EPA/600/R-17/028 | February 2017
www.epa.gov/homeland-security-research

United States Environmental Protection Agency

USGS science for a changing world

Processing Protocol for Soil Samples Potentially Contaminated With *Bacillus anthracis* Spores

Office of Research and Development
Homeland Security Research Program

This page left intentionally blank

EPA/600/R-17/028
February 2017

Processing Protocol for Soil Samples Potentially Contaminated *With Bacillus anthracis* Spores

U.S. Environmental Protection Agency
Office of Research and Development
Cincinnati, Ohio 45268

U.S. Geological Survey
St. Petersburg, FL 33701

This page left intentionally blank

Disclaimer

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, collaborated on the project described herein under an Interagency Agreement with the U.S. Geological Survey (USGS; IA #DW 14957748 and DW 92401101) and through Pegasus Technical Services, Inc., a contractor to the EPA (Contract # EP-C-11-006). This protocol has been subjected to the Agency's review and has been approved for publication. The contents of this protocol reflect the views of the contributors and do not necessarily reflect the views of the Agency. This report has been peer reviewed and approved for publication consistent with USGS Fundamental Science Practices (http://pubs.usgs.gov/circ/1367/). Mention of trade names or commercial products in this protocol or in the literature referenced in the protocol do not constitute endorsement or recommendation for use by the U.S. Government.

It should be noted that at the time of publication, this protocol has not been validated. The processing protocol has been verified at EPA and USGS. The protocol will be updated or replaced with a validated protocol 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 (FEM-2010-01).

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

Erin Silvestri, MPH
U.S. Environmental Protection Agency
National Homeland Security Research Center
26 W. Martin Luther King Drive, MS NG16
Cincinnati, OH 45268
513-569-7619
Silvestri.Erin@epa.gov

Dale W. Griffin, Ph.D.
United States Geological Survey
600 4th Street South
St. Petersburg, FL 33701
727-502-8075
dgriffin@usgs.gov

Acknowledgements

The following individuals and organizations are acknowledged for their contributions to this protocol:

U.S. Environmental Protection Agency
Office of Research and Development
National Homeland Security Research Center
Erin E. Silvestri
Frank W. Schaefer III
Sanjiv Shah
Eugene W. Rice
Vincent Gallardo
Joseph Wood
Tonya Nichols

U.S. Geological Survey
Toxic Substances Hydrology Program
Dale Griffin
John Lisle

Oak Ridge Institute for Science and Education
Douglas Hamilton

Pegasus, Contractor for the U.S. Environmental Protection Agency
David Feldhake
Brian Morris
Adin Pemberton

The following individuals are recognized for their technical input during the initial development of the protocol:

Centers for Disease Control and Prevention
Cari Beesley
William Bower
Alex Hoffmaster
Chung Marston
Stephen Morse
Laura Rose
Sean Shadomy
Angela Weber

Executive Summary

This protocol describes the processing steps for 45g and 9 g soil samples potentially contaminated with *Bacillus anthracis* spores. The protocol is designed to separate and concentrate the spores from bulk soil down to a pellet that can be used for further analysis. Soil extraction solution and mechanical shaking are used to disrupt soil particle aggregates and to aid in the separation of spores from soil particles. Soil samples are washed twice with soil extraction solution to maximize recovery. Differential centrifugation is used to separate spores from the majority of the soil material. The 45 g protocol has been demonstrated by two laboratories using both loamy and sandy soil types. There were no significant differences overall between the two laboratories for either soil type, suggesting that the processing protocol would be robust enough to use at multiple laboratories while achieving comparable recoveries. The 45 g protocol has demonstrated a matrix limit of detection at 14 spores/gram of soil for loamy and sandy soils.

Table of Contents

List of Figures

List of Acronyms

ACS	American Chemical Society
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BSL-3	Biological Safety Level 3
BSL-2	Biological Safety Level 2 Laboratory
BSC	Biological Safety Cabinet
CDC	Centers for Disease Control and Prevention
CFR	Code of Federal Regulations
EPA	United States Environmental Protection Agency
FSAP	Federal Select Agent Program
g	gravity force
HASP	Health and safety plan
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OSHA	Occupational Safety and Health Administration
PPE	Personal protective equipment
qPCR	Quantitative polymerase chain reaction
RV-PCR	Rapid viability-polymerase chain reaction
SES	Spore Extraction Solution
SHMP	Sodium hexametaphosphate
USGS	U.S. Geological Survey

1.0 Scope and Application

The purpose of this protocol is to recover and concentrate *Bacillus anthracis* (*B. anthracis*) spores from bulk environmental soil samples using a spore extraction solution (SES), mechanical shaking and differential centrifugation. The protocol can be used with 9g or 45g soil samples characterized as loam or sandy soil as defined by the U.S. Department of Agriculture (Reference 13.1). This protocol only provides the steps for processing soil samples; an assay for detection or quantitation of *B. anthracis* spores such as rapid viability-polymerase chain reaction (RV-PCR) (Reference 13.2) or DNA extraction via a kit coupled to quantitative PCR (qPCR; Reference 13.3) is expected to be used in concert with the soil processing protocol.

