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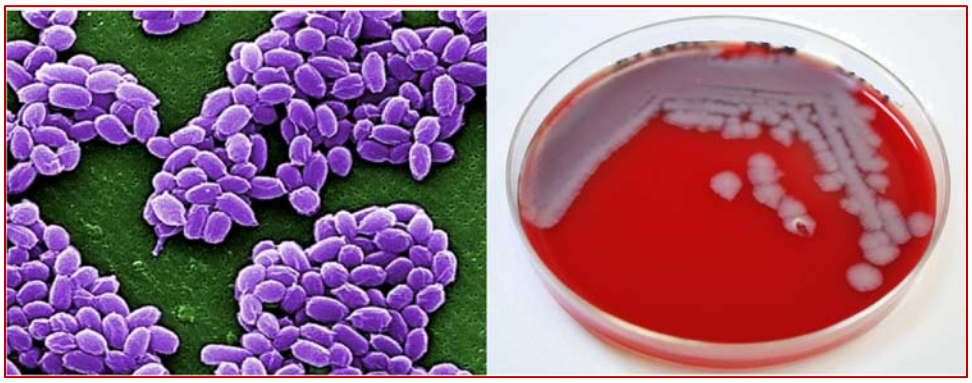
United States Environmental Protection Agency

Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Event



SCIENCE

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Foreword

Following the 2001 terrorist attacks and the anthrax bioterrorism event, several Presidential Directives broadened the U.S. Environmental Protection Agency's (EPA's) mission to include key aspects of homeland security. EPA was directed to protect human health and the environment from harmful effects of chemical, biological and radiological contamination. To effectively respond to such events, EPA established the Environmental Response Laboratory Network (ERLN) and Water Laboratory Alliance (WLA), the laboratory network responsible for analyses of environmental and water samples. In the event of the release of a bioterrorism agent such as *Bacillus anthracis* (*B. anthracis*), hundreds to thousands of environmental samples will need to be processed and analyzed in a timely manner to support decontamination planning efforts. Analytical results, in addition to technical expertise, will be provided to those responsible for making clearance decisions. To address these critical needs, EPA's National Homeland Security Research Center (NHSRC), in collaboration with the Centers for Disease Control and Prevention (CDC) and Lawrence Livermore National Laboratory (LLNL), has developed this protocol for detection of *B. anthracis* in environmental samples, including drinking water.

The "Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Event" includes response-phase-appropriate sample processing and analytical procedures for determining the presence or absence of *B. anthracis* spores in a cost-effective and time-efficient manner. This protocol includes sample processing procedures appropriate for the real-time polymerase chain reaction (PCR) analytical technique used during the site characterization phase. Since determination of whether viable *B. anthracis* spores are present in the samples will be required during the post decontamination phase of the response, sample processing methods appropriate for traditional microbiological culture and EPA's Rapid Viability (RV)-PCR analytical procedures are also included.

Although Laboratory Response Network (LRN) laboratories will provide analytical support to EPA by analyzing environmental samples, they will be using LRN-specific protocols as opposed to this protocol. NHSRC has made this protocol available to ERLN and WLA labs for the analysis of samples to assist in preparing for and recovering from disasters involving contamination from *B. anthracis* spores. This milestone specifically represents a strong and continuous commitment of NHSRC's research in support of the ERLN and WLA. It also exhibits EPA'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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Acronyms

ABI	Applied Biosystems®
BD	Becton, Dickinson and Company
BHI	Brain heart infusion
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biological safety level
CBR	Chemical, biological, radiological
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
C_T	Cycle threshold
DI	Deionized
DNA	Deoxyribonucleic acid
DQO	Data quality objectives
EDTA	Ethylenediaminetetraacetic acid
EIC	External inhibition control
EPA	United States Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
FEM	Forum on Environmental Measurement
ICLN	Integrated Consortium of Laboratory Networks
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
LLNL	Lawrence Livermore National Laboratory
LRN	Laboratory Response Network
MPN	Most probable number
MSDS	Material safety data sheets
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
NTC	No template controls
OSHA	Occupational Safety and Health Administration
PAPR	Powered air purifying respirator
PBST	Phosphate buffered saline with Tween® 20
PC	Positive control
PCR	Polymerase chain reaction
PES	Polyethersulfone
PMP	Paramagnetic particle
PNC	[Sample] processing negative control (Blank)
PPE	Personal protective equipment
psi	Pounds per square inch
PT	Proficiency testing
QA	Quality assurance
QC	Quality control
RNA	Ribonucleic acid
rpm	Revolutions per minute
RV-PCR	Rapid Viability-polymerase chain reaction
SBA	Sheep blood agar
SOP	Standard operating procedure
T_0	Time zero (no incubation)

T_9	Nine hour incubation
TE	Tris(hydroxymethyl)aminomethane-HCL-EDTA
TNTC	Too numerous to count
UV	Ultraviolet
WLA	Water Laboratory Alliance

Trademarked Products

Trademark	Holder	Location
Acrovent™	Pall Corporation	Ann Arbor, MI
BD Clay Adams™ Nutator Mixer	BD Diagnostics	Sparks, MD
Biopur® Safelock®	Eppendorf	United States
Applied BioSystems®	Life Technologies™	Carlsbad, CA
Autocup™	Whatman™ Ltd.	Maidstone, United Kingdom
BBL™	BD Diagnostics	Sparks, MD
Black Hole Quencher®	Biosearch Technologies, Inc.	Novato, CA
Costar®	Corning	Tewksbury, MA
Cole Parmer®	Cole Parmer®	Vernon Hills, IL
Dispatch®	Clorox Company	United States
Dynamag™	Life Technologies™	Carlsbad, CA
Fluoropore™	EMD Millipore	Billerica, MA
GN-6 Metricel®	Pall Corporation	Ann Arbor, MI
Invitrogen®	Life Technologies™	Carlsbad, CA
Jiffy-Jack®	Cole Parmer®	Vernon Hills, IL
Kendall™	Covidien, Inc.	Mansfield, MA
Life Technologies™	Life Technologies™	Carlsbad, CA
MagneSil® Blood Genomic	Promega	Madison, WI
Masterflex®	Cole Parmer®	Vernon Hills, IL
MaxQ™	Thermo Scientific Inc	Lenexa, KS
MicroFunnel™	Pall Corporation	Ann Arbor, MI
Microcon®	EMD Millipore	Billerica, MA
Nalgene®	Nalge Nunc Corporation	Rochester, NY
Stomacher®	Seward	United Kingdom
TaqMan®	Life Technologies™	Carlsbad, CA
Trypticase™ soy agar	BD Diagnostics	Sparks, MD
Tween®	Sigma-Aldrich	St. Louis, MO
Vacushield™	Pall Corporation	Ann Arbor, MI
Versalon™	Covidien, Inc.	Mansfield, MA
Whatman™	Whatman™ Ltd.	Piscataway, NJ

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Introduction

The series of 2001 terrorist attacks and the anthrax bioterrorism incidents that resulted in human casualties, and public and private facility closures, prompted enhanced and expanded national safeguards. Multiple Presidential Directives have designated the U.S. Environmental Protection Agency (EPA) as the primary federal agency responsible for the protection and decontamination of indoor/outdoor structures and water infrastructure vulnerable to chemical, biological and radiological (CBR) terrorist attacks. EPA's mission, to protect human health and the environment, was thereby expanded to address critical homeland security related needs.

The National Homeland Security Research Center (NHSRC) within the Office of Research and Development is EPA's hub for providing expertise on CBR agents and for conducting research to meet EPA's homeland security mission needs. A focus of NHSRC's research is to support the EPA's Environmental Response Laboratory Network (ERLN), a nationwide network of federal, state, local and commercial environmental laboratories which includes the Water Laboratory Alliance (WLA). Along with the Centers for Disease Control and Prevention's (CDC's) Laboratory Response Network (LRN), the ERLN can be activated in response to a large-scale environmental disaster to provide analytical capability, increased capacity and 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. Based on the realities of response activities after the 2001 anthrax event and continued preparedness efforts since then, it is anticipated that in the event of an intentional (bioterrorist attack) or accidental release of *Bacillus anthracis* (*B. anthracis*) spores, hundreds to thousands of diverse environmental sample (e.g., aerosols, particulates and drinking water) will need to be rapidly processed and analyzed in order to assess the extent of contamination and support the planning of decontamination efforts. A similar number of samples will also need to be analyzed to determine the efficacy of decontamination activities during the remediation phase of the response. During an anthrax event, EPA's decision makers will need timely results from rapid sample analyses for planning the decontamination efforts. To address these critical needs, NHSRC, in collaboration with CDC and Lawrence Livermore National Laboratory (LLNL), has generated this protocol for detection of *B. anthracis* in environmental samples.

To complement an effective sample collection strategy during an anthrax event, a systematic approach for timely and cost-effective sample analyses is critical. Such a systematic approach also helps in effectively managing and increasing the analytical laboratory capacity. This protocol attempts to present such an approach for the detection of *B. anthracis* spores in various environmental samples (e.g., aerosol, particulate [surface swabs, wipes, vacuum socks and filters, and Sponge-Sticks], drinking water). During the remediation phase, prior to decontamination activities, EPA has to quickly determine the extent of contamination in an affected building, facility, area or water system. Thus, for sample analyses during the site characterization phase, detecting presence or absence of the deoxyribonucleic acid (DNA) of *B. anthracis* by real-time polymerase chain reaction (PCR) is usually the most appropriate, and both time- and cost-effective technique. Since determination of whether viable *B. anthracis* spores are present in the samples will be required during the post decontamination phase of the response, either traditional microbiological culture or EPA's Rapid Viability-PCR (RV-PCR) analytical procedures must be used. Accordingly, the protocol presented here includes response-phase-appropriate sample processing and analytical procedures. It should be noted that LRN laboratories will support EPA environmental sample analyses during remediation using LRN-specific protocols.

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Sample processing procedures are provided for real-time PCR analyses conducted during the site characterization phase. In addition, sample processing procedures appropriate for RV-PCR and culture methods (for the post decontamination phase) are included. For drinking water samples, large volume samples may need to be analyzed to detect low concentrations of *B. anthracis* spores or vegetative cells. Therefore, the protocol also includes an ultrafiltration-based concentration procedure. For the post decontamination phase culture analyses, selected isolated colonies will be analyzed using real-time PCR to confirm the identity of *B. anthracis* as opposed to traditional biochemical and serological testing.

Many sample processing and analysis procedures in this document have been derived from LRN protocols. However, these procedures have been modified, as necessary, to address EPA's homeland security mission needs during the remediation phase of an anthrax event. Therefore, these modified procedures or this protocol itself must not be designated, referred to, or misconstrued as LRN procedures or protocol.

Since this protocol was developed to include the analyses of diverse environmental samples, it emphasizes appropriate sample processing as well as the DNA extraction and purification steps to significantly remove any growth- and/or PCR-inhibitory materials present in the samples. This protocol will be revised as better sample processing procedures and real-time PCR assays become available.

It should be noted that as of the publication date of this protocol has not been validated. During any *B. anthracis* related emergency situations, EPA's use of non-validated methods in the absence of validated methods must adhere to the EPA's Forum on Environmental Measurement (FEM) policy directive on method validation:

According to Agency Policy Directive FEM-2010-01, *Ensuring the Validity of Agency Methods Validation and Peer Review Guidelines: Methods of Analysis Developed for Emergency Response Situations:*

> It is EPA's policy that all methods of analysis (e.g., chemical, radiochemical, microbiological) must be validated and peer reviewed prior to issuance as Agency methods. There are emergency response situations that require methods to be developed and utilized, which may or may not have previously been validated or peer reviewed prior to use. This policy directive addresses those situations in which a method must be developed, validated and/or peer reviewed expeditiously for utilization in an emergency response situation. Also, in such emergency response situations only, an analytical method may be employed that has been validated by another established laboratory network (e.g., the Center for Disease Control and Prevention's Laboratory Response Network, the U.S. Department of Agriculture/Food and Drug Administration's Food Emergency Response Network). In those instances, the responsible federal agency will indicate that the level of validation and/or peer review that their analytical method underwent is consistent with the Integrated Consortium of Laboratory Networks (ICLN) *Guidelines for Comparison of Validation Levels between Networks.*[5] The responsible federal agency may also refer to the *Validation Guidelines for Laboratories Performing Forensic Analysis of Chemical Terrorism*[6] in order for the receiving federal agency to determine if the analytical method meets the intended purpose.

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> Any EPA regional or program office that proposes to utilize a method in an emergency response situation is responsible for establishing and documenting to what level and by what process the method has been validated and/or peer reviewed in accordance with this policy. A regional or program office may determine the level of validation and/or peer review that is necessary to provide the objective evidence that a method is suitable for its intended purpose; however, the office must document the validation and/or peer review information supporting use of the method. All documentation should be preserved in accordance with the Agency's records management policy.
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> [5] U.S. Department of Homeland Security, Integrated Consortium of Laboratory Networks (ICLN), *ICLN Guidelines for Comparison of Validation Levels between Networks*, Original Version, http://www.icln.org/docs/sop.pdf.
>
> [6] Federal Bureau of Investigation (FBI), Scientific Working Group on Forensic Analysis of Chemical Terrorism (SWGFACT), *Validation Guidelines for Laboratories Performing Forensic Analysis of Chemical Terrorism*, Forensic Science Communications, Volume 7, Number 2, April 2005.

The above policy is available at:

http://www.epa.gov/fem/pdfs/FEM_Policy2010-01_Emergency_Response_Methods_Final_July_2010.pdf

Also, EPA recognizes that having analytical data of known and documented quality is critical in making proper decisions during all phases of a response to an anthrax event and strives to establish data quality objectives (DQOs) for each response activity. [1] These DQOs are based upon needs for both quality and response time. EPA's ERLN, which is tasked with providing laboratory support following homeland security-related events, also has established data reporting procedures. Requirements for receiving, tracking, storing, preparing, analyzing and reporting data are specified in the *Environmental Response Laboratory Network Laboratory Requirements Document* at: http://epa.gov/erln/techsupport.html; project-specific requirements also are included in individual Analytical Service Requests.

[1] Information regarding EPA's DQO process, considerations and planning is available at: http://www.epa.gov/QUALITY/dqos.html

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Protocol for Detection of *Bacillus anthracis* in Environmental Samples During the Remediation Phase of an Anthrax Event

December 2012

1.0 Scope and Application

The purpose of this protocol is to detect *Bacillus anthracis* (*B. anthracis*) spores in environmental samples using real-time polymerase chain reaction (PCR) during the site characterization phase and to detect viable *B. anthracis* spores using either Rapid Viability-PCR (RV-PCR) or culture followed by isolate confirmation by real-time PCR during the post decontamination phase of the response. The real-time PCR assays included in this protocol have been only partially characterized for specificity, however, the use of these assays is currently recommended. These assays will be replaced with fully characterized and validated assays upon availability. This protocol is intended for the analyses of swabs, wipes, Sponge-Sticks, vacuum socks and filters, air filters, drinking water and decontamination waste water for *B. anthracis* spores.

2.0 Summary of Methods

2.1 Site Characterization Phase – Sample Analysis for Detection (Real-time PCR): After samples have been appropriately processed, the deoxyribonucleic acid (DNA) is extracted and purified. DNA extracts are analyzed by real-time PCR using the Applied Biosystems® (ABI) 7500 Fast Real-Time PCR System thermocycler. Direct DNA-based analysis of samples allows for high throughput and rapid results. Unless advised otherwise, real-time PCR should be performed using only the most sensitive assay, EPA-2 (Section 6.15).

2.2 Post Decontamination Phase – Sample Analyses for Viability: After samples have been appropriately processed, they are cultured by either inoculating into nutrient rich broth (RV-PCR procedures) or plating on sheep blood agar (SBA), culture procedures, to allow for germination of viable spores.

2.2.1 RV-PCR Procedure

The RV-PCR is a combination of rapid broth culture and real-time PCR. Culturing the samples allows the germination of viable *B. anthracis* spores recovered from processed samples. Real-time PCR, via change in cycle-threshold (C_T) value, offers a rapid determination of the viability and identity of *B. anthracis* bacteria that grow from germinated spores in broth culture. Samples (air filter, wipe, Sponge-Stick, swab, vacuum sock or filter, or drinking water) are processed in multiple spore extraction and wash steps.

Recovered spores are incubated in brain heart infusion (BHI) broth for optimum growth of *B. anthracis*. After vortexing, an aliquot is withdrawn for baseline analysis. This is the Time Zero (T_0) aliquot and is stored at 4°C. Then the broth culture remaining in the filter cup is incubated at 36°C for 9 hours on a rotary shaker incubator. After the broth culture has incubated for 9 hours, another aliquot is withdrawn. This is the T_9 aliquot. Both the T_0 and T_9 aliquots are processed to extract and purify total DNA. The T_0 and T_9 DNA extracts, in triplicate, are then analyzed in real-time PCR assay(s) to detect

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B. anthracis DNA. The C_T values for both the T_0 and T_9 DNA extracts are recorded and compared. Unless advised otherwise, real-time PCR should be performed using only the most sensitive assay, EPA-2 (Section 6.15).

2.2.2 Culture Procedure

The culture option includes plating serial dilutions of the sample on a non-selective agar SBA followed by rapid confirmation of typical isolated colonies using real-time PCR. Unless advised otherwise, real-time PCR should be performed using only the most sensitive assay, EPA-2 (Section 6.15).

3.0 Interferences and Contamination

3.1 Low recoveries of *B. anthracis* spores may be caused by the presence of high numbers of competing or inhibitory organisms, background debris, or toxic substances (e.g., metals or organic compounds).

3.2 Metals and organic compounds may also inhibit PCR reactions.

3.3 Problems related to sample processing, such as clogging of filters and inefficient extraction, may also result in low spore recoveries.

4.0 Safety

Note: This protocol should not be misconstrued as a laboratory standard operating procedure (SOP) that addresses all aspects of safety; the laboratory should adhere to their established safety guidelines.

4.1 Laboratory Hazards

Direct contact of skin or mucous membranes with infectious materials, accidental parenteral inoculation, ingestion, and exposure to aerosols and infectious droplets has resulted in *B. anthracis* infection. Due to the infectious nature of this organism, all samples should be handled using biosafety requirements as dictated by *Biosafety in Microbiological and Biomedical Laboratories* [BMBL], 5th Edition, CDC 2009. (Reference 16.1) and/or organizational health and safety plans.

4.2 Recommended Precautions

4.2.1 A biological safety level (BSL)-3 laboratory is required for handling and manipulating samples and cultures presumptive for *B. anthracis*. *B. anthracis* analyses should be conducted using BSL-3 practices, containment and facilities (BMBL, 5th Edition, CDC 2009. [Reference 16.1]). Additional biosafety and select agent information, as well as statutory requirements for possession, use and transfer of select agents, can be found in the Code of Federal Regulations (42 CFR part 73).

4.2.2 All drinking water concentration activities should be performed within a BSL-3 facility using BSL-3 practices. If a biological safety cabinet (BSC) is not used, due to space limitations, approved respiratory equipment should be used while concentrating samples within the BSL-3 suite. Caution should be used throughout the process (e.g., switching carboys, manipulation of the apparatus, adding wash solutions) to minimize spills/leaks.

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Analysts should be properly trained prior to concentrating samples using the protocol provided in Appendix A.

4.2.3 BSL-3 Practices

The laboratory supervisor must ensure that laboratory personnel demonstrate proficiency in standard and special microbiological practices before working with BSL-3 agents. All procedures involving manipulation of infectious material must be conducted within a BSC (preferably Class II or Class III) or other physical containment device. Protective clothing (e.g., laboratory coats, gloves and respirator) should be worn while processing and analyzing samples. Personal protective equipment (PPE) should never be worn outside the laboratory.

4.2.4 Disposable materials are recommended for sample manipulations.

4.2.5 The analyst must know and observe normal safety procedures required in a microbiology laboratory while preparing, using and disposing of media, cultures, reagents and materials. Analysts must be familiar with the operation of sterilization equipment.

4.2.6 Personal Protective Equipment (PPE)

- Laboratory personnel processing and conducting analyses of samples for *B. anthracis* place themselves at risk for exposure. However, the use of appropriate PPE can reduce the exposure risk. Laboratory personnel should familiarize themselves with the specific guidance for levels of protection and protective gear developed by the U.S. Department of Labor, Occupational Safety and Health Administration (OSHA), as provided in Appendix B of 29 CFR 1910.120 (http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9767). In addition to OSHA guidance, the Centers for Disease Control and Prevention (CDC) has developed recommendations for PPE based on BSL (Reference 16.1, http://www.cdc.gov/biosafety/publications/bmbl5/index.htm).

- Goggles or a face shield should be used for protection against splashes, spills and sprays. Protective coats, gowns, smocks or uniforms designated for laboratory use must be worn while working with samples and potentially contaminated materials. It may also be necessary to use a powered air purifying respirator (PAPR) to reduce the risk of inhalation. After use, protective clothing should be placed in sealed bags for appropriate decontamination, disposal or laundering. Disposable gloves should be worn to protect hands from contact with potentially contaminated samples. Wearing two pairs of gloves may be appropriate, but should not compromise needed dexterity.

Note: ***Gloves should be removed appropriately to avoid contaminating hands and surfaces between processing of each sample to prevent cross-contamination and disposed of whenever they become visibly contaminated or the integrity of the gloves is compromised. After all work with potentially infectious materials is completed, gloves should be removed and hands should be washed with soap and water.***

4.2.7 Depending on each laboratory's biosafety requirements, analysts may be required to be vaccinated prior to working with samples that could contain *B. anthracis*.

4.2.8 This protocol does not address all safety issues associated with its use. Please refer to BMBL, 5th Edition, CDC 2009. (Reference 16.1) for additional safety information. A reference file of Material Safety Data Sheets (MSDS) should be available to all personnel involved in analyses.

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5.0 Supplies and Equipment

Note: Refer to Appendix A for supplies and equipment for large volume drinking water sample processing.

