EPA/600/R-17/212 August 2017
www.epa.gov/homeland-security-research

United States
Environmental Protection
Agency

Field Application of Emerging Composite Sampling Methods

Office of Research and Development
National Homeland Security Research Center

This page left intentionally blank

EPA 600/R-17/212

Field Application of Emerging Composite Sampling Methods

Technical Report

U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

Disclaimer

This document has been internally and externally peer-reviewed in accordance with U.S. Environmental Protection Agency's (EPA's) Peer Review Handbook, 4th Edition 2015 (EPA/100/B15/001) and has been approved for publication. Note that approval does not signify that the contents necessarily reflect the views of the Agency. This procedure includes photographs of commercially available products. The photographs are included for the purposes of illustration only. Any mention of trade names, products, or services does not imply an endorsement by the U.S. government or EPA. The EPA does not endorse any commercial products, services, or enterprises.

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

Sang Don Lee, Ph.D.
National Homeland Security Research Center
Office of Research and Development
U.S. Environmental Protection Agency (E343-06)
109 T.W. Alexander Drive
Research Triangle Park, NC 27711
Telephone No.: (919) 541-2973
E-mail Address: Lee.Sangdon@epa.gov

Acknowledgments

The authors would like to thank the Department of Homeland Security (DHS) for their support of this work, in particular, Dr. Donald Bansleben. Dr. Sang Don Lee is the project lead for this document. The individuals and organizations listed below contributed to the development of this document.

EPA

M. Worth Calfee, National Homeland Security Research Center
Leroy Mickelsen, EPA Office of Emergency Management
John Archer, National Homeland Security Research Center
Timothy Boe, National Homeland Security Research Center

Oakridge Research Institute for Science and Education

Douglas Hamilton

Jacobs Technology, Inc.

Laurie Brixey
Abderrahmane Touati
Jerome Gilberry
Ryan Stokes

Contents

Tables

Figures

Acronyms and Abbreviations

°C	Degree(s) Celsius
× g	times gravity
µm	micrometer(s)
AA	aggressive air
ADA	aerosol deposition apparatus
ANOVA	analysis of variance
APS	Aerodynamic Particle Sizer
AREMA	American Railway Engineering and Mining Association
ATCC	American Type Culture Collection
AWTC	Asymmetric Warfare Training Center
Ba	*Bacillus anthracis*
Bg	*Bacillus atrophaeus* var. *globigii*
Biolab	ORD NHSRC Biocontaminant Laboratory
BSC	biosafety cabinet
Btk	*Bacillus thuringiensis* var. *kurstaki*
CBRN	Chemical, Biological, Radiological and Nuclear
CCTV	closed-circuit television
CDC	Centers for Disease Control and Prevention
CFM	cubic foot/feet per minute
CFU	colony-forming unit(s)
cm	centimeter(s)
CMAD	Consequence Management Advisory Division
CV	coefficient of variation
DCMD	Decontamination and Consequence Management Division
DFU	dry-filter unit
DHS	Department of Homeland Security
DI	deionized
DOD	Department of Defense
DPG	Dugway Proving Ground
DQI	data quality indicator
ECBC	Edgewood Chemical Biological Center
EPA	U.S. Environmental Protection Agency
FAPH	Fort A.P. Hill
ft	foot/feet
ft^2	square foot/feet
ft^3	cubic foot/feet
g	gram(s)
GIS	geographic information system
GSD	geometric standard deviation
HEPA	high-efficiency particulate air
h	hour
HSRP	Homeland Security Research Program
in.	inch(es)
ISO	International Organization for Standardization
L	liter(s)