It should be noted that at the time of publication, this protocol has not been validated. The processing protocol has been verified at EPA and USGS. The protocol will be updated or replaced with a validated protocol 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 (FEM-2010-01) (Reference 13.4).

According to Agency Policy Directive FEM-2010-01 (Reference 13.4)*:*

> 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* (Reference 13.5*).* The responsible federal agency may also refer to the *Validation Guidelines for* Laboratories Performing Forensic Analysis of Chemical Terrorism (Reference 13.6) 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.

2.0 Summary of Protocol

2.1 This protocol assumes soil samples to be analyzed have been collected and shipped to the laboratory under ambient conditions according to U.S. Environmental Protection Agency (EPA)/U.S. Geological Survey (USGS) sample collection protocol (Reference 13.7) and stored at 4°C until analysis can be conducted. Samples should ideally be analyzed within 5 days of receipt.

2.2 The initial protocol steps include the use of SES along with mechanical shaking. This is designed to disrupt soil particle aggregates and to aid in the separation of spores from soil particles. Soil samples are washed twice with SES in order to maximize *B. anthracis* spore recovery.

2.3 Differential centrifugation is used to separate spores from the majority of soil material. A low speed centrifugation (100 × *g*) sedimentates heavier and denser soil material. Supernatant is removed via a pipette and centrifuged again at a higher speed (5,900 *x g*) to sedimentate spores along with soil particles of similar densities. After the final centrifugation, the procedure is completed and samples can be stored at 4°C overnight. Although culturing (using spread plates) was used for detection or quantitation during the development of this protocol, it is anticipated that spore detection assays such as qPCR or RV-PCR will be used with environmental samples. This is due to the expected high concentration of "background" organisms in soil that could overwhelm the spread plate if used.

2.4 This protocol includes steps for 45 g soil samples and 9 g soil samples. The 45 g protocol was demonstrated at the EPA and USGS using sterile loam and sandy soil (see Reference 13.8 and Section 10.0). While the 9 g was demonstrated during development, a laboratory comparison was not completed.

3.0 Interferences and Contamination

3.1 The presence of a significant quantity of clay particles reduces the effectiveness of this protocol since clay particles co-sedimentate with spores during centrifugation, resulting in more soil material in the final pellet and therefore a lower concentration of spores per unit volume.

3.2 Low recoveries of *B. anthracis* spores can result from problems during soil processing. For example, the volume of SES and soil in a centrifuge bottle (250 or 50 ml tubes dependent on weight of soil screened) or tube cannot exceed 75% of the total bottle volume in order to optimize the effectiveness of mechanical mixing. Soil samples with high water content may necessitate adjusting

the volume of SES added to insure that there is 25% headspace available during the mixing procedure.

3.3 Due to variability in soil chemistry, the SES solution may not be equally effective in disrupting soil particle aggregates in all soil types.

4.0 Safety

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

4.1 Laboratory Hazards

Direct contact of skin or mucous membranes with infectious materials, accidental parenteral inoculation, ingestion, and exposure to aerosols and infectious droplets has resulted in *B. anthracis* infection. Due to the infectious nature of this organism, all samples should be handled using biosafety requirements as dictated by *Biosafety in Microbiological and Biomedical Laboratories* [BMBL], 5th Edition, CDC [Centers for Disease Control and Prevention] 2009 (Reference 13.9) and/or organizational health and safety plans.

4.2 Recommended Precautions

Samples collected as part of a survey with no expectation of high levels of *B. anthracis* spores can be processed using Biological Safety Level 2 (BSL-2) practices as described in BMBL, 5th Edition, CDC 2009 (Reference 13.9). However, all manipulations should occur within a certified Biological Safety Cabinet (BSC) and exterior surfaces of objects such as centrifuge tubes should be decontaminated with a bleach disinfection wipe following the manufacturer's or the laboratory health and safety plan (HASP) instructions, or other suitable reagent, followed by application of 70% ethanol to remove the bleach, prior to removal from the BSC for mixing, centrifugation, or refrigeration.

4.2.1 A Biological Safety Level 3 (BSL-3) laboratory is required for handling and manipulating samples and cultures presumptive for *B. anthracis*. *B. anthracis* analyses should be conducted using BSL-3 practices, containment and facilities (BMBL, 5th Edition, CDC 2009. [Reference 13.9]). Additional biosafety and select agent information, as well as statutory requirements for possession, use and transfer of select agents, can be found in the Code of Federal Regulations (42 CFR part 73) (Reference 13.10).

4.2.2 BSL-3 Practices

The laboratory supervisor must ensure that laboratory personnel demonstrate proficiency in standard and special microbiological practices before working with

BSL-3 agents. All procedures involving manipulation of infectious material must be conducted within a BSC (preferably Class II or Class III) or other physical containment device. Protective clothing (e.g., laboratory coats, gloves and respirator) should be worn while processing and analyzing samples. Personal protective equipment (PPE) should never be worn outside the laboratory.

4.2.3 Disposable materials are recommended for sample manipulations.

4.2.4 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.