5.1 General Laboratory Supplies

5.1.1 Gloves (e.g, latex, vinyl, or nitrile)

5.1.2 Sterile gloves (e.g., latex, vinyl, or nitrile)

5.1.3 Bleach wipes (Dispatch® Cat. No. 69150 or equivalent)

5.1.4 Wipes

5.1.5 Ziplock bags (large ~20" × 28", medium ~12" × 16", small ~7" × 8")

5.1.6 Sharps waste container

5.1.7 Absorbent pad

5.1.8 Medium and large biohazard bag(s) and rubber band(s)

5.1.9 Sterile scalpels

5.1.10 Sterile stainless steel scissors

5.1.11 Squeeze bottle with 70% isopropyl alcohol

5.1.12 Squeeze bottle with deionized (DI) water

5.1.13 Autoclave tape

5.1.14 Autoclave bags, aluminum foil, or kraft paper

5.1.15 Large photo-tray or similar tray for transport of racks

5.1.16 Laboratory marker

5.1.17 Timer

5.1.18 Disposable aerosol barrier pipet tips: 1000 µL, 200 µL, 10 µL (Rainin Cat. No. SR-L1000F, SR-L200F, GP-10F or equivalent)

5.1.19 1.5 mL Eppendorf Snap-Cap Microcentrifuge Biopur® Safe-Lock® tubes (Fisher Scientific Cat. No. 05-402-24B or equivalent)

5.1.20 50 mL conical tubes (Fisher Scientific Cat. No. 06-443-18 or equivalent)

5.1.21 15 mL conical tubes (Fisher Scientific Cat. No. 339650 or equivalent)

5.1.22 250 mL and 1 L filter systems, polyethersulfone (PES), 0.2 µm (Fisher Scientific Cat. No. 09-741-04, 09-741-03 or equivalent)

5.1.23 0.1 µm Ultrafree-MC, VV filter unit (Millipore Cat. No. UFC30VV00 or equivalent)

5.1.24 Tubes, sterile 2 mL DNase, RNase-free, gasketed, screw caps (National Scientific Cat. No. BC20NA-PS or equivalent)

5.1.25 Glass Petri dishes, 100 × 15 mm

5.1.26 Glass beads, acid washed, 106 µm and finer (Sigma Cat. No. G4649 or equivalent)

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5.1.27 Glass beads, acid washed, 425 – 600 µm and finer (Sigma Cat. No. G8772 or equivalent)

5.1.28 PCR 8 cap strips (VWR Cat. No. 83009-684 or equivalent)

5.1.29 Microcon® Centrifugal Filter Devices, Micron YM-100, blue Microcon® filters, (Amicon/Millipore Cat. No. 42413)

5.1.30 Wide mouth screw cap containers (Fisher Scientific Cat. No. 4-375-459 or equivalent)

5.1.31 Gauze wipes, 2" × 2" 50% rayon/50% polyester (Kendall™ Versalon™ Cat. No. 8042 or equivalent)

5.1.32 Air filters, 37 mm, Fluoropore™ (Millipore Cat. No. FSLW04700 or equivalent)

5.1.33 Swabs, macrofoam (VWR Cat. No. 10812-016 or equivalent)

5.1.34 Vacuum socks (Midwest Filtration Co. Cat. No. FAB-07-03-006PS or equivalent)

5.1.35 Vacuum filters (3M Forensic, Precision Data Products Cat. No. FF-1 with 4" diameter filter or equivalent)

5.1.36 Sponge-Stick sampling tools (3M Inc. Cat. No. SSL100 or equivalent).

5.2 Supplies for RV-PCR Analysis

5.2.1 30 mL screw cap tubes (E&K Scientific Cat. No. EK-T324S or equivalent)

5.2.2 Disposable nylon forceps (VWR Cat. No. 12576-933 or equivalent)

5.2.3 Monofilament polyester mesh disc (McMaster Carr Cat. No. 93185T17 or equivalent) or 2" × 2" cut squares from mesh sheets (McMaster Carr Cat. No. 9218T13 or equivalent)

5.2.4 Whatman™ Autocup™, filter cups (VWR Cat. No. 1602-0465 or equivalent)

5.2.5 Polyethylene caps, blue with pull-tabs (McMaster Carr Cat. No. 94075K56 or equivalent), for vortexing and incubation steps

5.2.6 50 mL Tube Cap (E&K Scientific, Cat. No. T3251-C or equivalent)

5.2.7 Polyethylene quick turn tube fittings (Ark-Plas Products Cat. No. 51525K365 or equivalent)

5.2.8 50 mL conical tubes, skirted, sterile (VWR Cat. No. 82050-322 or equivalent)

5.2.9 Disposable serological pipets: 25 mL, 10 mL, 5 mL

5.2.10 Single 50 mL conical tube holder (Bel-Art Cat. No. 187950001 or equivalent)

5.2.11 Screw cap tubes, 2 mL (VWR Cat. No. 89004-298 or equivalent)

5.2.12 96-well tube rack(s) for 2 mL tubes (8 × 12 layout) (Bel-Art Cat. No. 188450031 or equivalent)

5.2.13 2 mL Eppendorf tubes (Fisher Scientific Cat. No. 05-402-24C or equivalent)

5.2.14 96-well 2 mL tube rack (8 × 12 format) (Bel-Art Cat. No. 188450031)

5.3 Supplies for Real-time PCR Analysis

5.3.1 96-well PCR plates (ABI Cat. No. 4346906 or equivalent)

5.3.2 96-well plate holders, Costar®, black (VWR Cat. No. 29442-922 or equivalent)

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5.3.3 Edge seals for 96-well PCR plates (Adhesive Plate Sealers, Edge Bio Cat. No. 48461 or equivalent)

5.3.4 Foil seals for 96-well PCR plates (Polar Seal Foil Sealing Tape, E& K Scientific Cat. No. T592100 or equivalent) – for longer storage of the plates

5.3.5 Optical seals (ABI Cat. No. 4311971 or equivalent)

5.4 Supplies for Culture

5.4.1 Petri dishes, sterile, disposable, 100 × 15 mm

5.4.2 Inoculating loops and needles, sterile, disposable

5.4.3 Disposable cell spreaders (such as L-shaped, Fisher Scientific Cat. No. 03-392-150 or equivalent)

5.4.4 MicroFunnel™ Filter Funnels, 0.45 µm pore-size (VWR Cat. No. 55095-060 or equivalent)

5.4.5 Racks for 15 mL and 50 mL centrifuge tubes

5.4.6 Sterile, plastic, screw cap 50 mL centrifuge tubes (Becton, Dickinson and company [BD] Cat. No. 352070 or equivalent)

5.4.7 Sterile, plastic, screw cap 15 mL centrifuge tubes (BD Cat. No. 352097 or equivalent)

5.4.8 Pipet tips with aerosol filter for 1000 µL and 100 µL (Rainin Cat. No. SR-L1000F and GP-100F or equivalent)

5.4.9 Biotransport carrier (Nalgene® , Thermo Scientific Cat. No. 15-251-2 or equivalent)

5.5 Equipment

5.5.1 Biological Safety Cabinet (BSC) - Class II or Class III

5.5.2 PCR preparation hood (optional)

5.5.3 Shaker incubator for RV-PCR (Thermo Scientific, MaxQ™ 4000 Cat No. SHKE4000 or equivalent) and Universal 18" × 18" shaker platform (Thermo Scientific, MaxQ™ Cat. No. 30110)

5.5.4 Balance, analytical, with Class S reference weights, capable of weighing 20 g ± 0.001 g

5.5.5 Applied Biosystems® (ABI) 7500 Fast Real-Time PCR System (Life Technologies™)

5.5.6 Refrigerated centrifuge with PCR plate adapter and corresponding safety cups (Eppendorf Cat. No. 5804R, 5810R or equivalent) or PCR plate spinner (placed in BSC) (VWR, Cat. No. 89184-608 or equivalent)

5.5.7 Refrigerated micro-centrifuge for Eppendorf tubes with aerosol-tight rotor (Eppendorf Cat No. 5415R or equivalent)

5.5.8 Filter cup manifold-top and bottom for RV-PCR (DV Manufacturing. Cat. No. DVM-OSH-24 and DVM-BCP-24)

5.5.9 Allen wrench for manifold for RV-PCR (9/64 Hex key)

5.5.10 Capping tray for RV-PCR (DV Manufacturing Cat. No. DVM-LB-24) and screws to attach to shaker platform (DV Manufacturing)

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5.5.11 30 mL tube rack for RV-PCR (DV Manufacturing Cat. No. DVM-24 VC)

5.5.12 Vacuum pump with gauge (Cole Parmer® Model EW-07061-40 or equivalent) or vacuum source capable of < 10 pounds per square inch (psi)

5.5.13 Vacuum pump filters for pump (Acrovent™ Cat. No. 4249 or equivalent)

5.5.14 Vacuum trap accessories

5.5.15 Platform vortexer for RV-PCR (VWR Cat. No. 58816-115 or equivalent) with velcro straps

5.5.16 Single-tube vortexer (Fisher Scientific Cat. No. 02-215-365 or equivalent)

5.5.17 Heating block for RV-PCR (VWR Cat. No. 12621-096 or equivalent) and 2 mL tube blocks (VWR Cat. No. 12985-048 or equivalent) or water bath set at 95°C

5.5.18 Single-channel micropipettors (1000 µL, 200 µL,100 µL, 20 µL, 10 µL)

5.5.19 Serological pipet aid

5.5.20 Dynamag™ magnetic racks for RV-PCR (Invitrogen® Cat. No. 123-21D or equivalent)

5.5.21 Incubator(s), microbiological type, maintained at 37.0°C

5.5.22 Autoclave or steam sterilizer, capable of achieving 121°C (15 psi) for 30 minutes

5.5.23 Manifold incubator rack to hold up to 4 manifold/capping trays (DV Manufacturing Cat. No. 1701190**)** and/or peg kit for securing individual manifold/capping trays to the shaking incubator platform (Shaker Kit, DV Manufacturing Cat. No. 1701189) for RV-PCR

5.5.24 Cold block for 2 mL tubes (Eppendorf Cat. No. 3880 001.018 or equivalent)

5.5.25 Bead-beater (BioSpec Products, Inc. Cat. No. 693 [8 place] or 607 [16 place] or equivalent)

5.5.26 Tube racks, 80 place (VWR Cat. No. 30128-282 or equivalent)

5.5.27 40 kHz Sonicator bath (Branson Ultrasonic Cleaner Model 1510, Process Equipment and Supply, Inc. Cat. No. 952-116 or equivalent)

5.5.28 Stomacher® 400 Circulator (Seward Cat. No. 0400/001/AJ or equivalent) with closure bags (Cat. No. BA6141/CLR or equivalent) and rack (Cat. No. BA6091 [1 place] and BA6096 [10 place] or equivalent)

6.0 Reagents and Standards

6.1 Reagent-grade chemicals must be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (Reference 16.2). For suggestions regarding the testing of reagents not listed by the American Chemical Society, see '*AnalaR' Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K. (Reference 16.3); and *United States Pharmacopeia and National Formulary 24*, United States Pharmacopeial Convention, Md. (Reference 16.4).

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6.2 10X Wash Buffer for RV-PCR (250 mM KH_2PO_4, pH 7.4)

6.2.1 Composition:

KH_2PO_4	34 g
Reagent-grade water	~1 L

6.2.2 Dissolve KH_2PO_4 in 500 mL of reagent-grade water. Addition of NaOH is required if reagent-grade water has a low pH (pH ~5). Add 1 N NaOH to bring to pH 7.4 (≥ 100 mL of 1 N NaOH). Bring volume to 1 L with reagent-grade water. Filter sterilize using 250 mL, 0.22 µm PES filtering system. Store solution at 4°C until time of use for a maximum of 90 days.

6.3 1X Wash Buffer for RV-PCR (Low Salt Buffer)

6.3.1 Composition:

10X wash buffer	100 mL
Reagent-grade water	900 mL

6.3.2 Mix with magnetic stirrer: when mixed, measure pH. Final pH should be 7.4. Filter sterilize using a 1 L, 0.22 µm PES filtering system with disposable bottle. Store solution at 4°C until time of use for a maximum of 90 days.

6.4 Tween® 80 (Fisher Cat. No. T164 or equivalent)

6.5 Extraction Buffer with Tween® 80 for RV-PCR

6.5.1 Composition:

10X wash buffer	1 mL
200 proof ethanol	300 mL
Tween® 80	0.5 mL
Reagent-grade water	698.5 mL

6.5.2 Add 10X wash buffer, ethanol and Tween® 80 to 500 mL of reagent-grade water and mix well. Bring volume to 1 L with reagent-grade water; mix well. Filter sterilize using a 1 L, 0.22 µm PES filtering system with disposable bottle. Store solution at 4°C until time of use for a maximum of 90 days.

6.6 Extraction Buffer *without* Tween® 80 for RV-PCR

6.6.1 Composition:

10X wash buffer	1 mL
200 proof ethanol	300 mL
Reagent-grade water	699 mL

6.6.2 Add 10X wash buffer and ethanol to 500 mL of reagent-grade water mix well. Bring volume to 1 L with reagent-grade water; mix well. Filter sterilize using a 1 L, 0.22 µm PES filtering system with disposable bottle. Store solution at 4°C until time of use for a maximum of 90 days.

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6.7 High Salt Wash Buffer for RV-PCR

6.7.1 Composition:

KH_2PO_4	28.2 g
Reagent-grade water	1 L

6.7.2 Dissolve KH_2PO_4 in 500 mL of reagent-grade water. Addition of NaOH is required if reagent-grade water has low pH (pH ~5). Add 1 M NaOH to bring to pH 6.0 (≥ 100 mL of 1 M NaOH). Bring volume to 1 L with reagent-grade water. Filter sterilize using a 1 L, 0.22 µm PES filtering system with disposable bottle. Store solution at 4°C until time of use for a maximum of 90 days.

6.8 70% Ethanol for RV-PCR - Aseptically mix 70 mL of ethanol (100%) with 30 mL of sterile PCR-grade water. Dispense into 2 – 3, sterile 50 mL conical tubes and store at 4°C, for a maximum of one week. Unopened tubes may be stored for up to one month at 4°C.

6.9 PCR-grade water, sterile (Teknova Cat. No. W3350 or equivalent)

6.10 Phosphate buffered saline Tween® 20 (PBST) buffer for RV-PCR (Teknova Cat. No. P0201 or equivalent)

6.11 PBST buffer with 0.005% Anti-Foam reagent (Section 6.14) for Ultrafiltration secondary water sample processing; prepare aseptically mix well by vortexing before use.

6.12 0.1 M Sodium Phosphate/10 mM EDTA buffer/0.01% Tween-20, pH = 7.4 (Teknova Cat. No. S2216 or equivalent)

6.13 TE buffer (1X Tris-HCl-EDTA [Ethylenediaminetetraacetic acid]) buffer, pH 8.0 (Fisher Scientific Cat. No. BP2473-500 or equivalent)

6.14 Promega reagents for DNA extraction and purification procedure for RV-PCR:

- MagneSil® Blood Genomic, Max Yield System, Kit (Promega Cat. No. MD1360; VWR Cat. No. PAMD1360)
- Salt Wash (VWR Cat. No. PAMD1401 or equivalent)
- Magnesil Paramagnetic Particles (PMPs) (VWR Cat. No. PAMD1441 or equivalent)
- Lysis Buffer (VWR, Cat. No. PAMD1392 or equivalent)
- Elution Buffer (VWR Cat. No. PAMD1421 or equivalent)
- Alcohol Wash, Blood (VWR Cat. No. PAMD1411 or equivalent)
- Anti-Foam Reagent (VWR Cat. No. PAMD1431 or equivalent)

6.15 TaqMan® Fast Advanced PCR Master Mix (Life Technologies™ Cat. No. 4444557)

6.16 Primers and probe for EPA-2 PCR assay targeting the *cap*B gene on the pXO2 plasmid (Francy *et al.,* 2009 [Reference 16.5])

- Forward Primer (BA-EPA-2F) – 5'-TGCGCGAATGATATATTGGTTT-3'
- Reverse Primer (BA-EPA-2R) – 5'-GCTCACCGATATTAGGACCTTCTTTA-3'
- Probe (BA-EPA-2Pr) – 5'-6FAM-TGACGAGGAGCAACCGATTAAGCGC-BHQ1-3'

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6.17 Optional Real-time PCR Assays

EPA-1 targeting the *pag*A gene on pXO1 plasmid (Francy *et al.,* 2009 [Reference 16.5])

- Forward Primer (BA-EPA-1F) – 5'-GCGGATAGCGGCGGTTA-3'
- Reverse Primer (BA-EPA-1R) – 5'-TCGGTTCGTTAAATCCAAATGC-3'
- Probe (BA-EPA-1Pr) –

  5'-6FAM-ACGACTAAACCGGATATGACATTAAAAGAAGCCCTTAA-BHQ1-3'

BC3 targeting a hypothetical gene on the chromosome of *B. anthracis*

- Forward Primer (BA-BC3-F) – 5'-TTTCGATGATTTGCAATGCC-3'
- Reverse Primer (BA-BC3-R) – 5'-TCCAAGTTACAGTGTCGGCATATT-3'
- Probe (BA-BC3-Pr) – 5'-6FAM-ACATCAAGTCATGGCGTGACTACCCAGACTT-BHQ1-3'

6.18 Trypticase™ Soy Agar with 5% Sheep Blood (SBA)

6.18.1 The use of commercially prepared media plates is recommended (VWR Cat. No. 90001-276 or 90001-282 or equivalent), however dehydrated media (BBL™ Cat. No. 227300 or equivalent), with the addition of sheep blood (Oxoid Cat. No. SR0051 or equivalent), may be used. If commercially prepared media are not available, prepare media using procedures in Sections 6.17.2 − 6.17.4.

6.17.2 Composition:

Tryptone H	15 g
Soytone	5 g
Sodium chloride	5 g
Agar	12 g
Sheep blood	50 mL
Reagent-grade water	~900 mL

6.18.3 Add reagents except sheep blood to 850 mL of reagent-grade water and mix thoroughly using a stir bar and hot plate. Boil for 1 minute with rapid stir bar agitation to dissolve completely. Adjust pH to 7.3 ± 0.2 with 1.0 N HCl or 1.0 N NaOH and bring to 950 mL with reagent-grade water. Autoclave at 121°C (15 psi) for 15 minutes. Do not overheat. Cool to 45°C – 50°C in a water bath.

6.18.4 Prepare plates by aseptically adding 50 mL of sterile sheep blood (5.0% final concentration) to the cooled media and mix well. Aseptically pour 12 – 15 mL into each 15 × 100 mm sterile Petri dish. Store at 4°C for a maximum of two weeks.

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6.19 Brain Heart Infusion Broth for RV-PCR (BHI broth, Fisher Scientific Cat. No. DF0037-15-0)

6.19.1 Composition:

Calf brains, infusion from 200 g	7.7 g
Beef heart, infusion from 250 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride	5.0 g
Disodium phosphate (Na_2HPO_4)	2.5 g
Dextrose	2.0 g
Reagent-grade water	1.0 L

6.19.2 Add reagents to 1 L of reagent-grade water, mix thoroughly and heat to dissolve completely. Autoclave at 121°C (15 psi) for 15 minutes. Final pH should be 7.4 ± 0.2. Store at 4°C for a maximum of three months in screw cap containers.

6.20 10% Bleach-pH amended (prepared daily)

6.20.1 Prepare bleach solution by adding 1 part bleach, 1 part acetic acid and 8 parts reagent-grade water as described below.

6.20.2 Add 2 parts water to 1 part bleach, then add 5% acetic acid (1 part) and remaining water (6 parts). Measure pH and add bleach (to increase pH) or acetic acid (to decrease pH) as needed to obtain a final pH between 6 and 7. A pH meter should be used to measure pH as opposed to pH strips or kit. When mixed, place a lid on the mixture to reduce chlorine escape and reduce worker exposure.

7.0 Calibration and Standardization

7.1 Check temperatures in incubators twice daily with a minimum of four hours between each reading to ensure operation within stated limits. Record the temperature in a log book.

7.2 Check temperature in refrigerators/freezers at least once daily to ensure operation is within stated limits of the method. Record daily measurements in a refrigerator/freezer log book.

7.3 Check thermometers including those on instrumentation (e.g., digital display) at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check columns for breaks.

7.4 Calibrate pH meter prior to each use with two of three standards (e.g., pH 4.0, 7.0 or 10.0) closest to the range being tested.

7.5 Micropipettors should be calibrated at least annually and tested for accuracy on a weekly basis.

7.6 Follow manufacturer instructions for calibration of real-time PCR instruments.

7.7 Re-certify BSCs annually. Re-certification must be performed by a qualified technician.

7.8 Autoclave maintenance should be conducted at least annually. Autoclave temperature and total sterilization cycle time should be checked on a quarterly basis. Record the data in a log book. Spore strips or spore ampules should be used monthly as bioindicators to confirm sterilization.

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7.9 Refrigerated centrifuges should be checked to confirm temperature and revolutions per minute (rpm) on a quarterly basis. Record the data in a log book.

8.0 Quality Control (QC)

8.1 Each laboratory that uses this protocol is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. International Organization for Standardization (ISO)/International Electrotechnical Commission (IEC) 17025 (International Standard: General requirements for the competence of testing and calibration laboratories, Section Edition 2005-05-15) provides a quality framework that could be used to develop a formal QA program.

8.2 Sample integrity — Samples should be checked for integrity (e.g., improperly packaged, temperature exceedance, leaking). Samples may be rejected if the integrity has been compromised. Alternately, if sample integrity has been compromised it may be analyzed and the data qualified and marked accordingly (e.g., sample exceeded temperature during transport - data is flagged and marked as exceeding temperature), so that a decision can made regarding whether the data should be considered valid/invalid.

8.3 Analyst qualifications — Only those analysts that have been trained and have demonstrated proficiency with these analytical techniques should perform this procedure.

8.4 Proficiency testing (PT) — The laboratory should have analysts analyze test samples annually at a minimum to ensure they are maintaining proficiency. In addition, analysts should analyze PT samples to demonstrate proficiency prior to analyzing field samples. For laboratories not routinely using this protocol, analysts should analyze PT samples biannually. In the event of a PT failure the laboratory should identify and resolve any issues and then request and analyze additional PT samples. Field samples should not be analyzed until the laboratory passes the PT.

8.5 Media sterility check — The laboratory should test media sterility by incubating a single unit (tube or Petri dish) from each batch of medium (BHI broth or SBA) at 37ºC ± 2ºC for 24 ± 2 hours and observe for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform a media sterility check every day that samples are analyzed.

8.6 PCR: Positive control (PC) — The laboratory should analyze a PC in triplicate reactions with each PCR run. Prepare the PC at a concentration of 50 pg of purified *B. anthracis* total DNA per 5 µL of PCR-grade water. All PCs should result in a $C_T \leq 40$ and replicates should be within ± 1 C_T of each other.

8.7 Culture: Positive control (PC) — The laboratory should analyze PCs (known quantity of viable spores) to ensure that all media and reagents are performing properly. PCs should be analyzed whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory should run a PC every day that samples are analyzed.

8.8 External inhibition control (EIC) 50 pg genomic DNA from *B. anthracis* Ames — The laboratory should analyze an EIC for site characterization phase sample DNA extracts to determine if the matrix is causing inhibition potentially resulting in false negative results. Prepare the EIC at a concentration of 50 pg of purified *B. anthracis* DNA per 1 µL of PCR-grade water. Using a 10 µL pipettor, carefully add 1 µL of the DNA to the EIC wells on a PCR plate and then add 5 µL of sample DNA extract to each well and mix thoroughly. The PCR results from the PC and EICs

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(both containing 50 pg of *B. anthracis* DNA) are then compared. Lower or similar C_T values for the EIC indicate there is no inhibition. A higher C_T value for the EIC (>3 C_T values) is indicative of matrix inhibition.

Note: To minimize cross contamination the EICs should not be placed next to the field samples when setting up the PCR plate.

8.9 No template controls (NTC) — The laboratory should analyze NTCs (5 µL of PCR-grade water is added to the NTC wells on a PCR plate in place of the DNA or the sample DNA extract) to ensure that reagents are not contaminated. On an ongoing basis, the laboratory should analyze NTCs in triplicate PCR reactions with each PCR run. The negative controls (NTCs) must not exhibit fluorescence above the background level (i.e., no C_T value). If C_T values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis.

8.10 Field blank — The laboratory should request that the sampling team provide a field blank with each batch of samples. A field blank is defined as either a sample collection tool (e.g., wipe, swab) or sterile reagent-grade water that is taken out to the field, opened and exposed to the environment, but not used to collect a sample, and then placed in a bag and sealed and transported to the laboratory along with the field samples. The field blank is treated as a sample in all respects, including exposure to sampling location conditions, storage, preservation and all analytical procedures. Field blanks are used to assess any contamination contributed from sampling location conditions, transport, handling and storage. The laboratory should process and analyze this control along with each batch of environmental samples. The field blanks should not exhibit fluorescence (i.e., no C_T value).

