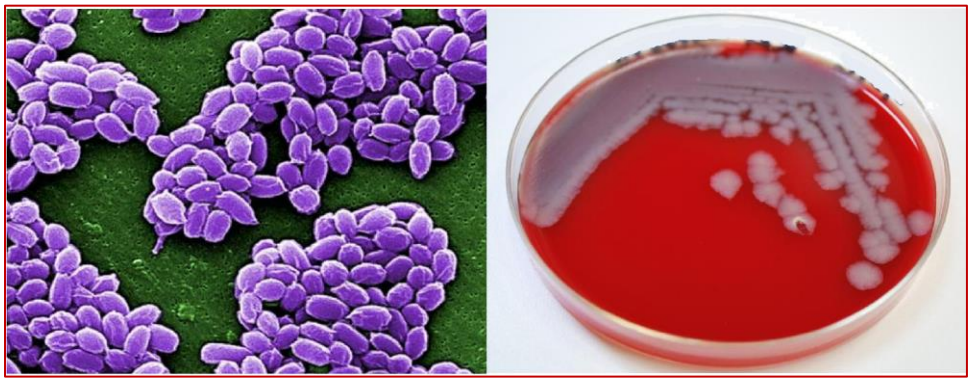
EPA/600/R-17/213 | July 2017
www.epa.gov/homeland-security-research

United States Environmental Protection Agency

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

Second Edition



SCIENCE

Office of Research and Development
Homeland Security Research Program

This page is intentionally left blank

EPA/600/R-17/213
July 2017

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

Second Edition

by

Sanjiv R. Shah, Ph.D.
Threat and Consequence Assessment Division
National Homeland Security Research Center
Cincinnati, OH 45268

EPA EP-C-15-012

U.S. Environmental Protection Agency Project Officer
Office of Research and Development
Homeland Security Research Program
Cincinnati, OH 45268

Disclaimer

The U.S. Environmental Protection Agency through its Office of Research and Development funded and managed the effort on the protocol described here under a contract (EPA EP-C-15-012) to CSRA. The contractor role did not include establishing Agency policy. This protocol has been subjected to the Agency's review and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. Mention of trade names, products, or services does not convey official EPA approval, endorsement, or recommendation.

Questions concerning this document or its application should be addressed to:

Sanjiv R. Shah, Ph.D.
National Homeland Security Research Center (NHSRC)
U.S. Environmental Protection Agency
1300 Pennsylvania Avenue, NW
USEPA-8801RR
Washington, DC 20460
shah.sanjiv@epa.gov

Table of Contents

List of Tables

List of Figures

Acronyms

ABI	Applied Biosystems®
ACS	American Chemical Society
BD	Becton, Dickinson and Company
BHI	Brain heart infusion
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biosafety level
CBR	Chemical, biological, radiological
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
CFU	Colony forming unit
C_T	Cycle threshold
DI	Deionized
DNA	Deoxyribonucleic acid
DQO	Data quality objectives
EDTA	Ethylenediaminetetraacetic acid
EIC	External inhibition control
EPA	U.S. Environmental Protection Agency
ERLN	Environmental Response Laboratory Network
FBI	Federal Bureau of Investigation
FEM	Forum on Environmental Measurement
HCL	Hydrochloric acid
ICLN	Integrated Consortium of Laboratory Networks
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
LLNL	Lawrence Livermore National Laboratory
LRN	Laboratory Response Network
MCE	Mixed cellulose ester
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
NG	No growth
NTC	No template control
OEM	Office of Emergency Management
ORD	Office of Research and Development
OSHA	Occupational Safety and Health Administration
oz.	Ounce
PBS	Phosphate buffered saline
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
RCF	Relative centrifugal force
rpm	Revolutions per minute
RV-PCR	Rapid viability-polymerase chain reaction
SBA	Sheep blood agar
SDS	Safety data sheet
SOP	Standard operating procedure
SWGFACT	Scientific Working Group on Forensic Analysis of Chemical Terrorism
T_0	Time zero (no incubation)
T_9	Nine-hour incubation
T_f	Time Final (total number of hours incubated)
TE	Tris(hydroxymethyl)aminomethane- hydrochloric acid -EDTA
TNTC	Too numerous to count
TSB	Trypticase™ soy broth
UF	Ultrafiltration
UNG	Uracil-N-Glycosylase
UV	Ultraviolet
WLA	Water Laboratory Alliance

Trademarked Products

Trademark	Holder	Location
Acrovent™	Pall Corporation	Ann Arbor, MI
Amicon®	MilliporeSigma Corporation	Billerica, MA
Applied Biosystems®	Thermo Fisher Scientific Inc.	Carlsbad, CA
Autovial™	Whatman™ Ltd.	Maidstone, United Kingdom
BBL™	BD Diagnostics Corporation	Sparks, MD
Biopur® Safe-lock®	Eppendorf NA	United States
Black Hole Quencher®	Biosearch Technologies, Inc.	Novato, CA
Clay Adams™	BD Diagnostics Corporation	Sparks, MD
Cole Parmer®	Cole Parmer® LLC	Vernon Hills, IL
Costar®	Corning Inc.	Tewksbury, MA
Dispatch®	Clorox Company	United States
Durapore®	MilliporeSigma Corporation	Billerica, MA
Dynamag™	Thermo Fisher Scientific Inc.	Carlsbad, CA
Fluoropore™	MilliporeSigma Corporation	Billerica, MA
GN-6 Metricel®	Pall Corporation	Ann Arbor, MI
Invitrogen®	Thermo Fisher Scientific Inc.	Carlsbad, CA
Jiffy-Jack®	Cole Parmer® LLC	Vernon Hills, IL
Kendall™	Covidien, Inc.	Mansfield, MA
Kimwipes™	Kimberly-Clark Corporation	Dallas, TX
Life Technologies™	Thermo Fisher Scientific Inc.	Carlsbad, CA
MagneSil®	Promega Corporation	Madison, WI
Masterflex®	Cole Parmer® LLC	Vernon Hills, IL
MaxQ™	Thermo Scientific Inc.	Lenexa, KS
MicroFunnel™	Pall Corporation	Ann Arbor, MI
Nalgene®	Nalge Nunc Corporation	Rochester, NY
Parafilm®	Bemis, Inc.	Neenah, WI
Sigma-Aldrich®	MilliporeSigma Corporation	St. Louis, MO
Stomacher®	Seward Ltd.	United Kingdom
TaqMan®	Thermo Fisher Scientific Inc.	Carlsbad, CA
Trypticase™	BD Diagnostics Corporation	Sparks, MD
Tween®	MilliporeSigma Corporation	St. Louis, MO
Ultracel®	EMD Millipore® Corporation	Billerica, MA
Ultrafree®	EMD Millipore® Corporation	Billerica, MA
Vacushield™	Pall Corporation	Ann Arbor, MI
Velcro®	Velcro Companies	Manchester, NH
Versalon™	Covidien, Inc.	Mansfield, MA
Whatman™	Whatman™ Ltd.	Piscataway, NJ

Acknowledgements

This protocol was developed under the technical leadership and direction of Sanjiv R. Shah of the National Homeland Security Research Center (NHSRC) within the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (ORD).

The following organizations and persons are gratefully acknowledged for their help in the preparation of this protocol:

Centers for Disease Control and Prevention (CDC), Laboratory Response Network (LRN)

- Richard Kellogg and Stephen Morse, for support and cooperation in the preparation and review of this protocol
- Laura Jevitt for providing the LRN protocols that were adapted for inclusion in this protocol

Lawrence Livermore National Laboratory (LLNL)

- Staci Kane, for technical contribution and critical review of the protocol

CSRA

Yildiz Chambers-Velarde and Emily King, for technical review and editing of the protocol

Technical Reviewers

- Laura Rose and Vincent Hill (CDC)
- Sonia Létant (LLNL)
- James Anthony (Department of Homeland Security)
- Douglas Anders (Federal Bureau of Investigation [FBI])
- Stephanie Harris (EPA Region 10)
- Jafrul Hasan (EPA Office of Pesticides Program Microbiology Laboratory)
- Marissa Mullins (EPA, Office of Emergency Management)
- Frank Schaefer, Gene Rice, Worth Calfee and Vincent Gallardo (EPA, ORD, NHSRC)

Quality Assurance Reviewers

- Eletha Brady-Roberts and Ramona Sherman (EPA, ORD, NHSRC)

Cover Photos: Left – Scanning electron micrograph of *Bacillus anthracis*; Right – *Bacillus anthracis* on blood agar (Source: CDC/Laura Rose - Public Health Image Library)

Section 11 Figure 6: *Bacillus anthracis* on blood agar (Source: CDC/Megan Mathias and J. Todd Parker-Public Health Image Library)

This page is intentionally left blank

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. Accordingly, EPA's mission to protect human health and the environment was expanded to address critical homeland security related needs.

The National Homeland Security Research Center (NHSRC) within the Office of Research and Development (ORD) is EPA's hub for providing expertise on CBR agents and for conducting 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) and Water Laboratory Alliance (WLA), an integrated nationwide network of federal, state, local, and commercial environmental testing laboratories. Along with the Centers for Disease Control and Prevention's (CDC) Laboratory Response Network (LRN), the ERLN/WLA can be activated in response to a large-scale environmental disaster to provide analytical capability, increase capacity, and produce quality data in a systematic and coordinated manner. Preparedness against potential indoor or outdoor wide-area anthrax attacks is one of the highest priorities for the ERLN/WLA. Based on the realities of response activities after the 2001 anthrax incident and continued preparedness efforts since then, it is anticipated that during an intentional (bioterrorist attack) or accidental release of *Bacillus anthracis* (*B. anthracis*) spores, several hundreds to thousands of diverse environmental samples (e.g., aerosol, particulates [surface swabs, wipes, 37-mm filter cassettes and filters, and Sponge-Sticks], air filters, 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 large number of samples may also need to be analyzed to determine the efficacy of decontamination activities during the remediation phase of the response. During an anthrax incident, EPA's decision makers will need timely results from rapid sample analyses for planning and assessing the decontamination efforts. To address these critical needs, NHSRC, in collaboration with CDC and Lawrence Livermore National Laboratory (LLNL), generated this protocol for detection of *B. anthracis* spores in environmental samples.

To complement an effective sample collection strategy during a suspected *B. anthracis* release incident, 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. Availability of a common analytical protocol for participant laboratories can be a significant part of such an approach. This protocol includes three analytical methods for the detection of *B. anthracis* spores in various environmental samples (e.g., aerosols [air filters], particulates [surface swabs, wipes, 37-mm filter cassettes and filters, and Sponge-Sticks], and drinking water). To simply detect the presence of the deoxyribonucleic acid (DNA) of *B. anthracis*, real-time polymerase chain reaction (PCR) based sample analysis method is included. To detect whether viable *B. anthracis* spores are present in the samples, microbiological culture and Rapid Viability-PCR (RV-PCR) analytical methods are included. This protocol has been specifically developed for use by ERLN and WLA laboratories for the analysis of environmental samples during an incident involving contamination from *B. anthracis* spores. It should be noted that LRN laboratories providing support to EPA for environmental sample analyses may use LRN-specific protocols.

Sample processing procedures are also provided for respective analytical methods for all sample types listed earlier. 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 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.

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 water sample concentration procedure. For 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.

Several sample processing and analysis procedures in this protocol 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 incident. Therefore, these modified procedures or this protocol itself must not be designated, referred to, or misconstrued as LRN procedures or as an LRN protocol.

It should be noted that at the time of publication and revision, this protocol has not been validated. The real-time PCR assays included in this protocol have been only partially characterized for specificity. These assays will be updated or replaced with fully characterized and validated assays upon availability. 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.

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.

[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:

https://www.epa.gov/sites/production/files/2015-01/documents/emergency_response_validity_policy.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 incident 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 incidents, 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: https://www.epa.gov/emergency-response/environmental-response-laboratory-network-erln-laboratory-requirements; project-specific requirements also are included in individual Analytical Service Requests.

[1] Information regarding EPA's DQO process, considerations and planning is available at: https://www.epa.gov/fedfac/guidance-systematic-planning-using-data-quality-objectives-process

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

1.0 Scope and Application

The purpose of this updated protocol is to provide methods that can be used to detect *Bacillus anthracis* (*B. anthracis*) spores in environmental samples. To simply detect the presence of the deoxyribonucleic acid (DNA) of *B. anthracis,* this protocol includes a real-time polymerase chain reaction (PCR) based method. Since traditional real-time PCR methods cannot determine viability of *B. anthracis* spores, this protocol includes two additional methods, Rapid Viability-PCR (RV-PCR) and culture/plating followed by confirmation of isolate by real-time PCR. Depending upon the laboratory capability, either of these two methods can be performed to detect viable *B. anthracis* spores in environmental samples. The real-time PCR assays included in this protocol have been only partially characterized for specificity. PCR assays included in this protocol will be updated or replaced with fully characterized and validated assays upon availability. During an actual incident, validated assays from other sources (e.g., Department of Defense Biological Products Assurance Office or Laboratory Response Network [LRN]) may be used.

This protocol will be periodically updated to include advances in sample processing and nucleic acid extraction-purification procedures. This protocol is intended for the analyses of swabs, wipes, Sponge-Sticks, 37-mm filter cassettes and filters, air filters, and water for *B. anthracis*.

2.0 Summary of Methods

2.1 Sample Analysis for Detection of *B. anthracis* DNA (Real-time PCR): Following sample processing including DNA extraction and purification, the DNA extracts are analyzed by real-time PCR using the Applied Biosystems® (ABI) 7500 Fast Real-Time PCR System. Direct DNA-based analysis of samples allows for high throughput and rapid results. Unless advised otherwise, for post-incident-recognition sample analysis and depending on the purpose of sample analyses, real-time PCR should be performed using only the most sensitive assay, EPA-2, which targets the capB gene (on pXO2 plasmid; Section 6.15) or an equivalent assay.

2.2 Sample Analyses for Detection of Viable *B. anthracis* Spores: After samples have been appropriately processed, they are cultured by either inoculating into nutrient rich broth (Reference 15.1 [RV-PCR procedure]) or plating on sheep blood agar (Reference 15.2 [culture procedure]), to allow for germination of viable spores and growth.

2.2.1 RV-PCR Procedure (updated)

The RV-PCR procedure serves as an alternative to the traditional culture-based methods for detection of viable pathogens. The RV-PCR procedure integrates high-throughput sample processing, short-incubation broth culture, and highly sensitive and specific real-time PCR assays to detect low concentrations of viable bacterial threat agents.

Specifically, the procedure uses the change in real-time PCR response, referred to as the change in cycle threshold, or ΔC_T, between the initial cycle threshold (C_T) at time 0 (T_0) (just before sample incubation) and the C_T after final incubation time (T_f). Example PCR response curves are shown in **Figure 3** along with the criteria for positive detection, namely $\Delta C_T \geq 9$. Unless advised otherwise, for post-incident-recognition sample analysis

and, real-time PCR based analysis should be performed using only the most sensitive assay, EPA-2, or all three assays included in Section 6.15, depending on the purpose of sample analyses.

2.2.2 Culture Procedure

The culture option includes sample processing and plating serial dilutions of the processed sample and membrane filters on a non-selective sheep blood agar (SBA) followed by rapid confirmation of typical isolated colonies using *B. anthracis* specific real-time PCR. Unless advised otherwise, for post-incident-recognition sample analysis, real-time PCR should be performed using only the BC3 PCR assay that targets the marker gene on the *B. anthracis* genome (Section 6.15) or all three assays included in Section 6.15, depending on the purpose of sample analyses.

3.0 Interferences and Contamination

3.1 Poor 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 inhibit PCR reactions. After spore recovery during sample processing, samples suspected of containing iron or rust particles should be placed on a magnetic rack (Invitrogen® Cat. No. 123-21D or equivalent) to separate out the particulates from the samples. The supernatant should be transferred to a clean sterile bottle or tube, using care not to transfer any of the particulates.

3.3 Problems related to sample processing, such as clogging of filters and inefficient extraction, may also result in poor 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 including biosafety while working with the Biological Select Agents; the laboratory should adhere to safety guidelines and requirements established by their organization or facility as well as the CDC. All wastes should be handled according to CDC & Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition, (Reference 15.3), waste management and disposal requirements.***

4.1 Safety Precautions

Direct contact of skin or mucous membranes with infectious materials, accidental parenteral inoculation, ingestion, and exposure to aerosols and infectious droplets have resulted in *B. anthracis* infection. Due to the infectious nature of this organism, all samples should be handled and analyzed using biosafety requirements as dictated by BMBL (Reference 15.3), or the most recent version and/or organizational health and safety plans. The CDC requires biosafety level (BSL)-3 handling of this organism.

4.2 Additional Recommended Precautions

4.2.1 To the extent possible, disposable materials (e.g., pipets, loops) should be used for sample manipulations.

4.2.2 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.

4.2.3 Personal Protective Equipment (PPE)

Laboratory personnel processing and conducting analyses of samples for *B. anthracis* must use appropriate PPE (e.g., gloves, lab coat). Also, 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, CDC developed recommendations for PPE based on biosafety level (BSL) (Reference 15.3, http://www.cdc.gov/biosafety/publications/bmbl5/index.htm).

Note: ***Remove used gloves and don new ones, as appropriate, to avoid contaminating hands and surfaces between processing of each sample and to prevent cross-contamination. Gloves should be disposed of (in an autoclavable biohazard bag) 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, properly disposed, and hands should be washed with soap and water.***

4.2.4 This protocol does not address all safety issues associated with its use. Refer to 1) BMBL, 5th Edition, CDC 2009 (Reference 15.3) for additional safety information; 2) organization specific Health and Safety guidelines; 3) Select Agent Program Requirements; and 4) a reference file of Safety Data Sheets (SDS).