4.2.5 Personal Protective Equipment (PPE)

- Laboratory personnel processing and conducting analyses of samples for *B. anthracis* place themselves at risk for exposure. However, the use of appropriate PPE can reduce the exposure risk. Laboratory personnel should familiarize themselves with the specific guidance for levels of protection and protective gear developed by the U.S. Department of Labor, Occupational Safety and Health Administration (OSHA) (Reference 13.11). In addition to OSHA guidance, the Centers for Disease Control and Prevention (CDC) has developed recommendations for PPE based on BSL (Reference 13.9). Laboratory personnel should also refer to the laboratory's own approved health and safety plan.
- Goggles or a face shield should be used for protection against splashes, spills and sprays. If a BSC is used due to space limitations during processing which requires a BSL-3, approved respiratory equipment should be used. Protective coats, gowns, smocks or uniforms designated for laboratory use must be worn while working with samples and potentially contaminated materials. It may also be necessary to use a powered air purifying respirator to reduce the risk of inhalation. After use, protective clothing should be placed in sealed bags for appropriate decontamination, disposal or laundering. Disposable gloves should be worn to protect hands from contact with potentially contaminated samples. Wearing two pairs of gloves may be appropriate, but should not compromise needed dexterity.

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

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

4.2.7 This protocol does not address all safety issues associated with its use. Please refer to BMBL, 5th Edition, CDC 2009. (Reference 13.9) for additional safety

information. A reference file of Safety Data Sheets should be available to all personnel involved in analyses.

5.0 Supplies and Equipment

5.1 General Laboratory Supplies

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

5.1.2 Goggles, a face shield, or appropriate approved respiratory protection depending on BSL (Laboratory personnel should also refer to their approved Health and Safety Plan)

5.1.3 Disposable lab gown, (Kappler PreVent 10,000 #MN101, Kappler Corporation, Guntersville, AL; or equivalent)

5.1.4 Autoclavable biohazard bags

5.1.5 Sodium hypochlorite bleach disinfection wipes (Fisher Scientific 9-898-971, ThermoFisher Scientific, Waltham, MA; or equivalent)

5.1.6 Absorbent pad (Fisher Scientific 19-140-911, or equivalent)

5.1.7 Polypropylene conical centrifuge 50 mL conical tubes (Fisher Scientific 05-526-B, or equivalent) as well as Styrofoam™ pack material (or equivalent)

5.1.8 Polypropylene wire racks for 50 mL conical tubes (Fisher Scientific #14-809-119, or equivalent)

5.1.9 Polypropylene 250 mL centrifuge bottles with sealing caps (Fisher Scientific No. 05-562-23, or equivalent)

5.1.10 Sterile 10 mL polystyrene serological pipets

5.1.11 Sterile 25 mL polystyrene serological pipets

5.1.12 Sterile 50 mL polystyrene serological pipets

5.1.13 Sterile, wide bore genomic pipet tips 200 µL (ThermoFisher Scientific No. #2069G, or equivalent)

5.1.14 Sterile disposable bacterial transfer loops, 1 to 10 µL (Fisher Scientific No. 22-363-602, or equivalent)

5.1.15 Sealable biological material transport carrier (Fisher Scientific #15-251-2, or equivalent)

5.1.16 Disposable polystyrene bottle top filters, I L capacity, with cellulose acetate 0.22 µm pore size filters and 45 mm neck size, (Fisher Scientific 09-761-38 or equivalent)

5.1.17 Pyrex® bottles with 45mm neck size; screw cap lid (250 mL, 500 mL, 1 L)

5.1.18 1 L graduated cylinder

5.1.19 Pyrex 1 L or 500 mL bottles or equivalent for liquid waste collection

5.1.20 Magnetic stir bar

5.1.21 Plastic weight boats

5.1.22 Pyrex 1L flask

5.2 Laboratory Equipment

5.2.1 Biological Safety Cabinet (BSC) – Class II or Class III

5.2.2 High speed centrifuge or equivalent and appropriate swinging bucket rotors for 250 mL centrifuge bottles (Thermo Scientific Sorvall® Evolution™ RC, Waltham, MA; or equivalent)

5.2.3 Platform shaker (New Brunswick™ Innova® 2100 Benchtop Platform Shaker, Eppendorf, Hauppauge, New York; or equivalent), and appropriate carriers for 250 mL centrifuge bottles (New Brunswick Utility Carrier for Platform Shaker, and New Brunswick Scientific Rod Kit for Utility Carrier; or equivalent)

5.2.4 Manual pipette filling device (Drummond Scientific Pipet-Aid, Multi-speed XP, Broomall, PA; or equivalent)

5.2.5 Vortex mixer

5.2.6 Digital top-loading balance accurate to +/- 0.01 g

5.2.7 Stir/hot plate

5.2.8 Vacuum source (house or pump)

5.2.9 pH meter

6.0 Reagents

6.1 Reagent-grade chemicals must be used in all solutions. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (ACS; Reference 13.12). 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 13.13); and *United States Pharmacopeia and National Formulary 24*, United States Pharmacopeial Convention, MD. (Reference 13.14).