8.11 Sample processing negative control (PNC) or method blank — The laboratory should process and analyze a PNC in the same manner as a sample to verify the sterility of equipment, materials and supplies. Absence of growth indicates lack of contamination from the target organism. Please refer to **Table 1** for appropriate PNC.

Table 1. Sample Processing Negative Controls

Matrix	PNC
Wipes	Clean (unused) wipe
Swabs	Clean (unused) swab
Vacuum socks/filters	Clean (unused) vacuum sock/filter
Air filters	Clean (unused) air filter
Sponge-Sticks	Clean (unused) Sponge-Stick
Drinking water and decontamination waste water	100 mL of sterile reagent-grade water

8.12 For RV-PCR based analysis, the T_0 and T_9 extracts are analyzed (in triplicate). PCR positive and negative controls must be analyzed using the same preparation of the PCR Master Mix and must be run on the same 96-well plate as the T_0 and T_9 extracts.

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9.0 Site Characterization Procedures

For sample analyses during the site characterization phase of the response to an anthrax event, only real-time PCR based analytical procedures will be used. Accordingly, this section includes appropriate sample processing and real-time PCR procedures.

Acceptable sample types: Gauze wipes (2" × 2" 50% rayon/50% polyester), air filters (37 mm), swabs (macrofoam), vacuum socks (large dust collection sample bags, 9 1/8" × 4", mean pore size 6.7 µm), vacuum filters (4" diameter filter), Sponge-Stick sampling tools, drinking water and decontamination waste water.

Note: For laboratories with RV-PCR capability please follow the sample processing procedures for spore recovery and concentration as outlined below (Section 9.1). For all other laboratories, either the same procedures (Section 9.1) could be followed or those described for culture analyses as outlined in Sections 11.1 – 11.5 could be used.

9.1 Sample Processing for Laboratories With RV-PCR Capability

Note: Sterile gloves should be used and changed between samples and as indicated below.

Prepare monofilament polyester mesh (Section 5.2.3) supports by cutting 2" × 2" squares using sterile scissors and place squares into a clean ziplock bag. Since the supports are not sterilized prior to use, ensure that the working surface has been disinfected and sterile gloves are worn during the process.

Fill sample tube rack with 30 mL or 50 mL screw cap conical tubes as appropriate. All sample types (except water samples) may be placed behind a mesh support in the tube to prevent interference from pipetting activities and to improve efficiency of spore extraction during vortexing. Using two pairs of sterile forceps, coil the mesh support and then grasp both ends of the coil with one pair of forceps. Place the support into the tube by holding the sample to the side of the tube with one pair of sterile forceps and placing the coiled mesh support on top with the other set of forceps.

9.1.1 *Wipe and Air Filter Samples*

Place mesh support over wipe or air filter samples in 30 mL tube by holding the wipe or air filter to the side of the tube with sterile forceps and placing the coiled mesh support on top (Section 9.1). Ensure the sample and mesh are in the bottom half of the tube (avoiding the conical portion). Change gloves in between each sample. The support keeps the wipe or air filter from interfering with pipetting activities and also improves efficiency of spore extraction during vortexing. Proceed to Section 9.1.7.

9.1.2 *Vacuum Samples (Socks and Filters)*

For vacuum socks, using sterile scissors, cut the top blue portion off and discard prior to folding the sock from the top opening down. Make four 1/2" folds in order to reduce the sock size to about 1" × 2". Cut the sock five times above the 30 mL tube using scissors, making sure to cut through all the folds. Place cut, folded sock in bottom half of tube (avoiding conical portion) and using two pairs of sterile forceps, place the coiled mesh support on top of sock (Section 9.1). Bleach the BSC working surface and don a fresh pair of gloves in between samples. Proceed to Section 9.1.7.

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Note: ***The sample processing procedure provided is for up to 1 tablespoon (~1 g of test dust) of debris per sock sample. If socks received contain higher levels of debris, follow procedure in Section 11.4.1.***

For vacuum filters, ensure that the exposed filter surface (with debris) is facing up and carefully cut through the evidence tape with a sterile scalpel in order to remove the top of the cartridge. Using a pair of sterile forceps, transfer large pieces of debris into the appropriate 50 mL tube, then fold filter in half with dirty, exposed filter side in, and then fold in half again in order to fit it into the 50 mL tube. Place folded filter in bottom half of tube (avoiding conical portion) and using two pairs of sterile forceps place the coiled mesh support on top of filter (Section 9.1). Proceed to Section 9.1.7.

9.1.3 ***Sponge-Stick Sampling Tools***

- If the Sponge-Sticks/wipes are not in Stomacher® bags, aseptically transfer each sample to a Stomacher® bag using sterile forceps. Change forceps between samples.
- Add 90 mL of phosphate buffered saline with Tween® 20 (PBST) to each bag. Set Stomacher® (Section 5.5.28) to 260 rpm.
- Place a bag containing a sample into the Stomacher® (Section 5.5.28) so the Sponge-Stick/wipe rests evenly between the homogenizer paddles and stomach each sample for 1 minute.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the wipe from the outside of the bag with hands. With the bag closed, move the Sponge-Stick/wipe to the top of the bag while using hands to squeeze excess liquid from the Sponge-Stick/wipe.
- Open the bag, remove and discard the Sponge-Stick/wipe using sterile forceps.
- Repeat steps described above for each sample.
- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- Gently mix the elution suspension in the Stomacher® bag up and down 3 times with a sterile 50 mL pipet. Remove half of the suspension volume (~45 mL) and place it in a 50 mL screw capped centrifuge tube. Place the remaining suspension (~45 mL) into a second 50 mL tube.
- Record suspension volumes on tubes and data sheet.
- Process elution suspension for each sample as described above.
- Place 50 mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.
- Centrifuge tubes at 3500 × g, with the brake off, for 15 minutes in a swinging bucket rotor.

Note: ***A higher × g is preferred as long as the speed is within the tube specifications.***

- Remove the supernatant from each tube with a 50 mL pipet and discard leaving approximately 3 mL in each tube. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the tube bottom. Use a sterile 50 mL pipet for each sample.
- Set the vortexer (Section 5.5.16) to high intensity. Set the sonicator water bath to high.

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- Vortex the tubes for 30 seconds and transfer the tubes to the sonicator bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles twice.
- Remove suspension from one tube with a sterile 5 mL pipet and combine it with the suspension in the other tube from the same sample. Measure final volume of suspension with 5 mL pipet and record the result on the tube and data sheet.
- Repeat vortexing and sonication steps for each sample.
- Proceed to Section 9.1.19.

9.1.4 ***Swabs***

Place swab into the 30 mL tube and cut handle with sterile scissors if necessary to fit into the tube. Using sterile forceps, place the coiled mesh support over the swab (Section 9.1). Proceed to Section 9.1.7.

9.1.5 ***Water Samples (Large Volume [10 L – 100 L], Drinking Water)***

Please see Appendix A for primary (Section 2.0) and secondary (Section 3.0) concentration of large volume (10 L – 100 L) water samples. For water samples < 10 L, please refer to Appendix A, Section 3.0, secondary concentration.

- Add 15 mL of PBST buffer to the 50 mL conical tube with membrane (Appendix A, Section 3.5).
- Set vortexer (Section 5.5.16) to high intensity.
- Vortex membrane in 10 second bursts for 2 minutes to dislodge spores.
- Remove membrane and centrifuge at 3500 × g, with the brake off, for 30 minutes at 4°C.

Note: ***A higher × g is preferred as long as the speed is within the tube specifications.***

- Remove 12 mL of the supernatant without disturbing/dislodging the pellet; resuspend the pellet by vortexing in the remaining volume.
- Use a 0.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.2.

9.1.6 ***Water Samples (Small Volume [< 50 mL], Surface or Drinking Water)***

- Transfer 30 mL of water sample into a 50 mL screw cap conical tube.
- Add 10 mL of PBST buffer (Section 6.10) and mix by vortexing for 30 seconds.
- Centrifuge at 3500 × g, with the brake off, for 30 minutes at 4°C.

Note: ***A higher × g is preferred as long as the speed is within the tube specifications.***

- Remove 37 mL of the supernatant without disturbing/dislodging the pellet. The volume of supernatant remaining should not be below the conical portion of the tube. Resuspend the pellet by vortexing in the remaining volume.
- Use a 0.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.2.

9.1.7 Add 20 mL (5 mL for swabs) of cold (4°C) extraction buffer with Tween® 80 to environmental samples (Sections 9.1.1, 9.1.2, 9.1.4) placed in 30 mL tubes in tube rack. Use a new serological pipet to transfer buffer from a sterile, 250 mL screw capped bottle to each tube (keep bottle cap loosely over opening between transfers). Uncap one tube at

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a time, add 20 mL extraction buffer with Tween® 80, close tube, and place it back in tube rack. Repeat for each sample tube. Check that all caps are on tubes securely. Label tubes, as appropriate, and document location in rack.

9.1.8 Place tube rack in plastic bag, seal, bleach bag and double bag, prior to transferring to platform vortexer (outside BSC).

9.1.9 Vortex samples for 20 minutes on platform vortexer (Section 5.5.15), with speed set to 7.

9.1.10 After vortexing, transfer sample tube rack to BSC. Remove tube rack from plastic bag.

9.1.11 Vortex one sample tube on single-tube vortexer (Section 5.5.16), in the BSC, for 3 – 5 seconds. For samples containing large amounts of debris, let sample sit for 30 seconds to allow large particles to settle prior to aliquoting.

9.1.12 Uncap tube. Using a 25 mL serological pipet, transfer as much liquid volume as possible (while avoiding any particles) to a fresh appropriately labeled 50 mL conical tube and place in tube rack. Dispose pipets in waste container. Cap sample tube and place tube back in rack. Change gloves.

9.1.13 Repeat Sections 9.1.11 − 9.1.12 for each sample tube.

9.1.14 Perform second spore extraction. Uncap one sample tube at a time.

9.1.15 Add 14 mL (5 mL for swabs) of cold (4°C) extraction buffer without Tween® to each sample tube, one at a time, with a new 25 mL serological pipet and a fresh pair of gloves for each sample. Keep buffer bottle loosely covered between transfers. Recap sample tube after buffer addition.

9.1.16 After adding extraction buffer to all tubes, check that all caps are on securely. Place rack in plastic bag, seal and bleach bag. Transfer bagged tube rack to platform vortexer (Section 5.5.15) outside BSC.

9.1.17 Vortex rack for 10 minutes, with speed set to 7.

9.1.18 Repeat Sections 9.1.11 – 9.1.12.

9.1.19 Centrifuge the 50 mL conical tube containing the combined suspension at 3500 × g, with the brake off, for 30 minutes at 4°C.

Note: A higher × g is preferred as long as the speed is within the tube specifications.

9.1.20 Leaving approximately 3 mL in the tube, carefully discard the supernatant using a serological pipet without disturbing/dislodging the pellet. Ensure that the volume of liquid remaining is not below the conical portion of the tube. Resuspend the pellet by vortexing.

9.1.21 Add 25 mL of Phosphate/EDTA/Tween buffer (Section 6.11) and mix the suspension by vortexing.

9.1.22 Centrifuge the suspension at 3500 × g, with the brake off, for 30 minutes at 4°C.

Note: A higher × g is preferred as long as the speed is within the tube specifications.

9.1.23 Carefully discard 22 mL of supernatant without disturbing/dislodging the pellet. Resuspend the pellet by vortexing in the remaining volume.

9.1.24 Use a 0.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.2.

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9.2 DNA Extraction and Purification

9.2.1 Using the 8 cap strips, transfer one level capful (~50 mg) of the 106 µm glass beads and one level capful (~50 mg) of the 425 – 600 µm glass beads (using a clean strip of caps between bead sizes) into each gasketed, capped bead-beating tube.

9.2.2 In the BSC, pipet 0.5 mL of the suspension (Section 9.1.23) into pre-labeled, gasketed, capped bead-beating 2 mL tube containing glass beads. Replace cap on tube securely. Wipe outside of tube with bleach wipe.

9.2.3 Insert tube in tube holder of the bead-beater set at 4800 rpm for 3 minutes (180 seconds). Instrument settings: Speed = 48; Time = 18. Press start. Bead-beating disrupts the cells and spores to release the DNA.

9.2.4 Remove tube from bead-beater (tube will be warm), place in a cold block for 2 minutes (or until cool to touch). If tube leaks during bead-beating, wipe tube and bead-beater thoroughly with bleach wipe.

9.2.5 Supernatant separation and transfer

- Set up tubes; label one, 1.5 mL microcentrifuge tube, one yellow-top filter unit, two blue Microcon® filter inserts and five blue Microcon® collection tubes with sample ID.
- In BSC, centrifuge the screw capped tube containing sample at 7000 rpm for 1 minute in a microcentrifuge to pellet beads and particulate matter.
- Using a micropipettor, carefully transfer supernatant to yellow top filter collection tube (try to avoid beads and particulate matter at bottom of tube).
- Centrifuge at 7000 rpm for 4 minutes.
- Open tube; remove the yellow top filter with sterile disposable forceps, gripping only on the sides. Transfer filtrate to blue Microcon® filter insert. Do not transfer any particulate matter that may be evident at bottom of tube. Replace filter and cap tube.
- Centrifuge at 7000 rpm for 1 minute.
- Remove the blue Microcon® filter insert with disposable forceps (gripping only the sides) and transfer to a new collection tube.

9.2.6 First wash

- Add 400 µL of 1X TE buffer to the filter.
- Centrifuge at 7000 rpm for 2 minutes.
- Carefully remove retentate from the top of the blue Microcon® filter insert, avoiding any particulate matter visible on filter surface (tilt the tube for better viewing) and transfer liquid into a new blue Microcon® filter insert and collection tube.

9.2.7 Second wash

- Add 400 µL 1X TE buffer to the filter.
- Centrifuge at 7000 rpm for 2 minutes.
- Transfer blue Microcon® filter insert with disposable forceps (gripping only the sides) to a new collection tube.

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9.2.8 Third wash

- Add 400 µL 1X TE buffer to the filter.
- Centrifuge at 7000 rpm for 2 minutes.
- Transfer blue Microcon® filter insert with disposable forceps (gripping only the sides) to a new collection tube.

9.2.9 Fourth wash

- Add 400 µL PCR-grade water to the filter.
- Centrifuge at 7000 rpm for 1 minute.
- Check fluid level in blue Microcon® filter insert. If above 200 µL, pulse spin for about 10 seconds (or less) until about 100 µL of fluid is retained on top of white base.

9.2.10 Sample retrieval from the filter unit

- Use a micropipettor to carefully remove all of the retentate from the blue Microcon® filter insert and transfer into clean, labeled 1.5 mL microcentrifuge tube. Avoid any particulate matter.
- If there is less than 100 µL of extract, transfer extract back to the same blue Microcon® filter insert and add 100 µL PCR-grade water and pulse spin to obtain about 100 µL on filter. Transfer retentate into clean, labeled 1.5 mL microcentrifuge tube.
- Using clean gloves, place tubes containing filter extracts in DNA loading station/hood in preparation for PCR analyses (Section 9.3).

9.3 Real-time PCR Analyses

As compared to traditional PCR, real-time PCR uses a sequence-specific hybridization probe sequence internal to the amplification primers, in addition to two target gene-specific amplification primers. The probe is fluorescently labeled at the 5' end with a reporter dye/fluorophore and at the 3' end with a quencher dye (usually, Black Hole Quenchers). The emission of light/fluorescence by the reporter dye is normally quenched by virtue of its proximity to the quencher dye. At the annealing step in a PCR, along with the amplification primers, depending upon its orientation, the probe sequence also hybridizes to its target site on the DNA strand downstream from the binding site of one of the primers. During the enzymatic extension step when the probe comes in the way of Taq DNA polymerase enzyme, the 5' exonuclease activity of the enzyme hydrolyzes the probe sequence by cleaving individual nucleotides from the 5' end. Cleavage of the probe releases the reporter dye from the proximal quencher allowing emission of measurable fluorescence. Therefore, this assay is also known as the 5' exonuclease assay as it relies on the 5' to 3' exonuclease activity of the Taq DNA polymerase enzyme to hydrolyze the probe. Thus, the PCR amplification of a specific gene sequence can be detected by monitoring the increase in fluorescence (**Figure 1**).

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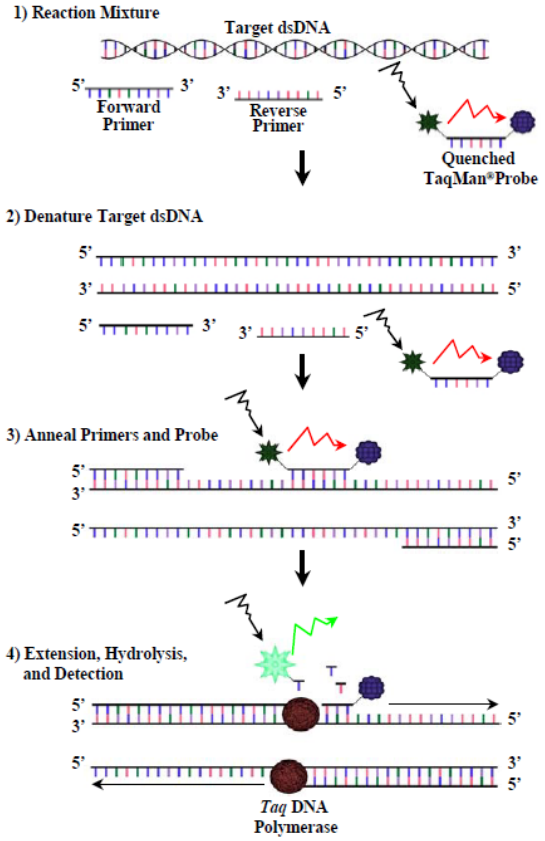

Figure 1. PCR Amplification.

As the amplification reaction proceeds, more amplicons become available for probe binding and hydrolysis, and consequently, the fluorescence signal intensity per cycle increases. The increase in fluorescence can be detected in real time on PCR thermocyclers. When the fluorescence level crosses a set threshold value at a certain cycle number during the PCR, the result indicates the presence of the target gene sequence in the DNA in the sample, which in turn indicates the presence of a target pathogen in the sample. The PCR can specifically amplify a single copy of target gene sequence and generate millions of copies in a matter of minutes.

The TaqMan® fluorogenic probe hydrolysis-based real-time PCR assays are commonly used in biodetection. Using established computer software (e.g., Primer Express) and genome sequence databases, bioagent-specific primers and probe nucleotide sequences for these assays are selected in such a way that they are present only in a specific location on the unique gene and/or virulence factor gene of interest for the detection and identification of a specific pathogen. These primers and probe sequences are absent in any other gene of that pathogen or in the genes of any near neighbor organisms. The primers generate a PCR product (amplicon) of a definite length/size. For a high-confidence identification of pathogens, PCR assays for multiple pathogen-specific genes are usually used. For example, for detection of *B. anthracis*, real-time PCR assays generally target three separate genes. They include one gene each on pXO1 and pXO2 plasmids (usually targeting virulence genes) and one gene on the chromosome/genome. An algorithm based on the positive detection of all three gene targets in a sample indicates the presence of virulent *B. anthracis* spores. However, since this protocol is developed for the remediation phase of the response where the *B. anthracis* strain has usually been identified and characterized, real-time

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PCR using only the EPA-2 assay targeting the capsular antigen gene on the pXO2 plasmid is recommended. Although, if needed, all three assays or any combination thereof can be performed using the same sample DNA extract. Accordingly, the primers and probe sequences, and PCR conditions for the two optional assays, EPA-1 (targeting the protective antigen gene on the pXO1 plasmid) and BC3 (targeting a hypothetical gene on the *B. anthracis* chromosome) are also included.

The real-time PCR assays included in this protocol have been only partially characterized for specificity, however, the use of these assays is currently recommended. These assays will be replaced with fully characterized and validated assays upon availability.

Note: ***This procedure is to be carried out in an area designated for PCR only. A PCR-workstation that is equipped with an ultraviolet (UV) light for sterilization must be used for PCR Master Mix preparation. Micropipets and corresponding sterile, aerosol-resistant pipet tips are used throughout this procedure for the addition of reagents. Aseptic technique must be used throughout and all reagents must be kept at or near 4°C.***

9.3.1 Decontaminate the PCR workstation by treating all work surfaces with a 10% pH amended bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. Turn on UV light for 15 minutes. After decontamination, discard gloves and replace with a new clean pair.

Note: ***If gloves become contaminated, they should be disposed of and fresh gloves donned. Only open one tube at a time throughout the process. At no point, should more than one tube be open. Do not allow hands (gloved or otherwise) to pass over an open tube, PCR plate, or any reagent container. All used pipet tips, gloves and tubes must be discarded in a biohazard autoclave bag.***

9.3.2 Determine the number of reactions that are to be run. Include four replicate reactions each (for each assay) for a NTC, PC and three replicates of the PNC (blank) per run. In addition, include three reactions for each sample and two reactions for the EIC for each sample. Prepare a sufficient volume of Master Mix to allow for one extra reaction for every ten reactions, so that there is enough Master Mix regardless of pipetting variations. *For example, if ten samples are to be analyzed a total of 61 reactions would be included in the run [4-NTC, 4-PC, 3-PNC, 30-samples and 20-EICs]. The amount of PCR Master Mix prepared should be sufficient to run 70 reactions.*

9.3.3 Based on the example provided above (i.e., 10 samples) the amount of Master Mix required would be as indicated in **Table 2**.

Table 2. Example EPA-2 PCR Assay Master Mix Preparation for 70 Reactions

Reagent	Volume (µL)	Total Volume (µL)	Final Concentration (µM)
TaqMan® 2X Universal Master Mix	12.5	875	1X
Forward primer, 25 µM	0.3	21	0.3
Reverse primer, 25 µM	0.3	21	0.3
Probe, 2 µM	1	70	0.08
PCR-grade water	5.9	413	N/A
Total Volume	20	1400	

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Optional PCR Assays

If required the Master Mix for the optional assays should be prepared according to **Table 3** based on the example provided in Section 9.3.2.

Table 3. Example EPA-1 and BC3 PCR Assay Master Mix Preparation for 70 Reactions

Reagent	Volume (µL)	Total Volume (µL)	Final Concentration (µM)
TaqMan® 2X Universal Master Mix	12.5	875	1X
Forward primer, 25 µM	1	70	1.0
Reverse primer, 25 µM	1	70	1.0
Probe, 2 µM	1	70	0.08
PCR-grade water	4.5	315	N/A
Total Volume	20	1400	

Note: ***The PC and NTC controls must be analyzed prior to sample analyses to verify that the Master Mix works properly and is free of contamination.***

9.3.4 In a clean PCR-preparation hood, pipet 20 µL of Master Mix to four wells of the PCR plate. Label two wells as NTC and two as PC.

9.3.5 Add 5 µL of PCR-grade water into the NTC wells.

9.3.6 Cover the plate with edge seal and transfer the PCR plate to the BSC. Remove the seal and add 5 µL of the PC (*B. anthracis* DNA [10 pg/µL]) to the PC wells.

Note: ***This step must be performed in the BSC outside the PCR clean room set-up area. Change gloves.***

9.3.7 Seal PCR plate with optical seal, using plate sealer for good contact. Change gloves.

9.3.8 Centrifuge sealed PCR plate for 1 minute at 2000 rpm and 4ºC, using the PCR plate safety cups or mini-plate centrifuge in the BSC.