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 Ziplock bags (large ~20” × 28” [inches], medium ~12” × 16”, small ~7” × 8”)

5.1.5 Sharps waste container

5.1.6 Absorbent pad, bench protector (Lab Source Cat. No. L56-149 or equivalent)

5.1.7 Medium and large autoclavable biohazard bags and wire twist ties

5.1.8 Sterile scalpels

5.1.9 Sterile stainless steel scissors

5.1.10 Sterile disposable forceps (Cole Parmer® Cat. No. U-06443-20 or equivalent)

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

5.1.15 Aluminum foil or kraft paper

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

5.1.17 Laboratory marker

5.1.18 Timer

5.1.19 Sterile disposable serological pipets: 5 mL and 50 mL

5.1.20 Sterile disposable aerosol barrier pipet tips: 1000 µL, 200 µL, 20 µL, 10 µL (Rainin Cat. No. SR-L1000F, SR-L200F, GP-20F, GP-10F or equivalent)

5.1.21 1.5 mL Eppendorf Snap-Cap Microcentrifuge Biopur® Safe-Lock® tubes (Fisher Scientific Cat. No. 05-402-24B or equivalent)

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

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

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

5.1.25 Sterile 2 mL tubes, DNase, RNase-free, gasketed, screw caps (National Scientific Cat. No. BC20NA-PS or equivalent)

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

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

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

5.1.29 Amicon® Ultra-0.5 Centrifugal Filter Concentrator with Ultracel® 100 Regenerated Cellulose Membrane (Millipore® Cat. No. UFC503096 or equivalent); Amicon® collection tubes (Millipore® Cat. No. UFC50VL96 or equivalent)

5.1.30 Sterile 0.22µm Ultrafree®-MC GV 0.5 mL Centrifugal Filter Unit with Durapore® PVDF Membrane, Yellow Color Coded (Millipore® Cat. No. UFC30GV0S or equivalent)

5.1.31 0.1 µm Ultrafree®-MC, VV Centrifugal Filter Device (Millipore® Cat. No. UFC30VV00 or equivalent)

5.1.32 Sterile wide mouth screw cap containers, 120 mL (Fisher Scientific Cat. No. 14-375-459 or equivalent)

5.1.33 Racks for 15 mL and 50 mL conical tubes

5.1.34 Sterile 2 ounce (oz., 1 ounce~30 mL) polypropylene cups with lids (Container & Packaging Supply; Cat. No. J037 and Cat. No. L208, or equivalent)

5.1.35 Plastic lidded box (Fisher Scientific Cat. No. 03-484-23 with lid Cat. No. 03-484-24, or equivalent)

5.1.36 Cassette opening tool (SKC Cat. No. 225-8372)

5.2 Supplies for Real-time PCR Method Based Sample Analysis

5.2.1 96-well PCR plates (Applied Biosystems® [ABI] Cat. No. 4346906 or equivalent)

5.2.2 96-well plate holders, Costar®, black (VWR Cat. No. 29442-922 or equivalent)

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

5.2.4 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

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

5.3 Supplies for RV-PCR Method Based Sample Analysis

5.3.1 30 mL screw cap tubes (E&K Scientific Cat. No. T324S or equivalent)

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

5.3.3 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)

5.3.4 GE Healthcare (Whatman™) Autovial 12 Syringeless Filter™, filter vials (GSS Cat. No. AV125NPUPSU or equivalent)

5.3.5 Pull-Tab Plug, orange cap (Caplugs Cat. No. ECP-M24 or equivalent), for vortexing and incubation steps

5.3.6 Pull-Tab Tapered Plug, red cap (Caplugs Cat. No. CPT-10 or equivalent), to cover vial while pipetting to prevent cross-contamination

5.3.7 Polyethylene female luer plug (Ark-Plas Products Cat. No. LPC14-PP0 or equivalent)

5.3.8 50 mL conical tubes, skirted (VWR Cat. No. 82050-322 or equivalent)

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

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

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

5.3.12 96-well tube rack(s) for 2 mL tubes (8 × 12 layout) (Bel-Art Cat. No. 188450031 or equivalent)

5.3.13 2 mL Eppendorf tubes (Fisher Scientific Cat. No. 05-402-24C or equivalent)

5.3.14 96-well 2 mL tube rack (8 × 12 format) (Bel-Art Cat. No. 188450031 or equivalent)

5.3.15 Adhesive tape (3M, Inc. Heavy Duty Scotch Tape Cat. No. 34-8711-4279-9, or equivalent), to seal unused manifold openings.

5.4 Supplies for Culture Method Based Sample Analysis

5.4.1 Sterile disposable Petri dishes, 100 mm × 15 mm

5.4.2 Sterile disposable inoculating loops (10 µL) and needles

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

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

5.4.5 Specimen Cups, 4.5 oz. (Kendall Cat. No. 17099 or equivalent)

5.4.6 Racks for 15 mL and 50 mL centrifuge tubes

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

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

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

5.4.10 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/Work station

5.5.3 Shaker incubator for RV-PCR (New Brunswick Innova 40, Eppendorf Cat. No. M1299-0080; or Thermo Scientific, MaxQ™ 4000 Cat No. SHKE4000; or equivalent) and Universal 18" × 18" shaker platform (New Brunswick, Cat. No. M1250-9902 or VWR, Cat. No. 89173-848; or Thermo Scientific, MaxQ™ Cat. No. 30110; or equivalent)

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

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

5.5.6 Refrigerated centrifuge with PCR plate adapter and corresponding safety cups and rotors for 5 mL and 50 mL tubes (Eppendorf Cat. No. 5804R, 5810R or equivalent) or PCR plate spinner (placed in BSC [VWR Cat. No. 89184-608 or equivalent])

Note: Swinging bucket and fixed angle rotors for the refrigerated centrifuge may also be necessary.

5.5.7 Refrigerated microcentrifuge for Eppendorf tubes with aerosol-tight rotor (Eppendorf Cat. No. 5415R/5424R or equivalent)

5.5.8 Filter vial manifold-top and bottom for RV-PCR (Pacon Manufacturing. Cat. No. 1701232-1 and 1701232-2 with Allen screws) and 2 top brackets (Pacon Manufacturing. Cat. No. PART 1 with Allen screws). The bottom manifold includes an elbow port for attaching tubing to a vacuum source.

5.5.9 T-Handle Hex Key, 6" with Cushion Grip, 9/64", Red (All-Spec Cat. No. 57306-12200 or equivalent)

5.5.10 Capping tray for RV-PCR (Pacon Manufacturing Cat. No. 1701233)

5.5.11 30 mL tube rack for RV-PCR (Pacon Manufacturing Cat. No. 1701234)

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) (68.95 kilopascals)

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 (VWR Cat. No. 58816-115 or equivalent)

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 (Pacon Manufacturing Cat. No. 1701190) and/or peg kit (Pacon Manufacturing Cat. No. 1701189-8 for New Brunswick Innova incubator and platform or Cat. No. 1701189-10 for Thermo-Fisher MaxQ incubator and platform) for securing individual manifold/capping trays to the shaking incubator platform for RV-PCR

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

5.5.25 Mini-Bead-beater (BioSpec Products, Inc. Cat. No. 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 (ACS) (Reference 15.4). For suggestions regarding the testing of reagents not listed by the ACS, see *AnalaR Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K. (Reference 15.5); and *United States Pharmacopeia and National Formulary 24*, United States Pharmacopeial Convention, Md. (Reference 15.6).

6.2 1X phosphate buffered saline with 0.05% Tween® 20 (PBST), pH 7.4, (Teknova Cat. No. P0201 or equivalent)

6.3 1X phosphate buffered saline (PBS), pH 7.4, (Teknova Cat. No. P0261 or equivalent)

6.4 Extraction Buffer with Tween® 20 for RV-PCR

6.4.1 Composition:

1X PBS 0.05% Tween® 20, pH 7.4	700 mL
200 proof ethanol	300 mL

6.4.2 Add 1X PBS 0.05% Tween® 20 to ethanol and 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.

6.5 Extraction Buffer *without* Tween® 20 for RV-PCR

6.5.1 Composition:

1X PBS, pH 7.4	700 mL
200 proof ethanol	300 mL

6.5.2 Add 1X PBS to ethanol and 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 High Salt Wash Buffer (10X PBS, pH 6.5, Teknova Cat. No. P0185 or equivalent) for RV-PCR

6.7 Low Salt Wash Buffer (1X PBS, pH 7.4, Teknova Cat. No. P0261 or equivalent) for RV-PCR

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 0.1 M sodium phosphate/10 mM EDTA (Ethylenediaminetetraacetic acid) buffer/0.01% Tween® 20, pH = 7.4 (Teknova Cat. No. S2216 or equivalent)

6.11 TE buffer (1X Tris [10 mM]-HCl-EDTA [1 mM]) buffer, pH 8.0 (Fisher Scientific Cat. No. BP2473-500 or equivalent)

6.12 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)
- MagneSil® Paramagnetic Particles (PMPs) (VWR Cat. No. PAMD1441)
- Lysis Buffer (VWR, Cat. No. PAMD1392)
- Elution Buffer (VWR Cat. No. PAMD1421)
- Alcohol Wash, Blood (VWR Cat. No. PAMD1411)
- Anti-Foam Reagent (VWR Cat. No. PAMD1431)

6.13 TaqMan® Universal PCR Master Mix (Thermo Fisher/Life Technologies, Cat. No. 4304437)

6.14 Platinum® *Taq* DNA Polymerase (Thermo Fisher/Life Technologies, Cat. No. 10966034)

6.15 PCR Assays

EPA-2 PCR assay targeting the *cap*B gene on the pXO2 plasmid (Reference 15.7)

- 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'

EPA-1 targeting the *pag*A gene on pXO1 plasmid (Reference 15.7)

- 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'

6.15.1 Preparation of concentrated and primer and probe working stocks

Prior to PCR analyses lyophilized primers and probes should be rehydrated in PCR-grade water to prepare concentrated stocks. Primary concentrated storage stocks should initially be prepared to obtain 100 µM (0.1 nmoles/µL) and 20 µM (0.02 nmoles/µL) solutions of primers and probes, respectively. These primary (concentrated) stocks will be used to prepare working stock solutions which will then be used to prepare PCR assay mixes (Section 9.3.3) on the day of use. Examples of rehydration of lyophilized primers/probes and dilution of rehydrated stocks to prepare working stocks are provided in **Tables 1** and **2**, respectively.

Table 1. Example Concentrated Stock Preparation

Lyophilized Primer/Probe (nmoles)		PCR-grade water (µL)	Concentration	
			nmoles/µL	µM
FWD Primer	29	290	0.1	100
RV Primer	35	350	0.1	100
Probe	10	500	0.02	20

Table 2. Example Working Stock Preparation

Concentrated Stock (µL)		PCR-grade water (µL)	Dilution	Concentration	
				nmoles/µL	µM
FWD Primer	50	150	0.25	0.025	25
RV Primer	50	150	0.25	0.025	25
Probe	20	180	0.1	0.002	2

Working stocks will be used to prepare master mix on the day of use (Section 9.3.3).

6.16 Positive Control (PC) –DNA isolated from an appropriate virulent *B. anthracis* strain containing all of the plasmids (pXO2, pXO1). For culture analyses, *B. anthracis* Sterne (BSL-2 organism) or other avirulent strains may be used as a PC to meet the laboratory's BSL.

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

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

6.17.2 Medium Composition:

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

6.17.3 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.

6.17.4 Aseptically add 50 mL of sterile sheep blood (5.0% final concentration) to the cooled medium and mix well. Aseptically pour 12-15 mL medium into each 100 mm × 15 mm sterile Petri dish. After agar solidifies, store at 4°C for a maximum of two weeks.

6.18 Brain Heart Infusion Broth for RV-PCR (BHI broth)

6.18.1 The use of commercially prepared medium is recommended (Fisher Scientific Cat. No. DF0037-15-0 or equivalent), however dehydrated medium (BBL™ Cat. No. 237500 or equivalent), may be used. If commercially prepared medium is not available, prepare medium using procedures in Sections 6.18.2-6.18.3.

6.18.2 Medium 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

6.18.3 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.

6.19 Trypticase™ Soy Broth (TSB)

6.19.1 The use of commercially prepared TSB is recommended (BBL™ Cat. No. 221715 or equivalent), however dehydrated medium (BBL™ Cat. No. 211768 or equivalent), may

be used. If commercially prepared medium is not available, prepare medium using procedures in Sections 6.19.2-6.19.3.

6.19.2 Medium Composition:

Pancreatic digest of casein	17g
Papaic digest of soybean meal	3 g
Sodium chloride	5 g
Dipotassium phosphate	2.5g
Dextrose	2.5g
Reagent-grade water	1.0 L

6.19.3 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 7.3 ± 0.2. Store at 4°C for a maximum of three months in screw cap containers.

6.20 10% Bleach-pH amended (prepared daily), optional

Add 2 parts water to 1 part bleach, and 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, as opposed to pH strips or kit, should be used to measure pH. When mixed, place a lid on the mixture to reduce chlorine escape and worker exposure.

7.0 Calibration and Standardization

7.1 Check temperatures in incubators twice daily with a minimum of 4 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 the storage requirements for samples, reagents and media. Record daily measurements in a refrigerator or 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 at least two of three standards (e.g., pH 4.0, 7.0 or 10.0) closest to the range being tested.

7.5 Calibrate balances once per month with reference weights (e.g., ASTM Class 2).

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

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

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

7.9 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.

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

7.11 Vacuum pressure (e.g., pumps, in house system) should be checked on a regular basis to ensure that the pressure is < 10 psi. Higher or lower vacuum pressure could negatively impact recoveries.

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 the sample may be analyzed and the data qualified and marked accordingly (e.g., if a sample exceeded temperature during transport, the data would be flagged and marked as exceeding temperature), so that a decision can made regarding whether the data should be considered valid or 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 PT 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. If a PT failure occurs, 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, TSB and 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 media sterility checks every day that samples are analyzed.

8.6 PCR: Positive control (PC) − DNA isolated from an appropriate virulent *B. anthracis* strain containing all of the plasmids (pXO2, pXO1) should be used as the 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. *B. anthracis* Sterne (BSL-2) or other avirulent strains may be used as a PC to meet the laboratory's BSL. 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 virulent *B. anthracis* strain containing all of the plasmids (pXO2, pXO1) − For determination of presence of DNA by real-time PCR, the laboratory should analyze an EIC for each environmental sample DNA extract 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 (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 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 due to 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., C_T >45).

Note: The field blank for large volume water samples should also be concentrated using ultrafiltration (UF) prior to analyses. A smaller volume of water (e.g., 10-20 L) may be used for the field blank to minimize the burden on the laboratory.

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 3** for appropriate PNC.

8.12 For RV-PCR based analysis, the T_0 and T_9 or T_f 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 or T_f extracts.

Table 3. Sample Processing Negative Controls

Matrix	PNC
Wipes	Clean (unused) wipe
Swabs	Clean (unused) swab
37-mm filter cassette	Clean (unused) cassette

Matrix	PNC
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
Large volume water samples	10-20 L of sterile reagent-grade water

9.0 Real-time PCR Method

Real-time PCR allows for rapid detection of *B. anthracis* spores and cells in samples based simply on the presence of DNA. However, since the DNA from dead spores and cells can also be detected by this method, a positive sample result does not confirm the presence of viable spores or cells. Therefore, this method is usually used for a time- and cost-effective presumptive analysis of samples. This section includes real-time PCR method with appropriate sample processing procedures for detection of *B. anthracis* spores.

Acceptable sample types: Gauze wipes (2" × 2" 50% rayon/50% polyester [Kendall™ Versalon™ Cat. No. 8042 or equivalent]), air filters (37 mm Fluoropore™ [Millipore® Cat. No. FSLW04700 or equivalent]), swabs (macrofoam [VWR Cat. No. 89022-994 small swabs or 89022-984 extra-large swabs or equivalent]), Sponge-Stick sampling tools (3M Inc. Cat. No. SSL100 or equivalent), 37-mm mixed cellulose ester (MCE) filter cassettes (SKC Cat. No. 225-9543 or equivalent), vacuum filters (3M Forensic, Precision Data Products Cat. No. FF-1 with 4" diameter filter or equivalent), drinking water and decontamination waste water

9.1 Sample Processing: Spore Recovery

Note: All subsequent procedures involving manipulation of samples must be carried out in a BSC using appropriate PPE. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

Prepare monofilament polyester mesh (Section 5.3.3) 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. **Figure 1** identifies the appropriate sub-sections for sample processing and spore recovery for each sample type/tool.

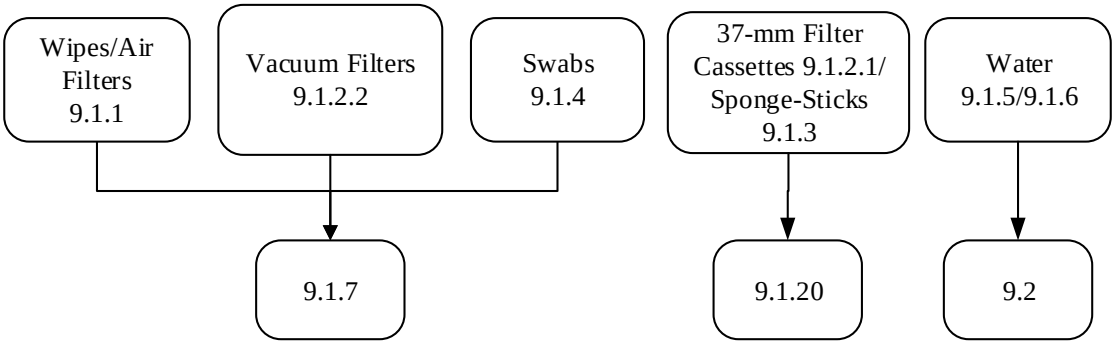


Figure 1. Sample processing and spore recovery steps for real-time PCR.

9.1.1 *Wipe and Air Filter Samples*

Place mesh support over wipe or air filter samples in 30 or 50 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 as described in Section 9.1. Ensure the sample and mesh are in the bottom half of the tube (avoiding the conical portion). Decontaminate workspace with 10% pH amended bleach (Section 6.20) or bleach wipes (Section 5.1.3) and don a fresh pair of gloves in between samples. Repeat process for each sample. The support keeps the wipe or air filter from interfering with pipetting activities and also improves efficiency of spore extraction during vortexing. Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3). Proceed to Section 9.1.7.

9.1.2 *Vacuum Samples (37-mm filter Cassettes and Filters)*

Note: All subsequent procedures involving manipulation of 37-mm filter cassettes must be carried out in a BSC using appropriate PPE.

9.1.2.1 37-mm filter Cassettes

- For each 37-mm filter cassette, prepare one 15 mL conical tube containing 11 mL of sterile Extraction Buffer with Tween and 30% (Section 6.4) and label one 2 oz. sterile cup (Section 5.1.34). In the BSC remove the conical tube containing the nozzle and the cassette from the containment bags and wipe the outside of the conical tube with a disinfectant and place it into a rack. Aseptically add 5 mL of buffer (from the 11 ml of a pre-measured aliquot of Extraction Buffer with Tween and 30%) to the conical tube containing the nozzle and tubing and set aside. Remove the band from around the cassette using a sterile scalpel or sterile pair of scissors. Wipe each cassette with 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) followed by a clean Kimwipe® and discard wipes into an autoclavable biohazard bag.
- Change gloves. Remove the red plug from the front filter side of the cassette; the plug on the back side should be kept in place. Using a transfer pipette dispense 1 mL of Extraction Buffer with Tween and 30% from the tube now containing the 6 mL into the cassette and replace plug. Roll the cassette around to allow the liquid to touch all surfaces of the inside of the cassette. If there is a large quantity of particulate matter, more Extraction Buffer with Tween and 30% may be required.

Particulate matter should be dampened enough to prevent aerosolization.

