottles and flasks with reagent name, date and initials.

1. Weigh 10 g NaOH
2. Transfer 10 g NaOH to 200 mL MilliQ H_2O
3. Mix with magnetic stirrer
4. After NaOH pellets are dissolved, bring final volume to 250 mL with MilliQ H_2O

10X Wash Buffer (250 mM KH_2PO_4, pH 7.4)

Note: Label all bottles and flasks with reagent name, date and initials.

1. Dissolve 34 g KH_2PO_4 in 500 mL MiliQ H_2O
2. Add enough 1 M NaOH to bring to pH 7.4 (≥ 200 mL of 1 M NaOH)
3. Bring to volume with MilliQ H_2O to 1 L
4. Filter sterilize using 1 L, 0.22 micron cellulose acetate filtering system with disposable bottle

1X Wash Buffer (25 mM KH_2PO_4, pH 7.4)

Note: Label all bottles and flasks with reagent name, pH level, date and initials.

1. 50 mL 10X wash buffer
2. 950 mL MilliQ H_2O
3. Mix with magnetic stirrer, when mixed, measure pH
4. Filter sterilize using 1 L, 0.22 cellulose acetate filtering system with disposable bottle

High Salt-Low pH Wash Buffer (207 mM KH_2PO_4, pH 6.0)

Note: Label all bottles and flasks with reagent name, date and initials.

Dissolve 28.2 g KH_2PO_4 in 500 mL MiliQ H_2O

1. Add enough 1 M NaOH to bring to pH 6.0 (≥ 100 mL of 1 M NaOH)
2. Bring volume with MilliQ H_2O to 1 L
3. Filter sterilize using 1 L, 0.22 micron cellulose acetate filtering system with disposable bottle

Extraction buffer (0.250 mM KH_2PO_4, 30 % EtOH, 0.1% Tween 80)

1. 698 mL MilliQ H_2O
2. 1 mL 10X wash buffer
3. 300 mL 200 proof ethanol
4. 1 mL Tween® 80
5. Filter sterilize using 1 L, 0.22 cellulose acetate filtering system with disposable bottle

Brain Heart Infusion Agar

1. 26 g Bacto Brain Heart Infusion agar powder
   500 mL MilliQ H_2O
2. Place on hotplate and gently mix with spin bar.
3. Autoclave
4. Place on hotplate and gently mix with spin bar. Allow agar to cool down to 45°C before pouring.
5. Pour 20 mL of solution in each petri dish using a serological pipette. Pouring is performed in a sterile BSC.

Appendix F:
PCR conditions

Reagents:

- Primers and probes
- TaqMan 2X Universal Master Mix with UNG and AmpliTaq Gold (ABI cat. Number 4305719)
- Molecular Biology grade distilled water, RNase- and DNase-free (Teknova cat. Number W3350)

PCR mix for pXO2 (EPA-2) primer/probe set.

Reagent	Volume (µL)	Final Concentration (µM)
TaqMan 2X Universal Master Mix	12.5	1X
Forward primer, 25 µM	0.3	0.3
Reverse primer, 25 µM	0.3	0.3
Probe, 2 µM	1	0.08
Molecular Biology Grade Water	5.9	N/A
Template DNA	5	Variable
TOTAL	25	

PCR mix for Chromosome (BC3) and pXO1 (EPA-1) primer/probe sets.

Reagent	Volume (µL)	Final Concentration (µM)
TaqMan 2X Universal Master Mix	12.5	1X
Forward primer, 25 µM	1	1.0
Reverse primer, 25 µM	1	1.0
Probe, 2 µM	1	0.08
Molecular Biology Grade Water	4.5	N/A
Template DNA	5	Variable
TOTAL	25	

Equipment:

- ABI 7500 fast thermocycler
- Optical Fast 96-well plates (ABI, cat. Number 4366932)
- Optical adhesive plate covers (ABI, cat. number 4311971)

PCR conditions for all three primer/probe sets:

STEPS	UNG incubation	AmpliTaq Gold activation	PCR , 45 cycles	
	HOLD	HOLD	Denaturation	Annealing/extension
Temperature	50°C	95°C	95°C	60°C
Time	2 min	10 min	5 sec	20 sec

*Fast Ramp: 3.5 oC/sec up and 3.5oC/sec down.