LLNL	Lawrence Livermore National Laboratory
m	meter(s)
m^3	cubic meter(s)
MDI	metered-dose inhaler
min	minute(s)
MITLL	Massachusetts Institute of Technology Lincoln Laboratory
mL	milliliter(s)
mm	millimeter(s)
MMAD	mass median aerodynamic diameter
NAM	negative air machine
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OLEM	Office of Land and Emergency Management
ORD	Office of Research and Development
OTD	Operational Technology Demonstration
PAPR	powered air-purifying respirator
PBST	phosphate-buffered saline with 0.05% Tween® 20
PNNL	Pacific Northwest National Laboratory
PPE	personal protective equipment
QA	quality assurance
QC	quality control
RFC	robotic floor cleaner
RH	relative humidity
rpm	rotation per minute
RTP	Research Triangle Park
SD	standard deviation
SS	stainless steel
TSA	tryptic soy agar
UTR	Underground Transport Restoration

Executive Summary

A release of hazardous biological material in an urban area would require decontamination of a wide range of surfaces to protect the public. The U.S. Environmental Protection Agency (EPA) is responsible for protecting human health and the environment from such contaminated areas. Accurate measurements of residual contamination are needed to (1) select methods, locations, and other considerations required for effective decontamination, and (2) inform decisions on re-entry and reoccupation of decontaminated spaces. Traditional surface sampling methods (such as wipe, swab, and vacuum sock sampling) are used for extent mapping, characterization, decontamination verification, and clearance sampling. For a wide-area contamination incident, these traditional sampling methods can become a critical bottleneck in the remediation process because they are time- and labor-intensive and may require large number of samples to achieve reasonable confidence in the results.

Innovative composite sampling techniques may prove useful as an addition to currently used surface sampling methods in a wide area biological incident. These composite sampling techniques include aggressive air (AA) sampling as well as sampling using readily available surface cleaning technologies such as robotic floor cleaners (RFCs) and wet vacuums. These methods will improve the sampling capability in addition to the traditional surface sampling methods responding to a wide area incident. The potential advantages of using these methods include the following:

- Reduced sampling time during a response
- Fewer samples requiring processing
- Detection of spore presence at unknown hot spots of contamination
- Improved detection of widespread contamination when concentrations are close to (or potentially below) detection limits for traditional surface sampling methods
- Shortened timeline to recovery.

AA, RFC, and wet vacuum sampling are suitable for use in many building interiors and can allow rapid sampling, requiring fewer personnel and fewer samples per unit area than current surface sampling methods.

The study discussed in this report tested the effectiveness of AA, RFC, and wet vacuum composite methods for sampling spores from a subway platform and rail surfaces. Specifically, this study consisted of a field sampling exercise and laboratory experiments that are discussed separately in this report. The field sampling exercise evaluated RFC, wet vacuum, and AA sampling. The field sampling exercise was designed to evaluate the performance of these composite sampling methods for post-decontamination sampling and sampling with the presence of multiple contamination hot spots. The separate laboratory experiments evaluated AA sampling operational parameters and efficacy under controlled conditions.

For this project, the field sampling exercise was conducted in a mock subway system at Fort A.P. Hill (FAPH) over a 24-hour (h) period. The AA, RFC, and wet vacuum sampling procedures were conducted on the concrete platform and track (only AA sampling) in the subway system. Post-decontamination sampling results showed that the wet vacuum and RFC methods can be used to sample areas containing viable spores at concentrations as low as the single-digit range per square foot. The study showed that

the RFC and wet vacuum sample processing procedures require improvements in environments with dusty surfaces. This study also showed that AA sampling methods require further development for large volumes of air in dusty environments to avoid the overloading of filters. Section 7 of this report provides specific recommendations, including the operational limitations of the sampling methods studied and recommendations for improving these methods based on input by operators and observers of the field sampling exercise and on the results of this study.

1 Introduction

The study discussed in this report tested the effectiveness of aggressive air (AA) sampling and other composite methods for sampling spores from subway platform and rail surfaces. This research supports the mission of the U.S. Environmental Protection Agency's (EPA's) National Homeland Security Research Center (NHSRC) by providing information pertinent to the decontamination of areas contaminated through an act of terrorism. This project supports the EPA's Homeland Security Research Program (HSRP) and NHSRC's strategic goals as described in detail in the Homeland Security Strategic Research Action Plan (EPA 2012a). This work is pertinent to Long-Term Goal 2, which states, "The Office of Land and Emergency Management (OLEM) and other clients use HSRP products and expertise to improve the capability to respond to terrorist attacks affecting buildings and the outdoor environments." This project specifically addresses a need expressed by OLEM's Chemical, Biological, Radiological and Nuclear (CBRN) Consequence Management Advisory Division (CMAD) to understand and optimize composite-based sampling for a wide-area anthrax incident.