9.3.9 Open the centrifuge safety cup and transfer PCR plate to the ABI 7500 Fast thermocycler.

9.3.10 The PCR cycling conditions on the ABI 7500 Fast include an initial cycle of 50ºC for 2 minutes, followed by one cycle of 95ºC for 10 minutes, followed by 45 cycles of 95ºC for 5 seconds, and 60ºC for 20 seconds (use Fast Temperature Ramp rate: 3.5ºC/s up and 3.5ºC/s down). Fluorescence is automatically measured at the end of the 60ºC annealing-extension combined step.

9.3.11 If the Master Mix test results show "True Positive" assay detection for the PC and "True Negative" assay detection for the NTC, then proceed with analyses of samples. If the results are not "True" then repeat the PCR Master Mix preparation and testing protocol and reanalyze.

9.3.12 In a clean PCR-preparation hood, pipet 20 µL of Master Mix into the required number of wells of a new PCR plate (as per the number of samples to be analyzed). An eight channel micropipettor can be used to add the Master Mix to the plate. Label two wells as NTC and two as PC. Label the rest of the wells such that there are five wells for each sample (three wells for actual sample analyses and two wells for EICs for each sample).

9.3.13 Add 5 µL of PCR-grade water into the NTC wells.

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9.3.14 Cover the plate with edge seal and transfer the PCR plate to the BSC. Remove the seal and add 5 µL of the PC (*B. anthracis* DNA [10 pg/µL]) to the PC wells.

Note: This step must be performed in the BSC outside the PCR clean room set-up area. Change gloves.

9.3.15 Add 5 µL of the PNC extract to the three PNC wells.

9.3.16 Add 5 µL of each sample DNA extract to the sample and EIC wells.

9.3.17 Add 1 µL of the PC (*B. anthracis* DNA [**50** pg/µL]) to all the EIC wells.

Note: To minimize cross contamination the EICs should not be placed next to the field samples when setting up the PCR tray.

9.3.18 Seal PCR plate with optical seal, using a plate sealer for good contact. Change gloves.

9.3.19 Centrifuge sealed PCR plate for one minute at 2000 rpm and 4ºC, using the PCR plate safety cups or mini-plate centrifuge in the BSC.

9.3.20 Transfer PCR plate to the ABI 7500 Fast thermocycler.

9.3.21 Run PCR using the thermocycling conditions as described in Section 9.3.10.

9.3.22 After completion of thermocycling, discard sealed PCR plate.

Note: PCR plates with amplified product should not be opened in the laboratory.

9.3.23 Laboratory clean-up procedures

- Dispose of all biological materials in autoclave bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with fresh bleach (Section 6.19), followed by 70% isopropyl and a DI water final rinse.

9.3.24 Refer to section 12.1 for Data Analyses and Calculations.

10.0 Post Decontamination Procedures for Rapid Viability-Polymerase Chain Reaction (RV-PCR) Analyses

Acceptable sample types: Gauze wipes (2" × 2" 50% rayon/50% polyester), air filters (37 mm), swabs (macrofoam), vacuum socks (large dust collection sample bags, 9 1/8" × 4", mean pore size 6.7 µm), vacuum filters (4" diameter filter), Sponge-Stick sampling tools, water and decontamination waste water.

***Note*: Neutralization of decontamination agent(s) may be required prior to sample processing and analyses.**

10.1 RV-PCR

The RV-PCR method (Figures 2 and 3) is most useful for the analyses of samples collected during and after decontamination because determination of the presence or absence of viable *B. anthracis* spores (in the presence of a large number of dead spores) is a key analytical requirement during this phase of the response. This method can be more sensitive than the traditional culture-based method because almost all the spores recovered from the sample are used for analysis. It is

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relatively rapid, cost-effective, less labor-intensive, less prone to inhibition by environmental matrices, and less prone to interferences from the outgrowth of other bacteria, fungi, other microbes and presence of other biological material in the sample. It also provides higher-throughput and generates significantly less biohazardous and general laboratory wastes than the culture-based method. Using the current version of the RV-PCR procedure a batch of 16 samples can be analyzed in 15 hours. If the laboratory is running 7/24 approximately 150 samples can be analyzed in 48 hours. If additional equipment and personnel are available, the throughput could be increased.

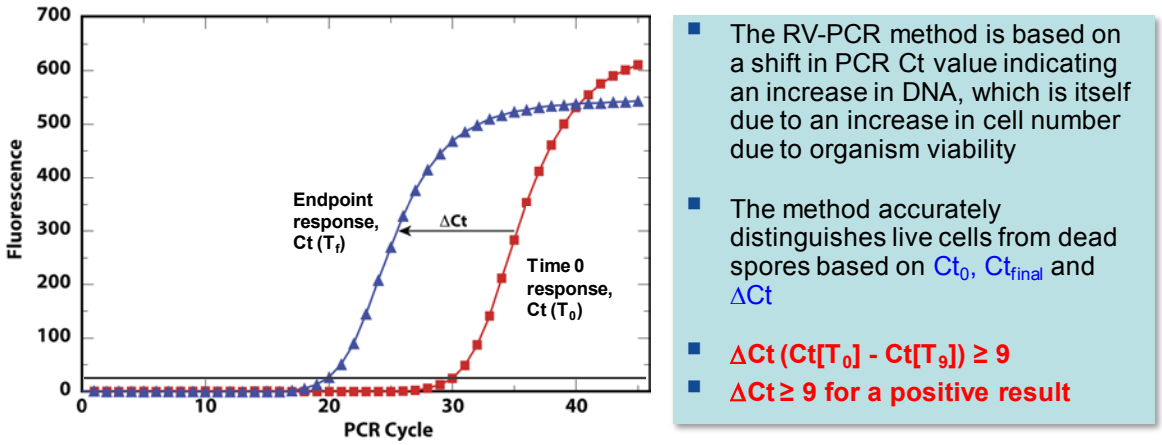

Figure 2. Example PCR Amplification Curves for the Initial T_0 Aliquot and the Endpoint (Final) Aliquot.

The RV-PCR method is a combination of culture and real-time PCR, a rapid, highly sensitive and specific analytical method to detect and identify bioterrorism agents. Culturing the samples allows the germination of viable *B. anthracis* spores recovered from processed samples. Real-time PCR via a change in the C_T value (Figure 2) offers a rapid determination of the viability and identity of *B. anthracis* bacteria that grow from germinated spores in broth culture. Samples (air filter, particulate [wipe, Sponge-Stick, swab, vacuum sock or filter], drinking water or decontamination waste water) are processed in multiple spore extraction and wash steps. Recovered spores are incubated in BHI broth for optimum growth of *B. anthracis*. After vortexing, an aliquot is withdrawn for baseline analysis before incubating the broth culture in the filter cup at 36°C for 9 hours on a rotary shaker incubator. This is the T_0 aliquot and is stored at 4°C for immediate processing or at -20°C for an extended period until analysis. After the broth culture is incubated for 9 hours, another aliquot is withdrawn. This is the T_9 aliquot. Both the T_0 and T_9 aliquots are then extracted and purified to obtain *B. anthracis* total DNA. The T_0 and T_9 DNA extracts are then analyzed, in triplicate, using real-time PCR to detect the presence of *B. anthracis* DNA. Figure 3 provides a flowchart for the RV-PCR analyses. The C_T values for both the T_0 and T_9 DNA extracts are recorded and compared. A change in C_T for the T_9 aliquot relative to the C_T for the T_0 aliquot is calculated as follows: ΔC_T (C_T [T_0] - C_T [T_9]). A $\Delta C_T \geq 9$ (i.e., the endpoint PCR C_T of ≤ 36 for the T_9 DNA extract in a 45-cycle PCR) is set as a cut-off value for a positive detection of viable *B. anthracis* spores in the sample. The $\Delta C_T \geq 9$ criterion represents an approximate three log increase in DNA concentration at T_9 relative to T_0. The increase in DNA concentration at T_9 is as a result of the presence of viable spores in the sample that germinated and grew during the 9 hours of incubation in growth medium. Depending upon the end user's requirement, sample complexity (dirtiness) and the phase of response during an event, a lower ΔC_T criterion of ≥ 6 (a two log difference in DNA concentration) and a corresponding higher endpoint

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PCR C_T of ≤ 39 could be set. The current protocol provides qualitative (presence or absence) results. However, introduction of the Most Probable Number (MPN) technique in the RV-PCR protocol (i.e., performing serial dilutions and replicate subsamples) could provide semi-quantitative results.

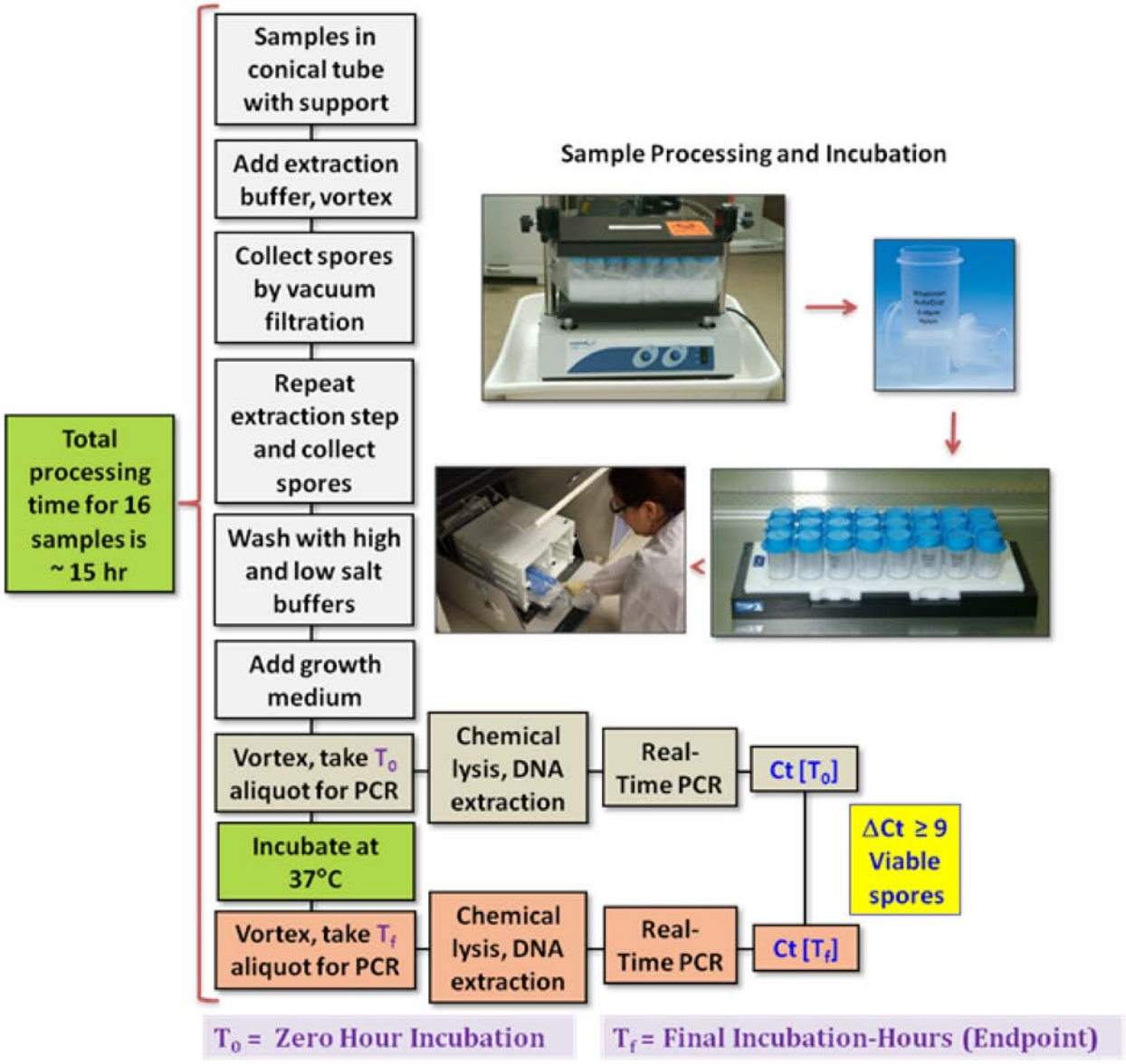

Figure 3. Flow Chart for RV-PCR Sample Analysis.

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10.2 RV-PCR Sample Processing: Spore Recovery

Note: Gloves should be used and changed between samples and as indicated below.

10.2.1 Prior to sample processing, prepare the following items:

- Fill sample tube rack with 30 mL or 50 mL screw cap conical tubes and label as appropriate.
- In a BSC, assemble manifold by connecting upper part (with 24 openings as 3 columns of 8 openings each) to lower part (with port) using six Allen screws. Place filter cups into the manifold in the two outer columns (leaving the center column vacant). Verify that all filter cups are completely pushed down in manifold such that the filter cup bottom is touching the top surface of the manifold. Place a 50 mL tube cap (Section 5.2.6) on each filter cup. Place blue pull-tab caps in a beaker (one for each filter cup with a couple extra) and put inside a 37°C incubator (caps are easily to apply when pre-warmed).
- Vacuum: Prepare vacuum pump or house vacuum source; connect vacuum source to in-line filter and to waste container filled with ~150 mL of fresh undiluted bleach resulting in a final concentration of approximately 10%, once filtration is complete (final waste volume will be ~1.5 L).

Note: If using external vacuum pump, tape pump exhaust tube to BSC to vent exhaust inside BSC.

- Capping tray set up: Add bottom caps to capping tray.
- For T_0 and T_9 sample aliquots: In the BSC, for each sample 96-well tube rack or control, set up the following tubes, each labeled with the sample ID, the time-point (T_0 or T_9), the date, and the operator's initials:
  - One 2 mL screw cap tube
  - One 2 ml Eppendorf tube
  - Two 1.5 mL Eppendorf tubes

  Use a different tube rack (96-well tube rack in 8 × 12 format) for T_0 and T_9 tubes, following the sample layout.
- Tape filter cup layout on outside glass window of the BSC.
- Special Instructions
  - All procedures in the laboratory are to follow protocols that maintain a safe and clean environment for the operators and reagents.
  - All the procedures must be carried out in a BSC.
  - Before and after sample analysis, decontaminate the BSC, pipet aids, centrifuge and other equipment. Also, decontaminate all working areas suspected to be contaminated.
  - When operating in a BSC and throughout the laboratory, perform all steps of the process using aseptic techniques. These precautionary techniques are to be used to prevent contamination of equipment and individual reagents.
  - Wear safety glasses, a lab coat, and gloves throughout the process.

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- If the gloves have somehow become contaminated, the gloves must be disposed of and fresh gloves must be donned.
- Open one tube at a time throughout the process. At no point may more than one tube be opened. Do not allow hands (gloved or otherwise) to pass over an open tube or container.
- All used pipette tips, gloves, and tubes must be discarded in a biohazard autoclave bag in the biosafety cabinet for later disposal after autoclaving.

10.2.2 *Wipe and Air Filter Samples*

Place mesh support over wipe or air filter samples in 30 mL tube by holding the wipe or air filter to the side of the tube with sterile forceps and placing the coiled mesh support on top (Section 9.1). Ensure the sample and mesh are in the bottom half of the tube (avoiding the conical portion). Change gloves in between each sample. The support keeps the wipe or air filter from interfering with pipetting activities and also improves efficiency of spore extraction during vortexing. Proceed to Section 10.2.8.

10.2.3 *Vacuum Samples (Socks and Filters)*

For vacuum socks, using sterile scissors, cut top blue portion off and discard prior to folding the sock from the top opening down. Make four, 1/2" folds in order to reduce the sock size to about 1" × 2". Cut the sock five times above the 30 mL tube using scissors, making sure to cut through all the folds. Place cut, folded sock in bottom half of tube (avoiding conical portion) and using sterile forceps, place mesh support on top of sock (Section 9.1). Bleach the BSC working surface and don a fresh pair of gloves in between samples. Proceed to Section 10.2.8.

Note: ***The sampling processing procedure provided is for up to 1 tablespoon (~1 g of test dust) of debris per sock sample. If socks received contain higher levels of debris, follow procedure in Section 11.4.1.***

For vacuum filters, ensure that the exposed filter surface (with debris) is facing up and carefully cut through the evidence tape with a sterile scalpel in order to remove the top of the cartridge. Using a pair of sterile forceps, transfer large pieces of debris into the appropriate 30 mL tube, then fold filter in half with dirty, exposed filter side in, and then fold in half again in order to fit it into the 30 mL tube. Place folded filter in bottom half of tube (avoiding conical portion) and using two pairs of sterile forceps, place mesh support on top of filter (Section 9.1). Proceed to Section 10.2.8.

10.2.4 *Sponge-Stick Sampling Tools*

- If the Sponge-Sticks/wipes are not in Stomacher® bags, aseptically transfer each sample to a Stomacher® bag using sterile forceps. Change forceps between samples.
- Add 90 mL of PBST to each bag. Set Stomacher® (Section 5.5.28) to 260 rpm.
- Place a bag containing a sample into the Stomacher® (Section 5.5.28) so the Sponge-Stick/wipe rests evenly between the homogenizer paddles and stomach each sample for 1 minute.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the wipe from the outside of the bag with hands. With the bag closed, move the Sponge-

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Stick/wipe to the top of the bag while using hands to squeeze excess liquid from the Sponge-Stick/wipe.

- Open the bag, remove and discard the Sponge-Stick/wipe using sterile forceps.
- Repeat steps (Section 10.2.4) described above for each sample.
- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- Process one stomacher bag sample at a time, transfer 20 mL at a time to the corresponding filter cup (with vacuum set to 5 – 10 psi), allowing complete filtration prior to the subsequent addition, until entire liquid volume has been processed. Use a new 25 mL serological pipet for each transfer.
- Turn off vacuum pump.
- Add 20 mL of cold (4°C) extraction buffer without Tween® 80 to each filter cup, and wait for 5 minutes prior to turning on the vacuum to 5 – 10 psi. Complete filtration through the filter cups. Washing the filter with extraction buffer containing 30% ethanol may reduce the number of vegetative cells that may compete with *B. anthracis* spore outgrowth.
- Proceed to Section 10.3.

10.2.5 ***Swabs***

Place swab into the 30 mL tube and cut handle with sterile scissors if necessary to fit into the tube. Using sterile forceps, place the mesh support over the swab (Section 9.1). Proceed to Section 10.2.8.

10.2.6 ***Water Samples (Large volume [10 L – 100 L], drinking water)***

- Please see Appendix A for primary (Section 2.0) and secondary (Section 3.0) concentration of large volume (10 L – 100 L) water samples. For water samples < 10 L, please refer to Appendix A, Section 3.0, secondary concentration.
- After secondary water concentration, using a 25 mL serological pipette add 15 mL of PBST buffer to the 50 mL conical tube with membrane filter (Appendix A, Section 3.5). Repeat for each tube.
- Set vortexer (Section 5.5.16) to high intensity.
- Vortex membrane in 10 second bursts for 2 minutes to dislodge spores, taking care to prevent the liquid from entering the tube cap. Repeat for each tube.
- Let tubes settle for 2 minutes.
- Using a new 25 mL serological pipette, transfer as much liquid as possible to the corresponding filter cup while avoiding any settled particles during pipetting.
- Repeat for each sample tube.
- Turn on vacuum pump to 5 – 10 psi in order to collect spores onto filter cups.
- Repeat extraction of membrane filter by adding another 15 mL of PBST buffer to the 50 mL conical tube with membrane and vortex for 2 minutes with 10 second bursts, as described above.

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- After 2 minute settling, transfer as much liquid as possible to the corresponding filter cup using a 25 mL serological pipet while avoiding any settled particle.
- Complete filtration of liquid through filter cups. Turn off the vacuum pump.
- Add 20 mL of cold (4°C) extraction buffer without Tween® 80 to each filter cup, and wait for 5 minutes prior to turning on the vacuum to 5 – 10 psi. Complete filtration through the filter cups. Washing the filter with extraction buffer containing 30% ethanol may reduce the number of vegetative cells that may compete with *B. anthracis* spore outgrowth.
- Proceed to Section 10.3.

10.2.7 ***Water Samples (small volume [< 50 mL], surface or drinking water)***

- Place manifold and filter cups in BSC. Label all filter cups, following the sample tube rack layout. Document filter cup and sample tube labels.
- Turn on vacuum pump to 5 − 10 psi.
- Mix water sample by vortexing 5 − 10 seconds, then using a 25 mL serological pipet transfer 20 mL of water to the corresponding filter cup.
- Dispose of pipet in waste container. Perform second transfer of 20 mL to same filter cup using a new serological pipet.

Note: Additional 20 mL transfers may be conducted.

- Cap sample tube. Change gloves.
- After performing transfers to the corresponding filter cup for each water sample, check that all sample tube caps are secure. Place tube rack in plastic bag, seal and bleach bag.
- Store water sample tubes at 4°C as an archive until analyses are completed or until directed to discard samples.
- Turn off the vacuum pump.
- Add 20 mL of cold (4°C) extraction buffer without Tween® 80 to each filter cup, and wait for 5 minutes prior to turning on the vacuum to 5 – 10 psi. Complete filtration through the filter cups. Washing the filter with extraction buffer containing 30% ethanol may reduce the number of vegetative cells that may compete with *B. anthracis* spore outgrowth.
- Proceed to Section 10.3.

10.2.8 Place manifold and filter cups with 50 mL tube caps in BSC. Label all filter cups, following the sample tube rack layout. Document filter cup and sample tube labels.

10.2.9 Add 20 mL of cold (4°C) extraction buffer with Tween® 80 to samples (use 5 mL for swabs) placed in 30 mL conical tubes (50 mL conical tubes for Sponge-Stick samples) in tube rack (up to 16 tubes per rack). Use a new serological pipet to transfer buffer from a sterile, 250 mL screw capped bottle to each tube (keep bottle cap loosely over opening between transfers). Uncap one tube at a time, add extraction buffer, close tube and place it back in tube rack. Check that all caps are on tubes securely. Label tubes as appropriate and document location in rack.

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10.2.10 Place tube rack in plastic bag, seal, double bag and bleach the bag prior to transferring to the platform vortexer located outside the BSC.

10.2.11 Vortex samples for 20 minutes on platform vortexer (Section 5.5.15), with the speed set to 7.

10.2.12 After vortexing, transfer sample tube rack to the BSC. Remove tube rack from plastic bag and discard the bag.

10.2.13 Vortex up to 8 sample tubes on a single-tube vortexer (Section 5.5.16) in the BSC, for 3 – 5 seconds each. Let sit for at least 2 minutes to allow large particles to settle prior to aliquoting (for samples containing debris). If required, allow the tubes to settle up to 5 minutes.

10.2.14 Uncap tube one at a time. Using a 25 mL serological pipet (10 mL pipet for swabs) carefully transfer 13 mL (5 mL for swabs) to corresponding filter cup by lifting 50 mL tube cap slightly (same position in the tube rack as in filter cup manifold). Dispose of pipet in waste container. Cap sample tube and place tube back in rack. Change gloves. Turn on vacuum pump at 5 − 10 psi.

10.2.15 Repeat Section 10.2.14 for each sample tube using a new serological pipet for each sample, processing one sample at a time and up to 8 samples as a set, not exceeding 2 sets of 8 (16 sample) per manifold (in rows 1 and 3). Repeat Section 10.2.13 for second set of up to 8 sample tubes.