6.1.1 Ultra-filtered deionized water or equivalent

6.1.2 Sodium hexametaphosphate (Sigma Aldrich 305553, St. Louis, MO, or equivalent)

6.1.3 Tween® 80 (Sigma Aldrich P1754 or equivalent)

6.1.4 Sodium hydroxide (NaOH), (Fisher S318-1 or equivalent)

6.1.5 3 N Hydrochloric acid (HCl), (Fisher RBSH165 or equivalent)

6.1.6 pH standards; pH 4, pH 7, pH 10

6.1.7 70% ethanol

6.2 Spore Extraction Solution (SES)

The solution is prepared from stock solutions of 20% Tween 80 and 20% sodium hexametaphosphate (SHMP) to a final concentration of 1% Tween 80 and 2% SHMP, and the pH is adjusted with NaOH or HCl to pH 7-7.2. Fifty mL of the 20% Tween 80 stock and 100 mL of the 20% SHMP stock are added to 350 mL Milli-Q or Super-Q water, pH is adjusted and the volume is adjusted to 1.0 L. The solution is then filter sterilized using a 1 L capacity disposable polystyrene bottle top filter with cellulose acetate 0.22 µm pore size filters and 45 mm neck size and a vacuum source. The solution is stored at 4◦C. A manual pipette filling device can be used when transferring SES and emptying bottles. The SES should be made fresh for each use.

6.2.1 Preparation of 1.0 L 20% Tween 80 (w/v): Two hundred g of Tween 80 is weighed out in weight boats and dissolved in approximately 700 ml Milli-Q or Super-Q water in a graduated cylinder or Pyrex bottle, with stirring and gentle heat using a stir bar and hot plate. Once the Tween 80 is completely mixed (approximately one hour), adjust the volume to 1.0 L and store at room temperature. This solution is stable for at least three months.

6.2.2 Preparation of 1.0 L 20% SHMP (w/v): Prepare 40 mL of 2 N NaOH by weighing out 3.2g NaOH in plastic weigh boats and dissolving in 40 mL Milli-Q or Super-

Q water in a flask. Weigh out 200g SHMP in plastic weight boats and slowly add to approximately 800 mL Milli-Q or Super-Q water in a Pyrex flask container. After about 25% of the SHMP is in solution, add the 40 mL of 2 N NaOH followed by the remaining SHMP. Using a pH meter, check to see that the pH should be between 7.1 and 7.4. Add NaOH or HCL 10 mL disposable pipette dropwise if the pH is outside this range. This solution can be stored at room temperature in a sterile 1 L Pyrex bottle for one month.

7.0 Calibration and Standardization

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

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

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

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

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

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

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

8.0 Quality Control and Quality Assurance

8.1 Each laboratory that uses this protocol should consider use of a formal quality assurance program that addresses and documents the maintenance and performance of instrument and equipment, quality and performance of reagents, training and certification of analysts, and storage and retrieval of records. 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 quality assurance program.

8.2 Sample integrity — Samples should be checked for integrity (e.g., improperly packaged, temperature exceedance [samples should be stored at 4°C if not analyzed upon receipt], leaking). Samples may be rejected if the integrity has been compromised. Alternately, if sample integrity has been compromised it may be analyzed and the data qualified and marked accordingly (e.g., sample exceeded temperature during transport - data is flagged and marked as exceeding temperature), so that a decision can made regarding whether the data should be considered valid/invalid.

8.3 Reagent sterility check — The laboratory should test SES sterility by incubating three trypticase soy agar plates spread with 100 µL of SES and incubated at 37ºC ± 2ºC for 24 ± 2 hours and observe for growth. Absence of growth indicates SES sterility. On an ongoing basis, the laboratory should perform a reagent sterility check for each liter of reagent prepared.

9.0 Soil Processing Protocol

The soil processing procedure is outlined in Figure 1. Soils are not pre-processed prior to use of this protocol, however, environmental soil samples should have debris such as sticks, large leaves and stones removed aseptically when being weighed on a top-loading balance. If BSL-2 laboratory safety practices are utilized, laboratory policy based on the source of the samples determines whether samples can be weighed on the benchtop or inside a BSC. All materials removed from the BSC should be decontaminated using a bleach disinfecting wipe following the manufacturer or the laboratory HASP instructions or placed in an autoclave bag before being moved. The same procedure should be followed if a leak occurs during shaking; the biosafety carrier containing centrifuge bottles or tubes should be immediately transferred to the BSC and decontaminated using bleach disinfecting wipes (following manufacturer's instructions) followed by cleaning with 70% ethanol to remove bleach residue. If BSL-3 safety practices are utilized, the samples are weighed and shaken inside the BSC.

9.1 Protocol for 45 g Samples

The procedure that follows is for 45g soil samples processed in 250 mL centrifuge bottles. For development of this protocol, aspiration of the supernatant was completed by pipetting with the tip placed just below the meniscus of the liquid and aspirating while continuously moving the tip of the pipette slightly below the meniscus, until the tip is approximately the width of the pipette tip above the surface of the pellet. However, the method of aspiration that is best for the operator should be used.