This project consisted of a field sampling exercise conducted in a mock subway system and laboratory experiments to evaluate AA sampling operational parameters and efficacy under controlled conditions. The field sampling exercise evaluated the use of three composite sampling methods for detecting spore contamination: robotic floor cleaner (RFC), wet vacuum, and AA sampling. The field sampling exercise was designed to evaluate the performance of these composite sampling methods for post-decontamination sampling and sampling with the presence of hot spot contamination. The separate laboratory tests evaluated AA sampling operational parameters and efficacy under controlled conditions.

The project background, description, and objectives are discussed below.

1.1 Project Background

A release of hazardous biological material in an urban area would require decontamination of a wide range of surfaces to protect the public. EPA is responsible for protecting human health and the environment from such contaminated areas. Accurate measurements of residual contamination are needed to: (1) select methods, locations, and other considerations required for effective decontamination, and (2) inform decisions on re-entry and reoccupation of decontaminated spaces. Traditional surface sampling methods (such as wipe, swab, and vacuum sock sampling) are used for extent mapping, characterization, decontamination verification, and clearance sampling. For a wide-area contamination incident, these traditional sampling methods can become a critical bottleneck in the remediation process because they are time- and labor-intensive and may require large numbers of samples to achieve reasonable confidence.

Innovative composite sampling techniques may prove useful as an addition to currently used surface sampling methods. These composite sampling techniques include AA sampling as well as sampling using readily available surface cleaning technologies such as RFCs and wet vacuums. The potential advantages of these methods include the following:

- Reduced sampling time during a response
- Fewer samples requiring processing

- Detection of spore presence at unknown hot spots of contamination
- Improved detection of widespread contamination when concentrations are close to (or potentially below) detection limits for traditional surface sampling methods
- Shortened timeline to recovery.

AA, RFC, and wet vacuum sampling are suitable for use in many building interiors and can allow rapid sampling requiring fewer personnel and fewer samples per unit area than current surface sampling methods.

EPA has conducted prior studies (Lee et al. 2013) to test commercial floor cleaning devices such as RFCs and wet vacuum cleaners for sampling *Bacillus* spores. The devices were evaluated for their usability on various surface types and under different contamination scenarios. Commercial floor cleaning devices can sample a wider area per sampling event than traditional surface sampling methods, thereby reducing labor. The sampling efficacy of the RFCs and wet vacuums used in this study is comparable to currently used sampling methods such as wipe and vacuum sock sampling.

During this project, sampling procedures were developed for RFCs and wet vacuums to provide methods for trained incident responders to collect environmental samples from flat, contiguous surfaces after a biological contamination incident. Appendix A and Appendix B detail the sampling and sample retrieval procedures for RFCs, respectively, and Appendix C details the sampling procedures for wet vacuums. Data from the collected samples are intended to allow determination of the presence or absence of pathogenic microorganisms and the contamination level after natural outbreaks and intentional or accidental releases of pathogenic microorganisms.

1.2 Project Description

As indicated above, this project consisted of a field sampling exercise and laboratory experiments. Each project component is discussed below.

1.2.1 Field Sampling Exercise

The field sampling exercise evaluated the RFC, wet vacuum, and AA sampling methods in a mock subway system over a 24-hour (h) period during the Operational Technology Demonstration (OTD) project, part of the Department of Homeland Security (DHS)-funded Underground Transport Restoration (UTR) program.