10.2.16 Complete filtration of liquid through filter cups. Change gloves.

10.2.17 Perform the second spore extraction. Uncap one sample tube at a time.

10.2.18 Add 20 mL (5 mL for swabs) of cold (4°C) extraction buffer without Tween® 80 to each sample tube, one at a time with a new 25 mL serological pipet for each sample. Keep the buffer bottle loosely covered between transfers. Recap the sample tube after each buffer addition.

10.2.19 After adding extraction buffer to all of the tubes, check that all caps are tight. Place rack in plastic bag, seal and bleach the bag. Transfer bagged tube rack to platform vortexer (Section 5.5.15) located outside the BSC.

10.2.20 Vortex rack for only 10 minutes, with speed set to 7.

10.2.21 Repeat sections 10.2.12 – 10.2.16 except transfer 15 – 16 mL to the corresponding filter cup instead of 13 mL, taking care to avoid settled particles during aliquoting. Proceed to RV-PCR processing section (Section 10.3) below, with filter cup manifold.

10.2.22 Check that all caps are on sample tubes securely. Place tube rack in a plastic bag, seal and bleach the bag. Store the samples at 4°C as an archive until analyses are completed or until directed to discard samples.

10.3 RV-PCR Sample Processing: Buffer Washes and Broth Culture

10.3.1 Place into a 37°C incubator: Blue filter cup caps, one for each filter cup, in a ziplock bag.

Note: Caps are easier to place on filter cups when pre-warmed.

10.3.2 Place into BSC: 25 mL and 10 mL (for swab samples) serological pipets and cold (4°C) high salt wash buffer (pH 6.0) in 250 mL screw cap bottle.

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10.3.3 To each filter cup, transfer 20 mL (10 mL for swab samples) of cold (4°C) high salt wash buffer (pH 6.0) using a 25 mL serological pipet (10 mL pipet for swabs) and lifting 50 mL tube cap slightly, keeping the bottle covered between transfers. Use new pipet for each filter cup. Complete filtration of liquid through the filter cups. Change gloves.

10.3.4 Place into the BSC: 10 mL serological pipets and cold (4°C) 1X low salt wash buffer, pH 7.4, in 250 mL screw cap bottle.

10.3.5 Transfer 20 mL of cold (4°C) 1X low salt wash buffer (pH 7.4) to each filter-cup using a 25 mL serological pipet (by lifting 50 mL tube cap slightly). Keep the bottle covered between transfers and use new pipet for each filter cup.

10.3.6 Complete filtration of liquid through filter cups. Remove 50 mL tube caps and dispose to waste. Turn off vacuum pump. Change gloves.

10.3.7 Unscrew the manifold top using an Allen wrench. Break the seal on manifold to ensure there is no vacuum by inserting a plate sealer between manifold top and bottom. Using gloves hold the sides of the filter cup manifold top and remove it from the bottom vacuum manifold and place on top of the capping tray, fitted with bottom caps (Section 10.2.1). Press down firmly to ensure caps are securely fastened to filter cup bottom ports. Place bleach wipes over the manifold bottom until it can be disinfected. Change gloves.

10.3.8 Place into the BSC: 5 mL serological pipets, 200 µL pipettor, 200 µL tips, cold (4°C) BHI broth aliquoted in 50 mL conical tubes, sharps container and blue filter cup caps (pre-warmed in 37°C ± 1°C incubator).

10.3.9 Pipet 3.5 mL of cold BHI broth into each filter cup using a 5 mL serological pipet. Use a new pipet for each filter cup.

10.3.10 Firmly press blue caps onto filter cups prior to vortexing. Record the time of the BHI broth addition since this represents T_0.

10.3.11 Place the capped filter cup manifold in a plastic bag, seal, double bag and bleach the bag.

10.3.12 Vortex the filter cups for 10 minutes on the platform vortexer (Section 5.5.15), setting 7.

10.3.13 Place 2 mL screw cap tubes for T_0 aliquots into the cold block in the BSC.

10.3.14 After vortexing, transfer filter cup manifold in capping tray to the BSC. Remove bag.

10.3.15 Uncap one filter cup at a time and open the corresponding 2 mL tube. Using a 1 mL pipettor while gently pipetting up and down 10 or more times to mix the sample (and to avoid aerosol generation), transfer 1 mL (T_0 aliquot) from each cup to the corresponding pre-chilled (cold block) 2 mL tube. Cap the tube and place it back into the cold block.

10.3.16 Repeat Section 10.3.15 for each filter cup.

10.3.17 After transferring the T_0 aliquot for all of the samples, place the capped filter cup manifold in a plastic bag, seal, double bag and bleach the outer bag.

10.3.18 Transfer bagged filter cup manifold in capping tray to the shaker incubator. Secure the manifold using manifold rack or pins (for single manifold). Incubate at 37ºC ± 1ºC at 230 rpm, for 9 hours (i.e., 9 hours from the addition of BHI broth to the filter cups).

10.3.19 Process 1 mL T_0 aliquots in 2 mL screw cap tubes using the Manual DNA Extraction and Purification Protocol (starting from Section 10.4.9), below.

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10.4 Manual DNA Extraction and Purification

Prepare lysis buffer with anti-foam according to manufacturer's instructions in the Magnesil Blood Genomic, Max Yield System, Kit. Prepare the alcohol wash solution by adding ethanol and isopropyl alcohol according to manufacturer's instructions. Transfer sufficient volume of buffer to sterile, 100 mL reservoir immediately before use. ***Pre-heat heat block to 80°C prior to Section 10.4.8.***

Note: 1 mL T_0 and T_9 aliquots are processed in the same manner, as described below.

10.4.1 The T_0 aliquots can be extracted and purified during incubation of the T_9 aliquots.

10.4.2 After the 9 hour incubation, remove the filter cup manifold from the shaker incubator.

10.4.3 Vortex filter cups for 10 minutes on platform vortexer (Section 5.5.15) with speed set to 7.

10.4.4 Transfer the filter cup manifold to the BSC, remove and discard bags.

10.4.5 Set up 2 mL screw cap tubes for T_9 aliquots in a 96-well tube rack (8 × 12) and verify that 2 mL tube labels match the filter cup layout. Maintain the tube layout when transferring tubes between the magnetic stand and the 96-well tube rack. Do not use 1.5 mL tubes. Transfer T_9 aliquot screw cap tubes to the BSC.

10.4.6 Uncap one filter cup at a time and open the corresponding 2 mL tube. Using a 1 mL pipettor, swirl pipet tip gently in filter cup, while gently pipetting up and down 10 or more times to mix sample (and to avoid aerosol generation), transfer 1 mL (T_9 aliquot) from each cup, and transfer to corresponding T_9 aliquot tube in the 96-well tube rack; cap the tubes.

10.4.7 Repeat Section 10.4.6 for each filter cup.

10.4.8 Centrifuge 2 mL screw cap tubes at 14,000 rpm for 10 minutes (4°C). Remove 800 µL of the supernatant from each tube, using a 1000 µL pipettor and dispose to waste. Do not disturb the pellet (use a new tip for each sample and discard tips in a sharps container). If processing for DNA extraction immediately, proceed to Section 10.4.11.

10.4.9 Store T_9 aliquots at -20°C until further processing, if the remaining steps of the protocol cannot be conducted immediately.

Note: T_0 and T_9 extractions can be completed separately.

10.4.10 Thaw T_0 and T_9 aliquots if they were stored at -20°C.

10.4.11 Add 800 µL of lysis buffer using a 1000 µL pipettor with a new tip for each sample. Cap the tubes and mix by vortexing on high (~1800 rpm) in 10 second pulses for a total of 60 seconds and place in 96-well tube rack at room temperature. Change gloves in between the samples.

10.4.12 Vortex each screw-cap tube briefly (low speed, 5 − 10 seconds) and transfer the sample volume to a 2 mL Eppendorf tube (ensure the tubes are labeled correctly during transfer). Change gloves in between each sample. Incubate the T_0 and T_9 lysate tubes at room temperature for 5 minutes.

10.4.13 Vortex the PMPs on high (~1800 rpm) for 30 – 60 seconds, or until they are uniformly resuspended. Keep PMPs in suspension by briefly vortexing (3 – 5 seconds) before adding to each T_0 and T_9 lysate tube.

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10.4.14 Uncap one tube at a time and add 600 µL of PMPs to each T_0 and T_9 lysate (containing 1 mL sample), hereafter referred to as "T_0 and T_9 tubes" using a new pair of gloves for each tube. Mix by briefly vortexing (use a new tip for each sample and discard used tips in a sharps container). Change gloves in between each tube.

10.4.15 Repeat Section 10.4.14 for all T_0 and T_9 tubes, vortexing the PMPs suspension (10.4.13) between each T_0 and T_9 tube.

10.4.16 Vortex each T_0 and T_9 tube for 5 − 10 seconds (high), incubate at room temperature for 5 minutes, briefly vortex, and then place on the magnetic stand with hinged-side of the tube facing toward the magnet. After all the tubes are in the stand, invert tubes 180 degrees (upside-down) turning away from you, then right side-up, then upside down toward you, then right side-up (caps up) position. This step allows all PMPs to contact the magnet. Check to see if any beads are in the caps and if so, repeat the tube inversion cycle again. Let the tubes sit for 5 − 10 seconds before opening. Maintain the tube layout when transferring tubes between the magnetic stand and the 96-well tube rack.

10.4.17 Uncap each tube, one at a time and withdraw all liquid using a 1000 µL pipettor with the pipet tip placed in the bottom of 2 mL tube, taking care not to disturb the PMPs. Ensure that all the liquid is removed. Use a new pipet tip to remove any residual liquid, if necessary. If liquid remains in the tube cap, remove by pipetting. Dispose tip and liquid in a sharps container. Recap tube. Change gloves.

Note: ***Section 10.4.17 can be combined with Section 10.4.18. After withdrawing the liquid in Section 10.4.17, add 360 µL of Lysis buffer using a separate pipettor and new tip.***

10.4.18 Uncap each T_0 and T_9 tube, one at a time, and add 360 µL of lysis buffer using a 1000 µL pipettor. Use a new tip for each sample and discard tips in a sharps container. Cap and vortex on low setting for 5 − 10 seconds, and transfer to 96-well tube rack.

10.4.19 After adding lysis buffer to all of the T_0 and T_9 tubes, vortex each tube for 5 − 10 seconds (low) and place back on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Section 10.4.16.

10.4.20 Remove all the liquid as described in Section 10.4.17, except that a glove change between samples is not required. Use a new tip for each T_0 and T_9 tube (discard used tips in a sharps container). Recap the tube.

Note: ***Section 10.4.20 can be combined with Section 10.4.21. After withdrawing the liquid in Section 10.4.20, add 360 µL of Lysis buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.21 Repeat Sections 10.4.18 − 10.4.20 for all tubes.

Note: ***Section 10.4.21 can be combined with Section 10.4.22. After withdrawing the liquid in Section 10.4.17, add 360 µL of Salt Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.22 1st Salt Wash: Uncap each T_0 and T_9 tube, one at a time, and add 360 µL of Salt Wash solution (VWR Cat. No. PAMD1401 or equivalent). Use a new tip for each T_0 and T_9 tube and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.

10.4.23 After adding the Salt Wash solution to all of the T_0 and T_9 tubes, vortex each tube for 5 − 10 seconds (low) and place on the magnetic stand. After all tubes are in the stand, follow tube inversion cycle, as described in Section 10.4.16.

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10.4.24 Remove liquid as described in Section 10.4.17, except that a glove change between T_0 and T_9 tubes is not required. Use a new tip for each T_0 and T_9 tube and discard used tips in a sharps container. Recap the tube. Repeat for all T_0 and T_9 tubes.

Note: ***Section 10.4.24 can be combined with Section 10.4.25. After withdrawing the liquid in Section 10.4.24, add 360 µL of Salt Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.25 2nd Salt Wash: Repeat Sections 10.4.22 – 10.4.24 for all T_0 and T_9 tubes.

Note: ***Section 10.4.25 can be combined with Section 10.4.26. After withdrawing the liquid in Section 10.4.25, add 500 µL of Alcohol Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.26 1st Alcohol Wash: Uncap each T_0 and T_9 tube, one at a time, and add 500 µL of alcohol wash solution. Use a new tip for each sample and discard used tips in a sharps container. Cap and transfer to 96-well tube rack.

10.4.27 After adding alcohol wash solution to all of the T_0 and T_9 tubes, vortex each tube for 5 − 10 seconds (low speed) and place on the magnetic stand. After all T_0 and T_9 tubes are in the stand, follow the tube inversion cycle, as described in Section 10.4.16.

10.4.28 Remove liquid as described in Section 10.4.17, except that a glove change between T_0 and T_9 tubes is not required. Use a new tip for each T_0 and T_9 tube and discard used tips in a sharps container. Recap the tube.

Note: ***Section 10.4.28 can be combined with Section 10.4.29. After withdrawing the liquid in Section 10.4.28, add 500 µL of Alcohol Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.29 2nd Alcohol Wash: Repeat Sections 10.4.26 – 10.4.28 for all T_0 and T_9 tubes.

Note: ***Section 10.4.29 can be combined with Section 10.4.30. After withdrawing the liquid in Section 10.4.29, add 500 µL of Alcohol Wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.30 3rd Alcohol Wash: Repeat Sections 10.4.26 – 10.4.28 for all T_0 and T_9 tubes.

Note: ***Section 10.4.30 can be combined with Section 10.4.31. After withdrawing the liquid in Section 10.4.30, add 500 µL of 70% ethanol wash buffer using a separate pipettor and new tip. If the steps are combined, cap the tube after the buffer addition.***

10.4.31 4th Alcohol Wash: Repeat Sections 10.4.26 – 10.4.28 except use 70% ethanol wash solution for all tubes. After the liquid is removed, recap the tube and transfer to the 96-well tube rack.

10.4.32 Open all T_0 and T_9 tubes and air dry for 2 minutes.

10.4.33 Heat the open T_0 and T_9 tubes in the heat block at 80°C until the PMPs are dry (~20 minutes). Allow all the alcohol solution to evaporate since alcohol may interfere with analysis.

10.4.34 DNA elution: While they are in the heating block add 300 µL of elution buffer to each T_0 and T_9 tube, and close tube.

10.4.35 Vortex for 10 seconds and let the tubes sit in the heating block for 80 seconds.

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10.4.36 Briefly vortex the tubes (5 − 10 seconds) taking care to prevent the liquid from entering the tube cap and let the tube sit in the heating block for 1 minute.

10.4.37 Repeat Section 10.4.36 four more times.

10.4.38 Remove the tubes from the heating block, place them in a 96-tube rack in the BSC, and let them sit at room temperature for at least 5 minutes.

10.4.39 Briefly vortex each tube (5 − 10 seconds) on low speed and centrifuge at 2000 rpm at 4ºC for 1 minute. Place tube in 96-well tube rack.

10.4.40 Briefly vortex each tube and place on the magnetic stand for at least 30 seconds. Bring the cold block to the BSC.

10.4.41 Collect liquid from each T_0 or T_9 tube with a micropipettor and transfer to a clean, labeled, 1.5 mL tube (~170 µL) on a cold block (check tube labels to ensure the correct order). Use a new tip for each tube and discard tips in a sharps container. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place 1.5 mL tube on magnet, collect liquid, and transfer to a clean, labeled, 1.5 mL tube (ensure the tubes are labeled correctly during transfer).

10.4.42 Centrifuge tubes at 14,000 rpm at 4°C for 5 minutes to pellet any particles remaining with the eluted DNA; carefully remove supernatant and transfer to a new 1.5 mL tube using a new tip for each tube (ensure the tubes are labeled correctly during transfer).

10.4.43 Store T_0 and T_9 DNA extract tubes "referred to as T_0 and T_9 DNA extracts" at 4ºC until PCR analysis (use photo-tray to transport 1.5 mL tubes in a rack).

Note: If PCR cannot be performed within 24 hours, freeze DNA extracts at -20ºC.

10.4.44 Laboratory Clean-up procedures

- Dispose of all biological materials in autoclave bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with fresh pH amended bleach (Section 6.19), followed by 70% isopropyl and a DI water final rinse.

10.5 Real-time PCR Analysis of T_0 and T_9 DNA Extracts

For real-time PCR, follow Sections 9.3 − 9.3.16 with the following exceptions and changes:

- No EIC control is required for the samples.
- PCR Master Mix for 6 reactions per sample is required to accommodate the T_0 and T_9 DNA extracts.
- For each batch of samples, PCR Master Mix should be made for 4 PCs, 4 NTCs, 3 PNCs and 6 DNA extracts per sample (3 for T_0 and 3 for T_9 DNA extracts).

10.5.1 *T_0 DNA extracts*: Label 1.5 mL tubes with the sample identifier and "10-fold dilution". Add 90 µL of PCR-grade water to the tubes.

10.5.2 Mix T_0 DNA extracts by vortexing (3 − 5 seconds), spin at 14,000 rpm for 2 minutes, and transfer 10 µL of supernatant to 1.5-mL Eppendorf tubes with 90 µL of PCR-grade water, maintaining the plate layout.

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Note: ***No centrifugation is required (Section 10.5.2) if PCR analysis is conducted immediately after DNA elution (Section 10.4.42).***

10.5.3 Mix diluted T_0 DNA extracts by vortexing (5 seconds at low speed), and transfer 5 µL from the tubes to the PCR plate (with PCR Master Mix).

10.5.4 *T_9 DNA extracts*: Label 1.5 mL tubes with the sample identifier and "10-fold dilution". Add 90 µL of PCR-grade water to the tubes.

10.5.5 Mix T_9 DNA extracts by vortexing (3 − 5 seconds), spin at 14,000 rpm for 2 minutes, and transfer 10 µL of supernatant to 1.5-mL Eppendorf tubes with 90 µL of PCR-grade water, maintaining the plate layout.

Note: ***No centrifugation is required (Section 10.5.5) if PCR analysis is conducted immediately after DNA elution (Section 10.4.42).***

10.5.6 Mix diluted T_9 DNA extracts by vortexing (5 seconds), and transfer 5 µL from the tubes to the PCR plate (with PCR Master Mix).

10.5.7 Seal PCR plate with optical seal, using a plate sealer to ensure good contact. Change gloves.

10.5.8 Centrifuge sealed PCR plate for 1 minute at 2000 rpm and 4°C using the PCR plate safety cups or mini-plate centrifuge in the BSC.

10.5.9 Open the centrifuge safety cup and transfer PCR plate to ABI thermocycler.

10.5.10 Transfer the PCR plates to the ABI 7500 Fast thermocycler.

10.5.11 Run PCR using the thermocycler conditions described in Section 9.3.10.

10.5.12 After the PCR run, discard sealed PCR plate.

Note: ***PCR plates with amplified product should not be opened in the laboratory.***

10.5.13 Follow laboratory cleanup protocol provided in Section 10.4.44.

10.5.14 Refer to Section 12.2 for Data Analyses and Calculations.

11.0 Post Decontamination Procedures for Culture Analyses

Acceptable sample types: Gauze wipes (2" × 2", 50% rayon/50% polyester), air filters (37 mm), swabs (macrofoam), vacuum socks (large dust collection sample bags, 9 1/8" x 4", mean pore size 6.7 µm), vacuum filters (4" diameter filter), Sponge-Stick sampling tools, drinking water and decontamination waste water.

Note: ***Neutralization of decontamination agent(s) may be required prior to sample processing and analyses.***

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11.1 Sample Processing and Plating for Sponge-Sticks and Wipes

Note: Sterile gloves should be used and changed between samples and as indicated below.

11.1.1 Dislodge spores from the sample (Sponge-Sticks and wipes)

Note: All subsequent procedures involving manipulation of Sponge-Sticks, wipes, or spore suspensions must be carried out in a BSC using appropriate PPE (e.g., gloves, lab coat).

- If the Sponge-Sticks/wipes are not in Stomacher® bags, aseptically transfer each sample to a Stomacher® bag using sterile forceps. Change forceps between samples.
- Add 90 mL of phosphate buffered saline with Tween® 20 (PBST) to each bag. Set Stomacher® (Section 5.5.28) to 260 rpm.
- Place a bag containing a sample into the Stomacher® (Section 5.5.28) so the Sponge-Stick/wipe rests evenly between the homogenizer paddles and stomach each sample for 1 minute.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the wipe from the outside of the bag with hands. With the bag closed, move the Sponge-Stick/wipe to the top of the bag while using hands to squeeze excess liquid from the Sponge-Stick/wipe.
- Open the bag, remove and discard the Sponge-Stick/wipe using sterile forceps.
- Repeat steps (Section 11.1.1) described above for each sample.
- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.

Note: For RV-PCR analysis, proceed to Section 10.2.4. Do not proceed to Section 11.1.2.

11.1.2 Concentrate elution suspension

- Gently mix the elution suspension in the Stomacher® bag up and down 3 times with a sterile 50 mL pipet. Remove half of the suspension volume (~45 mL) and place it in a 50 mL screw capped centrifuge tube. Place the remaining suspension (~45 mL) into a second 50 mL tube.
- Record suspension volumes on tubes and data sheet.
- Repeat steps above (Section 11.1.2) for each sample.
- Place 50 mL tubes into sealing centrifuge buckets and decontaminate centrifuge buckets before removing them from the BSC.
- Centrifuge tubes at 3500 × g, with the brake off, for 15 minutes in a swinging bucket rotor.

Note: A higher × g is preferred as long as the speed is within the tube specifications.

- Remove the supernatant from each tube with a 50 mL pipet and discard leaving approximately 3 mL in each tube. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the tube bottom. Use a sterile 50 mL pipet for each sample.
- Set the vortexer (Section 5.5.16) to high intensity. Set the sonicator water bath to high.

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- Vortex the tubes for 30 seconds and transfer the tubes to the sonicator bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles twice.
- Remove suspension from one tube with a sterile 5 mL pipet and combine it with the suspension in the other tube from the same sample. Measure final volume of suspension with 5 mL pipet and record the result on the tube and data sheet.
- Repeat vortexing and sonication steps for each sample.

Note: ***For real-time PCR analyses (for the site characterization phase) proceed to Sections 9.2 (DNA Extraction and Purification) and 9.3 (Real-time PCR analyses). For culture analyses proceed to 11.1.3, below.***

11.1.3 Serially dilute the spore elution suspension in PBST

- Vortex the elution suspension on high for 30 seconds.

  a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL containing 9 mL of PBST. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

  b. Open the cap of the 10^{-1} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the tube and vortex on high for 30 seconds. This is the 10^{-2} suspension.

  c. The above results in 3 spore suspensions: the initial wipe elution suspension (undiluted) and 2 serial dilutions of the suspension in PBST (10^{-1} and 10^{-2}).

- Repeat steps (a) through (b) for each sample.

11.1.4 Culture spore suspensions on SBA

Note: ***Plating of 100 µL results in an additional 1:10 dilution of each of the suspensions.***

Each of the following will be conducted in triplicate, resulting in the evaluation of 9 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA plate (labeled 10^{-1}).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried SBA plate (labeled 10^{-2}).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried SBA plate (labeled 10^{-3}).

- After pipetting the 3 spread plates for each dilution, use a sterile L-Shaped spreader to distribute the inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over the entire surface of the plate. Repeat for the next 2 replicate plates.
- Allow inoculum to absorb into the medium completely.

11.1.5 Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance. Comma-shaped projections may arise from the colony edge.

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B. anthracis is not β-Hemolytic. However, weak hemolysis may be seen under areas of confluent growth in aging cultures and should not be confused with β-hemolysis.

a. If the number of colonies is ≤ 300/plate, record actual number.

b. If the number of colonies is > 300/plate, record as "too numerous to count" (TNTC).

c. If no target colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.1.7).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 11.6).