9.1.1 Wash 1

9.1.1.1 Working in a BSC, add 175 mL SES to each 45g soil sample in 250 mL centrifuge bottles (labeled with Bottle 1 and unique sample code for each sample) using a 50 mL pipette and seal the lid securely. Suspend the soil by inverting repeatedly. Decontaminate the outside of the bottles using bleach disinfection

wipes (following the manufacturer's or the laboratory HASP instructions) followed by 70% ethanol (decontaminate in this protocol always refers to a bleach wipe/ethanol treatment unless stated otherwise).

9.1.1.2 Invert each "Bottle 1" labeled centrifuge bottle several times to suspend the soil while placing samples in a biotransport carrier on the shaker. Secure the container on the center of shaker platform with the platform load-bars by additionally bracing the container with a suitable relatively non-absorbant material, for example broken up pieces of styrofoam packing material. Cushion the centrifuge bottles inside the carrier with absorbent pads in order to keep them in place while shaking. Using an orbital shaker, shake samples for 20 minutes at 450 rpm (420 rpm minimum). Stop the shaker if any leaks occur and reseal the bottle(s) or if the container becomes unstable. Dispose of packing material after used.

9.1.1.3 In a BSC, weigh each "Bottle 1" labeled bottle to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the bottles at 100 × *g* for 5 minutes in a swinging bucket rotor using minimal braking to settle large or very dense particles.

9.1.1.4 Working in a BSC, pipet off the supernatant (Supernatant 1) using a 25 mL or 50 mL pipet and transfer this supernatant into new sterile 250 mL centrifuge bottle (labeled as Bottle 2 plus a unique sample code corresponding to the Bottle 1 sample code).

9.1.1.5 In a BSC, weigh the "Bottle 2" labeled centrifuge bottles to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the bottles at 5,900 x *g* within minimal braking for 30 minutes to pellet spores (Pellet 1). Pipet off the supernatant using a 50 mL pipette and discard into a waste bottle. Save Pellet 1 in the "Bottle 2" labeled bottles.

9.1.2 Wash 2

9.1.2.1 Working in a BSC, add 150 mL SES using a 50 mL pipette to Pellet 2 in the original sample bottles (Bottle 1) and resuspend pellets by repeatedly inverting. Wide bore pipette tips can also be used to resuspend the soil in the SES. Make sure the bottle threads are free of debris. Decontaminate the exterior of the bottles as previously described (see Section 4.2).

9.1.2.2 Invert the centrifuge the "Bottle 1" labeled centrifuge bottles several times to suspend the soil while placing samples in a biotransport carrier on the shaker. Secure the container on the center of shaker platform with the platform load-bars by additionally bracing the container with foam, broken up Styrofoam pieces, or equivalent. Cushion the centrifuge bottles inside the carrier with absorbent pads in order to keep them in place while shaking. Using the Innova 2100 platform shaker, shake samples for 20 minutes at 450 rpm (420 rpm minimum). Stop the

shaker if any leaks occur and reseal the bottle(s) or if the carrier is unstable. Dispose of Styrofoam pieces after use.

9.1.2.3 In a BSC, weigh the "Bottle 1" labeled centrifuge bottles to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the bottles at 100 × *g* for 5 minutes in a swinging bucket rotor using minimal braking to settle large or very dense particles.

9.1.2.4 Working in a BSC, pipet off the supernatant (Supernatant 2) using a 50 mL pipet and add to Pellet 1 in Bottle 2 (could have approximately 147-150 mL of supernatant), and decontaminate outside of the bottles as previously described (see Section 4.2).

9.1.2.5 In a BSC, weigh the "Bottle 2" labeled bottles containing the now combined Pellet 1 and Supernatant 2 to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the bottles at 5,900 × *g* using a minimal braking setting for 30 minutes to pellet spores. Working in a BSC, pipet off the supernatant using a 50 mL pipette and discard into a waste bottle. Save the combined pellet for further processing.

9.1.3 Transfer the Pellet

9.1.3.1 In a BSC, add 15 mL of SES to the 250 mL centrifuge bottles using a 25 mL pipette. Suspend the combined pellet using a sterile disposable bacterial transfer loop. Transfer this suspension by pipetting with a sterile 25 mL pipette into a labeled sterile 50 mL centrifuge tube (Tube 1).

9.1.3.2 Repeat step 9.1.3.1 two times using 15 mL and 10 mL volumes SES until the final volume of approximately 40 mL is in the 50 mL centrifuge tubes. Decontaminate the exterior of the 50 mL tubes as previously described for the centrifugation bottles (see Section 4.2).

9.1.3.3 In a BSC, weigh tubes to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the tubes at 5,900 × *g* using a minimal braking setting for 30 minutes to pellet spores.

9.1.3.4 Pipet the supernatant using 25 or 50 mL pipette and discard into a waste bottle. Decontaminate the outside of the 50 mL centrifuge tubes as previously described (see Section 4.2).