- Using the cassette tool (Section 5.1.36) pry open the top section of the cassette, using care not to spill the Extraction Buffer inside the cassette. Set the bottom portion containing the filter aside carefully (filter side up), and using a transfer pipette rinse the walls of the cassette with 1-2 mL of Extraction Buffer with Tween and 30%. Transfer the rinsate using the same pipette to the appropriately labelled 2 oz. sterile cup (Section 5.1.34). Using the same transfer pipette repeat the rinsing process for the bottom portion of the cassette and transfer the rinsate to the 2 oz. cup.
- Using the cassette tool remove the middle section of the cassette (this piece is holding the filter in place). Using sterile forceps aseptically remove the filter without picking up the support filter underneath. Place the filter in the 2 oz. cup with the rinsates. Use the remainder of the 6 mL Extraction Buffer with Tween and 30% to rinse walls and bottom section of the cassette and transfer to the 2 oz. cup. Discard the cassette sections, support filter, plugs, and transfer pipette in an autoclavable biohazard bag.
- Disinfect the outside of the 2 oz. cup with 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and place in rack. Decontaminate the BSC with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and don a fresh pair of gloves in between samples in between samples. Repeat procedure described above for each 37-mm filter cassette.
- Seal the conical tubes containing the nozzle and tubing in 5 mL Extraction Buffer with Tween and 30%, tubing and nozzle with Parafilm®. Place the rack of conical tubes into the sonicating bath to a level that allows at least 1 inch (~2.5 cm) of tube to be above the water line. Place a rectangular weight on top of the tubes to prevent them from floating or tipping over. Sonicate for 1 minute and remove tubes from the sonicating bath. Dry and disinfect each tube with a 10% amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Vortex the conical tubes on high for 2 minutes and transfer the 5 mL suspension to the appropriate 2 oz. cup.
- Seal all of the 2 oz. cups with Parafilm®. Place the rack of 2 oz. cups in the sonicating bath and cover with a rectangular weight on top of the cups to prevent them from floating or tipping over. There should be 1 inch (~2.5 cm) between the level of the water and the cup lids. Sonicate for 3 minutes without heat. Remove rack from the bath and dry each cup with a Kimwipe® and place in the BSC. Place cups in a sealable plastic lidded box (Section 5.1.35). Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Using a 10 mL serological pipet, transfer as much suspension as possible from each 2 oz. cup to the corresponding labeled 50 mL conical tubes.
- Proceed to Section 9.1.20.

9.1.2.2 Vacuum filters

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 (Section 9.1) on top of filter. Repeat for each sample. Decontaminate the BSC with 10% amended bleach (Section 6.20) or bleach wipes (5.1.3) and don a fresh pair of gloves in between samples. Proceed to Section 9.1.7.

9.1.3 ***Sponge-Stick Samples***

Note: All subsequent procedures involving manipulation of Sponge-Sticks samples must be carried out in a BSC using appropriate PPE.

- If the Sponge-Stick sponge is not in a Stomacher® bag, holding the plastic handle of the Sponge-Stick with one gloved hand, carefully remove the sponge using sterile forceps and aseptically transfer it to a Stomacher® bag. 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 rests evenly between the homogenizer paddles and stomach each sample for 1 minute at 260 rpm.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the sponge from the outside of the bag with hands. With the bag closed, move the sponge to the top of the bag while using hands to expel liquid from the sponge.
- Open the bag, remove and discard the sponge in an autoclavable biohazard bag, using sterile forceps.
- Repeat steps described above for each sample, changing forceps and gloves between samples.
- 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 three times with a sterile 50 mL pipet. Remove half of the suspension volume (~45-46 mL) and place it in a labeled 50 mL screw capped centrifuge tube. Place the remaining suspension (~45-46 mL) into a second 50 mL tube. Adjust the suspension volumes in both the tubes to ensure they are similar.
- 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 and rotor specifications.

- Using a sterile 50 mL pipet for each sample, carefully remove ~43-44 mL leaving approximately 2 mL of the supernatant from each of the two 50 mL tubes and discard it in an autoclavable biohazard bag. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube.
- Set the vortexer (Section 5.5.16) to high intensity. Set the sonicator bath (Section 5.5.27) to high.
- Vortex the tubes for 30 seconds to resuspend the pellets and transfer the tubes to the sonicator bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles two more times.

Note: As an alternative to sonication, tubes may be vortexed for 2 minutes in 10 second bursts using a multi-tube vortexer.

- 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.
- Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Proceed to Section 9.1.20.

9.1.4 ***Swab Samples***

Note: All subsequent procedures involving manipulation of Swab samples must be carried out in a BSC using appropriate PPE.

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). Repeat process for each sample. Discard all waste in an autoclavable biohazard bag and decontaminate counters and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3). 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 and ≥ 50 mL, please refer to Appendix A, Section 3.0, secondary concentration.

- Add 15 mL of sodium phosphate/EDTA/Tween® 20 buffer (Section 6.10) 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.
- Using sterile forceps remove membrane from the tube and discard in an autoclavable biohazard bag. Centrifuge suspension at 3500 × g, with the brake off, for 15 minutes at 4°C.

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

- Remove 12 mL of the supernatant without disturbing/dislodging the pellet; resuspend the pellet by vortexing in the remaining volume.
- Repeat for each sample.
- Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Use a 1.5 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 sodium phosphate/EDTA/Tween® 20 buffer (Section 6.10) and mix by vortexing for 30 seconds.
- Centrifuge at 3500 × g, with the brake off, for 15 minutes at 4°C.

Note: A higher × g is preferred as long as the speed is within the tube and rotor 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 for 30 seconds in the remaining volume.
- Repeat for each sample.
- Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Use a 1.5 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® 20 (Section 6.4) to environmental samples (Sections 9.1.1, 9.1.2, 9.1.4) placed in 30 mL tubes (50 mL tubes for vacuum filters) 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 a time, add 20 mL extraction buffer with Tween® 20, 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. If needed, the tube caps can be sealed with Parafilm.

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 dispensing aliquots.

9.1.12 Open the tube. Using a 25 mL serological pipet, transfer as much liquid volume as possible (while avoiding settled particles) to a fresh appropriately labeled 50 mL conical tube. Discard pipets in an autoclavable biohazard 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® 20 (Section 6.5) 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 tube rack in plastic bag, seal, bleach bag and double bag, prior to transferring to platform vortexer (outside BSC).

9.1.17 Vortex rack for 10 minutes on platform vortexer (Section 5.5.15), with speed set to 7.

9.1.18 Repeat Sections 9.1.10-9.1.11.

9.1.19 Open the tube. Using a 25 mL serological pipet, transfer as much liquid volume as possible (while avoiding settled particles) to the original 50 mL tube containing the first extraction suspension to combine the extracts. Discard pipets in an autoclavable biohazard waste container. Cap sample tube and place tube back in rack. Change gloves.

9.1.20 Centrifuge the 50 mL conical tubes containing the suspension at 3500 × g, with the brake off, for 15 minutes at 4°C.

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

9.1.21 Leaving approximately 3 mL in the tube, carefully discard the supernatant in an autoclavable biohazard bag, 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.

9.1.22 Add 25 mL (10 mL for swabs) of sodium phosphate/EDTA/Tween® 20 buffer (Section 6.10), tightly cap the tube, and mix the suspension by vortexing.

9.1.23 Repeat Sections 9.1.21-9.1.22 for each sample.

9.1.24 Centrifuge the suspension at 3500 × g, with the brake off, for 15 minutes at 4°C.

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

9.1.25 Carefully discard 25 mL (10 mL for swabs) of supernatant in an autoclavable biohazard bag without disturbing/dislodging the pellet. Resuspend the pellet by vortexing in the remaining volume. Repeat process for each sample.

9.1.26 Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).

9.1.27 Use a 1.5 mL aliquot for DNA extraction using bead-beating, as described in Section 9.2.

9.2 Sample Processing: DNA Extraction and Purification

Note: Alternate DNA extraction-purification procedures may be used (e.g., MagNA-Pure LC instrument).

9.2.1 In a clean room, using the 8 cap strips, transfer two level capfuls (~100 mg) of the 106 µm glass beads and two level capfuls (~100 mg) of the 425-600 µm glass beads (using a clean strip of caps between bead sizes) into each gasketed, capped 2 mL bead-beating tube.

9.2.2 In the BSC, pipet 1.0 mL of the suspension (sample eluent, Section 9.1.24) into pre-labeled, gasketed, capped bead-beating 2 mL tube containing glass beads. Replace cap on tube securely. Wipe outside of tube with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3). Store the remaining suspension at 4ºC.

9.2.3 Insert tubes in tube holders of the bead-beater (Section 5.5.25) and set the timer for 3 minutes (180 seconds). Bead-beat at 3450 oscillations/minute to disrupt spores to release the DNA.

9.2.4 Remove tubes from bead-beater (tubes will be warm), and place in a cold block for 2 minutes (or until cool to touch). If any tubes leak during bead-beating, wipe tubes and bead-beater thoroughly with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).

To remove potential PCR inhibitors in very dirty samples, in the BSC centrifuge the bead-beating tubes at 7000 rpm for 2 minutes in a microcentrifuge using a fixed angle rotor to pellet beads and particulate matter. Using a micropipettor, carefully remove 1.0 mL and follow the Manual DNA Extraction and Purification Procedure (Section 10.4). Then after, proceed to Section 9.2.10 and onwards.

9.2.5 Supernatant Separation and Transfer

- Set up tubes; for each sample, label one 1.5 mL microcentrifuge tube, two yellow-top 0.22µm Ultrafree®-MC filter units (Section 5.1.30; Millipore® Cat. No. UFC30GV0S), one Amicon® Ultra filter insert (Section 5.1.29; Millipore® Cat. No. UFC503096), and six Amicon® Ultra collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96) with sample ID for each bead beating tube (Section 9.2.4); and one 0.1 µm Ultrafree®-MC filter device (Section 5.1.31; Millipore® Cat. No. UFC30VV00).

Note: It may not be necessary to label all the collection tubes as long as the Amicon Ultra filter insert is clearly labeled.

- In a BSC, centrifuge the bead-beating tubes (Section 9.2.4) at 7000 rpm for 2 minutes in a microcentrifuge using a fixed angle rotor to pellet beads and particulate matter.
- Using a micropipettor, carefully transfer 0.4 mL of the supernatant from the bead-beating tube to each of the two yellow-top filter units (Section 5.1.30; Millipore® Cat. No. UFC30GV0S). Avoid beads and particulate matter at bottom of bead-beating tube). Cap the filter units.
- Centrifuge at 7000 revolutions per minute (rpm) for 3 minutes at 4ºC.

Note: Ensure that the supernatant has been filtered. Centrifuge for an additional 2 minutes if there is any supernatant in the filter.

- Open the filter units; remove the yellow-top filter inserts with sterile disposable forceps (gripping only on the sides) and discard in an autoclavable biohazard bag. Transfer 0.4 mL of the filtrate from the collection tubes to Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096). Do not transfer any particulate matter that may be evident at bottom of the tubes. Place filter inserts into new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Cap the filter units.
- Centrifuge at 7000 rpm for 2 minute at 4ºC.
- Open the filter units. Remove the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Dispose of old collection tubes with filtrate in an autoclavable biohazard bag.
- Transfer the remaining (0.4 mL) filtrate from all of the second set of yellow-top filter units to the corresponding sample Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096). Do not transfer any particulate matter that may be evident at bottom of tubes. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.
- Open the filter units. Remove the Amicon® Ultra filter inserts using disposable forceps (gripping only the sides) and transfer to new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Dispose of old collection tubes with filtrate in an autoclavable biohazard bag.

9.2.6 First Wash

- Add 400 µL of 1X TE buffer (Section 6.11) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 2 minutes at 4ºC.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) to new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Dispose of used collection tubes with filtrate in an autoclavable biohazard bag.

9.2.7 Second Wash

- Add 400 µL of 1X TE buffer to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.
- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) to new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Dispose of used collection tubes with filtrate in an autoclavable biohazard bag.

9.2.8 Third Wash

- Add 400 µL of 1X TE buffer to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 3 minutes at 4ºC.

- Open the filter units. Transfer the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) with disposable forceps (gripping only the sides) to new collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96). Dispose of used collection tubes with filtrate in an autoclavable biohazard bag.

9.2.9 Fourth Wash

- Add 400 µL of PCR-grade water (Section 6.9) to the Amicon® Ultra filters. Cap the filter units.
- Centrifuge at 7000 rpm for 1 minute at 4ºC.
- Check fluid level in the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096). If fluid level is above 200 µL, pulse spin for about 10 seconds (or less) until about 100 µL of fluid is retained on top of white base.
- If there is less than 100 µL of extract, transfer DNA extract back to the same Amicon® Ultra filter insert (Section 5.1.29; Millipore® Cat. No. UFC503096) and add 100 µL PCR-grade water and pulse spin to obtain about 100 µL on filter.

Note: Very dirty samples may require additional washes to remove any potential inhibitors.

9.2.10 Filtration of DNA Extract using 0.1 µm Centrifugal Filter Device (Section 5.1.31)

Centrifugal filtration with 0.1-µm Ultrafree®-MC filter device following extraction of DNA allows for the removal of any *B. anthracis* spores which may have contaminated DNA preparations, making the samples safe without compromising the sensitivity of the real-time PCR assay (Reference 15.8).

- Using a micropipettor, carefully remove all of the retentate (~ 100 µL) from the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) and transfer to corresponding 0.1 µm Ultrafree®-MC filter devices (Section 5.1.31; Millipore® Cat. No. UFC30VV00). Do not allow the micropipettor tip to touch the filter membrane. Avoid transferring any particulate matter that may be evident at bottom of the tubes. Close the caps. Discard the Amicon® Ultra filter inserts (Section 5.1.29; Millipore® Cat. No. UFC503096) with collection tubes (Section 5.1.29; Millipore® Cat. No. UFC50VL96) in an autoclavable biohazard bag.
- Repeat the above step for all the samples/retentates.
- Place the Ultrafree®-MC filter devices (Section 5.1.31; Millipore® Cat. No. UFC30VV00) into a centrifuge (Section 5.5.7; Eppendorf 5415R/5424R) and balance the rotor head.
- Centrifuge at 8000 × g (approximately, 9200 rpm) for 2 minutes at 4ºC (Reference 15.8).
- Carefully open the caps and remove the Ultrafree®-MC filter inserts (Section 5.1.31; Millipore® Cat. No. UFC30VV00) using disposable forceps (gripping only the sides), cap the collection tubes and dispose of the Ultrafree®-MC inserts (Section 5.1.31; Millipore® Cat. No. UFC30VV00) in an autoclavable biohazard bag. Place the collection tubes in a cold block.

- Carefully wipe the outside of the collection tubes containing sample DNA extract with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Using clean gloves, place the cold block with the tubes containing filter extracts in the 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 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 2**).

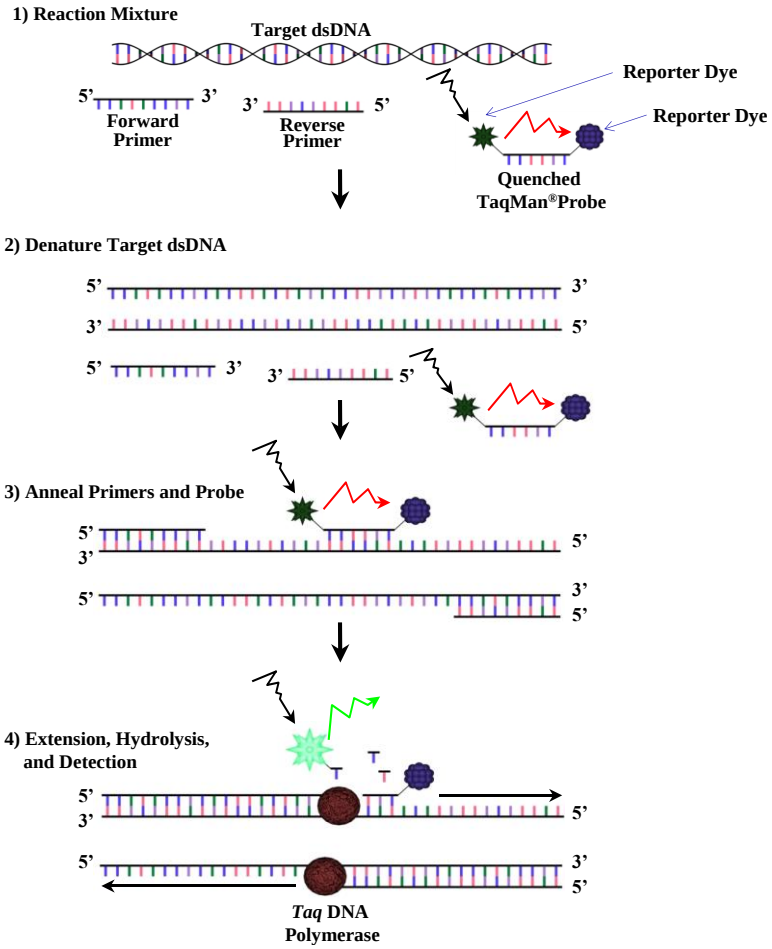


Figure 2. Real-time PCR amplification.

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, for sample analysis during a confirmed anthrax incident for which the *B. anthracis* strain has already been identified and characterized, only the most sensitive real-time single-plex PCR assay targeting a plasmid containing gene may be performed. 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 all three PCR assays, EPA-2 assay targeting the capsular antigen gene (capB) on the pXO2 plasmid, EPA-1 (targeting the protective antigen gene, pagA, on the pXO1 plasmid) and BC3 (targeting a hypothetical gene on the *B. anthracis* chromosome) are included.

It should be noted that the real-time PCR assays included in this protocol have been only partially characterized for specificity. These assays will be updated or 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 (Section 6.20) or bleach wipes (Section 5.1.3), 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 in an autoclavable biohazard bag and replace with a new, clean pair.

Note: If gloves become contaminated, they should be disposed of in an autoclavable biohazard bag 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 an autoclavable biohazard 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 (Section 8.9), PC (Section 8.6) and three replicates of the PNC (Section 8.11) per run. In addition, include three reactions for each sample including field blanks (Section 8.10) and two reactions for the EIC (Section 8.8) for each sample. Prepare a sufficient volume of Master Mix to allow for a minimum of one extra reaction for every 10 reactions, so that there is enough Master Mix regardless of pipetting variations. *For example, if 10 samples are to be analyzed for each PCR assay, a total of 61 reactions would be included in the run [e.g., 4-NTC, 4-PC, 3-PNC, 30-samples and 20-EICs]. Therefore, the volume of PCR Master Mix prepared should be sufficient to run 70 reactions per PCR assay.*

9.3.3 Based on the example provided above (i.e., 10 samples) the amount of Master Mix required would be as indicated in **Tables 4 and 5**.

Table 4. Example EPA-2 Single-plex PCR Master Mix Preparation for 70 Reactions

Reagent	Volume per reaction (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	5.65	395.5	N/A
Platinum® *Taq* DNA Polymerase	0.25	17.5	N/A
Total Volume	20	1400	

If required, the Master Mix for the other two assays should be prepared according to Table 5**,** based on the example provided in Section 9.3.2.

Table 5. Example EPA-1 or BC3 Single-plex PCR Master Mix Preparation for 70 Reactions

Reagent	Volume per reaction (µL)	Total Volume (µL)	Final Concentration (µM)
TaqMan® 2X Universal Master	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	4.25	297.5	N/A
Platinum® *Taq* DNA Polymerase	0.25	17.5	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 (Section 6.9) into the NTC wells.

9.3.6 Cover the plate with adhesive plate sealer 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.***

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 thermocycler are provided in **Table 6**. Fluorescence is automatically measured at the end of the 60ºC annealing-extension combined step.