11.1.6 Capture spores on MicroFunnel™ filter membranes and culture on SBA

- Place 3, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.4.4) on the vacuum manifold and moisten membrane with 5 mL PBST. All filtering should be done with a vacuum pressure < 20 mm Hg.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBST into each filter cup. Add 1.0 mL of the undiluted elution suspension each to two MicroFunnel™ cups and to the third cup add the remainder.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBST and filter. Open the valve and complete filtration.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup. Remove each membrane with sterile forceps and place it grid-side up on a SBA plate. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the undiluted elution suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 11.1.6) described above for each sample.

11.1.7 Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. Confirm 1 – 3 colonies using real-time PCR (Section 11.6).

11.2 Sample Processing and Plating for Swabs

11.2.1 Dislodge spores from swabs

Note: ***All subsequent procedures involving manipulation of swabs or spore suspensions must be carried out in a BSC using appropriate PPE (e.g., gloves, lab coat).***

- If the swabs are not in screw cap centrifuge tubes, transfer each swab to sterile, plastic 15 mL screw cap centrifuge tube using sterile forceps.
- If necessary, cut the handle of the swab to fit into the tube using sterile scissors. Use sterile forceps and scissors for each sample.

Note: ***Flaming scissors with an alcohol lamp will not be sufficient to sterilize scissors in between samples due to the presence of Bacillus spores in the samples.***

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- Add 5 mL of PBST to each tube.
- Set vortexer (Section 5.5.16) to high intensity.
- Vortex swab in 10 second bursts for 2 minutes to dislodge spores from swab.
- Using sterile forceps, remove the swab from the 15 mL centrifuge tube. Use the forceps to press the tip of the swab against the inside of the tube to remove extra liquid from the foam tip before discarding the swab.
- Repeat vortexing steps for each swab sample.

Note: ***For real-time PCR analyses (for the site characterization phase) proceed to Section 9.1.19. For culture analyses proceed to 11.2.2, below.***

11.2.2 Serially dilute the spore elution suspension in PBST

- Vortex the elution suspension on high for 30 seconds.

  a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBST. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

  b. Open the cap of the 10^{-1} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the tube and vortex on high for 30 seconds. This is the 10^{-2} suspension.

  c. The above results in 3 spore suspensions: the initial wipe elution suspension (undiluted) and 2 serial dilutions of the suspension in PBST ($10^{-1}$and 10^{-2}).

- Repeat steps (a) through (b) for each sample.

11.2.3 Culture spore suspensions on SBA

Note: ***Plating of 100 µL results in an additional 1:10 dilution of each of the suspensions.***

Each of the following will be conducted in triplicate, resulting in the evaluation of 9 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA plate (10^{-1} mL of the original elution suspension).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried SBA plate (10^{-2} mL of the original elution suspension).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried SBA plate (10^{-3} mL of the original elution suspension).

- After pipetting the 3 spread plates for each dilution, use a sterile L-shaped spreader to distribute inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over entire surface of the plate. Repeat for the next 2 dilutions.
- Allow inoculum to absorb into the medium completely.

11.2.4 Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a

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"ground glass" appearance. Comma-shaped projections may arise from the colony edge. *B. anthracis* is not β-Hemolytic. However, weak hemolysis may be seen under areas of confluent growth in aging cultures and should not be confused with β-hemolysis.

a. If the number of colonies is ≤ 300/plate, record actual number.

b. If the number of colonies is > 300/plate, record as "too numerous to count" (TNTC).

c. If no target colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.2.6).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 11.6).

11.2.5 Capture spores on MicroFunnel™ filter membranes and culture on SBA

- Place 3, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.4.4) on the vacuum manifold and moisten membrane with 5 mL PBST. All filtering should be done with a vacuum pressure < 20 mm Hg.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBST into each filter cup. Add 1.0 mL of the undiluted elution suspension each to two MicroFunnel™ cups and to the third cup add the remainder.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBST and filter. Open the valve and complete filtration.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup. Remove each membrane with sterile forceps and place it grid-side up on a SBA plate. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the undiluted elution suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 11.2.5) described above for each sample.

11.2.6 Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. Confirm 1 – 3 colonies using real-time PCR (Section 11.6).

11.3 Sample Processing and Plating for Air Filters

11.3.1 Dislodge spores from air filter

Note: ***All subsequent procedures involving manipulation of filters or spore suspensions must be carried out in a BSC using appropriate PPE (e.g., gloves, lab coat).***

- Add 5 mL of sterile PBST into a leak-proof, 50 mL conical tube containing an air filter, and close tube.
- Set vortexer (Section 5.5.16) to high intensity.
- Vortex membrane in 10 second bursts for 2 minutes to dislodge spores.

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- Using sterile forceps remove the air filter from the conical tube and discard.
- Repeat the vortexing steps for each air filter.

Note: ***For real-time PCR analyses (for the site characterization phase) proceed to Sections 9.2 (DNA Extraction and Purification) and 9.3 (Real-time PCR analyses). For culture analyses proceed to 11.3.2, below.***

11.3.2 Serially dilute the suspension in PBST

- Vortex the elution suspension on high for 30 seconds.

  a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBST. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

  b. Open the cap of the 10^{-1} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the tube and vortex on high for 30 seconds. This is the 10^{-2} suspension.

  c. Open cap of the 10^{-2} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the PBST tube and vortex on high for 30 seconds. This is the 10^{-3} suspension.

  d. The above results in 4 spore suspensions: the initial sock elution suspension (undiluted) and three serial dilutions of the suspension in PBST (10^{-1}, 10^{-2} and 10^{-3}).

- Repeat steps (a) through (c) for each sample.

11.3.3 Culture spore suspensions on SBA

Note: ***Plating of 100 µL results in an additional 1:10 dilution of each of the suspensions.***

Each of the following will be conducted in triplicate, resulting in the evaluation of 12 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA plate (10^{-1} mL of the original elution suspension).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried SBA plate (10^{-2} mL of the original elution suspension).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried SBA plate (10^{-3} mL of the original elution suspension).

d. After vortexing tubes, pipet 0.1 mL of 10^{-3} suspension onto surface of pre-dried SBA plate (10^{-4} mL of the original elution suspension).

- After pipetting the 3 spread plates for each dilution, use a sterile L-shaped spreader to distribute inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over entire surface of the plate. Repeat for next 3 dilutions.
- Allow inoculum to absorb into the medium completely.

11.3.4 Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. *B. anthracis* produces flat

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or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance. Comma-shaped projections may arise from the colony edge. *B. anthracis* is not β-Hemolytic. However, weak hemolysis may be seen under areas of confluent growth in aging cultures and should not be confused with β-hemolysis.

a. If the number of colonies is ≤ 300/plate, record actual number.

b. If the number of colonies is > 300/plate, record as "too numerous to count" (TNTC).

c. If no target colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.3.6).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 11.6).

11.3.5 Capture spores on MicroFunnel™ filter membranes and culture on SBA

- Place 3, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.4.4) on the vacuum manifold and moisten membrane with 5 mL PBST. All filtering should be done with a vacuum pressure < 20 mm Hg.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBST into each filter cup. Add 1.0 mL of the undiluted elution suspension each to two MicroFunnel™ cups and to the third cup add the remainder.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBST and filter**.** Open the valve and complete filtration.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup. Remove each membrane with sterile forceps and place it grid-side up on a SBA plate. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the undiluted elution suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 11.3.5) described above for each sample.

11.3.6 Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. Confirm 1 – 3 colonies using real-time PCR (Section 11.6).

11.4 Sample Processing and Plating for Vacuum Socks and Filters

11.4.1 Dislodge spores from the vacuum socks and concentrate elution suspension

Note: ***All subsequent procedures involving manipulation of vacuum socks or spore suspensions must be carried out in a BSC using appropriate PPE (e.g., gloves, lab coat).***

- Place 50 mL of PBST into sterile, leak-proof, wide-mouth screw cap plastic container.
- Remove the sock from the bag by holding onto the upper blue plastic material. Wet the sock by dipping the lower 1" of the vacuum sock into the PBST in the container.

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- While holding the sock over the container, cut lower edge of sock as close to the lower edge seam as possible with disposable sterile scissors. Use sterile scissors for each sample.

Note: ***Flaming scissors with an alcohol lamp will not be sufficient to sterilize scissors in between samples due to the presence of Bacillus spores in the samples.***

- Submerge the sock in the PBST so that the liquid is allowed to enter the opening and wet the contents inside.
- When the liquid appears to have wet the sock beyond about 1" from the bottom, cut a 1" vertical slit up the center from the bottom of the sock. Then cut horizontally from side to side, about 1" from the bottom, allowing the two pieces to fall into the container with PBST.
- Submerge the lower edge of the sock again to allow wetting of the contents inside. Again cut a 1" vertical slit up the center and horizontally from side to side to allow two additional sections to fall into the container with PBST.
- Continue to submerge and cut the sock until all of the white filter part of the sock is in pieces in the jar.
- Discard the upper blue portion of the vacuum sock and change gloves.
- Tightly close the container, seal with parafilm, and place on a platform shaker/rotator with lock bars. Agitate samples at 300 rpm for 30 minutes.

Note: ***If shaker/rotator is outside of the BSC, the containers should be enclosed in plastic bags and a sealed biotransport carrier.***

- Remove the biotransport carrier from the shaker and place it in the BSC. Allow settling of samples for 1 minute, then transfer 30 mL of supernatant from each sample into corresponding 50 mL sterile, screw cap, conical tubes.
- Discard the settled material.
- Place the conical tubes into sealing centrifuge buckets within the BSC. Transport to centrifuge and place them in a swinging bucket rotor.
- Centrifuge the supernatant at 3500 × g, with the brake off, for 15 minutes.

Note: ***A higher × g is preferred as long as the speed is within the tube specifications.***

- After centrifugation, move the sealed centrifuge buckets back to the BSC.
- Carefully pipet off 25 mL of the supernatant and resuspend the pellet in the remaining 5 mL by vortexing the 5 mL sample for 1 minute with 10 second bursts.

11.4.2 Dislodge spores from the vacuum filters and concentrate the elution suspension

- Place 50 mL of PBST into a sterile, leak-proof, wide-mouth screw cap plastic container.
- Ensure that the exposed filter surface (with debris) is facing up and carefully cut through the evidence tape with a sterile scalpel in order to remove the top of the cartridge. Using a pair of sterile forceps, transfer large pieces of debris into the appropriate screw cap container.

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- Fold the filter in half with dirty, exposed filter side in, and then fold in half again in order to fit it into the screw cap plastic container.
- Place folded filter in screw cap plastic container.
- Submerge the filter in the PBST so that the liquid is allowed to wet the entire filter.
- Tightly close the container, seal with parafilm. Place on a platform shaker/rotator with lock bars. Agitate samples at 300 rpm for 30 minutes.

Note: ***If the shaker/rotator is outside of the BSC, the containers should be enclosed in plastic bags and a sealed biotransport carrier.***

- Remove biotransport carrier from the shaker and place in the BSC. Allow settling of samples for 1 minute, then transfer 30 mL of supernatant from each sample into corresponding 50 mL sterile, screw cap, conical tubes.
- Discard the settled material.
- Place conical tubes into sealing centrifuge buckets within the BSC. Transport them to centrifuge and place on swinging bucket rotor.
- Centrifuge the supernatant at 3500 × g, with the brake off, for 15 minutes.

Note: ***A higher × g is preferred as long as the speed is within the tube specifications.***

- After centrifugation, move the sealed centrifuge buckets back to the BSC.
- Carefully pipet off 25 mL of the supernatant and resuspend the pellet in the remaining 5 mL by vortexing the 5 mL sample for 1 minute with 10 second bursts.

Note: ***For real-time PCR analyses (for the site characterization phase) proceed to Sections 9.2 (DNA Extraction and Purification) and 9.3 (Real-time PCR analyses). For culture analyses proceed to 11.4.3, below.***

11.4.3 Serially dilute the spore elution suspension in PBST

- Vortex the elution suspension on high for 30 seconds.

  a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBST. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

  b. Open the cap of the 10^{-1} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the tube and vortex on high for 30 seconds. This is the 10^{-2} suspension.

  c. Open cap of the 10^{-2} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the PBST tube and vortex on high for 30 seconds. This is the 10^{-3} suspension.

  d. Resulting in 4 spore suspensions: the initial sock elution suspension (undiluted) and three serial dilutions of the suspension in PBST (10^{-1}, 10^{-2} and 10^{-3}).

- Repeat steps (a) through (c) for each sample.

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11.4.4 Culture spore suspensions on SBA

Note: Plating of 100 µL results in an additional 1:10 dilution of each of the suspensions.

Each of the following will be conducted in triplicate, resulting in the evaluation of 12 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA plate (10^{-1} mL of the original elution suspension).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried SBA plate (10^{-2} mL of the original elution suspension).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried SBA plate (10^{-3} mL of the original elution suspension).

d. After vortexing tubes, pipet 0.1 mL of 10^{-3} suspension onto surface of pre-dried SBA plate (10^{-4} mL of the original elution suspension).

- After pipetting the 3 spread plates for each dilution, use a sterile L-shaped spreader to distribute inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over entire surface of the plate. Repeat for next 3 dilutions.
- Allow inoculum to absorb into the medium completely.

11.4.5 Invert the plates and incubate at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance. Comma-shaped projections may arise from the colony edge. *B. anthracis* is not β-Hemolytic. However, weak hemolysis may be seen under areas of confluent growth in aging cultures and should not be confused with β-hemolysis.

a. If the number of colonies is ≤ 300/plate, record actual number.

b. If the number of colonies is > 300/plate, record as "too numerous to count" (TNTC).

c. If no target colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.4.7).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 11.6).

11.4.6 Capture spores on MicroFunnel™ filter membranes and culture on SBA

- Place 3, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.4.4) on the vacuum manifold and moisten membrane with 5 mL PBST. All filtering should be done with a vacuum pressure < 20 mm Hg.
- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBST into each filter cup. Add 1.0 mL of the undiluted elution suspension each to two MicroFunnel™ cups and to the third cup add the remainder.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBST and filter. Open the valve and complete filtration.

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- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup. Remove each membrane with sterile forceps and place it grid-side up on a SBA plate. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the undiluted elution suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 11.4.6) described above for each sample.

11.4.7 Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record. Confirm 1 – 3 colonies using real-time PCR (Section 11.6).

11.5 Sample Processing and Plating for Water Samples

Note: ***All water samples prior to 11.5.1 have been concentrated according to Appendix A Sections 2.0 and 3.0 (large volume) or Section 3.0 (small volume).***

11.5.1 Dislodge and elute spores from the MicroFunnel™ membrane (from secondary water concentration, Appendix A, Section 3.0)

Note: ***All subsequent procedures involving manipulation of membranes or spore suspensions must be carried out in a BSC using appropriate PPE (e.g., gloves, lab coat).***

- Add 5 mL of sterile PBST into a screw cap, 50 mL conical tube containing a membrane filter, and close tube.
- Set vortexer (Section 5.5.16) to high intensity.
- Vortex the membrane in 10 second bursts for 2 minutes to dislodge spores.
- Using sterile forceps remove membrane from conical tube and discard.
- Repeat the vortexing steps for each membrane.

Note: ***For real-time PCR analyses (for the site characterization phase) proceed to Sections 9.2 (DNA Extraction and Purification) and 9.3 (Real-time PCR analyses). For culture analyses proceed to 11.5.2, below.***

11.5.2 Serially dilute the suspension in PBST

- Vortex the elution suspension on high for 30 seconds.

a. Transfer 1 mL of the suspension from the 50 mL tube to a 15 mL tube containing 9 mL of PBST. Recap the tube and vortex it on high for 30 seconds. This is the 10^{-1} suspension.

b. Open the cap of the 10^{-1} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the tube and vortex on high for 30 seconds. This is the 10^{-2} suspension.

c. Open cap of the 10^{-2} suspension and transfer 1 mL of this suspension into a new 15 mL tube containing 9 mL of PBST. Recap the PBST tube and vortex on high for 30 seconds. This is the 10^{-3} suspension.

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d. The above results in 4 spore suspensions: the initial sock elution suspension (straight/neat undiluted) and 3 serial dilutions of the suspension in PBST (10^{-1}, 10^{-2} and 10^{-3}).

- Repeat steps (a) through (c) for each sample.

11.5.3 Culture spore suspensions on SBA

Note: ***Plating of 100 µL results in an additional 1:10 dilution of each of the suspensions.***

Each of the following will be conducted in triplicate, resulting in the evaluation of 12 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA plate (10^{-1} mL of the original elution suspension).

b. After vortexing tubes, pipet 0.1 mL of 10^{-1} suspension onto surface of pre-dried SBA plate (10^{-2} mL of the original elution suspension).

c. After vortexing tubes, pipet 0.1 mL of 10^{-2} suspension onto surface of pre-dried SBA plate (10^{-3} mL of the original elution suspension).

d. After vortexing tubes, pipet 0.1 mL of 10^{-3} suspension onto surface of pre-dried SBA plate (10^{-4} mL of the original elution suspension).

- After pipetting the 3 spread plates for each dilution, use a sterile L-shaped spreader to distribute inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Please ensure that inoculum is evenly distributed over entire surface of the plate. Repeat for next 3 dilutions.
- Allow inoculum to absorb into the medium completely.

11.5.4 Invert plates and incubate at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. *B. anthracis* produces flat or slightly convex, 2 – 5 mm colonies, with edges that are slightly irregular and have a "ground glass" appearance. Comma-shaped projections may arise from the colony edge.

B. anthracis is not β-Hemolytic. However, weak hemolysis may be seen under areas of confluent growth in aging cultures and should not be confused with β-hemolysis.

a. If the number of colonies is ≤ 300/plate, record actual number.

b. If the number of colonies is > 300/plate, record as "too numerous to count" (TNTC).

c. If no target colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.5.6).

A minimum of 3 typical colonies should be confirmed using real-time PCR (Section 11.6).

11.5.5 Capture spores on MicroFunnel™ filter membranes and culture on SBA

- Place 3, 0.45 µm (pore-size) MicroFunnel™ filter funnels (Section 5.4.4) on the vacuum manifold and moisten membrane with 5 mL PBST. All filtering should be done with a vacuum pressure < 20 mm Hg.

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- With the vacuum valve closed (and vacuum pressure released), place 10 mL of PBST into each filter cup. Add 1.0 mL of the undiluted elution suspension each to two MicroFunnel™ cups and to the third cup add the remainder.
- Open the vacuum valve and filter the suspension. Close the valve and release the vacuum pressure. Rinse the walls of each MicroFunnel™ cup with 10 mL of PBST and filter. Open the valve and complete filtration.
- Squeeze the walls of the MicroFunnel™ cup gently and separate the walls from the base holding the filter. Discard cup. Remove each membrane with sterile forceps and place it grid-side up on a SBA plate. Make sure that the filter is in contact with the surface of the agar. If an air pocket occurs under the filter, use the sterile forceps to lift the edge of the filter to release the air pocket.
- Record the exact volume of the undiluted elution suspension filtered (1.0 mL) on each plate.
- Repeat steps (Section 11.5.5) described above for each sample.

11.5.6 Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of 3 days. Plates should be examined within 18 – 24 hours after starting the incubation and at 72 hours of incubation. Count the number of colonies and record results. Confirm 1 – 3 colonies using real-time PCR (Section 11.6).

11.6 Confirmation of *B. anthracis* Colonies by Real-time PCR Analysis

11.6.1 Pipet 100 µL of PCR-grade water into a 1.5 mL Eppendorf microcentrifuge tube (Section 5.2.13).

11.6.2 Use a disposable 1 µL inoculating loop or pre-wetted swab to remove bacterial growth from a typical *B. anthracis* colony grown on SBA.

Note: ***In some cases, it may be difficult to remove the bacterial growth with a loop. If this happens, use a sterile applicator swab. Be sure to pre-wet the swab with PCR-grade water before removing the bacterial growth.***

11.6.3 Insert the loop or swab into the tube containing the PCR-grade water and immerse the in the liquid.

11.6.4 Gently spin the loop or swab in the liquid to remove and resuspend the bacterial growth in the water. Press the tip of the swab against the tube to remove the liquid from the tip prior to discarding.

11.6.5 Lysate preparation

- Cap the microcentrifuge tubes containing the bacterial suspension and briefly vortex.
- Place the capped tubes in a floating rack if using the water bath. Otherwise place the capped tube in the heat block at 95°C – 98°C.
- Heat the sample for 5 minutes.
- Remove the tubes from the water bath or heat block and place it directly in a cold block. Chill for a minimum of 2 minutes.
- Place the microcentrifuge tubes in the refrigerated microcentrifuge. Centrifuge at 12,000 rpm for 2 minutes.

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11.6.6 Filtration of lysate using 0.1 µm centrifugal filter unit (Section 5.1.23)

- Remove top cap from the filtrate collection tube.
- Hold the filter unit vertical with the filter cup opening facing up. Pipet the supernatant from each microcentrifuge tube into the corresponding filterate collection tube. Avoid removing any visible pellet material.
- Replace top cap onto the filtrate collection tube. Balance the tubes prior to placing the filter unit into the microcentrifuge fixed–angle rotor.
- Centrifuge the filter units for 2 minutes at 8000 rpm. Ensure all supernatant is collected in the filtrate cups.

Note: If the supernatant has not passed completely through the filter, centrifuge for an additional 2 minutes. Repeat as necessary until all the supernatant has passed through the filter.

- Remove top cap and discard the filtrate cup using sterile disposable forceps. Replace top cap.
- The liquid in the filtrate collection tube is the lysate for real-time PCR analysis.
- Wipe the outside of the tube containing lysate with bleach.
- It is safe to remove lysates from the BSL-3 after filtration and disinfecting the outside of the tube.
- Lysates must be stored in a cold block while preparing for the PCR analysis.
- If PCR analysis will not be completed the same day the lysates are prepared, aliquot and freeze them at -20°C.

Note: DNA extracted by this procedure should not be stored for more than one week.

11.6.7 Use 5 µL of the lysate as the DNA template to run the PCR analysis in triplicate.

11.6.8 For real-time PCR, follow Sections 9.3 − 9.3.23 with the following exceptions and changes:

- No PNC and EIC controls are required for the samples.
- For each batch of sample colonies, PCR Master Mix should be made for 4 PCs, 4 NTCs and 3 replicates for DNA extracts per colony.

11.6.9 Refer to Sections 12.1 and 12.3 for Data Analyses and Calculations.

12.0 Data Analysis and Calculations

12.1 Real-time PCR During the Site Characterization Phase

Calculate the average C_T from the replicate reactions for each sample, PC and the EIC, where applicable. The average $C_T \leq 40$ for the sample indicates a positive result suggesting the presence of *B. anthracis* spores in the sample. A minimum of two out of three replicates must show $C_T \leq 40$ for a sample result to be considered positive. If only one out of three PCR replicates for any sample gives $C_T \leq 40$, the PCR analysis of the DNA extract for that sample must be repeated.

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If the EIC for a sample results in a C_T value (≥ 3) compared to the C_T value for the positive control, there may be matrix inhibition. If the corresponding sample is negative ($C_T > 40$) for *B. anthracis*, the sample should be diluted 1:10 and the PCR assay should be repeated for that sample along with the EIC with diluted sample extract. Negative controls (NTCs) should not yield any measurable C_T values; if C_T values are obtained check for potential for cross-contamination and repeat analysis. In addition, field blank samples should not yield any measurable C_T values. If C_T values are obtained as a result of a possible contamination or cross-contamination, depending upon the C_T value, a careful interpretation of the C_T values for the sample DNA extracts must be done for the final result or the PCR analyses must be repeated.