9.1.3.5 Pellets can be stored at 4◦C until further analysis is conducted.

9.2 Protocol for 9 g Samples

The procedure that follows is for 9g samples processed in 50 mL centrifuge tubes. Polypropylene wire racks can be used to hold 50 mL conical tubes during processing. For development of this protocol, aspiration of the supernatant was completed by pipetting

with the tip placed just below the meniscus of the liquid and aspirating while continuously moving the tip of the pipette slightly below the meniscus, until the tip is approximately the width of the pipette tip above the surface of the pellet. However, the method of aspiration that is best for the operator should be used.

9.2.1 Wash 1

9.2.1.1 Working in a BSC, add 35 mL SES to each 9g soil sample in 50 mL centrifuge tubes (labeled with Tube 1 and unique sample code for each sample) using a 10 mL or 25 mL pipette and seal the lid securely. Suspend the soil by inverting repeatedly. Decontaminate the outside of the bottles using bleach disinfection wipes (following the manufacturer's or the laboratory HASP instructions) followed by 70% ethanol (decontaminate in this protocol always refers to a bleach wipe/ethanol treatment unless stated otherwise).

9.2.1.2 Invert each "Tube 1" labeled centrifuge tube several times to suspend the soil while placing samples in a biotransport carrier on the shaker. Secure the container on the center of shaker platform with the platform load-bars by additionally bracing the container with a suitable relatively non-absorbant material, for example broken up pieces of styrofoam packing material. Cushion the centrifuge tubes inside the carrier with absorbent pads in order to keep them in place while shaking. Using an orbital shaker, shake samples for 20 minutes at 450 rpm (420 rpm minimum). Stop the shaker if any leaks occur and reseal the bottle(s) or if the container becomes unstable. Dispose of packing material after used.

9.2.1.3 In a BSC, weigh each "Tube 1" labeled centrifuge tube to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the tubes at 100 × *g* for 5 minutes in a swinging bucket rotor using minimal braking to settle large or very dense particles.

9.2.1.4 Working in a BSC, pipet off the supernatant (Supernatant 1) using a 10 mL or 25 mL pipet and transfer this supernatant into new sterile 50 mL centrifuge tube (labeled as "Tube 2" with the sample code corresponding to the Tube 1 sample code).

9.2.1.5 In a BSC, weigh the "Tube 2" labeled centrifuge tubes to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the tubes at 5,900 x *g* within minimal braking for 30 minutes to pellet spores (Pellet 1). Pipet off the supernatant using a 10 or 25 mL pipette and discard into a waste bottle. Save Pellet 1 in the "Tube 2" labeled centrifuge tubes.

9.2.2 Wash 2

9.2.2.1 Working in a BSC, add 30 mL SES using a 10 or 25 mL pipette to Pellet 2 in the original sample tubes (Tube 1) and resuspend pellets by repeatedly inverting.

Wide bore pipette tips can also be used to resuspend the soil in the SES. Make sure the bottle threads are free of debris. Decontaminate the exterior of the bottles as previously described (see Section 4.2).

9.2.2.2 Invert the "Tube 1" labeled centrifuge tubes several times to suspend the soil while placing samples in a biotransport carrier on the shaker. Secure the container on the center of shaker platform with the platform load-bars by additionally bracing the container with foam, broken up Styrofoam pieces, or equivalent. Cushion the centrifuge tubes inside the carrier with absorbent pads in order to keep them in place while shaking. Using the Innova 2100 platform shaker, shake samples for 20 minutes at 450 rpm (420 rpm minimum). Stop the shaker if any leaks occur and reseal the bottle(s) or if the carrier is unstable. Dispose of Styrofoam pieces after use.

9.2.2.3 In a BSC, weigh the "Tube 1" labeled centrifuge tubes to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the tubes at 100 × *g* for 5 minutes in a swinging bucket rotor using minimal braking to settle large or very dense particles.

9.2.2.4 Working in a BSC, using a 10 or 25 mL pipet, pipet off the supernatant (Supernatant 2) and add to Pellet 1 in Tube 2 (could have approximately 30 mL using a 10 or 25 mL pipette), and decontaminate outside of the bottles as previously described (see Section 4.2).

9.2.2.5 In a BSC, weigh the "Tube 2" labeled centrifuge tubes containing the now combined Pellet 1 and Supernatant 2 to ensure a balanced centrifuge load, adding additional SES as necessary before centrifuging. Centrifuge the tubes at 5,900 × *g* using a minimal braking setting for 30 minutes to pellet spores. Working in a BSC, pipet off the supernatant using a 10 or 25 mL pipette and discard into a waste bottle. Save the combined pellets for further processing.