Table 6. ABI 7500 Fast Thermocycler PCR Cycling Conditions [a, b]

Steps	UNG [c] Incubation	AmpliTaq Gold Activation	PCR, 45 cycles [d]	
	Hold	Hold	Denaturation	Annealing/Extension
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 minutes	10 minutes	5 seconds	20 seconds [e]

[a] Run Mode: Fast 7500
[b] Reaction volume: 25 µL
[c] Uracil-N-Glycosylase
[d] Fast Ramp: 3.5°C/s up and 3.5°C/s down
[e] 30 seconds for ABI 7500 Fast Dx instrument

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.

9.3.14 Cover the plate with adhesive plate sealer 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. Change gloves.

Note: ***The following steps must be performed in the BSC outside the PCR clean room set-up area.***

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 respective sample wells 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 the sealed PCR plate in an autoclavable biohazard bag.

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 autoclavable biohazard bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3), followed by 70% isopropyl and a DI water final rinse.

9.3.24 Refer to section 12.1 for data analyses and calculations and record the results.

10.0 Rapid Viability-Polymerase Chain Reaction (RV-PCR) Method

Acceptable sample types: Gauze wipes (2" × 2" 50% rayon/50% polyester [Kendall™ Versalon™ Cat. No. 8042 or equivalent]), air filters (37 mm Fluoropore™ [Millipore® Cat. No. FSLW04700 or equivalent]), swabs (macrofoam [VWR Cat. No. 89022-994 small swabs or 89022-984 extra-large swabs or equivalent]), Sponge-Stick sampling tools (3M Inc. Cat. No. SSL100 or equivalent), 37-mm mixed cellulose ester (MCE) filter cassettes (SKC Cat. No. 225-9543 or equivalent), vacuum filters (3M Forensic, Precision Data Products Cat. No. FF-1 with 4" diameter filter or equivalent), drinking water and decontamination waste water

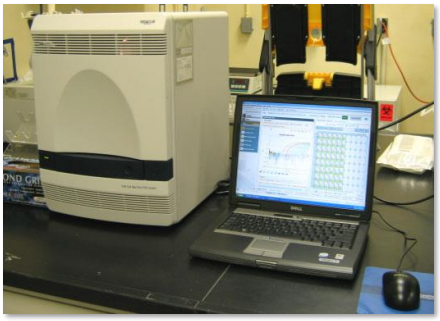
Note*: *Neutralization of decontamination agent(s) may be required prior to sample processing and analyses.

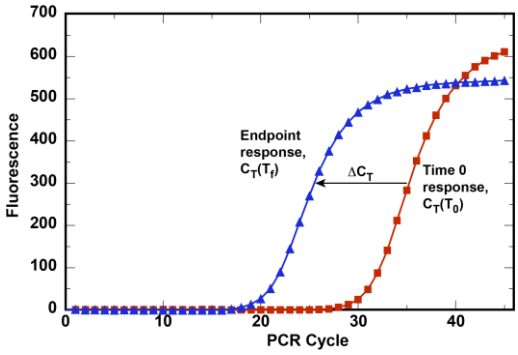
10.1 RV-PCR

Note:* *This procedure has been updated to incorporate the use of Whatman™ Autovial™ filter vials instead of the filter cups included in the 2012 edition. Filter cups are no longer available from the manufacturer.

The RV-PCR method (**Figures 3** and **4**) serves as an alternative to the traditional culture-based methods for detection of viable *B. anthracis* spores in the presence of a large number of dead spores. The RV-PCR method integrates high-throughput sample processing, short-incubation broth culture, and highly sensitive and specific real-time PCR assays to detect low concentrations of viable *B. anthracis* spores (Reference 15.1). This section includes a RV-PCR method with appropriate sample processing procedures for detection of *B. anthracis* in environmental samples.

The RV-PCR method is 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, including a large number of dead/killed *B. anthracis* spores, in the sample. The RV-PCR method not only generates rapid results, but also may provide a higher throughput capability compared to the traditional culture-based methods, and hence, increases the laboratory capacity for sample analysis (Reference 15.9). It also 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 22 samples can be analyzed in 17 hours. If the laboratory is operating in three shifts with adequate analysts, more than 200 samples can be analyzed in 48 hours. If additional equipment and personnel are available, the throughput could be further increased.





- RV-PCR is based on DNA analyses before (T_0) and after (T_f) enrichment incubation of sample
- A shift in PCR C_T value indicates an increase in DNA, which is itself due to an increase in cell number
- Algorithm for detection of viable *B. anthracis*:
  ΔC_T [$C_T(T_0)$ - $C_T(T_f)$] ≥ 9.0
  - $C_T(T_f)$ value ≤ 36.0
  - $C_T(T_0)$ value ≤ 45.0

  (If no C_T for T_0, it is set to 45 to calculate ΔC_T)
- The method accurately distinguishes live spores/cells from dead based on $C_T(T_0)$, $C_T(T_f)$ and ΔC_T

Figure 3. Example real-time PCR amplification curves for the initial T_0 aliquot and the T_f (Final) endpoint aliquot.

Specifically, the RV-PCR method is a combination of culture and real-time PCR, a rapid, highly sensitive and specific analytical method that can be used to detect and identify *B. anthracis*. Culturing the samples allows the germination and growth of viable *B. anthracis* spores recovered from processed samples. Real-time PCR, via a change in the C_T value (Figure 3), offers a rapid determination of the identity and viability of *B. anthracis* bacteria that grow from germinated spores in broth culture. Samples (air filter, particulate [wipe, Sponge-Stick, swab, 37-mm filter cassette or filter], drinking water or decontamination waste water) are processed in multiple spore extraction and wash steps and collected in filter vials. Recovered spores are incubated in filter vials with 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 vial at 37°C for 9 hours or longer 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 or longer, another aliquot is withdrawn. This is the T_9 or T_f aliquot. Both the T_0 and the T_9 or T_f aliquots are then extracted and purified to obtain *B. anthracis* total DNA. The T_0 and the T_9 or T_f DNA extracts are then analyzed, in triplicate, using real-time PCR to detect the presence of *B. anthracis* DNA. Figure 4 provides a flowchart for the RV-PCR analyses. The C_T values for both the T_0 and the T_9 or T_f DNA extracts are recorded and compared (Figure 3). A change in C_T for the T_9 or T_f 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 or T_f]). A $\Delta C_T \geq 9$ (i.e., the endpoint PCR C_T of ≤ 36 for the T_9 or T_f 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 $\Delta C_T \geq 9$ algorithm represents an approximate three log increase in DNA concentration at T_9 or T_f relative to T_0. The increase in DNA concentration at T_9 or T_f is as a result of the presence of viable spores in the sample that germinated and grew during the 9 or more hours of incubation in growth medium. Depending upon the end user's

requirement, sample complexity (dirtiness), and the phase of response, especially the post-decontamination phase during an incident, a lower ΔC_T algorithm of $\Delta C_T \geq 6$ (an approximate two log difference in DNA concentration) and a corresponding higher endpoint PCR C_T of ≤ 39 could be set.

Note*: *The incubation time could be extended to 15-18 hours to minimize the possibility of false negative results due to dirty and/or post-decontamination clearance samples.

As stated in Section 9.3, for a high-confidence identification of pathogens, PCR assays for multiple pathogen-specific genes are usually used. Therefore, in this protocol, three single-plex PCR assays for *B. anthracis* are included. 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. The BC3 PCR assay targets a hypothetical gene on the *B. anthracis* chromosome while the EPA-2 and EPA-1 PCR assays target the capB gene on the pXO2 plasmid and the pagA gene on the pXO1 plasmid, respectively. It should be noted that for sample analysis during a confirmed anthrax incident for which the *B. anthracis* strain has already been identified and characterized, only the most sensitive real-time PCR assay targeting a strain-specific gene on a plasmid may be performed. In the absence of such a gene, the assay targeting the chromosomal marker should be used. Although, if needed, all three assays or any combination thereof can be performed using the same sample DNA extract.

Note:* *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 for the EPA sample analysis needs. These assays will be updated or replaced with fully characterized and validated assays upon availability.

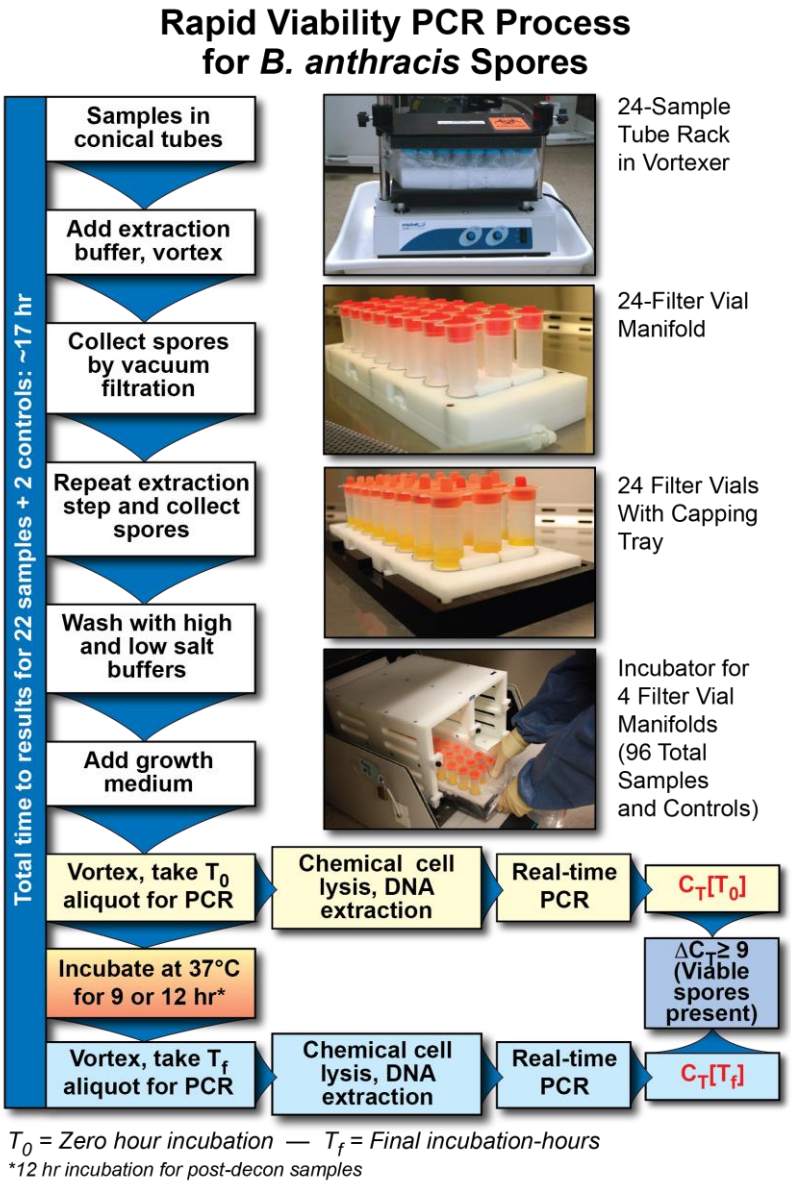


Figure 4. Flow chart for RV-PCR sample analysis.

10.2 RV-PCR Sample Processing: Spore Recovery

Note: ***All subsequent procedures involving manipulation of samples must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements. If any samples need to be removed from the BSC (e.g., for centrifugation, incubation) they should be decontaminated by wiping the outside of the sample container with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) prior to removing from the BSC.***

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 up to 24 Whatman™ Autovial™, filter vials (Section 5.3.4) into the manifold using the two outer columns first. Verify that all filter vials are completely pushed down in manifold. Place a red pull-tab tapered plug (referred to as "red cap") (Section 5.3.6) in each filter vial. Attach brackets (two per manifold) (Section 5.5.8) with Allen screws (two per bracket) to top of manifold to secure Autovials in the openings. Seal any unused openings with tape (Section 5.3.15).
- Vacuum: If using external vacuum pump, tape pump exhaust tube to BSC to vent exhaust inside BSC. 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).
- Capping tray set up: Add bottom caps to capping tray.
- For T_0 and T_9 or T_f 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 or T_f), 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 or T_f tubes, following the sample layout.

- Tape filter vial layout on outside glass window of the BSC.
- Special Instructions
  - All activities conducted in the laboratory should adhere to protocols and aseptic techniques 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.
  - If the gloves become contaminated for any reason, they must be disposed of in an autoclavable biohazard bag 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 pipet tips, gloves and tubes must be discarded in a biohazard autoclave bag in the biosafety cabinet for later disposal after autoclaving.

Figure 5 identifies the appropriate sub-sections for sample processing and spore recovery for each sample type/tool.

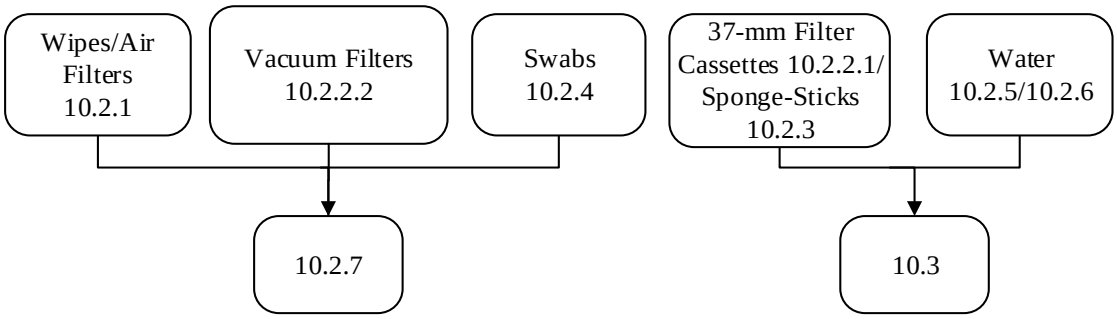


Figure 5. Sample processing and spore recovery steps for RV-PCR.

10.2.1 *Wipe and Air Filter Samples*

Place mesh support over wipe or air filter samples in 30 or 50 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 as described in 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 10.2.7.

10.2.2 *Vacuum Samples (37-mm Filter Cassettes and Filters)*

Note: All subsequent procedures involving manipulation of 37-mm filter cassettes must be carried out in a BSC using appropriate PPE.

10.2.2.1 37-mm Filter Cassettes

- For each 37-mm filter cassette, prepare one 15 mL conical tube containing 11 mL of sterile Extraction Buffer with Tween and 30% (Section 6.4) and label one 2 oz. sterile cup (Section 5.1.34). In the BSC remove the conical tube containing the nozzle and the cassette from the containment bags and wipe the outside of the conical tube with a disinfectant and place it into a rack. Aseptically add 5 mL of buffer (from the 11 ml of a pre-measured aliquot of Extraction Buffer with Tween and 30%) to the conical tube containing the nozzle and tubing and set aside. Remove the band from around the cassette using a sterile scalpel or sterile pair of scissors. Wipe each cassette with 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) followed by a clean Kimwipe® and discard wipes into an autoclavable biohazard bag.
- Change gloves. Remove the red plug from the front filter side of the cassette; the plug on the back side should be kept in place. Using a transfer pipette dispense 1 mL of Extraction Buffer with Tween and 30% from the tube now containing the 6 mL into the cassette and replace plug. Roll the cassette around to allow the liquid to touch all surfaces of the inside of the cassette. If there is a large quantity of particulate matter, more Extraction Buffer with Tween and 30% may be required.

Particulate matter should be dampened enough to prevent aerosolization.

- Using the cassette tool (Section 5.1.36) pry open the top section of the cassette, using care not to spill the Extraction Buffer inside the cassette. Set the bottom portion containing the filter aside carefully (filter side up), and using a transfer pipette rinse the walls of the cassette with 1-2 mL of Extraction Buffer with Tween and 30%. Transfer the rinsate using the same pipette to the appropriately labelled 2 oz. sterile cup (Section 5.1.34). Using the same transfer pipette repeat the rinsing process for the bottom portion of the cassette and transfer the rinsate to the 2 oz. cup.
- Using the cassette tool remove the middle section of the cassette (this piece is holding the filter in place). Using sterile forceps aseptically remove the filter without picking up the support filter underneath. Place the filter in the 2 oz. cup with the rinsates. Use the remainder of the 6 mL Extraction Buffer with Tween and 30% to rinse walls and bottom section of the cassette and transfer to the 2 oz. cup. Discard the cassette sections, support filter, plugs, and transfer pipette in an autoclavable biohazard bag.
- Disinfect the outside of the 2 oz. cup with 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and place in rack. Decontaminate the BSC with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and don a fresh pair of gloves in between samples in between samples. Repeat procedure described above for each 37-mm filter cassette.
- Seal the conical tubes containing the nozzle and tubing in 5 mL Extraction Buffer with Tween and 30%, tubing and nozzle with Parafilm®. Place the rack of conical tubes into the sonicating bath to a level that allows at least 1 inch (~2.5 cm) of tube to be above the water line. Place a rectangular weight on top of the tubes to prevent them from floating or tipping over. Sonicate for 1 minute and remove tubes from the sonicating bath. Dry and disinfect each tube with a 10% amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Vortex the conical tubes on high for 2 minutes and transfer the 5 mL suspension to the appropriate 2 oz. cup.
- Seal all of the 2 oz. cups with Parafilm®. Place the rack of 2 oz. cups in the sonicating bath and cover with a rectangular weight on top of the cups to prevent them from floating or tipping over. There should be 1 inch (~2.5 cm) between the level of the water and the cup lids. Sonicate for 3 minutes without heat. Remove rack from the bath and dry each cup with a Kimwipe® and place in the BSC. Place cups in a sealable plastic lidded box (Section 5.1.35). Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Place manifold and Whatman™ Autovial™ filter vials (Section 5.3.4) with red caps in BSC. Label all filter vials. Record filter vial labels.
- Turn on vacuum pump at 5-10 psi.
- Using a 10 mL serological pipet, transfer as much suspension as possible from each 2 oz. cup to the corresponding labeled filter vial by lifting red cap slightly. Use a new 10 mL serological pipet (with graduations up to 13 mL) for each new cup.

- Complete filtration of liquid through filter vials. Turn off vacuum pump.
- Proceed to Section 10.3.

10.2.2.2 Vacuum Filters

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 mesh support (Section 9.1) on top of filter. Proceed to Section 10.2.7.

10.2.3 *Sponge-Stick Samples*

- If the Sponge-Stick sponge is not in a Stomacher® bag, holding the plastic handle of the Sponge-Stick with one gloved hand, carefully remove the sponge using sterile forceps and aseptically transfer it to a Stomacher® bag. Change forceps between samples.
- Add 90 mL of cold (4°C) extraction buffer with Tween® 20 (Section 6.4) 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 rests evenly between the homogenizer paddles and stomach each sample for 1 minute at 260 rpm.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the sponge/wipe from the outside of the bag with hands. With the bag closed, move the sponge/wipe to the top of the bag while using hands to expel liquid from the sponge/wipe.
- Open the bag, remove and discard the sponge in an autoclavable biohazard bag using sterile forceps.
- Follow steps described above for each sample, changing forceps between samples.
- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- Gently mix the suspension in the Stomacher® bag (Section 5.5.28) up and down three times with a sterile 50 mL pipet. Remove half of the suspension volume (~45-46 mL) and place it in a 50 mL screw cap centrifuge tube. Place the remaining suspension (~45-46 mL) into a second 50 mL tube. Adjust the suspension volumes in both the tubes to ensure they are similar. Record suspension volumes on tubes and data sheet.
- Process the 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 at 4°C.