Appendix G: Consumables

Description	Catalog #	Units	Supplier	Item part Number
PCR Materials				
PCR plates	4346906	20/box	Applied Biosystems, INC.	4366932
PCR plates seals	4311971	100/box	Applied Biosystems, INC.	4311971
Universal PCR master mix	4305719	(10) 5 mL bottles	Applied Biosystems Inc (ABI)	4305719
PCR probes	custom		Biosearch Technologies	DLO-FB1-1
PCR primers	MD1360	96 reactions	Biosearch Technologies	P2C-1
96 Well Hard Plates Costar-black 100/cs	29442-922	cs	GSS	29442-922
2.0 mL Screw cap tubes (500 bag)	20170-237	bag	GSS	20170-237
Culture Materials				
BHI media	237500	500 g / bottle	VWR	90004-690
BHI agar	241830	500 g /bottle	VWR	90003-262
TSB Agar			VWR	200059-616
TSB Media			VWR	90004-270
Petri dishes 60x15	25373-085	cs	GSS	25373-085
LAZY-L-SPREADERS STERILE case of 500	101100-886	500/cs	Government Scientific source (GSS)	101100-886
Buffers				
Potassium phosphate, KH2PO4	22, 130-9	500 g / bottle	Aldrich	221309
Tween® 80 9005-65-6	103170	100 mL	MP Biomedicals	103170
EtOH 200proof absolute, anhydrous	111ACS200	1 L	TRANS MERIDIAN UCI/QUANTIUM CHEM	
Distilled Water	W3350	1 L	Quality Biological Inc.	351-029-721
DNA extraction and purification reagents				
Promega Kit			Promega	MD1360
Promega salt wash			Promega	MD1401
Promega beads			Promega	MD1441
Promega lysis buffer			Promega	MD1392
Promega anti-foam			Promega	MD1431

Vacuum filtration materials				
Tygon Tubing 1/4" ID 1/2" OD	BH-95636-00	bx	Cole-Parmer	BH-95636-00
Nalgene 2 Liter Bottle	BH06257-20	ea	Cole-Parmer	BH06257-20
Nalgene Venting Cap	BH06258-10	ea	Cole-Parmer	BH06258-10
3870E DOOR BELLOWS ASSEMBLY KIT - Part for vacuum pump	TUK030-2150	ea	A & A Dental & Medical Services	TUK030-2150
3870E AIR JET VALVE (BLACK TOP) - Part for vacuum pump	TUJ034-2149	ea	A & A Dental & Medical Services	TUJ034-2149
3870E DOOR GASKET (Door Seal) - Part for vacuum pump	TUG074-2146	ea	A & A Dental & Medical Services	TUG074-2146
3870E FILL/VENT MESH CHAMBER FILTER (Stainless Steel) - Part for vacuum pump	MIF062-2126	ea	A & A Dental & Medical Services	MIF062-2126
3870E PLUNGER VALVE KIT (3mm) - Part for vacuum pump	TUK082-2155	ea	A & A Dental & Medical Services	TUK082-2155
3870E PLUNGER VALVE KIT (6mm) - Part for vacuum pump	TUK086-2156	ea	A & A Dental & Medical Services	TUK086-2156
3870E SAFETY VALVE (40 PSI) - Part for vacuum pump	TUV065-2166	ea	A & A Dental & Medical Services	TUV065-2166
Sample processing materials				
30 mL Screw blue cap tube	EK-T3242S	100/cs	E & K Scientific	T3242S
Polyethylene caps	94075K56	pk	McMaster Carr	57935K16
Monofilament polyester mesh disc,	93185 T17	ea	McMaster Carr	3185T17
Quick turn tube fitting polypropylene, female cap	51525K365	pk	Ark-Plas Products, Inc.	AP17FLP00P
Whatman autocups [available from VWR under misc-supplies]	1602-0465		VWR	1502-0465(Whatman)
Disposable Nylon Forceps	12576-933	100/pack	Government Scientific source (GSS)	12576-933
100 mL Reagent Reservoirs (100/case)	8086	cs	Thermo Fisher	8086
Bioblocks for dilutions (96 wells/ 2mL per well)	662000	20/case	E & K Scientific	662000
Robotic materials				
Robotic tips	6001256	10 racks/ case	Perkin Elmer	6001256
Large reservoirs for robot	2035	25/case	E & K Scientific	EK-2035
Sharps container for Janus	33000-956	32/case	GSS	33000-956
Pipettors and tips for PCR and DNA extraction and purification				
1000 µL Filter LTS Tips	RT-L10F	cs	Rainin	RT-L20F
200 µL Filter LTS Tips	RT-L1000F	cs	Rainin	RT-L1000F
20 µL Filter LTS Tips	RT-L200F	cs	Rainin	RT-L200F
L-1000 LTS Pipettor	L1000 LTS	ea	Rainin	L1000 LTS
L-100 LTS Pipettor	L100 LTS	ea	Rainin	L100 LTS
L-200 LTS Pipettor	L200 LTS	ea	Rainin	L200 LTS
L-20 LTS Pipettor	L20 LTS	ea	Rainin	L20 LTS
L-10 LTS Pipettor	L10 LTS	ea	Rainin	L10 LTS
Carousel Stand	CR-7	ea	Rainin	CR-7