12.2 RV-PCR

Calculate an average C_T from the replicate reactions for T_0 and T_9 DNA extracts of each sample. Subtract the average C_T of the T_9 DNA extract from the average C_T of the T_0 DNA extract. If there is no C_T for the T_0 DNA extract (i.e., the T_0 is non-detect), use 45 (total number of PCR cycles used) as the C_T. The change (decrease) in the average C_T value from T_0 to T_9 (ΔC_T) ≥ 9 indicates a positive result suggesting the presence of viable *B. anthracis* spores in the sample. If an incubation time longer than 9 hours was used for the RV-PCR, instead of T_9, appropriate T_x (incubation time) should be used. However, (ΔC_T) ≥ 9 algorithm should still be used for a positive result. Depending upon the end user's requirement, sample complexity (dirtiness) and the phase of response during an event, a lower ΔC_T criterion of ≥ 6 (a two log difference in DNA concentration) and a corresponding higher endpoint PCR C_T of ≤ 39 could be set. A minimum of two out of three T_0 PCR replicates must result in C_T values ≤ 44 (in a 45-cycle PCR) to calculate the average C_T. A minimum of two out of three T_9 PCR replicates (or T_x for other incubation time) must result in C_T values ≤ 36 to calculate the average C_T for a sample result to be considered positive. Negative controls (NTCs) should not yield any measurable C_T values above the background level. If C_T values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat analysis. In addition, field blank samples should not yield any measurable C_T values. If C_T values are observed as a result of a possible contamination or cross-contamination, a careful interpretation of the C_T values for the sample DNA extracts and field blanks must be done to determine if the data is considered valid or if the PCR analyses must be repeated.

12.3 Culture

12.3.1 Serial dilution plating

Count the number of typical colonies on replicate culture plates and calculate the average number of colonies per plate. Apply the following (a − c) when counting the colonies and report results based on the number of confirmed colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on SBA plates from field blank samples. If growth is observed on plates, colony morphology should be evaluated to determine if contamination is due to the target organism and potential source of contamination. Samples should be reanalyzed if QC plates are contaminated with *B. anthracis.*

a. If the number of colonies is ≤ 250/plate, record actual number.

b. If the number of colonies is > 250/plate, record as "TNTC".

c. If no target colonies are observed, record as "None detected".

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To determine the number of spores per sample divide the total number of *B. anthracis* colonies by the dilution factor plated, and multiply by the conversion factor for 1 mL, and the total suspension volume. For example if 201 colonies were observed on the 10^{-1} dilution plate, and the total suspension volume was 5 mL, the number of colonies per sample would be 100,500.

$$[(201/0.1) \times 10] \times 5 = 100{,}500 \text{ spores per sample}$$

12.3.2 MicroFunnel™ filter plating

Count the number of typical colonies on each filter and record. Apply the following (a − c) when counting the colonies and report results based on the number of confirmed colonies.

Media sterility checks should not exhibit growth. Growth should also not be present on SBA plates from field blank samples. If growth is observed on filters, colony morphology should be evaluated to determine if contamination is due to the target organism and potential source of contamination. Samples should be reanalyzed if QC plates are contaminated with *B. anthracis.*

a. If the number of colonies is ≤ 80/plate, record actual number.

b. If the number of colonies is > 80/plate, record as "TNTC".

c. If no target colonies are observed, record as "None detected".

To determine the number of spores per 5 mL sample using the 1 mL aliquot plates, multiply the total number of *B. anthracis* colonies on each plate by 5 (total suspension volume). For example if 60 colonies were observed on one of the 1.0 mL plates, and the total suspension volume was 5 mL, the number of colonies per sample would be 300.

$$60 \times 5 = 300 \text{ spores per sample}$$

To determine the number of spores per 5 mL using the third plate (remainder of the volume, [1.7 – 2.7 mL) multiply the total number of *B. anthracis* colonies by the ratio of the total suspension volume (5 mL) to the actual volume plated. For example if 60 colonies were observed on the plate, the volume plated was 2.7 mL and the total suspension volume was 5 mL, the number of colonies per sample would be 111.

$$60 \times (5/2.7) = 111 \text{ spores per sample}$$

12.3.3 Confirmation of Colonies by Real-time PCR

Presence of *B. anthracis* typical colonies on the culture plate indicates the presence of viable *B. anthracis* spores or vegetative bacteria in the sample. A minimum of three typical colonies should be confirmed using real-time PCR. The $C_T \leq 40$ for the sample indicates a positive result suggesting the presence of *B. anthracis* in the respective sample. Report the results based on the number of confirmed colonies. Negative controls (NTCs) should not yield any measurable C_T values above the background level. If C_T values are obtained as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis.

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13.0 Method Performance

To be completed upon protocol verification and/or validation.

14.0 Pollution Prevention

14.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

14.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be discarded.

15.0 Waste Management

15.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management**,** especially the biohazard and hazardous waste rules and land disposal restrictions. Following these regulations protects the air, water and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

15.2 Samples, reference materials and equipment known or suspected to be contaminated with or to contain viable *B. anthracis* must be decontaminated prior to disposal.

15.3 Large volume water filtrates should be decontaminated using bleach (10% final concentration) for a minimum of 30 minutes prior to disposing to the sanitary sewer (e.g., pouring down the drain).

15.4 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 16.6) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 16.7), both authored by the American Chemical Society.

16.0 References

16.1 U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health. 2009. *Biosafety in Microbiological and Biomedical Laboratories* (BMBL), 5th Edition. http://www.cdc.gov/biosafety/publications/bmbl5/index.htm

16.2 American Chemical Society (ACS). 2005. *Reagent Chemicals: Specification and Procedures*, Oxford University Press (USA), New York.

16.3 British Drug Houses, Ltd. 1957. *AnalaR Standards for Laboratory Chemicals*. 5th Edition. BDH Ltd., Poole, Dorset, U.K.

16.4 United States Pharmacopeia. 2005. *United States Pharmacopeia and National Formulary 24*. United States Pharmacopeial Convention, Md.

16.5 Francy, D.S., Bushon, R.N., Brady, A.M., Bertke, E.E., Kephart, C.M., Likirdopulos, C.A., Mailot, B.E., Schaefer, F.W. III and Lindquist, H.D.A. 2009. "Performance of Traditional and Molecular Methods for Detecting Biological Agents in Drinking Water." U.S. Department of the Interior /U.S. Geological Survey. Scientific Investigations Report 2009–5097.

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16.6 American Chemical Society (ACS) 1990. *The Waste Management Manual for Laboratory Personnel*. American Chemical Society Department of Government Relations and Science Policy, Washington, DC.

16.7 American Chemical Society (ACS). 2002. *Less Is Better: Laboratory Chemical Management for Waste Reduction*. American Chemical Society Taskforce on RCRA (Resource Conservation and Recovery Act of 1976), Washington, DC. http://portal.acs.org/portal/fileFetch/C/WPCP_012290/pdf/WPCP_012290.pdf

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Appendix A

Ultrafiltration (UF) for the Detection of Bioterrorism Threat (BT) Agents in Potable Water Samples

Note: ***This protocol should not be misconstrued as a laboratory standard operating procedure (SOP) that addresses all aspects of safety; the laboratory should adhere to their established safety guidelines.***

At a MINIMUM these procedures should be performed in a Biological Safety Level (BSL)-3 facility using BSL-3 practices. It is recommended that all sample manipulations be performed within a Class II (or higher) biological safety cabinet (BSC).

1.0 Sample Preparation

Note: ***Water samples should be concentrated as soon as possible after collection and analyses should be initiated immediately, if possible. However, if analyses cannot be accomplished immediately, the concentrated sample may be stored at 2ºC − 8ºC for up to 24 hours.***

1.1 Laboratory Supplies

1.1.1 Asahi Kasai Rexeed 25SX Dialyzers (Dial Medical Supply Cat. No. 25SX or equivalent)

1.1.2 Masterflex® L/S #36 silicon tubing (Cole Parmer® Cat. No. EW-96410-36 or equivalent)

Note: ***An alternative to the Masterflex® tubing is #36 BioPharm Silicone tubing (Cole Parmer® Cat. No. EW-96420-36).***

1.1.3 Masterflex® L/S #24 silicon tubing (Cole Parmer® Cat. No. EW-96410-24 or equivalent)

1.1.4 Masterflex® tubing reducing connectors (Cole Parmer® Cat. No. EW-40610-08 or equivalent)

1.1.5 3-prong extension clamp (Cole Parmer® Cat. No. EW-08021-36 or equivalent)

1.1.6 Ring stand (Fisher Cat. No. 14-670C or equivalent)

1.1.7 Clamp connector/holder (Cole Parmer® Cat. No. EW-08041-20 or equivalent)

1.1.8 Nalgene® Analytical Filter Unit, 0.45 µm (Fisher Scientific Cat. No. 09-740-21B or equivalent)

1.1.9 Heavy duty pinchcock, metal clamp (Cole Parmer® Cat. No. EW-08126-0302 or equivalent)

1.1.10 GN-6 Metricel® Membrane Filters, 0.45 µm (VWR Cat. No. 28148-926 or equivalent)

1.1.11 Forceps, sterile, disposable (Cole Palmer® Cat. No. U06443-20 or equivalent)

1.1.12 Hose clamps (Cole Parmer®)

(a) Large: #10 and #12 white, plastic (Cat. No. EW-06832-10, EW-06832-12 or equivalent)

(b) Medium size: #6 and #8 white, plastic (Cat. No. EW-06832-06, EW-06832-08 or equivalent) or 7/32" to 5/8" stainless steel (Cat. No. EW-06403-11 or equivalent)

(c) Small: #4 white, plastic (Cat. No. EW-06832-04 or equivalent) or 7/32" to 5/8" stainless steel (Cat. No. EW-06403-11 or equivalent)

1.1.13 1 L heavy duty polypropylene vacuum bottle (Fisher Scientific Cat. No. 06-443A or equivalent)

1.1.14 3 port filling/venting closure cap with tubing (Fisher Scientific Cat. No. 02-923-13Y or equivalent)

1.1.15 Barbed reducing Y connector 1/4 × 3/8 (Cole Parmer® Cat. No. EW-30726-33 or equivalent)

1.1.16 DIN adapters (filter connectors)

(a) Small connectors for 24 tubing (Molded Products Cat. No. MPC-855NS.250PP or equivalent)

(b) Large connectors for 36 tubing (Molded Products Cat. No. MPC-855NS.375PP or equivalent)

1.1.17 Blood Port Storage Cap (screw cap), polypropylene (Molded Products Cat. No. MPC-40PP or equivalent)

1.1.18 Ice bucket and ice

1.1.19 Flow regulator (Keck) tubing clamps (Cole Parmer® Cat. No. A-06835-07 or equivalent)

1.1.20 Ziplock bags

1.1.21 Parafilm

1.1.22 Bleach Wipes (Dispatch® Cat. No. 69150 or equivalent)

1.1.23 Bottle, sterile, 100 mL

1.1.24 Tubes, sterile, 15 mL (Fisher Scientific Cat. No. 339650 or equivalent)

1.1.25 Bottle, 1-L sterile polypropylene (Thermo Scientific-Nalgene® Cat. No. 2105-0032 or equivalent)

1.1.26 Filter flask, sterile, 500 mL, glass or polypropylene (Fisher Scientific Cat. No. FB-300-500; 10-182-50A; or equivalent).

1.1.27 10 mL plastic pipets, sterile, T.D. bacteriological (Fisher Scientific Cat. No. 13-678-12E or equivalent)

1.1.28 50 mL plastic pipets, sterile, T.D. bacteriological (Fisher Scientific Cat. No. 13-678-14C or equivalent)

1.1.29 Pliers

1.1.30 Graduated cylinder (1 L) or graduated beaker (1 L)

1.1.31 Syringe, 60 mL

1.1.32 #7 rubber stopper, 500 mL flask, with hole (Fisher Scientific Cat. No. 14-135L or equivalent)

1.1.33 Collapsible containers, 10 L or 20 L (Cole Parmer® Cat. No. EW-06100-30 or EW-06100-40)

1.1.34 Vacushield™ Vent Device (HEPA filter) (VWR Cat. No. 55095-006 or equivalent)

1.2 Equipment

1.2.1 Masterflex® Console Drive (Cole Parmer® Cat. No. EW-07554-90 or equivalent)

1.2.2 Masterflex® EasyLoad II Pump Head (Cole Parmer® Cat. No. SI-77200-52 or equivalent)

1.2.3 Jiffy-Jack® apparatus positioner (Cole Parmer® Cat. No. A-08057-40 or equivalent)

1.2.4 BD Clay Adams™ Nutator Mixer (VWR Cat. No. 15172-203 or equivalent)

1.2.5 Biological safety cabinet (BSC) – Class II or Class III

1.2.6 Vortex mixer (Fisher Scientific Cat. No. 02-215-365 or equivalent)

1.3 Reagents

1.3.1 1000X (10%) Sodium Poly-Phosphate (NaPP): Add 10 g of NaPP per 100 mL of sterile reagent-grade water in a sterile 100-mL bottle. Cap the bottle and shake vigorously by hand for 1 minute to mix the solution. If the water is cold (e.g., from refrigerator), let the solution dissolve for 2 hours at room temperature, mixing for 1minute approximately every 15 minutes. If water is initially at room temperature, the NaPP should dissolve within 30 minutes (mixing vigorously by hand every 15 minutes). If the NaPP is not completely dissolved in the water, place the NaPP solution in a water bath at 50°C and incubate for 2 hours. Continue incubating until the NaPP is completely in solution. Store the 10% NaPP in refrigerator for up to 2 months.

1.3.2 10% Tween 80-1% Antifoam Y-30 solution: Pipet 0.1 mL Antifoam Y-30 Emulsion (Sigma Cat. No. A5758 or equivalent) and 1 mL Tween 80 (Fisher Cat. No. T164 or equivalent) into 15 mL conical tube containing 8.9 mL reagent-grade water. Vortex for 30 seconds to mix. Solution can be stored at room temperature for 1 month.

1.3.3 Elution solution (0.01% Tween® 80, 0.01% NaPP and 0.001% Antifoam Y-30): Add 0.5 mL of the 10% Tween 80-1% Antifoam Y-30 solution and 0.5 mL of 10% NaPP to 500 mL of sterile reagent-grade water. Swirl to mix. Solution can be made up to 24 hours in advance and stored in a refrigerator. Bring to room temperature prior to use.

Note: The elution solution should be made in or transferred to a sterile 1-L heavy duty polypropylene bottle with closed cover.

1.3.4 0.01% NaPP solution (filter wash) – 1 L: Add 1 mL of 10% NaPP solution to 999 mL of reagent-grade water and swirl to mix. Solution can be made up to 24 hours in advance and stored in a refrigerator. Bring to room temperature prior to use.

1.3.5 1% Bleach solution (for tubing decontamination) – 500 mL: Add 5 mL bleach to 495 mL reagent grade or deionized water and swirl to mix. Solution can be stored at room temperature for 1 week.

1.3.6 70% Ethanol solution (Fisher Scientific Cat. No. 04-355-56 or equivalent)

1.3.7 Sodium thiosulfate solution: Prepare 10% w/v sodium thiosulfate by adding 100 g sodium thiosulfate (Fisher Scientific Cat. No. S446 or equivalent) to 1 L of sterile reagent-grade water and mix well. Store at 4°C for up to 1 month.

1.3.8 Dialyzed Fetal Bovine Serum (FBS) (Fisher Scientific Cat. No. SH3007303 or equivalent)

1.3.9 Phosphate buffered saline (PBS) (Fisher Scientific Cat. No. BP3991 or equivalent)

1.4 Pre-treat the Rexeed ultrafilter with dialyzed 5% FBS in water to block non-specific protein binding.

1.4.1 To make 150 mL of 5% FBS, add 7.5 mL of FBS to 142.5 mL of reagent-grade water.

1.4.2 Before adding the FBS to the filter, secure the ultrafilter to ring stand with a 3 prong clamp. Ensure the bottom port is securely closed with a blood port storage screw cap. Place the white port caps that come with the ultrafilter onto both side ports, leaving them loosened to allow air to escape.

1.4.3 Load the ultrafilter with approximately 120 mL (no more than 150 mL) of 5% FBS by injecting it into the top port of the ultrafilter (a 60 mL syringe with no needle works well for this application). Attach 3" of #24 tubing to a small filter connector, secure with a #4 clamp, screw into the filter port, and insert the tip of the syringe into the #24 tubing. Pour 60 mL of FBS solution into the syringe and use the plunger to push the solution into the ultrafilter. Repeat for remaining volume.

1.4.4 When the ultrafilter is loaded, close the open end of the ultrafilter with a second blood port storage screw cap and ensure side ports are closed with white port caps (they will "click" into place).

1.4.5 Place the ultrafilter on a rocker panel at room temperature; rock for 30 minutes.

Note: Do not store the filter after pretreating, as this will encourage growth of contaminating bacteria which may clog the filter.

1.5 Pre-treat the Water Sample

Note: Any procedure in which sample containers are opened should be performed inside a BSC.

1.5.1 If sodium thiosulfate was not added to the sample at the time of collection, add 0.5 mL/L of a 10% w/v solution of sodium thiosulfate immediately upon receipt of the sample.

1.5.2 Pretreat the water sample with NaPP to reach a final concentration of 0.01%. To achieve a 0.01% concentration of NaPP, add 1 mL of 10% NaPP per 1 L of water sample.

1.6 Prepare the empty filtrate container(s) by adding a sufficient volume of bleach such that the final concentration is at least 1% bleach (e.g., 200 mL bleach in a 20 L container).

1.7 Assembly of the Sample Tubing Set

1.7.1 Remove both ends (tip and end containing cotton material) of a 10 mL pipet by carefully breaking off the ends while the pipet is within its plastic wrapping (Figure 1, a). Keep the plastic sleeve to place the sample pipet in during changing of sample containers.

1.7.2 Connect the 10 mL pipet to 14" of #24 Masterflex® (MF) tubing (Figure 1, b) and secure with a medium hose clamp (#8 plastic) (Figure 1, #1).

1.7.3 Connect the #24 tubing to one of the small 1/4" barbs on the Y connector (Figure 1, c) and secure with a small hose clamp (#4 plastic or 7/32" to 5/8" stainless steel) (Figure 1, #2).

1.7.4 Connect 21" of #36 tubing (Figure 1, d) to the end of the large filter connector (Figure 1, e) and secure connection with large hose clamp (#10 plastic) (Figure 1, #3).

1.7.5 Connect the other end of the #36 tubing to the large 3/8" barb on the Y connector (Figure 1, c) and secure with a large hose clamp (#8 plastic) (Figure 1, #4).

1.7.6 Connect 6" of #24 tubing (Figure 1, f) to the open small 1/4" barb on the Y connector (Figure 1, c) and secure with a small hose clamp (#4 plastic or 7/32 to 5/8" stainless steel) (Figure 1, #5). Connect the opposite end to the empty port on the 1 L vented cap bottle and secure with a stainless steel clamp (vented cap bottle not shown in Figure 1, #6; see Figure 7a, #10).

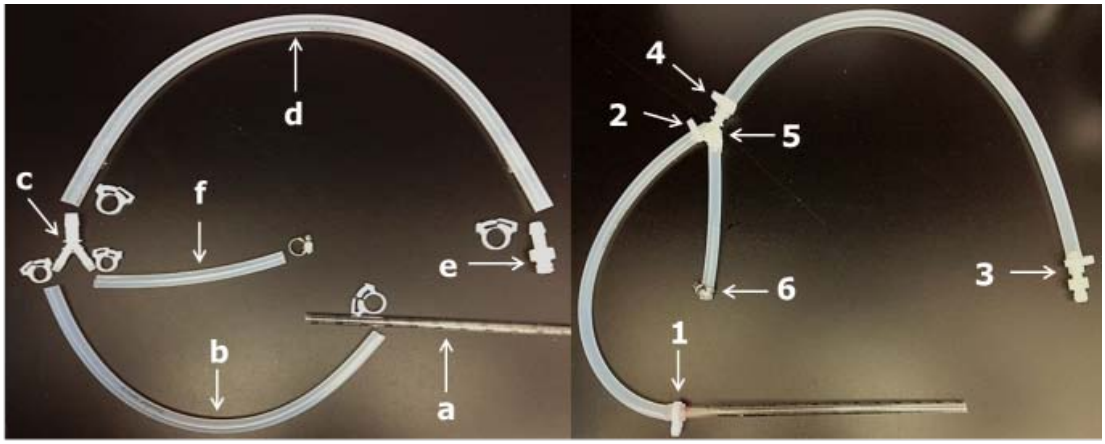


Figure 1. Sample tubing set assembly.

1.8 Assembly of the Retentate Return Tubing Set

1.8.1 Connect 15" of #24 tubing (Figure 2, a) to the small filter connector (Figure 2, b); secure connection with a small hose clamp (#4 plastic or 7/32" to 5/8" stainless steel) (Figure 2, c).

1.8.2 Attach a flow regulator tubing clamp (Figure 2, d) to the #24 tubing so that the wide end is facing the 1 L vented cap bottle (Figure 2, e).

1.8.3 Connect the opposite end of the #24 tubing to the port on the 1 L vented cap bottle that is attached to the shorter internal tubing (Figure 2, f; Figure 7a, #9); secure with a stainless steel clamp (Figure 2, g)..

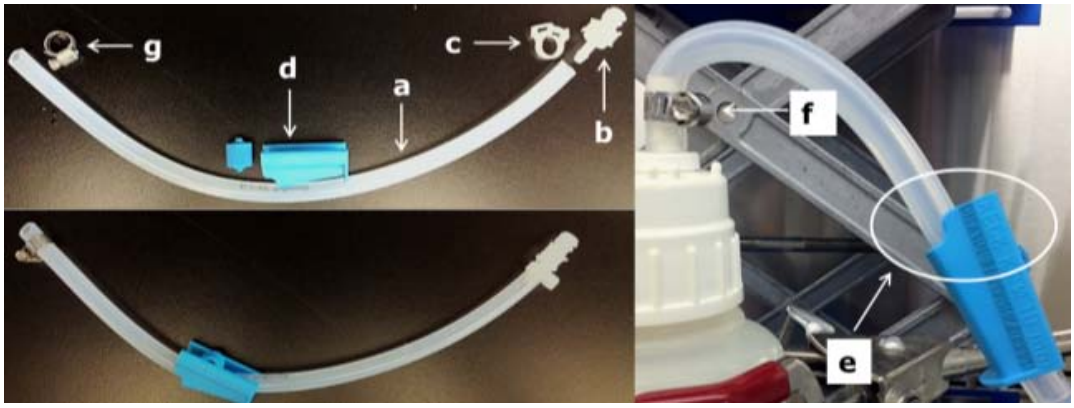


Figure 2. Retentate return tubing set assembly.

1.9 Assembly of the Filtrate Tubing Set

1.9.1 Remove both ends (tip and end containing cotton material) of a 10 mL pipet by carefully breaking off the ends while the pipet is within its plastic wrapping (Figure 3, a).

1.9.2 Connect the 10 mL pipet to 16" of #36 MF tubing and secure with a medium hose clamp (#8 plastic) (Figure 3, b).

Figure 3. Filtrate tubing set assembly.