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

- Place manifold and Whatman™ Autovial™ filter vials (Section 5.3.4) with red caps in BSC. Label all filter vials. Record filter vial labels.
- Turn on vacuum pump at 5-10 psi.
- Using a sterile 50 mL pipet for each sample, carefully remove 40 mL of the supernatant from each sample tube and discard it in an autoclavable biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube.
- Vortex each tube for 30 seconds to resuspend the pellets in the remaining volume of the supernatant.
- Using a sterile 10 mL pipet, transfer entire volume from each tube to the corresponding labeled filter vial by lifting red cap slightly.
- Complete filtration of liquid through filter vials. Turn off vacuum pump.
- Add 12.5 mL of cold (4°C) extraction buffer without Tween® 20 (Section 6.5) to each filter vial by lifting red cap slightly. Wait for 5 minutes prior to turning on the vacuum to 5-10 psi. Complete filtration through the filter vials. 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.

10.2.4 *Swab Samples*

Place swab into the 30 mL tube and snap off handle at notch to fit in the tube. Make sure that the swab head is at the bottom of the tube. Using sterile forceps, place the mesh support over the swab (Section 9.1). Proceed to Section 10.2.7.

10.2.5 *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 and ≥ 50 mL, please refer to Appendix A, Section 3.0, secondary concentration.

- After secondary water concentration, using a 10 mL serological pipet add 10 mL of PBST (0.025% Tween® 20, Section 6.2) 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.
- Place manifold and Whatman™ Autovial™, filter vials (Section 5.3.4) with red caps in BSC. Label all filter vials, following the sample tube rack layout. Record filter vial and sample tube labels.

- Using a new 10 mL serological pipet, transfer as much liquid as possible to the corresponding labeled filter vial by lifting red cap slightly, avoiding any settled particles.
- Repeat for each sample tube.
- Turn on vacuum pump to 5-10 psi in order to collect spores onto filter vials.
- Repeat extraction of each membrane filter by adding another 10 mL of PBST buffer to the 50 mL conical tube with membrane and vortex for 2 minutes with 10 second bursts, as described above.
- After 2 minutes of settling, using a 10 mL serological pipet, transfer as much liquid as possible from each 50 mL tube to the corresponding labeled filter vial by lifting red cap slightly, avoiding any settled particles.
- Complete filtration of liquid through filter vials. Turn off the vacuum pump.
- Add 12.5 mL of cold (4°C) extraction buffer without Tween® 20 (Section 6.5) to each filter vial, and wait for 5 minutes prior to turning on the vacuum to 5-10 psi. Complete filtration through the filter vials. Turn off the vacuum pump. 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.

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

- Place manifold and Whatman™ Autovial™, filter vials (Section 5.3.4) with red caps in BSC. Label all filter vials, following the sample tube rack layout. Document filter vial 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 12.5 mL of the water sample to the corresponding filter vial by lifting red cap slightly.
- Dispose of pipet in an autoclavable biohazard waste container. After completion of filtration, transfer remaining sample volume in 12.5 mL increments to corresponding labeled filter vial using a new serological pipet and complete filtration. Turn off vacuum.
- Cap sample tube. Change gloves.
- After performing transfers to the corresponding filter vial for each water sample, check that all sample tube caps are secure. Place tube rack in plastic bag, seal, bleach bag and double bag.
- Store water sample tubes at 4°C as an archive until analyses are completed or until directed to discard samples.
- Add 12.5 mL of cold (4°C) extraction buffer without Tween® 20 (Section 6.5) to each filter vial by lifting red cap slightly. Wait for 5 minutes prior to turning on the vacuum to 5-10 psi. Complete filtration through the filter vials and turn off the vacuum pump. Washing the filter with extraction buffer containing 30% ethanol

could reduce the number of vegetative cells that could compete with *B. anthracis* spore outgrowth.

- Proceed to Section 10.3.

10.2.7 Place manifold and Whatman™ Autovial™, filter vials (Section 5.3.4) with red caps in BSC. Label all filter vials, following the sample tube rack layout. Document filter vial and sample tube labels.

10.2.8 Add 12.5 mL of cold (4°C) extraction buffer with Tween® 20 (Section 6.4) to samples (use 5 mL for swabs) placed in 30 mL conical tubes (50 mL tubes for vacuum filters) in tube rack (up to 24 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. If needed, the tube caps can be sealed with Parafilm. Label tubes as appropriate and document location in rack.

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

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

10.2.11 After vortexing, transfer sample tube rack to the BSC. Remove tube rack from plastic bag and discard the bag in an autoclavable biohazard bag.

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

10.2.13 Uncap tubes one at a time. Using a 10 mL serological pipet, carefully transfer 12.5 mL suspension (5 mL for swabs) to corresponding labeled filter vial by lifting red cap slightly. Dispose of pipet in an autoclavable biohazard waste container. Cap sample tube and place tube back in rack. Change gloves.

10.2.14 Repeat Section 10.2.12 for each sample tube using a new serological pipet, processing one sample at a time and up to 8 samples as a set. Repeat Section 10.2.13 for each set of up to 8 sample tubes.

10.2.15 Turn on vacuum pump at 5-10 psi and filter suspensions. Turn off the vacuum pump. Change gloves.

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

10.2.17 Add 10 mL (5 mL for swabs) of cold (4°C) extraction buffer without Tween® 20 (Section 6.5) to each sample tube, one at a time with a new 10 mL serological pipet and a fresh pair of gloves for each sample. Keep the buffer bottle loosely covered between transfers. Recap the sample tube after each buffer addition.

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

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

10.2.20 Repeat Sections 10.2.11-10.2.13 except transfer as much remaining liquid as possible (approximately 5 mL for swabs) to the corresponding filter vial while taking care to avoid settled particles during the dispensing of aliquots. Proceed to RV-PCR processing section (Section 10.3) below, with filter vial manifold.

10.2.21 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 a ziplock bag with orange caps, one for each filter vial, into the BSC.

10.3.2 Place 10 mL serological pipets and cold (4°C) high salt wash buffer (Section 6.7) in 250 mL screw cap bottle in the BSC.

10.3.3 To each filter vial, transfer 12.5 mL (10 mL for swab samples) of cold (4°C) high salt wash buffer using a 10 mL serological pipet and lifting red cap slightly, keeping the bottle covered between transfers. Use a new pipet for each filter vial. Turn on vacuum pump at 5-10 psi. Complete filtration of the suspension. Turn off vacuum pump. Change gloves.

10.3.4 Place 10 mL serological pipets and cold (4°C) low salt wash buffer (Section 6.7) in 250 mL screw cap bottle in the BSC.

10.3.5 Transfer 12.5 mL of cold (4°C) low salt wash buffer to each filter vial using a 25 mL serological pipet (by lifting red cap slightly). Keep the bottle covered between transfers and use new pipet for each filter vial.

10.3.6 Turn on vacuum pump at 5-10 psi. Complete filtration of the suspension. 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 vial manifold top and remove it from the bottom vacuum manifold and place on top of the capping tray, fitted with bottom caps. Press down firmly on all sides of the top manifold to ensure caps are securely fastened to filter vial bottom ports. Place bleach wipes over the manifold bottom until it can be disinfected. Change gloves.

10.3.8 Place 5 mL serological pipets, 1 mL pipettor, 1 mL pipet tips, cold (4°C) BHI broth dispensed as aliquots into 50 mL conical tubes, sharps container and orange caps (required number in a ziplock bag) in the BSC.

10.3.9 Remove red cap and discard in an autoclavable biohazard bag. Pipet 5.0 mL of cold BHI broth (Section 6.18) into the filter vial using a 5 mL serological pipet. Firmly press orange caps with pull-tabs onto the filter vial.

10.3.10 Repeat 10.3.9 for each filter vial. Record the time of the BHI broth addition since this represents T_0.

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

10.3.12 Vortex the filter vials for 10 minutes on the platform vortexer (Section 5.5.15), with speed set to 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 vial manifold in capping tray to the BSC. Remove bag.

10.3.15 Uncap one filter vial 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 vial 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 vial.

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

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

Note*: *The incubation time could be extended to 15-18 hours to minimize the possibility of false negative results due to dirty and/or post-decontamination clearance samples.

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 10.4.11), below.

10.4 Manual DNA Extraction and Purification Using the MagneSil® Blood Genomic, Max Yield System, Kit

Note: Alternate DNA extraction-purification procedures may only be used (e.g., MagNA-Pure LC instrument) if the limit of detection is verified to be similar to the manual procedure described below.

Prepare Lysis Buffer with anti-foam according to manufacturer's instructions in the MagneSil® Blood Genomic, Max Yield System, Kit (Section 6.12). 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 or T_f aliquots are processed in the same manner, described below.

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

10.4.2 After the 9 hour or longer incubation, remove the filter vial manifold from the shaker incubator.

Note: For dirty and post-decontamination samples the incubation time could be extended to 15-18 hours.

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

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

10.4.5 Set up 2 mL screw cap tubes for T_9 or T_f aliquots in a 96-well tube rack (8 × 12) and verify that 2 mL tube labels match the filter vial 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 or T_f aliquot screw cap tubes to the BSC.

10.4.6 Uncap one filter vial at a time and open the corresponding 2 mL tube. Using a 1 mL pipettor, swirl pipet tip gently in filter vial while gently pipetting up and down 10 or more

times to mix sample (and to avoid aerosol generation); transfer 1 mL (T_9 or T_f aliquot) from each vial to the corresponding T_9 or T_f aliquot tube in the 96-well tube rack. Cap the tubes.

10.4.7 Repeat Section 10.4.6 for each filter vial.

10.4.8 Centrifuge tubes at 14,000 rpm (20,800 relative centrifugal force [RCF]) for 10 minutes at 4°C. Remove 800 µL of the supernatant from each tube using a 1000 µL pipettor and discard in an autoclavable biohazard bag. Do not disturb the pellet (use a new tip for each sample and discard tips in an autoclavable sharps container). If processing for DNA extraction immediately, proceed to Section 10.4.11.

10.4.9 Store pellets on ice or in cold block (4°C). Alternatively, pellets may be stored at -20°C until it is ready to be processed for DNA extraction.

Note: ***T_0 and T_9 or T_f DNA extractions can be completed separately.***

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

10.4.11 Add 800 µL of Lysis Buffer (Section 6.12; VWR, Cat. No. PAMD1392 or equivalent) using a 1000 µL pipettor with a new tip for each sample. Cap the tubes and mix by vortexing on high (~1800 rpm) for 30 seconds and place in 96-well tube rack at room temperature. Change gloves as necessary between 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 or T_f lysate tubes hereafter referred to as "T_0 and T_9 or T_f tubes" at room temperature for 5 minutes.

10.4.13 Vortex the MagneSil® paramagnetic particles (PMP) suspension on high (~1800 rpm) for 30-60 seconds, or until PMPs are uniformly resuspended. Keep PMPs in suspension by briefly vortexing (3-5 seconds) before adding to each T_0 and T_9 or T_f tube.

10.4.14 Uncap one tube at a time and add 600 µL of PMPs to each T_0 and T_9 or T_f tubes (containing 1 mL sample). Discard used tips in an autoclavable sharps container. Recap tube and mix by vortexing for 3-5 seconds.

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

10.4.16 Vortex each T_0 and T_9 or T_f tube for 5-10 seconds (high, ~1800 rpm), 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 an autoclavable 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 or T_f 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 used tips in an autoclavable sharps container. Cap and vortex on low setting for 5-10 seconds, then transfer to 96-well tube rack.

10.4.19 After adding Lysis Buffer to all of the T_0 and T_9 or T_f 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 or T_f tube (discard used tips in an autoclavable sharps container). Recap the tube.

10.4.21 Repeat Sections 10.4.18-10.4.20 for all tubes.

Note: Section 10.4.20 can be combined with Section 10.4.22. After withdrawing the liquid in Section 10.4.20, add 360 µL of Salt Wash solution 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 or T_f tube one at a time, and add 360 µL of Salt Wash solution (Section 6.12). Use a new tip for each T_0 and T_9 or T_f tube and discard used tips in an autoclavable 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 or T_f 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.

10.4.24 Remove liquid as described in Section 10.4.17, except that a glove change between T_0 and T_9 or T_f tubes is not required. Use a new tip for each T_0 and T_9 or T_f tube and discard used tips in an autoclavable sharps container. Recap the tube. Repeat for all T_0 and T_9 or T_f 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 solution 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 or T_f 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 or T_f tube, one at a time, and add 500 µL of Alcohol Wash (Section 6.12). Use a new tip for each sample and discard used tips in an autoclavable sharps container. Cap and transfer to 96-well tube rack.

10.4.27 After adding the Alcohol Wash (Section 6.12) to all of the T_0 and T_9 or T_f tubes, vortex each tube for 5-10 seconds (low speed) and place on the magnetic stand. After all T_0 and T_9 or T_f 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 or T_f tubes is not required. Use a new tip for each T_0 and T_9 or T_f tube and discard used tips in an autoclavable 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 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 or T_f 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 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 or T_f 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 (Section 6.8) 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 or T_f tubes and air dry for 2 minutes.

10.4.33 Heat the open T_0 and T_9 or T_f tubes in the heat block (placed in the BSC) 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 200 µL of Elution Buffer (Section 6.12) to each T_0 and T_9 or T_f tube, and close tube.

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

10.4.36 Briefly vortex the tubes (5-10 seconds), taking care to prevent the liquid from entering the tube cap. Let the tubes 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. *Optional: 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 elution liquid from each T_0 or T_9 or T_f tube with a micropipettor and transfer to a clean, labeled, 1.5 mL tube (~80-90 µL) on a cold block (check tube labels to ensure the correct order). Use a new tip for each tube and discard tips in an autoclavable sharps container. Visually verify absence of PMP carryover during final transfer. If magnetic bead carryover occurred, place the 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).

Note: If analyses need to be conducted outside of a BSL-3, the DNA extract may be filtered using a 0.1 µm Ultrafree®-MC filter insert as described in Section 9.2.10.

10.4.43 Store T_0 and T_9 or T_f DNA extract tubes 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 autoclavable biohazard bags (double bagged).
- Autoclave all waste materials at the end of the work day.
- Decontaminate counters and equipment with fresh 10% pH amended bleach solution (Section 6.20), followed by 70% isopropyl and a DI water final rinse.

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

Note: PCR Master Mix for 6 reactions per sample is required to accommodate the T_0 and T_9 or T_f 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 or T_f DNA extracts). No EIC control is required for the samples.

For real-time PCR, follow Section 9.3.

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 (Section 6.9) to the tubes.

Note: No centrifugation is required (Section 10.5.2) if PCR analysis is conducted immediately after DNA elution (Section 10.4.42). Each sample is analyzed in triplicate.

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.

10.5.3 Mix diluted T_0 DNA extracts by vortexing (5 seconds at low speed), and transfer 3, 5 µL aliquots from each tube to the PCR plate (with PCR Master Mix).

10.5.4 *T_9* or T_f *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.

Note: No centrifugation is required (Section 10.5.5) if PCR analysis is conducted immediately after DNA elution (Section 10.4.42). Each sample is analyzed in triplicate.

10.5.5 Mix T_9 or T_f 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.

10.5.6 Mix diluted T_9 or T_f DNA extracts by vortexing (5 seconds), and transfer 3, 5 µL aliquots from each tube 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 the PCR plate to the ABI® 7500 Fast thermocycler.

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

10.5.11 After the PCR run, discard sealed PCR plate in an autoclavable biohazard bag.

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

10.5.12 Follow laboratory cleanup protocol provided in Section 10.4.44.

10.5.13 Refer to Section 12.2 for Data Analyses and Calculations and record the results.

11.0 Culture Method

Acceptable sample types: Gauze wipes (2" × 2" 50% rayon/50% polyester [Kendall™ Versalon™ Cat. No. 8042 or equivalent]), air filters (37 mm Fluoropore™ [Millipore® Cat. No. FSLW04700 or equivalent]), swabs (macrofoam [VWR Cat. No. 89022-994 small swabs or 89022-984 extra-large swabs or equivalent]), Sponge-Stick sampling tools (3M Inc. Cat. No. SSL100 or equivalent), 37-mm mixed cellulose ester (MCE) filter cassettes (SKC Cat. No. 225-9543 or equivalent), vacuum filters (3M Forensic, Precision Data Products Cat. No. FF-1 with 4" diameter filter or equivalent), drinking water and decontamination waste water

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

Media sterility checks (Section 8.5) and positive controls (Section 8.7) should be analyzed every day that samples are analyzed, to ensure that all media and reagents are performing properly.

11.1 Sample Processing and Plating for Sponge-Sticks and Wipes

Note: All subsequent procedures involving manipulation of Sponge-Sticks and wipes must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

11.1.1 Recover Spores from Sponge-Sticks and Wipes

- If the Sponge-Stick sponge/wipe sample is not in a Stomacher® bag, aseptically transfer it to a Stomacher® bag using sterile forceps.

Note: In most cases, the Sponge-Stick handle will be removed in the field by the sampler. However, if the handle was not removed, proceed as follows. While holding the sponge-stick handle outside the Stomacher® bag, grip the Sponge-Stick head with your other hand outside the bag and twist the head to snap off stick at the crimp line near the handle base.

- Using aseptic technique, remove the plastic stick base holding the sponge together. Place gloved hands on the outside of the Stomacher® bag, grip the sponge-stick head on both sides and peel the sponge away from the base and unfold the sponge. Be careful not to puncture bag with edge of stick base. Using sterile forceps remove stick base from bag

and discard in an autoclavable biohazard bag. Change gloves and forceps between samples.

- Add 90 mL of PBST (0.05% Tween® 20, Section 6.2) to each bag. Set Stomacher® (Section 5.5.28) to 260 rpm.
- Place the bag containing the sample into the Stomacher® (Section 5.5.28) so the sponge/wipe rests evenly between the paddles and homogenize each sample for one minute at 260 rpm.
- Open the door of the Stomacher® (Section 5.5.28) and remove the bag. Grab the sponge/wipe from the outside of the bag with your hands. With the bag closed, move the sponge/wipe to the top of the bag while using your hands to squeeze excess liquid from the sponge/wipe.
- Open the bag and remove sponge/wipe using sterile forceps. Retain the sponge/wipe in a labeled specimen cup (Section 5.4.5).
- Follow the steps described above for each sample, changing forceps between samples.
- Allow bags to sit for 10 minutes to allow elution suspension foam to settle.
- Gently mix the suspension in the Stomacher® bag (Section 5.5.28) up and down three times with a sterile 50 mL pipet. Remove half of the suspension volume (~45-46 mL) and place it in a 50 mL screw cap centrifuge tube. Place the remaining suspension (~45-46 mL) into a second 50 mL tube. Adjust the suspension volumes in both the tubes to ensure they are similar. Record suspension volumes on tubes and data sheet.
- Process the 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 at 4°C.