9.2.2.6 Pellets can be stored at 4◦C until further analysis is conducted.

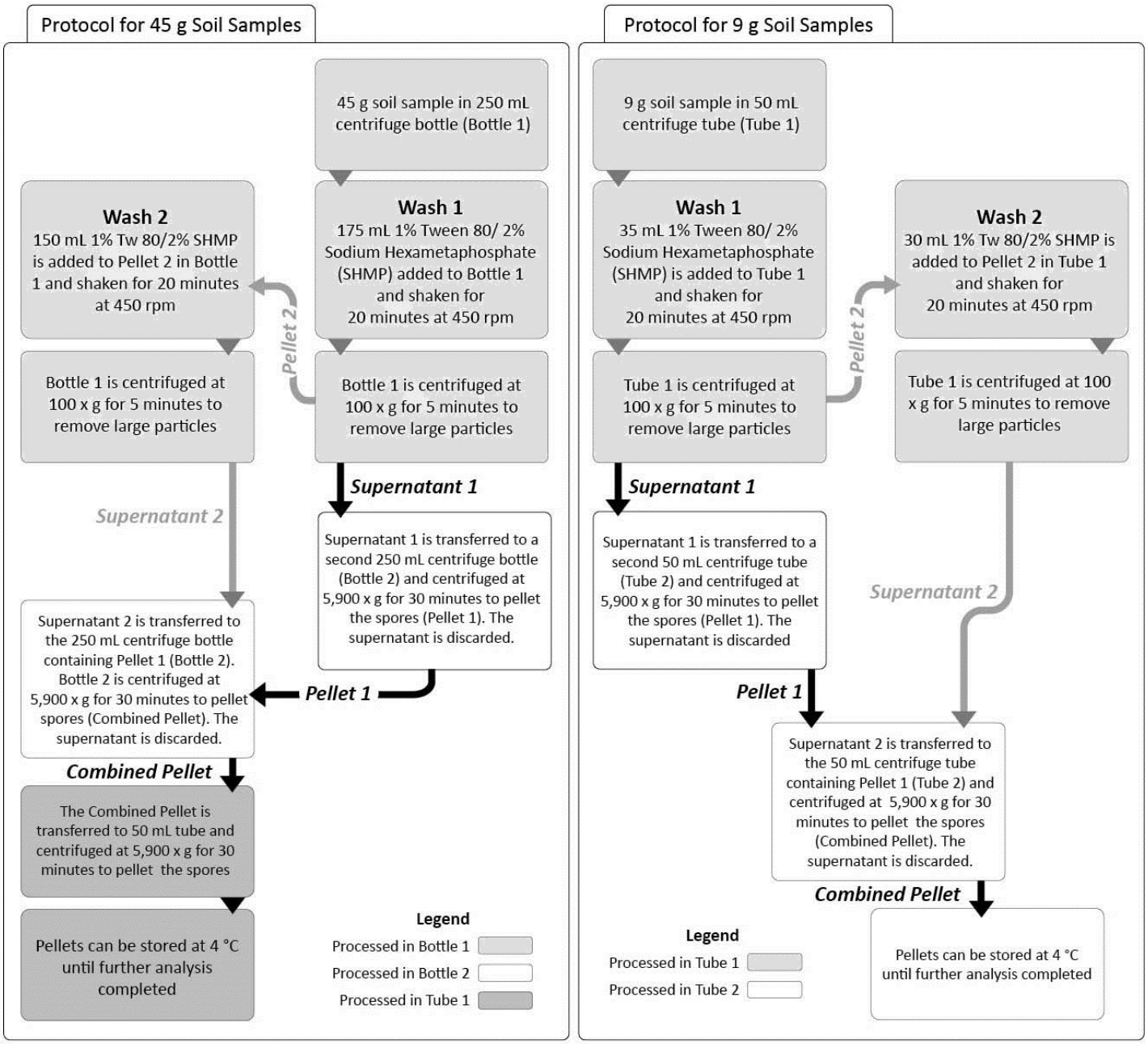

Figure 1. Protocol summary for 45 g and 9 g loam and sandy soil samples.

10.0 Protocol Performance

The 45 g protocol was demonstrated using sterile soil at the EPA and USGS laboratories. Results of that evaluation have been described in Reference 13.8. There was no significant difference overall between the laboratories for either soil type, suggesting the processing protocol is robust enough to use at multiple laboratories and still achieve similar recoveries. The protocol demonstrated a matrix limit of detection for loamy and sandy soils at 14 spores/gram of soil. Data tables and statistics from the two laboratory demonstration are included in Reference 13.8. while initial development work for the 9 g protocol indicated similar recoveries would be possible to the 45 g protocol, a laboratory comparison was not completed for the 9 g protocol.

11.0 Pollution Prevention

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

12.0 Waste Management

12.1 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.

12.2 Samples known to be contaminated with viable *B. anthracis* must be reported to the Federal Select Agent Program (FSAP) and transferred to an appropriate FSAP laboratory or destroyed on-site by a recognized sterilization or inactivation process within 7 days in accordance with the Select Agent regulations (Reference 13.10 and Reference 13.15).

12.3 Reference materials and equipment known or suspected to be contaminated with or to contain viable *B. anthracis* must be decontaminated prior to disposal. More information on environmental sample disposal can be found in EPA/600/R-10/092 (Reference 13.16), 40 CFR part 260 (Reference 13.17) and 40 CFR part 270 (Reference 13.18).

12.4 For further information on waste management, consult *The Waste Management Manual for Laboratory Personnel* (Reference 13.19) and *Less Is Better: Laboratory Chemical Management for Waste Reduction* (Reference 13.20), both authored by the ACS.