1.10 Connection of the Pumping Station

Note: ***The pumping station and ultrafilter setup may be assembled inside of a BSC or on the bench top (bench top allows ease of assembly, but it is recommended that this protocol not be performed on the bench). Additionally, absorbent underpads/diapers may be placed under the UF setup and containers to capture any potential leaks or spills that may occur during sample processing or disassembly.***

1.10.1 Raise the pump to a height equal to or above the top of the reservoirs by placing the pump on a shelf or raising it on a variable height platform such as the Jiffy-Jack® apparatus positioner. Also, secure the filter and the 1 L vented cap bottle in a vertical position by using adjustable metal clamps and a ring stand.

1.10.2 Connect the sample reservoir to the ultrafilter using the sample tubing set. Attach the large filter connector on the end of the sample tubing set to the top port of the ultrafilter. Feed the #36 tubing through the pump head, and ensure the tubing remains securely clamped to the large filter connector.

1.10.3 Attach the small filter connector on the end of the retentate return tubing set to the bottom port of the ultrafilter.

1.10.4 Connect the ultrafilter to the filtrate reservoir by connecting the filtrate return tubing set to the top side port of the ultrafilter (may secure with #12 plastic clamp, but not required) and placing the 10 mL pipet into the filtrate reservoir. Ensure the other side port is closed with the plastic cap provided with the filter.

1.10.5 The assembled UF setup should appear as in Figures 7a and 7b.

1.10.6 Place a 1 L beaker next to the UF setup and another 1 L beaker next to the filtrate setup. Use beakers to hold the sample and filtrate tubing pipets when changing sample and filtrate containers to prevent potential water droplets from dripping onto the BSC.

1.10.7 Verify that the MF tubing is threaded through the pump head correctly (Figure 4).

1.10.8 Set the pump to ~50% power. If using a digital pump, the flow rate should be set to 1450 mL/min.

1.10.9 If the pump has a flow direction toggle switch, confirm the flow direction is set to the right (Figure 4).

Figure 4. Photo of the pump head.

1.11 Washing the Ultrafilter

1.11.1 Wash the 5% FBS from the ultrafilter by placing the sample tubing end (Figure 5, #2) into the 1 L bottle containing 0.01% NaPP filter wash prepared in Section 1.3.3 (Figure 5, #1).

1.11.2 Detach the retentate return tubing set (Figure 5, #5) from the 1 L vented cap bottle (Figure 5, #7) and place the end in the filtrate reservoir (Figure 5, #6) so that the retentate return tubing set and the filtrate tubing are both in the filtrate container.

1.11.3 Apply the pinchcock to the tubing from port C (clamp the tubing from vented cap bottle to the Y connector, getting as close to the Y connector as possible) (Figure 7a, #1).

1.11.4 Start the pump (Figure 5, #3) and flush the 0.01% NaPP filter wash through the lines and the ultrafilter (Figure 5, #4).

Note: While flushing the NaPP filter wash, be sure to check the tubing and connections for any leaks or drips throughout the system.

1.11.5 When the filter wash is completed, reset the tubing as shown in Figure 7a.

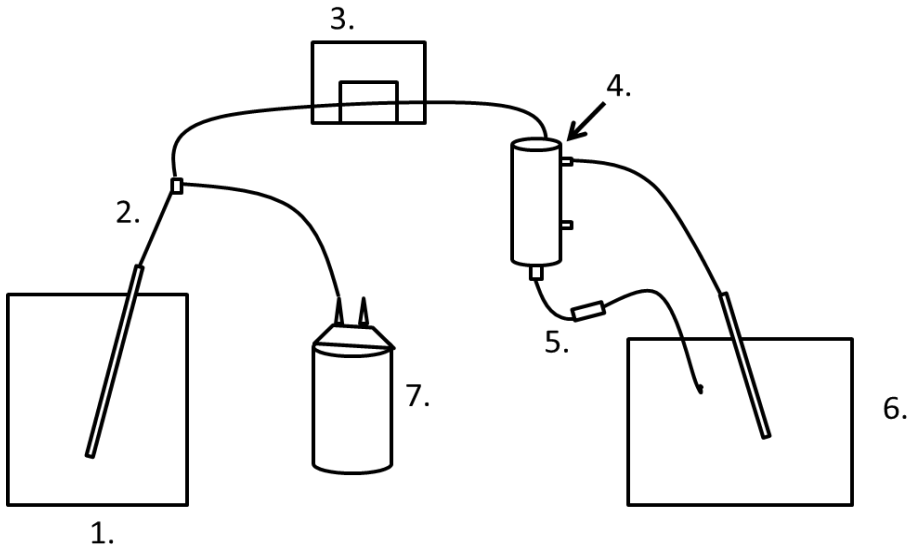


Figure 5. Schematic of Filter Wash

(1) Sterile water wash, (2) Sample tubing, (3) Pump, (4) Ultrafilter,

(5) Retentate tubing set, (6) Filtrate reservoir, (7) 1 L vented cap bottle

2.0 Primary Water Sample Concentration

2.1 With the sample container in place and the tubing set per Figure 7a, check to make sure the pinchcock is applied to the tubing from port C (Figures 7a and 7c, #1), the vented cap is securely tightened to the bottle, and the rubber cap is removed from the vented cap.

Note: ***When applying the pinchcock clamp to the tubing, make sure it is clamped as close to the Y connector as possible to completely block and/or stop the flow of water through tubing (Figure 7c, #1).***

2.2 Start the pump with the flow switch turned to the right and the pump speed set to the maximum (~ 2900 mL/min flow rate).

2.3 Once the 1 L retentate bottle is ~2/3 of the way full, quickly close the open port of the vented cap with the rubber cap and remove the pinchcock from the tubing (Figures 7a and 7c, #2). The pump will now be drawing water from both the sample container and the 1 L vented cap (retentate) bottle. Make sure the water level in the 1 L retentate bottle does not continue to rise. If the water level in the 1 L bottle does rise, remove the rubber cap and apply pinchcock to the tubing from port A (Figures 7a and 7c, #3). Once the water level in the 1 L bottle is 2/3 of the way full, close port with rubber cap and remove the pinchcock (Figures 7a and 7c, #2).

2.4 Tighten the flow regulator by rolling the knob to the right until the "K" in the "KT" lettering on the front face of the flow regulator is directly in the middle of the adjustment knob (Figure 6). The back pressure provided by the flow regulator should produce a filtrate rate between 1000 – 1400 mL/min.

Note: ***The flow rate does not need to be measured.***

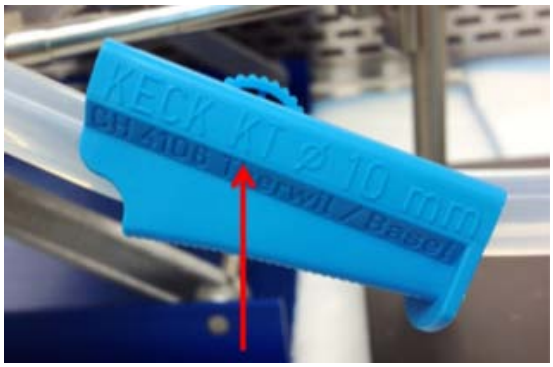
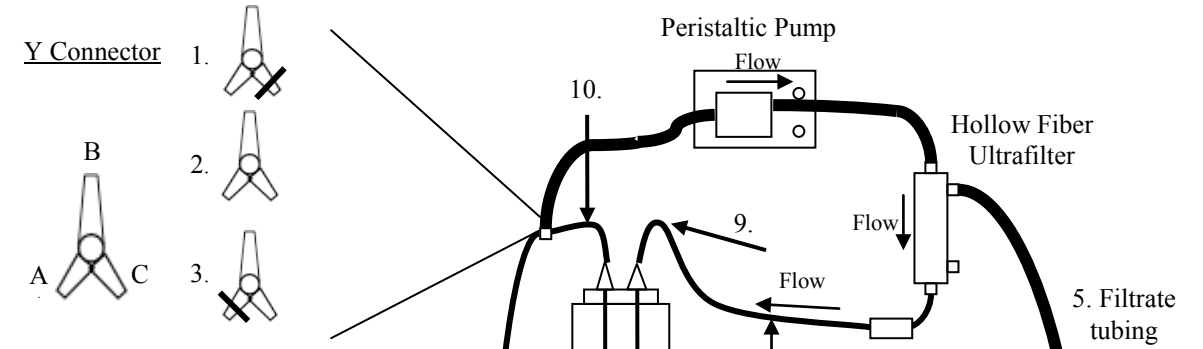


Figure 6. Position flow regulator rolling clamp over "K" in "KT".



Y Connector and pinchcock:

1. Clamp tubing from port C at beginning of filtration. Water flows from sample container through ultrafilter to fill 1L bottle.
2. Remove pinchcock when the volume in the 1L bottle is 2/3 full. The system then draws from both 1L bottle and sample container.
3. Clamp tubing from port A once the entire sample is drawn from the sample container. Sample is now being reduced in the 1L bottle only.

Figure 7a. Recirculating ultrafiltration assembly.

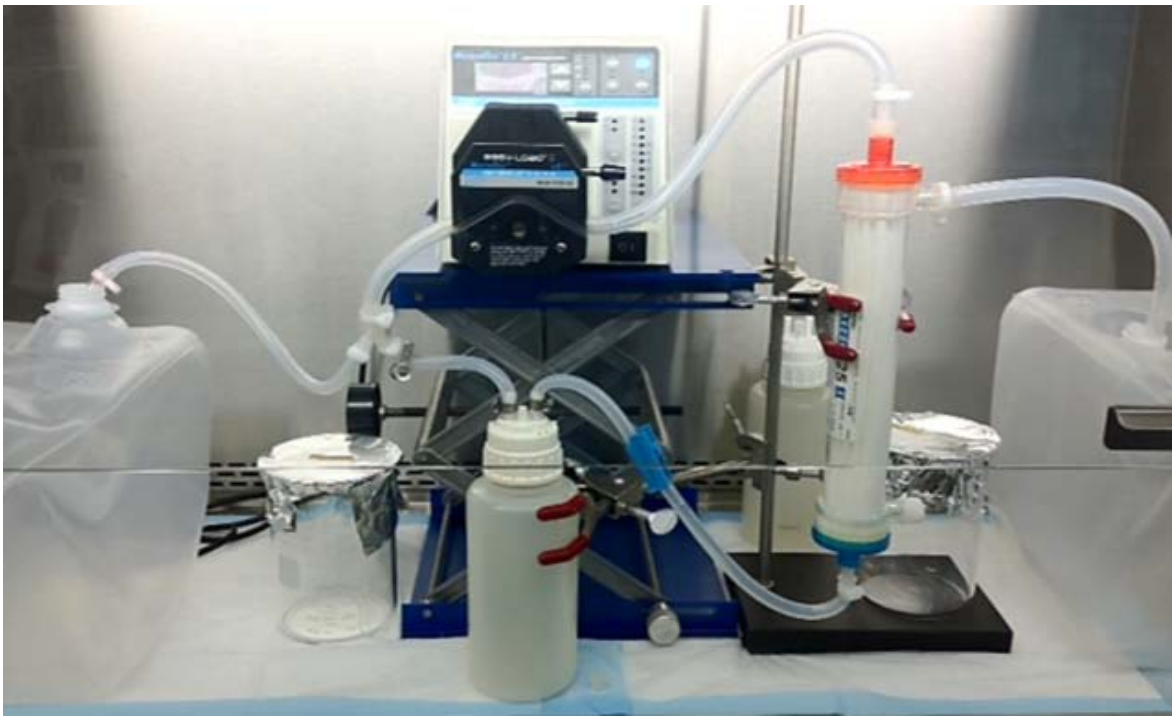


Figure 7b. Photograph of the ultrafiltration system set up inside a BSC.

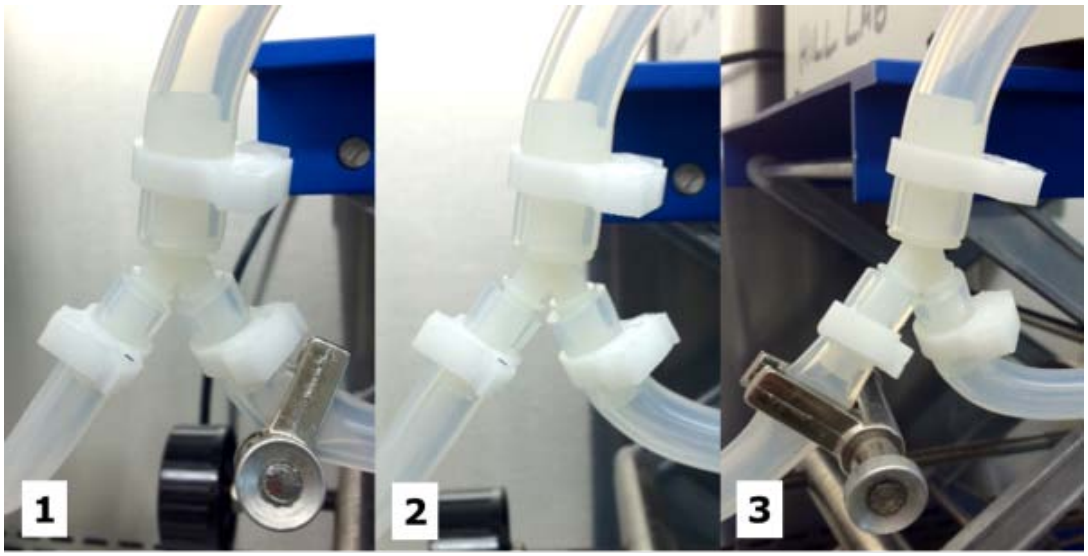


Figure 7c. Pinchcock positions.

2.5 When the sample container is empty, apply the pinchcock to clamp the tubing from port A (Figures 7a and 7c, #3), loosen the flow regulator and turn off the pump.

Note: ***Whenever the pump needs to be stopped, apply the pinchcock to clamp the tubing from port A so that the water containing microbes cannot flow back into the sample tank and then quickly press the stop button.***

2.6 If this is the only sample container, skip to step 2.13.

2.7 Remove the sample tubing set from the empty sample container and carefully place it in a sterile 1 L beaker. Remove the filtrate pipet in the same manner and place in a second sterile 1 L beaker.

Note: ***To prevent drips, ensure the pipet is free of any remaining sample while still inside the sample container. Labs may consider placing the pipet back into the plastic sleeve prior to placing it into the 1 L beaker. Alternatively, if sample container is disposable, the tubing may be cut and the pipet can be disposed of inside the sample container.***

2.8 Remove the empty sample container and replace with the next sample container to be filtered. Also replace the full filtrate container with an empty filtrate container [containing 1% bleach as directed in Section 1.3.4].

Note: ***Follow Biosafety in Microbiological and Biomedical Laboratories (BMBL) and lab-specific safety practices for BSL-3 working conditions while slowly moving containers into and out of the BSC. Let airflow re-establish for a minimum of 15 minutes each time the sash is lifted.***

2.9 Place the sample tubing set into the new sample container and the filtrate tubing set into the new, empty filtrate container.

2.10 Turn the pump back on and remove the pinchcock from the tubing from port A (Figures 7a and 7c, #2) so that the water is once again being drawn from both the container and the 1 L bottle.

2.11 Tighten the flow regulator to the same position described in Section 2.4 and continue filtration.

2.12 Repeat steps 2.7 – 2.11 for the rest of the containers.

2.13 When the last container has been emptied, apply the pinchcock to clamp the tubing from port A (Figures 7a and 7c, #3) so that the sample is only being re-circulated in the 1 L bottle, loosen the flow regulator, and remove the rubber cap from the 1 L bottle.

2.14 When the sample in the 1 L bottle draws down to about 1.5" from the bottom, move the pinchcock to clamp the tubing from port C (Figures 7a and 7c, #1), so that only air is being drawn into the filtration system by the pump.

Note: ***Continue flushing until all the retentate is out of the tubing and the filter. The retentate tubing may be lifted 2 - 3 times to help flush the water that has settled in the retentate tubing into the 1 L bottle.***

2.15 Turn off the pump when all of the retentate has been flushed from the tubing and the filter. The final volume should be approximately 200 mL – 250 mL.

2.16 Carefully unscrew the steel clamps from the ports on the vented cap bottle and remove the tubing connections from the top ports (containing the concentrated sample). Place them onto the ports of the second vented cap bottle containing the elution solution and secure the clamps (remove the closed cap from the elution solution bottle first).

2.17 Unscrew the vented cap from the first bottle containing the sample. Lift and securely hold the cap with one hand (tubing remains inside the bottle) while using a 25 mL or 50 mL pipet in the other hand to measure and transfer the sample retentate into a sterile, 1 L plastic bottle. Record the retentate volume.

2.18 Secure the second vented cap bottle containing the elution solution with the 3-pronged clamp from the first vented cap bottle. Check that the flow regulator is fully opened, the pinchcock is clamped to the tubing from port A (Figures 7a and 7c, #3) and the rubber cap is removed from the 1 L vented cap bottle. The assembly should now be ready for elution of the ultrafilter.

2.19 Turn on the pump, allowing the eluent volume to gradually reduce until the level draws down to 1.5" from the bottom of the bottle. Move the pinchcock to clamp the tubing from port C (Figure 7a and 7c, #1) to flush the rest of the eluent from the tubing and filter. Turn off the pump.

2.20 Unscrew the vented cap assembly from the second bottle containing the concentrated eluent. Lift and securely hold the cap with one hand (tubing remains inside the bottle) while using a 25 mL or 50 mL pipet in the other hand to measure and transfer the eluent to the 1 L plastic sample bottle containing the retentate. The total volume of the final UF concentrate should be 400 mL – 500 mL.

3.0 Secondary Water Concentration

3.1 Inside of a BSC, assemble the membrane filtration setup shown in Figure 9, ensuring the HEPA filter (Vacushield) is attached with #24 tubing (or alternative vacuum tubing) to the house vacuum and the side arm of the back-up flask. Connect the side arm of the main flask to the stopper of the back-up flask with vacuum tubing. This tubing length should be long enough such that the tubing rests on the surface of the BSC to help stabilize the flasks. Attach an additional piece of vacuum tubing to the side arm of the main flask for connection to the disposable filter units.

3.1.1 Use the piece of #24 tubing from the side arm of the main filter flask to attach the quick disconnect side arm on the filter unit (Figure 9, #5) and use sterile forceps to place a new filters onto the base of each filter unit (Figure 9, #3).

Note: ***Disposable filter units (Section 1.1.8) come with cellulose nitrate membrane filters, which must be replaced with the mixed ester cellulose filters. DO NOT remove the cellulose support pad on the base of the unit when replacing membrane filters. It is recommended to keep a small autoclave bag inside the BSC during processing for safe disposal of filter funnels.***

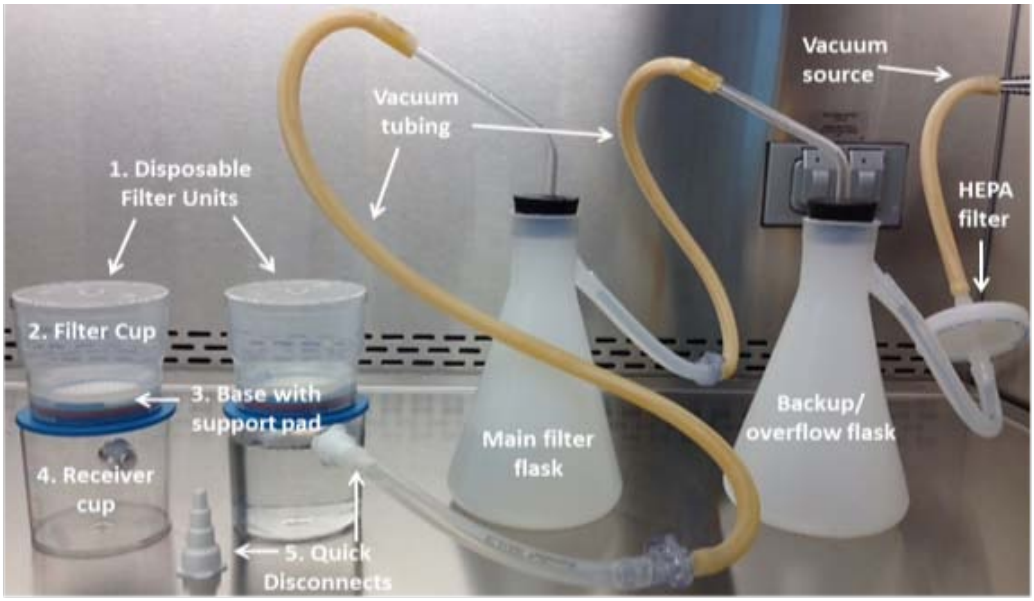


Figure 9. Membrane filtration setup.

3.2 Run a negative control before filtering any sample concentrate. Add 20 mL PBS to the filter cup (Figure 9, #2) and turn on the vacuum. Once the PBS has finished filtering, turn off the vacuum and remove the cup from the base of the filter unit (Figure 9, #3). Retrieve the membrane filter with sterile forceps and place onto a SBA plate. Incubate at 37ºC ± 1ºC for a maximum of 3 days.

3.3 Use sterile forceps to place a new 0.45 µm filter onto the support pad on the base of the filter unit. Reattach the filter cup to the base.

3.4 Add the retentate slowly to the filter unit (Figure 9, #2) and turn on the vacuum (not all the retentate will fit at once). Continue to add retentate to the filter unit taking care to avoid clogging the filter. Once the sample has finished filtering, rinse the filter cup 3 times with PBS in a squirt bottle. Turn off the vacuum; remove the filter cup from the bottom portion (Figure 9, #3) of the filter unit and set it on a sterile surface.

Note: ***If the final retentate is particularly cloudy or has noticeable sediment, it might be necessary to split the retentate volume between two or more filter units.***

3.5 Using sterile disposable forceps, grab the edge of the membrane at the filter unit base and fold it toward the other end. While holding the 2 edges together, take the forceps and place folded membrane into the bottom half of a 50 mL conical tube, avoiding the conical portion. Close tube.

3.6 Repeat steps 3.1 – 3.4 for the remaining samples.

Note: ***If multiple filtrations were run due to excess sediment, process all of the membrane-filters.***

3.7 For polymerase chain reaction (PCR) analysis, proceed to Section 9.1.5 of the protocol.

3.8 For Rapid Viability-PCR (RV-PCR) analysis, proceed to Section 10.2.6 of the protocol.

3.9 For culture analysis, proceed to Section 11.5 of the protocol.

4.0 Limitations

4.1 If the procedure is not performed correctly, it may result in false negative results.

4.2 Water from certain sources may contain higher levels of minerals, organic compounds or other substances which may affect the water concentration procedure and subsequent testing procedures for the detection of potential BT agents.

4.3 The presence of chlorine-based disinfectants in the water supply will inhibit or prevent the growth of most microorganisms. Sodium thiosulfate should be added as soon as possible to inactivate chlorine in the water. Addition of sodium thiosulfate at the time of collection of the water sample is recommended.

4.4 Even with the addition of sodium thiosulfate, some organisms may not survive the filtration process and may only be detected by real-time PCR.

5.0 Acronyms

BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biological safety level
BT	Bioterrorism threat
FBS	Fetal bovine serum
MF	Masterflex®
NaPP	Sodium poly-phosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RV-PCR	Rapid viability-polymerase chain reaction
SOP	Standard operating procedure
UF	Ultrafiltration

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