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

- Using a sterile 50 mL pipet for each sample, carefully remove ~42-43 mL of the supernatant from each sample tube and discard it in an autoclavable biohazard container. The pellet may be easily disturbed and not visible, so keep the pipet tip away from the bottom of the tube.
- Set the vortexer (Section 5.5.16) to high intensity. Set the sonicator water bath to high.
- Vortex the tubes for 30 seconds to resuspend the pellets and transfer the tubes to the sonicator bath and sonicate for 30 seconds. Repeat the vortex and sonication cycles two more times.
- 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: As an alternative to sonication, tubes may be vortexed for 2 minutes in 10 second

bursts using a vortexer with a multi-tube adapter.

11.1.2 Serially Dilute Spore Suspensions in PBST

- Vortex the 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 elution suspension (undiluted) and 2 serial dilutions of the suspension in PBST (10^{-1} and 10^{-2}).

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

11.1.3 Culture Spore Suspensions on SBA

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature, or dried using a laminar-flow hood.

Note: Plating of 0.1 mL 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 (Section 6.17) 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, beginning with the 10^{-3} 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. Use a different sterile spreader for each plate. Repeat for the next two dilutions 10^{-2} and 10^{-1}, in that order.

- Allow inoculum to absorb into the medium completely.

11.1.4 Incubate and Enumerate Plates

Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* 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 (**Figure 6**). 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.

Figure 6. *B. anthracis* colonies on SBA.

- Plate counts

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

  b. If the number of colonies is > 250/plate, record as "too numerous to count" (TNTC).

  c. If no typical colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (Section 11.1.5).

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

11.1.5 Capture Spores on MicroFunnel™ Filter Membranes and Culture

- Place 2, 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 < 10 psi.
- 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 (Section 11.1.1) to each of two MicroFunnel™ cups.
- 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 the cup in an autoclavable biohazard bag. Remove each membrane with sterile forceps and place it grid-side up on a labeled SBA plate. Make sure that the filters are 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 suspension filtered (e.g., 1.0 mL) on each plate.
- Repeat steps (Section 11.1.5) described above for each sample.
- Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* colonies and record results. Confirm 1-3 colonies using real-time PCR (Section 11.6).

Note: For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1} and 10^{-2} dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in TSB.

11.1.6 Enrich in TSB

- Add the remainder of the undiluted, 10^{-1} and 10^{-2} suspensions to the specimen cup containing the corresponding sponge/wipe. Add 25 mL of TSB (Section 6.19) to the specimen cup. Repeat for each sample. Incubate cups at 37ºC ± 1ºC for 24-48 hours.
- Evaluate the TSB Enrichment
  - **a.** If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is turbid, record as positive growth (G+) and proceed to next step.
  - **c.** Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates.
  - **d.** Incubate the isolation plates and TSB with growth at 37ºC ± 1ºC for a maximum of three days.
  - **e.** Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (Section 11.6).
  - **f.** If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section 11.6).

11.2 Sample Processing and Plating for Swabs

Note: All subsequent procedures involving manipulation of swabs must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

11.2.1 Recover Spores from Swabs

- 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.

- Add 5 mL of PBST (0.05% Tween® 20, Section 6.2) to each tube and vortex at the highest setting for 2 minutes.
- 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.
- Place the swab into a labeled 50 mL tube with 5 mL TSB and set aside.

- Repeat vortexing steps for each swab sample.

11.2.2 Serially Dilute Spore Suspensions in PBST

- Vortex the 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 elution suspension (undiluted) and 2 serial dilutions of the suspension in PBST ($10^{-1}$and 10^{-2}).
- Repeat steps (a) and (b) for each sample.

11.2.3 Culture Spore Suspensions on SBA

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

Note: Plating of 0.1 mL 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 (Section 6.17) 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, beginning with the 10^{-3} 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. Use a different sterile spreader for each plate. Repeat for the next two dilutions 10^{-2} and 10^{-1}, in that order.
- Allow inoculum to absorb into the medium completely.

11.2.4 Incubate and Enumerate Plates

Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* 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 (Figure 6). 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.

- Plate counts

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

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

c. If no typical colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.2.5).

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

11.2.5 Capture Spores on MicroFunnel™ Filter Membranes and Culture

- Place 2, 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 < 10 psi.
- 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 (Section 11.2.1) to each of two MicroFunnel™ cups.
- 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 the cup in an autoclavable biohazard bag. Remove each membrane with sterile forceps and place it grid-side up on a labeled SBA plate. Make sure that the filters are 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 suspension filtered (e.g., 1.0 mL) on each plate.
- Repeat steps (Section 11.2.5) described above for each sample.
- Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* colonies and record results. Confirm 1-3 colonies using real-time PCR (Section 11.6).

Note: ***For faster sample analysis results during the initial stages of an incident (e.g., incident characterization) and during post-decontamination/clearance phase, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1} and 10^{-2} dilutions) be filtered using an additional MicroFunnel™ and plated as described above, instead of proceeding with enrichment in TSB.***

11.2.6 Enrich in TSB

- Add the remainder of the undiluted, 10^{-1} and 10^{-2} suspensions to the 50 mL tube containing the corresponding swab. Add 25 mL of TSB (Section 6.19) to the tube. Repeat for each sample. Incubate tubes at 37ºC ± 1ºC for 24-48 hours.
- Evaluate the TSB Enrichment

a. If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.

b. If broth is turbid, record as positive growth (G+) and proceed to next step.

c. Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates.

d. Incubate the isolation plates and TSB with growth at 37ºC ± 1ºC for a maximum of three days.

e. Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (Section 11.6).

f. If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section 11.6).

11.3 Sample Processing and Plating for Air Filters

Note: All subsequent procedures involving manipulation of air filters must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

11.3.1 Recover Spores from Air Filter

- If the air filters are not in 50 mL tubes, aseptically transfer each sample to a sterile 50 mL tube using sterile forceps. Change gloves and forceps between samples.
- Add 5 mL of sterile PBST (0.05% Tween® 20, Section 6.2) to each tube and vortex on high for 2 minutes.
- Transfer liquid to a sterile, labeled 50 mL tube.
- Place air filter in a specimen cup and set aside.
- Repeat the steps described above for each air filter.

11.3.2 Serially Dilute Spore Suspensions 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 filter 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

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

Note: Plating of 0.1 mL 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 24 spread plates per sample:

a. After vortexing tubes, pipet 0.1 mL of undiluted suspension onto surface of pre-dried SBA (Section 6.17) 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 (labeled10^{-3}).

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

- After pipetting the 3 spread plates for each dilution, beginning with the 10^{-4} 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. Use a different sterile spreader for each plate. Repeat for the next three dilutions 10^{-3}, 10^{-2} and 10^{-1}, in that order.
- Allow inoculum to absorb into the medium completely.

11.3.4 Incubate and Enumerate Plates

Invert the plates and incubate them at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* 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 (Figure 6). 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.

- Plate counts

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

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

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

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

11.3.5 Capture Spores on MicroFunnel™ Filter Membranes and Culture

- Place 2, 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 < 10 psi.

- 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 (Section 11.3.1) to each of two MicroFunnel™ cups.
- 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 the cup in an autoclavable biohazard bag. Remove each membrane with sterile forceps and place it grid-side up on a labeled SBA plate. Make sure that the filters are 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 suspension filtered (e.g., 1.0 mL) on each plate.
- Repeat steps (Section 11.3.5) described above for each sample.
- Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* colonies and record results. Confirm 1-3 colonies using real-time PCR (Section 11.6).

Note: ***For sample analyses conducted during the post-decontamination/clearance phase of an incident, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1} 10^{-2} and 10^{-3} dilutions) be filtered using a third MicroFunnel™ and plated as described above instead of proceeding with enrichment in TSB.***

11.3.6 Enrich in TSB

- Add the remainder of the undiluted, 10^{-1}, 10^{-2} and 10^{-3} suspensions to the specimen cup containing the corresponding air filter. Add 30 mL of TSB (Section 6.19) to the specimen cup. Repeat for each sample. Incubate tubes at 37ºC ± 1ºC for 24-48 hours.
- Evaluate the TSB Enrichment
  - **a.** If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is turbid, record as positive growth (G+) and proceed to next step.
  - **c.** Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat 2 times for a total of three SBA isolation plates.
  - **d.** Incubate the isolation plates and TSB with growth at 37ºC ± 1ºC for a maximum of three days.
  - **e.** Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (Section 11.6).
  - **f.** If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section 11.6).

11.4 Sample Processing and Plating for 37-mm Filter Cassettes and Filters

Note: All subsequent procedures involving manipulation of 37-mm filter cassettes and filters must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

11.4.1 Recover Spores from 37-mm Filter Cassettes

Note: All subsequent procedures involving manipulation of 37-mm filter cassettes must be carried out in a BSC using appropriate PPE.

- For each 37-mm filter cassette, prepare one 15 mL conical tube containing 11 mL of sterile PBST (Section 6.2) and label one 2 oz. sterile cup (Section 5.1.34). In the BSC remove the conical tube containing the nozzle and the cassette from the containment bags and wipe the outside of the conical tube with a disinfectant and place it into a rack. Aseptically add 5 mL of PBST (from the 11 ml of pre-measured aliquots of PBST) to the conical tube containing the nozzle and tubing and set aside. Remove the band from around the cassette using a sterile scalpel or sterile pair of scissors. Wipe each cassette with 10% pH amended bleach solution (6.20) or bleach wipes (5.1.3) followed by a clean Kimwipe® and discard wipes into an autoclavable biohazard bag.
- Change gloves. Remove the red plug from the front filter side of the cassette; the plug on the back side should be kept in place. Using a transfer pipette dispense 1 mL of PBST from the tube now containing the 6 mL into the cassette and replace plug. Roll the cassette around to allow the liquid to touch all surfaces of the inside of the cassette. If there is a large quantity of particulate matter, more PBST may be required. Particulate matter should be dampened enough to prevent aerosolization.
- Using the cassette tool (Section 5.1.36) pry open the top section of the cassette, using care not to spill the PBST inside the cassette. Set the bottom portion containing the filter aside carefully (filter side up), and using a transfer pipette rinse the walls of the cassette with 1-2 mL of PBST. Transfer the rinsate using the same pipette to the appropriately labelled 2 oz. sterile cup. Using the same transfer pipette repeat the rinsing process for the bottom portion of the cassette and transfer the rinsate to the 2 oz. cup.
- Using the cassette tool remove the middle section of the cassette (this piece is holding the filter in place). Using sterile forceps aseptically remove the filter without picking up the support filter underneath. Place the filter in the 2 oz. cup with the rinsates. Use the remainder of the 6 mL PBST to rinse walls and bottom section of the cassette and transfer to the 2 oz. cup. Discard the cassette sections, support filter, plugs, and transfer pipette in an autoclavable biohazard bag.
- Disinfect the outside of the 2 oz. cup with 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and place in rack. Decontaminate the BSC with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3) and don a fresh pair of gloves in between samples in between samples. Repeat procedure described above for each 37-mm filter cassette.
- Seal the conical tubes containing 5 mL PBS, tubing and nozzle with Parafilm®. Place the rack of conical tubes into the sonicating bath to a level that allows at least 1 inch of tube to be above the water line. Place a rectangular weight on top of the tubes to prevent them

from floating or tipping over. Sonicate for 1 minute and remove tubes from the sonicating bath. Dry and disinfect each tube with a 10% amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).

- Vortex the conical tubes on high for 2 minutes and transfer the 5 mL PBST to the appropriate 2 oz. cup.
- Seal all of the 2 oz. cups with Parafilm®. Place the rack of 2 oz. cups in the sonicating bath and cover with a rectangular weight on top of the cups to prevent them from floating or tipping over. There should be 1 inch (~2.5 cm) between the level of the water and the cup lids. Sonicate for 3 minutes without heat. Remove rack from the bath and dry each cup with a Kimwipe® and place in the BSC. Place cups in a sealable plastic lidded box (Section 5.1.35). Discard all waste in an autoclavable biohazard bag and decontaminate workspace and equipment with a 10% pH amended bleach solution (Section 6.20) or bleach wipes (Section 5.1.3).
- Using a 10 mL serological pipet, transfer as much suspension as possible from each 2 oz. cup to the corresponding labeled 50 mL sterile, screw cap, conical tubes.
- Place 50 mL 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 and rotor specifications.

- After centrifugation, move the sealed centrifuge buckets back to the BSC.
- Using a 5 mL serological pipet, carefully discard 5 mL of the supernatant and resuspend the pellet in the remaining 5 mL by vortexing for 1 minute with 10 second bursts.

11.4.2 Recover Spores from Vacuum Filters

- Place 50 mL of PBST (0.05% Tween® 20, Section 6.2) into a sterile, leak-proof, wide-mouth screw cap plastic container (Section 5.1.32).
- 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 plastic container.
- 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 30-35 mL of suspension from each sample into

corresponding 50 mL sterile, screw cap, conical tubes.

- Transfer vacuum filter to a clean wide-mouth screw cap plastic container and set aside.
- Place 50 mL 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 and rotor specifications.

- After centrifugation, move the sealed centrifuge buckets back to the BSC.
- Using a 25 mL serological pipet, carefully discard 25-30 mL of the supernatant (leaving approximately 5 mL in the tube) and resuspend the pellet in the remaining 5 mL by vortexing for 1 minute with 10 second bursts.

11.4.3 Serially Dilute Spore Suspensions 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 four spore suspensions: the initial 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.

11.4.4 Culture Spore Suspensions on SBA

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

Note: Plating of 0.1 mL 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 (Section 6.17) 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 (labeled10^{-3}).

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

- After inoculating the three spread plates for the first suspension (e.g., 10^{-1}), 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 two dilutions 10^{-2} and 10^{-3}.
- Allow inoculum to absorb into the medium completely.

11.4.5 Incubate and Enumerate Plates

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 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* 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 (Figure 6). 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.

- Plate Counts

  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 typical colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.4.6).

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

11.4.6 Capture Spores on MicroFunnel™ Filter Membranes and Culture

- Place 2, 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 < 10 psi.
- 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 (Section 11.4.1) to each of two MicroFunnel™ cups.
- 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 labeled 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.

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

Note: For sample analyses conducted during the post-decontamination/clearance phase of an incident, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1} 10^{-2} and 10^{-3} dilutions) be filtered using a third MicroFunnel™ and plated as described above instead of proceeding with enrichment in TSB.

11.4.7 Enrich in TSB

- Add the remainder of the undiluted, 10^{-1}, 10^{-2} and 10^{-3} suspensions to the wide-mouth screw cap plastic container containing the corresponding vacuum filter/sock. Add 30 mL of TSB (Section 6.19) to the container. Repeat for each sample. Incubate containers at 37ºC ± 1ºC for 24-48 hours.
- Evaluate the TSB Enrichment
  - **a.** If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is turbid, record as positive growth (G+) and proceed to next step.
  - **c.** Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat 2 times for a total of three SBA isolation plates.
  - **d.** Incubate the isolation plates and TSB with growth at 37ºC ± 1ºC for a maximum of 3 days.
  - **e.** Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (Section 11.6).
  - **f.** If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section 11.6).

11.5 Sample Processing and Plating for Water Samples

Note: All subsequent procedures involving manipulation of water samples must be carried out in a BSC using appropriate PPE. Sterile gloves should be used and changed between samples and as indicated below. The CDC requires BSL-3 handling of this organism. All wastes should be handled according to CDC & BMBL waste management and disposal requirements.

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.

11.5.1 Recover Spores from the MicroFunnel™ Membrane (from secondary water concentration, Appendix A, Section 3.0)

- If the membranes are not in 50 mL tubes, aseptically transfer each membrane to a sterile 50 mL tube using sterile forceps. Change gloves and forceps between samples.
- Add 5 mL of sterile PBST (0.05% Tween® 20, Section 6.2) to each tube and vortex on high in 10 second bursts for 2 minutes to dislodge spores.

- Transfer liquid to a sterile, labeled 50 mL tube.
- Place the membrane in a specimen cup and set aside.
- Repeat the steps described above for each membrane.

11.5.2 Serially Dilute Spore Suspensions in PBST

- Vortex the 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 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.

11.5.3 Culture Spore suspensions on SBA

To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

Note: Plating of 0.1 mL 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 (Section 6.17) 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}).
  - **d.** After vortexing tubes, pipet 0.1 mL of 10^{-3} suspension onto surface of pre-dried SBA plate (labeled 10^{-4}).
  - After inoculating the three spread plates for the first suspension (e.g., 10^{-1}), 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 three dilutions 10^{-2} 10^{-3} and 10^{-4}.
- Allow inoculum to absorb into the medium completely.

11.5.4 Incubate and Enumerate Plates

Invert plates and incubate at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* 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 (Figure 6). 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.

- Plate counts

  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 typical colonies are observed, record as "None detected" and proceed to evaluation of growth on MicroFunnel™ plates (11.5.6).

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

11.5.5 Capture Spores on MicroFunnel™ Filter Membranes and Culture

- Place 2, 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 < 10 psi.
- 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 (Section 11.5.1) to each of two MicroFunnel™ cups.
- 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 labeled 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.
- Invert and incubate SBA plates with membranes at 37ºC ± 1ºC for a maximum of three days. Plates should be examined within 18-24 hours after starting the incubation and at 24 hour intervals for a maximum of 72 hours, if necessary. Count the number of *B. anthracis* colonies and record results. Confirm 1-3 colonies using real-time PCR (Section 11.6).

Note: ***For sample analyses conducted during the post-decontamination/clearance phase of an incident, it is recommended that the remainder of all suspensions (e.g., undiluted, 10^{-1}***

10^{-2} and 10^{-3} dilutions) be filtered using a third MicroFunnel™ and plated as described above instead of proceeding with enrichment in TSB.

11.5.6 Enrich in TSB

- Add the remainder of the undiluted, 10^{-1}, 10^{-2} and 10^{-3} suspensions to the specimen cup containing the corresponding membrane filter. Add 30 mL of TSB (Section 6.19) to the specimen cup. Repeat for each sample. Incubate tubes at 37ºC ± 1ºC for 24-48 hours.
- Evaluate the TSB Enrichment
  - **a.** If broth is not turbid, record as no growth (NG) and incubate for an additional 24 hours.
  - **b.** If broth is turbid, record as positive growth (G+) and proceed to next step.
  - **c.** Cap tightly and mix TSB with growth for 30 seconds. Remove a loopful of broth with a 10 µL loop and streak on a SBA plate for isolation. Repeat two times for a total of three SBA isolation plates.
  - **d.** Incubate the isolation plates and TSB with growth at 37ºC ± 1ºC for a maximum of three days.
  - **e.** Examine plates for *B. anthracis* colonies. If any colonies are isolated, proceed to PCR confirmation (Section 11.6).
    - **f.** If no *B. anthracis* colonies are observed, perform PCR on TSB with growth (Section 11.6).

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

11.6.1 DNA Preparation from Cultured Cells

- Cells grown on solid culture medium
  - **a.** Pipet 100 µL of PCR-grade water (Section 6.9) into a 1.5 mL Eppendorf microcentrifuge tube (Section 5.1.21).
  - **b.** 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.