General laboratory supplies				
Diamond Grip Latex Gloves X-Small	32916-498	cs	GSS	32916-498
Diamond Grip Latex Gloves Small	32916-506	cs	GSS	32916-506
Diamond Grip Latex Gloves Medium	32916-500	cs	GSS	32916-500
Diamond Grip Latex Gloves Large	32916-502	cs	GSS	32916-502
Diamond Grip Latex Gloves X-Large	32916-503	cs	GSS	32916-503
VWR Autoclave Bags 25x35	14220-042	cs	GSS	14220-032
VWR 5.0 mL Freezer Vials	66008-400	bag	GSS	66008-400
Corning 50 mL Conical centrifuge tubes	21008-714	cs	GSS	89004-367
Corning 15 mL Conical centrifuge tubes	21008-678	cs	GSS	89004-370
BD Sharps Containers	BD305551	cs	GSS	BD305551
Kaydry EX-L Wipers	21903-021	cs	GSS	21903-021
Bleach gallon bottles case	37001-060	cs	GSS	37001-060
Sleeves protectors	10832-668	cs	ValuMax International	1919W
Disposables lab. coats w/cuffs	CV9841N	cs	GSS	CV9841N
VWR, Bleach wipes 10 pkg/cs	47735-634	10pkg/cs	Government Scientific source (GSS)	37001-060
Heavy Duty Waste Bags	436188D4		Staples	Item 436188 Model WHD3339
Autoclave ampoules	101101-788	20 ampules/ box	Government Scientific source (GSS)	14220-030
Wipers			Government Scientific source (GSS)	37002-030
Serological pipettor and tip for manual RV-PCR only				
Portable pipet aid	4-000-100		VWR	4-000-100
50 mL serological pipettor tips	29442-440	bag	GSS	53283-712
10 mL serological pipettor tips	29442-430	bag	GSS	53283-708
25 mL serological pipettor tips	29442-436	bag	GSS	53283-710
5 mL serological pipettor tips	29442-422	bag	GSS	53283-706
Sample materials				
Aerosol filters	FSLW04700	100 filters/ box	Millipore	FSLW04700
Wipes	Kendall #8042	3000/case	GSS	89004-507

Acronyms: bx, box; cs, case; ea, each; pk, package

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