13.0 References

13.1 Benham, E., R.J. Ahrens, W.D. Nettleton. 2009. Clarification of soil texture class boundaries. U.S. Department of Agriculture, National Soil Survey Center. Lincoln, Nebraska. Last accessed online November 3, 2016 at http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_031477.pdf

13.2 U.S. Environmental Protection Agency. 2011. Development and verification of rapid viability polymerase chain reaction (RV-PCR) protocols for *Bacillus anthracis* – For application to air filters, water and surface samples. U.S. Environmental Protection Agency, Office of Research and Development, National Homeland Security Research Center, Cincinnati, Ohio. EPA 600/R-10/156

13.3 Kane, S.R., S.E. Létant, G.A. Murphy, T.M. Alfaro, P.W. Krauter, R. Mahnke, T.C. Legler, E. Raber. 2012. Rapid, high-throughput, culture-based PCR methods to analyze samples for viable spores of *Bacillus anthracis* and its surrogates. J. Microbiol Methods 76(3): 278-284.

13.4 Forum on Environmental Measurements. 2010. 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. U.S. Environmental Protection Agency. Accessed online December 21, 2016 at: https://www.epa.gov/sites/production/files/2015-01/documents/emergency_response_validity_policy.pdf

13.5 U.S. Department of Homeland Security, Integrated Consortium of Laboratory Networks (ICLN). 2008. ICLN Guidelines for Comparison of Validation Levels between Networks, Original Version, http://www.icln.org/docs/sop.pdf.

13.6 Federal Bureau of Investigation (FBI), Scientific Working Group on Forensic Analysis of Chemical Terrorism (SWGFACT). 2005. Validation Guidelines for Laboratories Performing Forensic Analysis of Chemical Terrorism. Forensic Science Communications 7(2).

13.7 U.S. Environmental Protection Agency and U.S. Geological Survey. 2014. USEPA/USGS sample collection protocol for bacterial pathogens in surface soil. U.S. Environmental Protection Agency and the U.S. Geological Survey. EPA/600/R-14/027.

13.8 Silvestri, E.E., D. Griffin, D. Feldhake, J. Lisle, A. Pemberton, T.L. Nichols, S. Shah, F.W. Schaefer, III. 2016. Optimization of sample processing protocol for recovery of *Bacillus anthracis* spores from soil. J Micro Methods 130:6-13.

13.9 Centers for Disease Control and Prevention. 2009. Biosafety in microbiological and biomedical laboratories (BMBL), 5th Edition. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health. Last accessed online November 3, 2016 at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm

13.10 CFR. Code of Federal Regulations 42 Part 73. Last accessed online November 3, 2016 at http://www.ecfr.gov/cgi-bin/text-idx?tpl=/ecfrbrowse/Title42/42cfr73_main_02.tpl

13.11 Occupational Safety and Health Administration. 1998. General description and discussion of the levels of protection and protective gear. Code of Federal Regulations 1910.120, Appendix B. United States Department of Labor, Occupational Safety and Health Administration. Last accessed online November 3, 2016 at http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9767

13.12 American Chemical Society. 2005. Reagent chemicals: Specification and procedures, 10th edition. American Chemical Society Oxford University Press (USA), New York.

13.13 British Drug Houses Ltd. (BDH). 1984. AnalaR standards for laboratory chemicals. 8th Edition. British Drug Houses, Ltd., Poole, Dorset, U.K.

13.14 United States Pharmacopeial Convention (USP). 2005. United States Pharmacopeia and National Formulary 24. United States Pharmacopeia Convention, Rockville, Maryland.

13.15 Centers for Disease Control and Prevention (CDC). 2016. Federal Select Agent Program regulations (7 CFR part 331, 9 CFR part 121, and 42 CFR parts 73). Centers for Disease Control and Prevention and the U.S. Department of Agriculture. Last accessed online November 3, 2016 at http://www.selectagents.gov/regulations.html

13.16 U.S. Environmental Protection Agency. 2010. Laboratory environmental sample disposal information document. Companion to the Standardized Analytical Methods for Environmental Restoration Following Homeland Security Events (SAM) - Revision 5.0. U.S. Environmental Protection Agency. EPA/600/R-10/092.

13.17 U.S. Environmental Protection Agency. 40 CFR part 260. Hazardous waste management system: General. Last accessed online November 3, 2016 at http://www.access.gpo.gov/nara/cfr/waisidx_07/40cfr260_07.html

13.18 U.S. Environmental Protection Agency.40 CFR part 270. EPA administered permit programs: The Hazardous Waste Permit Program. Last accessed online November 3, 2016 at http://www.access.gpo.gov/nara/cfr/waisidx_07/40cfr270_07.html

13.19 American Chemical Society. 1990. The waste management manual for laboratory personnel. American Chemical Society, Department of Government Relations and Science Policy, Washington, DC.

13.20 American Chemical Society. 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. Last accessed online November 3, 2016 at https://www.acs.org/content/dam/acsorg/about/governance/committees/chemicalsafety/publications/less-is-better.pdf

This page left intentionally blank.

United States
Environmental Protection
Agency

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

Office of Research and Development (8101R)
Washington, DC 20460

Official Business
Penalty for Private Use
$300