  - **c.** Insert the loop or swab into the tube containing the PCR-grade water and immerse the bacterial growth in the liquid.
  - **d.** 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 in an autoclavable biohazard bag. Proceed to Section 11.6.2.
- Cells grown in liquid culture medium
  - **a.** Transfer 50 µL of broth with growth to a microcentrifuge tube.
  - **b.** Place tube into a refrigerated microcentrifuge and spin at 12,000 × g for 2 minutes.

c. Remove and discard the supernatant in an autoclavable biohazard container. Add 100 µL of PCR-grade water to the tube containing the bacterial pellet.

d. Resuspend the pellet by flicking the tube. Proceed to Section 11.6.2.

11.6.2 Preparation of Lysate

- Cap the microcentrifuge tubes containing the bacterial suspension with cap-holding tabs to prevent the tubes from popping open during heating, and briefly vortex.
- Place the capped tubes in a heat block at 95°C – 98°C.
- Heat the sample for 20 minutes. Heating for 20 minutes will ensure all organisms are killed; this allows the sample to be handled outside of the BSL-3 laboratory.
- Remove the tubes from the water bath or heat block and place them directly in a cold block. Chill the tubes for a minimum of 2 minutes.
- Remove the cap-holding tabs and place the microcentrifuge tubes in the refrigerated microcentrifuge. Centrifuge at 12,000 × g for 2 minutes.

11.6.3 Filtration of Lysate Using 0.1 µm Centrifugal Filter Device (Section 5.1.31)

Centrifugal filtration with 0.1-µm Ultrafree®-MC filter devices following extraction of DNA allows for the removal of *B. anthracis* spores which may contaminate DNA preparations, making the samples safe without compromising the sensitivity of the real-time PCR assay (Reference 15.8).

- Remove top cap from the 0.1 µm Ultrafree®-MC filter device (Section 5.1.31; Millipore® Cat. No. UFC30VV00).
- Hold each filter device vertical with the filter cup opening facing up. Using a micropipettor tip, transfer the supernatant from each microcentrifuge tube into the corresponding 0.1 µm Ultrafree®-MC filter device (Section 5.1.31; Millipore® Cat. No. UFC30VV00). Do not allow the micropipettor tip to touch the filter membrane. Avoid transferring any particulate matter that may be evident at the bottom of the tube. Close the cap. Discard the microcentrifuge tube in an autoclavable biohazard bag.
- Place the Ultrafree®-MC filter devices into a centrifuge (Section 5.5.7; Eppendorf 5415R/5424R) and balance the rotor head.
- Centrifuge at 8000 × g (approximately, 9200 rpm) for 2 minutes at 4ºC (Reference 15.8).

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.***

- Carefully open the caps and remove the Ultrafree®-MC filter inserts (Section 5.1.31; Millipore® Cat. No. UFC30VV00) using disposable forceps (gripping only the sides), close the caps of the collection tubes and dispose of the Ultrafree®-MC inserts in an autoclavable biohazard bag.
- If there is concern regarding the biosafety of the filtered material, it is recommended that the laboratory perform a sterility check on the filtered material according to internal laboratory procedures.

- Wipe the outside of the tubes containing lysates with 10% pH amended beach (Section 6.20) or bleach wipes (Section 5.1.3). Samples lysates are safe to remove from the BSL-3 after filtration and disinfection of the tube.
- Using clean gloves, place the cold block with the tubes containing the lysates in DNA loading station/hood in preparation for PCR analyses (Section 9.3).
- If PCR analysis will not be completed the same day the lysates are prepared, split into aliquots and freeze them at -20°C.

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

11.6.4 Use 5 µL of the lysate as the DNA template to run the PCR analysis in triplicate using the BC3 assay. Optionally, any combination or all three PCR assays can be used. If *B. anthracis* Sterne is used as the PC, the EPA 2 assay should not be used.

Note: DNA obtained from cell lysates should be diluted (e.g., 1:10 or 1:100) prior to testing to avoid excess DNA template which can cause false negative results.

11.6.5 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.

11.6.6 Refer to Sections 12.1 and 12.3 for data analyses and calculations.

12.0 Data Analysis and Calculations

12.1 Real-time PCR Analysis

Calculate the average C_T from the replicate reactions for each sample DNA extract, PC and the EIC, where applicable.

$$\frac{\sum_{j=1}^{N} C_T(j)}{N} = Average\ C_T,$$

$$where\ N\ is\ the\ number\ of replicate\ reactions$$

Example:

$$Where\ 3\ replicate\ reactions\ produce\ C_T\ values\ of\ 20, 21, and\ 19,$$

$$\frac{\sum_{j=1}^{3} C_T(1)+C_T(2)+C_T(3)}{3} = \frac{\sum 20+21+19}{3} = \frac{60}{3} = 20 = Average\ C_T$$

The average $C_T \leq 40$ and the presence of exponential amplification in the real-time graph for the sample DNA extract indicates a positive result, suggesting the presence of *B. anthracis* 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 DNA extract gives $C_T \leq 40$, the PCR analysis of the DNA extract for that sample must be repeated in triplicate. If the repeated PCR analysis gives similar data (zero or one replicate with $C_T \leq 40$), the sample result is considered negative. If two or three PCR replicates for a sample DNA extract give C_T values >

40 but ≤ 41, or the average C_T value > 40 but ≤ 41, the PCR analysis must be repeated in triplicate. If the repeated PCR analysis gives the same data, a careful interpretation of the data should be made in consideration/conjunction with data from relevant samples. Also, a recommendation can be made for additional sample collection and analysis. NTCs should not yield any measurable C_T values above the background level. If C_T values are obtained for NTCs as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis. In addition, field blank samples should not yield any measurable C_T values. If C_T values are obtained for field blank samples as a result of 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.

If the EIC (with 50 pg of the *B. anthracis* DNA) for a sample results in a C_T value (≥ 3) compared to the C_T value for the positive control (also with 50 pg of the *B. anthracis* DNA), there may be matrix inhibition. If the corresponding sample DNA extract is negative (C_T > 40) for *B. anthracis*, the sample DNA extract should be diluted 1:4 and 1:10 and the PCR assay should be repeated in triplicate for that sample along with the EIC with diluted sample DNA extracts.

12.2 RV-PCR Analysis

RV-PCR Analysis Algorithm for a Positive Result:

$\Delta C_T = C_T (T_0) - C_T (T_9 \text{ or } T_f) \geq 9$ (Default algorithm), where, $C_T (T_0)$ and $C_T (T_9 \text{ or } T_f)$, each, represent an average C_T of PCR replicates

$\Delta C_T = C_T (T_0) - C_T (T_9 \text{ or } T_f) \geq 6$ (Optional algorithm depending upon the end user's requirement, sample matrix complexity, and the response phase during an incident)

a. Calculate an average C_T from the triplicate reactions for T_0 and T_9 or T_f DNA extracts of each sample. To calculate the average C_T (T_0), a minimum of two out of three T_0 PCR replicates must result in C_T values ≤ 45 (in a 45-cycle PCR). If two or all three T_0 PCR replicates do not give a C_T value, use 45 (total number of PCR cycles used) as the average C_T (T_0) value to calculate the ΔC_T for the sample. To calculate the average C_T (T_9 or T_f), a minimum of two out of three T_9 or T_f PCR replicates must result in C_T values ≤ 36 (for the $\Delta C_T \geq 9$ algorithm) or ≤ 39 (for the $\Delta C_T \geq 6$ algorithm). If only one out of three T_9 or T_f PCR replicates for any DNA extract gives C_T values ≤ 36 (for the $\Delta C_T \geq 9$ algorithm) or ≤ 39 (for the $\Delta C_T \geq 6$ algorithm), the PCR analysis of (both) the T_0 and T_9 or T_f DNA extracts for that sample must be repeated in triplicate. If the repeated PCR analysis gives the same data, the sample result is considered negative. For the $\Delta C_T \geq 9$ algorithm, if two or three PCR replicates of a T_9 or T_f sample DNA extract give C_T values > 36 but ≤ 37, the PCR analysis of the T_0 and T_9 or T_f DNA extracts of that sample must be repeated in triplicate. Similarly, for the $\Delta C_T \geq 6$ algorithm, if two or three PCR replicates of a T_9 or T_f sample DNA extract give C_T values > 39 but ≤ 40, the PCR analysis of the T_0 and T_9 or T_f DNA extracts of that sample must be repeated in triplicate. If the repeated PCR analysis of the DNA extracts gives the same data, a careful interpretation of the data should be made in consideration/conjunction with data from relevant samples. Also, a recommendation can be made for additional sample collection and analysis.

b. Subtract the average C_T of the T_9 or T_f DNA extract from the average C_T of the T_0 DNA extract. The change (decrease) in the average C_T value from T_0 to T_9 or T_f (ΔC_T) ≥ 9 (for the $\Delta C_T \geq 9$ algorithm) or $\Delta C_T \geq 6$ (for the $\Delta C_T \geq 6$ algorithm) 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 RV-PCR analysis, the appropriate T_f (incubation time) should be indicated. However, the $\Delta C_T \geq 9$ algorithm should still be used for a positive result. Depending upon the end user's requirements, sample matrix complexity, and the phase of response during an incident (i.e., especially for the post-decontamination phase to avoid false-negative results), a $\Delta C_T \geq 6$ (an approximate two log difference in DNA concentration) and a corresponding higher endpoint PCR C_T of ≤ 39, could be set. For the $\Delta C_T \geq 9$ algorithm, if the ΔC_T is < 9 but not < 8, the PCR analysis of the T_0 and T_9 or T_f DNA extracts of that sample must be repeated in triplicate. Similarly, in case of the $\Delta C_T \geq 6$ algorithm use, if the ΔC_T is < 6 but not < 5, the PCR analysis of the T_0 and T_9 or T_f DNA extracts of that sample must be repeated in triplicate. If the repeated PCR analysis of the DNA extracts gives the same data, a careful interpretation of the data should be made in consideration/conjunction with data from relevant samples. Also, a recommendation can be made for additional sample collection and analysis.

c. NTCs should not yield any measurable C_T values above the background level. If C_T values are obtained for NTCs as a result of a possible contamination or cross-contamination, prepare fresh PCR Master Mix and repeat the analysis. In addition, field blank samples should not yield any measurable C_T values. If C_T values are obtained for field blank samples as a result of 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 Analysis

12.3.1 Serial Dilution Plating

Count the number of typical colonies (Figure 6) on replicate culture plates and calculate the average number of colonies per plate. Apply the following guidelines (a − e, below) when counting the colonies and report results based on the number of characteristic *B. anthracis* 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. Depending on the situation, results should be qualified if QC plates are contaminated with *B. anthracis.*

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

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

c. Ideally, plates with 25−250 colonies should be used to calculate the number of spores per sample as described below.

d. If there are no plates with 25−250 colonies, chose the plates with the counts closest to the acceptable range of 25−250 colonies. For example, if all plate counts are greater than 250 chose the plates that have counts closest to 250. Likewise, if all of the plate counts are less than 25, the plates with counts closet to 25 would be used to calculate the number of spores per sample.

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

To determine the number of spores per sample, add the number of colonies from the plates with 25−250 colonies/plate and divide by the total volume plated. In this case assume the colony counts were 210, 193, and 200 for the 10^{-1} dilution and 25, 19, and 26 for the 10^{-2}. Divide the total number of colonies on plates with 25-250 colonies by the total volume plated to obtain the number of colonies in 1 mL, and then multiply by the total suspension volume, as in the following equation:

Example:

$$\left[\left(\frac{210 + 193 + 200}{0.1 + 0.1 + 0.1}\right)\right] = 2{,}010 \text{ CFU per mL}$$

$$\left[\left(\frac{25 + 26}{0.01 + 0.01}\right)\right] = 2{,}550 \text{ CFU per mL}$$

$$\left[\left(\frac{2010 + 2550}{2}\right)\right] = 2{,}280 \text{ CFU } x \text{ 5 mL} = 11{,}400 \text{ CFU per sample}$$

12.3.2 MicroFunnel™ Filter Plating

Count the number of typical colonies (Figure 6) on each filter and record. Apply the following (a − e) when counting the colonies and report results based on the number of characteristic *B. anthracis* 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. Depending on the situation, results should be qualified if QC plates are contaminated with *B. anthracis.*

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

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

c. Ideally, plates with 20−80 colonies should be used to calculate the number of colonies/CFUs per sample, as described below.

d. If there are no plates with 20−80 colonies, choose the plates with the counts closest to the acceptable range of 20−80 colonies. For example, if all plate counts are greater than 80 choose the plates that have counts closest to 80. Likewise, if all of the plate counts are less than 20, the plates with counts closet to 20 would be used to calculate the number of spores per sample.

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

To determine the number of spores per sample, average the number of colonies on the duplicate filters which had 20−80 colonies/plate. In this case, assume the colony counts were 57 colonies/filter and 63 colonies/filter on the 2 respective filters. Since each filter received 1.0 mL of the suspension, then the average colony count for the filters would then be 60 colonies/mL. Multiply by the average colony count per mL by the total suspension volume per sample, as in the following equation:

Example:

$$\left[\left(\frac{57+63}{2}\right)\right] = 60 \text{ CFU per mL}$$

$$[60\ x\ 5] = 300 \text{ CFU per sample}$$

12.3.3 Enrichment in TSB

Evaluate post-enrichment streaked SBA plates for the presence of *B. anthracis* colonies (Figure 6). If no suspect colonies are observed, broth should be evaluated for the presence of *B. anthracis*. Typical isolates or TSB with growth must be confirmed using real-time PCR prior to reporting results. Since the sample was enriched, only qualitative (presence/absence) results can be reported.

Media sterility checks should not exhibit growth. Growth should also not be present in TSB 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 the potential source of contamination. Depending on the situation, results should be qualified if QC plates are contaminated with *B. anthracis.*

12.3.4 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 the BC3 real-time PCR assay (or any combination or all three PCR assays). A $C_T \leq 40$ and the presence of exponential amplification in the real time graph 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. Please refer to 12.1 for detailed data analysis and calculations, as appropriate.

13.0 Method Performance

To be completed upon protocol verification and/or validation.

14.0 Pollution Prevention

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

Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be discarded. If there is any possibility of the materials having been contaminated, they must be disposed of according to CDC BSL-3 procedure (in an autoclavable biohazard container).

14.0 Waste Management

14.1 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.

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

14.3 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).

14.4 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 15.10) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 15.11), both authored by the American Chemical Society.

15.0 References

15.1 Létant, S., Murphy, G. Alfaro T., Avila, J. Kane, S. Raber, E. Bunt, T. and Shah, S. 2011. "Rapid Viability Polymerase Chain Reaction for detection of Virulent *Bacillus anthracis* from Environmental Samples." *Applied and Environmental Microbiology*, 77: 6570-6578, 2011. http://aem.asm.org/content/77/18/6570.long [Accessed March 3, 2017]

15.2 American Society for Microbiology. 2016. Sentinel Level Clinical Laboratory Guidelines for Suspected Agents of Bioterrorism and Emerging Infectious Diseases – *Bacillus anthracis*. Washington, DC. https://www.asm.org/images/PSAB/LRN/Anthrax316.pdf [Accessed March 3, 2017]

15.3 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

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

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

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

15.7 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.

15.8 Dauphin, L. A., and Bowen, M. D. 2009. "A simple method for the rapid removal of *Bacillus anthracis* spores from DNA preparations." *Journal of Microbiological Methods*, 76: 212–214.

15.9 Kane, S., Shah, S., Létant, S., Murphy, G., Alfaro, T., Avila, J., Salazar, E., Mullins, M., and Nichols, T. 2013. "Operational Evaluation of the Rapid Viability PCR Method for Post-

Decontamination Clearance Sampling." *Journal of Bioterrorism & Biodefense* S3: 016. doi:10.4172/2157-2526.S3-016

[Accessed March 3, 2017]

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

15.11 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://goo.gl/2yIHPr

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

Large volume water samples (10 L-100 L) may need to be concentrated using ultrafiltration (UF) to detect low levels of biothreat agents prior to sample analysis. UF is an integral component of the sample processing procedures provided in Sections 9 and 10 of the protocol and should not be misconstrued as sample collection.

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 25S Dialyzers (Dial Medical Supply Cat. No. 25S 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, 47 mm mixed ester cellulose (VWR Cat. No. 28148-926 or equivalent)

1.1.11 Forceps, sterile, disposable (Cole Parmer® 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 inch (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, 1L 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 1L 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 (DI) 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.

The ultrafilter comes filled with sterile water that must be removed prior to use. Prepare the ultrafilter by removing all caps from top, bottom and side ports. Hold the ultrafilter upright to allow all sterile water to settle on one end. The sterile water can be poured out the side ports into a sink until all visible water is removed.

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"-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"- 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, #8).

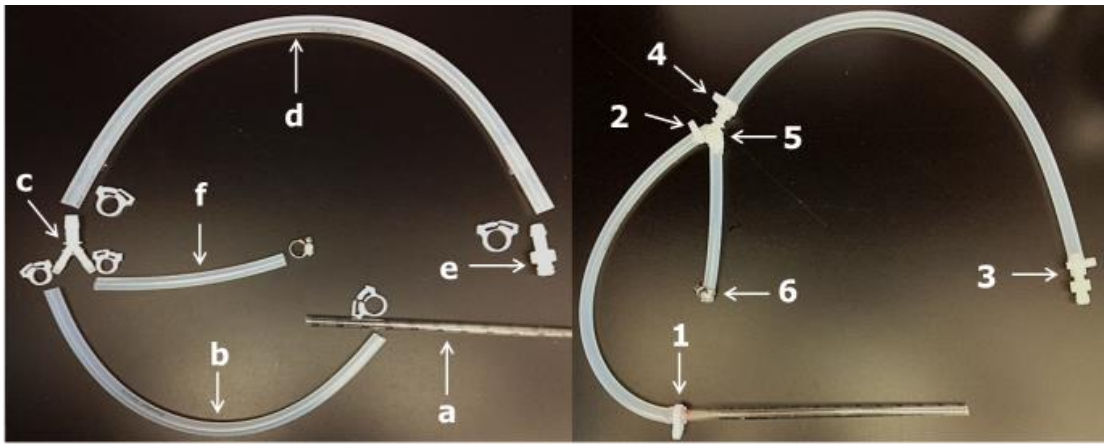


Figure 1. Sample tubing set assembly.

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"-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).

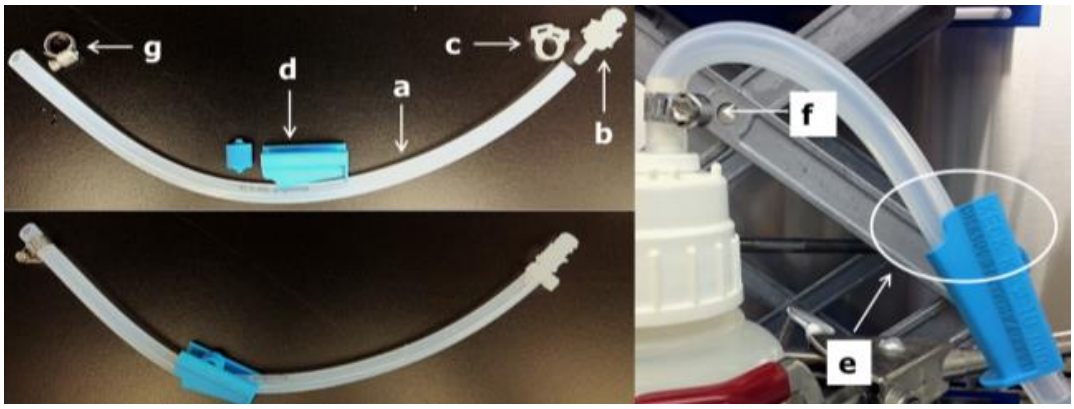


Figure 2. Retentate return tubing set assembly.

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).

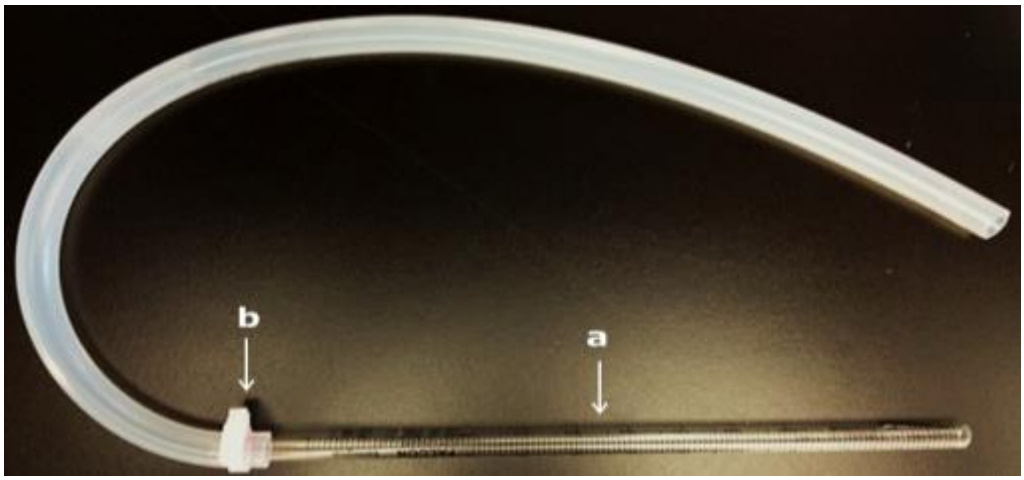


Figure 3. Filtrate tubing set assembly.

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).

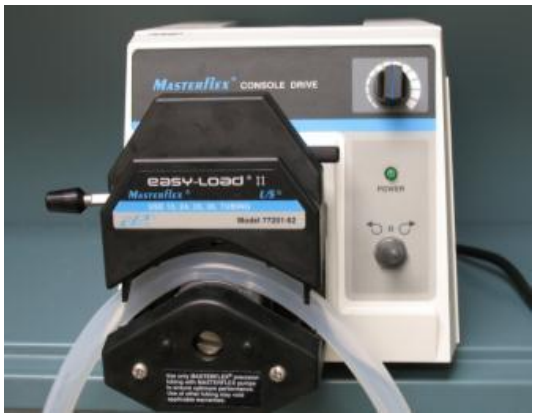


Figure 4. Photo of the pump head.

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 1.3.4 (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.

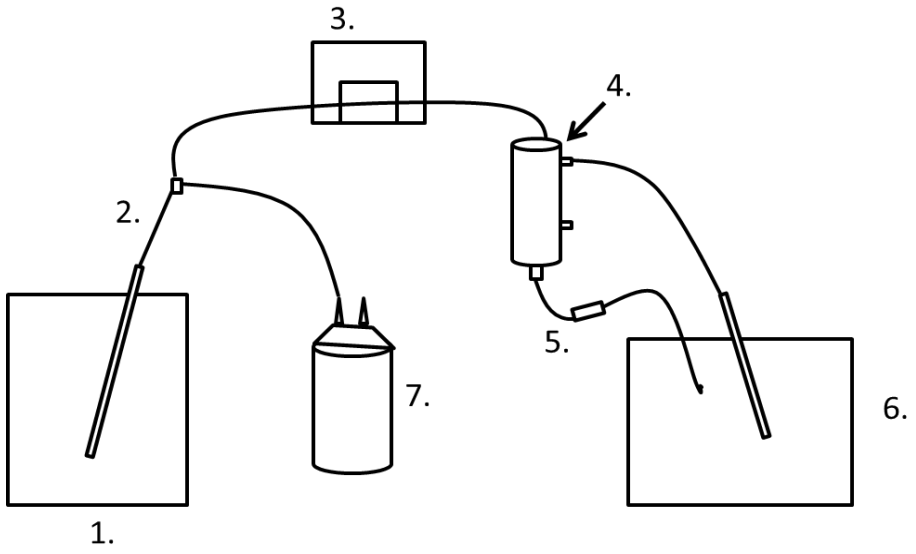


Figure 5. Schematic of filter wash

(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).

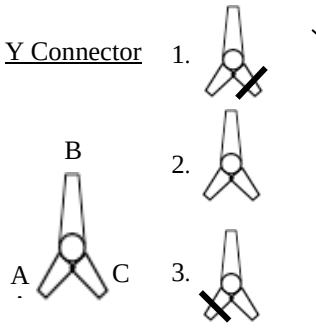
2.2 Start the pump with the flow switch turned to the right and the pump speed set to the maximum (~ 2900 mL/minute flow rate).

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.

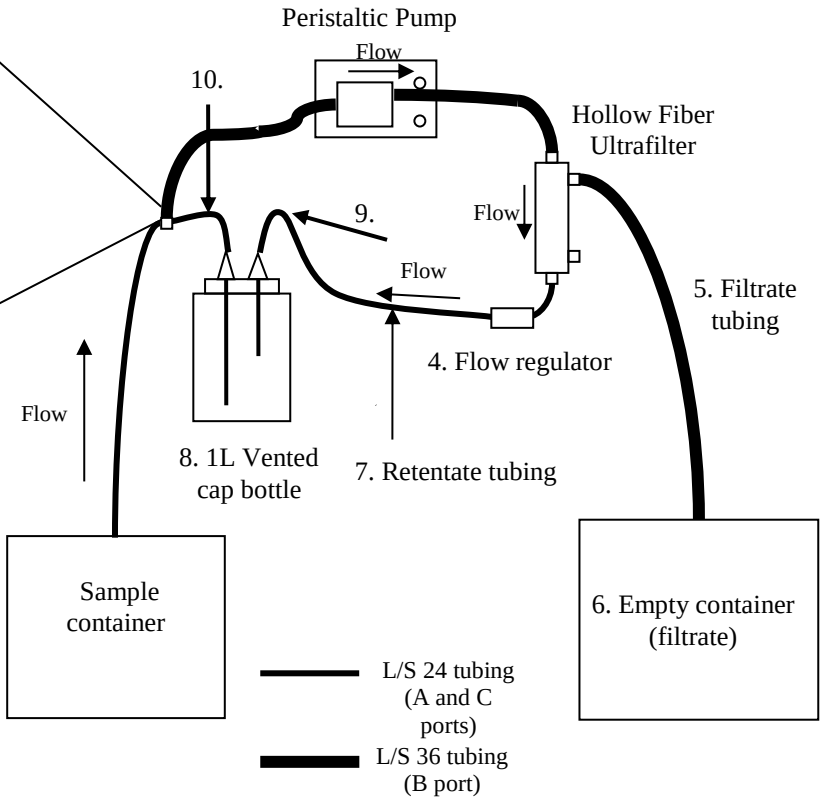


Figure 7a. Recirculating UF assembly.

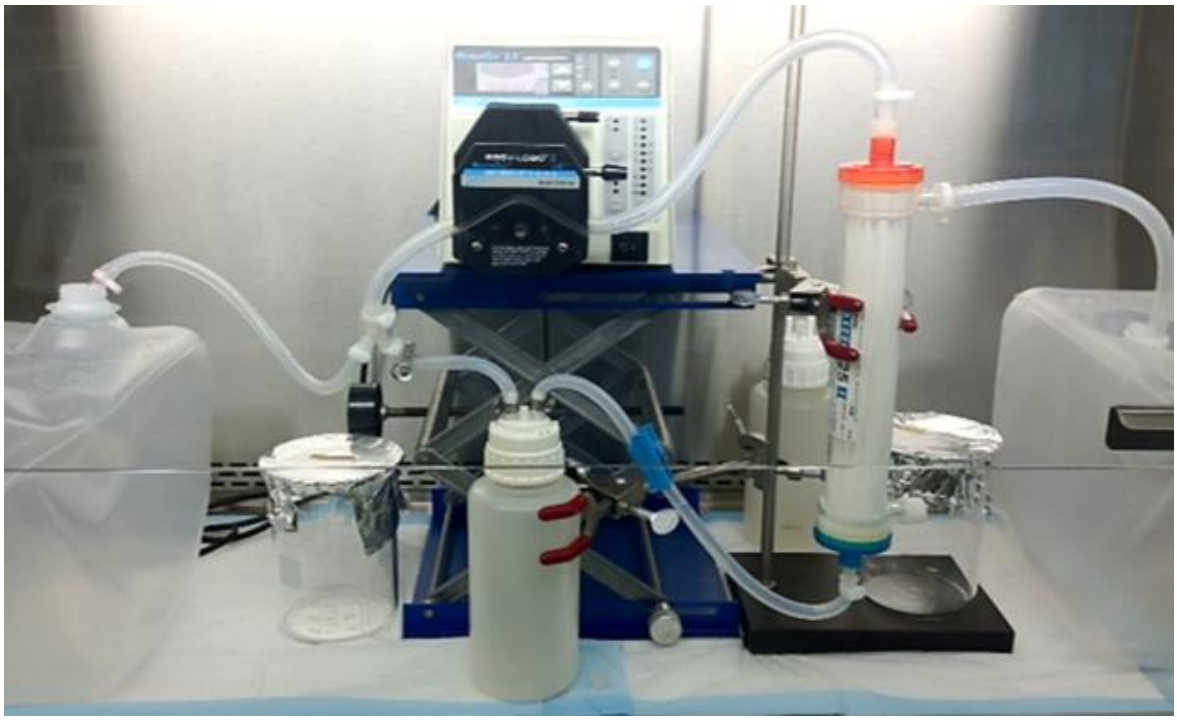


Figure 7b. Photograph of the UF system set up inside a BSC.

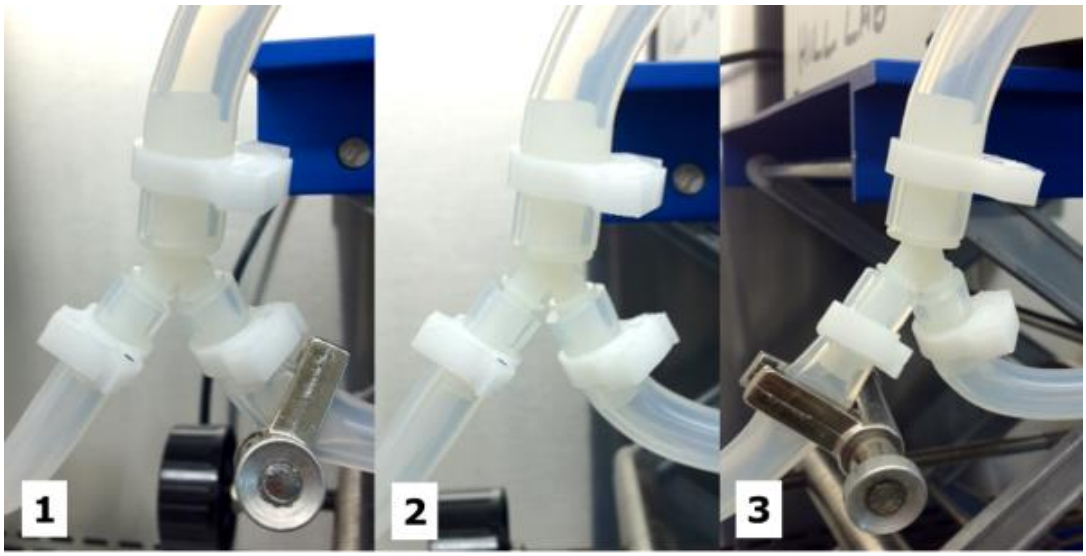


Figure 7c. Pinchcock positions.

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 1.3.5].

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 through 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 8, 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 8, #5) and use sterile forceps to place new filters onto the base of each filter unit (Figure 8, #3).

Note: ***Disposable filter units (Section 1.1.8) come with cellulose nitrate membrane filters (Section 1.1.10), 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.***

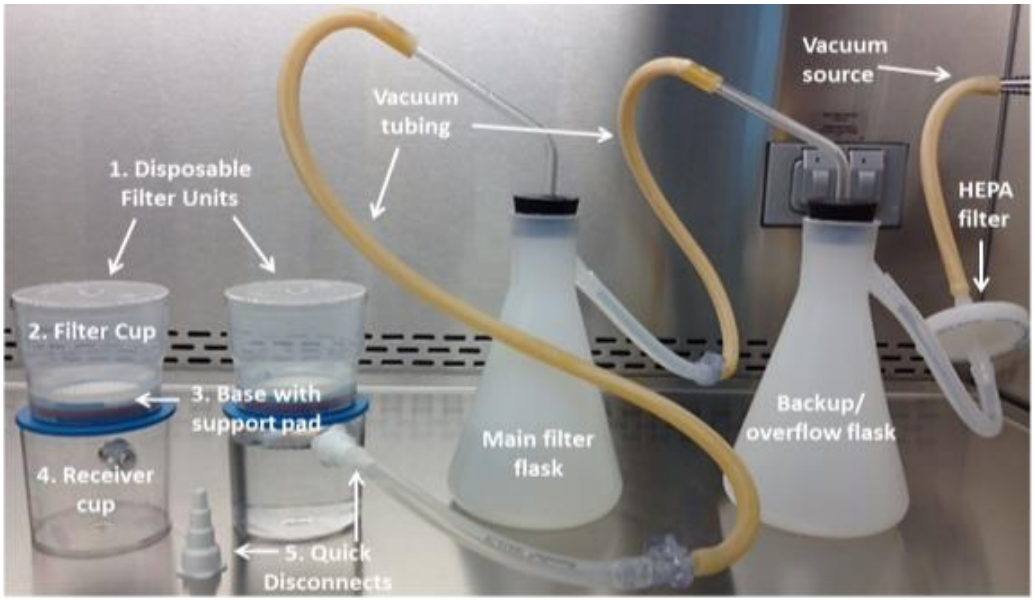


Figure 8. Membrane filtration setup.

3.2 Run a negative control before filtering any sample concentrate. Add 20 mL PBS to the filter cup (Figure 8, #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 8, #3). Retrieve the membrane filter with sterile forceps and place onto a SBA plate. Incubate at 37ºC ± 1ºC for a maximum of 3 days.

Note: If it is anticipated that there is a high concentration of B. anthracis and other background organisms in the sample, it may be necessary to filter aliquots of the retentate. In this case, split the retentate into aliquots (2.0, 20, and 2 × 200 mL) and filter each aliquot as described below. A single filter unit may be used to filter all of the aliquots from the same sample as long as the aliquots are filtered in the order of smallest to largest volume (e.g., 2.0 mL → 20 mL → 200 mL).

3.3 Use sterile forceps to place a new 0.45 µm filter onto the support pad on the base of either a new filter unit or the one used for the filtration blank. Reattach the filter cup to the base.

3.4 Add the retentate slowly to the filter unit (Figure 8, #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 8, #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 through 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 10.2.5 of the protocol.

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

4.0 Decontamination and Disposal and/or Reuse

4.1 Filtrates should be collected in a container containing sufficient bleach such that the final concentration is at least 1% (e.g., 200 mL bleach in a 20 L container). After 30-minute contact time, the treated filtrate can be poured down the drain.

4.2 Ultrafiltration Supplies

4.2.1 Disposable sample containers (e.g., Cubitainers™) can be flattened by slowly compressing the container while inside BSC. Secure the cap, decontaminate the surface and slowly remove from the BSC and place in biohazard waste container. Reusable containers should be capped, surface-decontaminated and removed from BSC for autoclaving.

4.2.2 Prior to disconnecting the ultrafiltration assembly, flush 500 mL of 1% bleach solution through the entire assembly so that all tubing is in contact with the bleach.

4.2.2.1 Place sample pipet in 500 mL of 1% bleach solution and adjust pump speed to 50% (~ 1450 mL/min).

4.2.2.2 Ensure rubber stopper is off the vented cap, tubing from port C is clamped with pinchcock (Fig. 7c, #1) and turn on pump.

4.2.2.3 Pump the bleach until approximately 100 mL remains in the 500 mL bleach bottle (tubing should be filled with solution, 1L bottle should be ~1/2 - 2/3 full).

4.2.2.4 Move pinchcock to clamp the tubing from port A (Fig. 7c, #3) and turn off the pump.

4.2.2.5 Ensure a minimum contact time of 30 minutes. Once this is completed, flush all of the bleach solution out of the system so that it is collected in the 1L vented cap bottle, then place the tubing assembly except the pinchcock and stainless steel clamps and ultrafilter, into a biohazard waste container.

4.2.3 The vented cap bottle assemblies (including inner tubing and rubber caps), 1 L bottles and 1 L beakers should be autoclaved for a minimum of 30 minutes at 121°C /15 psi. After sterilization, bottles should be washed and re-autoclaved prior to reuse.

4.2.4 Wipe down the pinchcock with 10% bleach followed by 70% ethanol before removing from BSC.

4.2.5 Soak steel clamps in a beaker of 0.1N NaOH solution for 15 minutes, followed by soaking in 10% bleach (made fresh) for 15 minutes and then rinse thoroughly with DI water prior to reuse.

4.3 Secondary Concentration

4.3.1 Filter units and Centricons should be autoclaved and disposed of properly.

4.3.2 Filter flasks/stopper set-ups for membrane filtration should be autoclaved and reused.

4.4 Surfaces and/or spills should be decontaminated by the following three-step process:

- Wet and wipe down with 0.1 N NaOH, let sit for 15 minutes
- Wet and wipe down with 10% bleach, let sit for 15 minutes
- Wipe with 70% ethanol

Note: It is recommended to have these solutions readily available in squeeze bottles during processing for immediate surface decontamination.

5.0 Limitations

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

5.2 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 bioterrorism threat (BT) agents.

5.3 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.

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

6.0 Acronyms

BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSC	Biological safety cabinet
BSL	Biological safety level
BT	Bioterrorism threat
DI	Deionized
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

7.0 Reference

7.1 U.S. Environmental Protection Agency and U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. 2011. Comparison of Ultrafiltration Techniques for Recovering Biothreat Agents in Water. EPA 600/R-11/103 http://cfpub.epa.gov/si/si_public_record_report.cfm?address=nhsrc/&dirEntryId=238310

This page is intentionally left blank

This page is intentionally left blank

SCIENCE

PRESORTED STANDARD
POSTAGE & FEES PAID
EPA
PERMIT NO. G-35

Office of Research and Development
Homeland Security Research Program

Cincinnati, OH 45268