The UTR-OTD project is an interagency effort involving the following federal agencies and National Laboratories: EPA, DHS, Lawrence Livermore National Laboratory (LLNL), Massachusetts Institute of Technology Lincoln Laboratory (MITLL), and Pacific Northwest National Laboratory (PNNL). The overall purpose of the UTR-OTD project was to conduct and evaluate field-level mass transportation and biological remediation of two decontamination technologies directed at the intentional release of a biological agent such as *Bacillus anthracis* (*Ba*).

The OTD was conducted at a Department of Defense (DOD) mock subway tunnel at Fort A.P. Hill (FAPH) in Bowling Green, VA. The UTR-OTD involved all aspects of remediation of a subway system

contaminated with a biological agent, including pre-decontamination and post-decontamination verification sampling and waste management. However, the aspects of the UTR-OTD pertinent to this project are limited to the first dissemination of *Bacillus atrophaeus* var. *globigii* (*Bg*) spores in the tunnel and decontamination of the tunnel by fogging with diluted bleach. The UTR-OTD report (EPA 2017) discusses in detail the methods of spore dissemination, decontamination, sampling, and analysis conducted before the activities described in this report.

The AA sampling method involves forced aerosolization of particles from a surface using a leaf blower, collection of aerosolized particles by air sampling, followed by quantitative analysis of collected samples for bacterial spores. During the field sampling exercise, the air samplers used were dry-filter units (DFUs) and negative air machines (NAMs) equipped with prefilters. Traditional surface sampling techniques can provide a measure of the surface contamination from a fraction of potentially contaminated surface area. AA sampling can provide a collective measure of contamination in the impacted area regardless of surface types with small number of samples compared to the traditional sampling methods.

Composite sampling was conducted on September 27 and 28, 2016, after the first round of decontamination (by fogging) and post-decontamination sampling using conventional sampling methods. Table 1-1 describes the composite sampling campaign, which was divided into five stages.

Table 1-1. FAPH Composite Sampling Stages

Stage	Details	Purpose
1	RFC and wet vacuum sampling on platform	Evaluate post-decontamination sampling
2	AA sampling	Evaluate post-decontamination sampling
3	AA sampling with hot spot *Bg* contamination	Assess detection of hot-spot contamination that could be missed by traditional surface sampling methods
4	Overnight settling of *Bg* spores on agar plates	Assess redistribution of hot spots from AA sampling
5	RFC and wet vacuum sampling on platform	Assess redistribution of hot spots from AA sampling

During Stage 1, RFCs and wet vacuums were used to conduct post-decontamination sampling in subway platform floor areas. During Stage 2, post-decontamination AA sampling was conducted using leaf blowers to aerosolize particles from all of the floor and track surfaces that could be reached. During Stage 3, trays of ballast rocks inoculated in the laboratory with *Bg* spores were placed in the subway tunnel to create “hot spots” of concentrated spore contamination, and the AA sampling procedure was repeated. Stage 4 consisted of an overnight settling period for the *Bg* spores using 200 agar plates distributed throughout the subway tunnel. Finally, during Stage 5, RFCs and wet vacuums were used to sample the same areas of the subway platform floor that were sampled during Stage 1.

1.2.2 Laboratory Experiments

The laboratory experiments included a DFU filter loading evaluation, NAM prefilter comparison, and forced aerosol evaluation. These experiments were aimed to provide information that the NHSRC can use to assess and improve the AA sampling method for *Ba* spores and to evaluate equipment and methods used in the field sampling exercise.

1.3 Project Objectives

The purpose of the field sampling exercise was to evaluate AA, RFC, and wet vacuum sampling techniques for detecting spore contamination. The laboratory experiments were designed to evaluate AA sampling operational parameters and efficacy under controlled conditions. The specific project objectives included the following:

- During the field sampling exercise, characterize the aerosolization of surrogate spores from ballast rock material used around train tracks in subway systems.
- During the field sampling exercise, test the hypothesis that using the AA sampling method in a large area will distribute hot spot contamination throughout the entire area so that the contamination is more likely to be detected by surface sampling methods.
- During the laboratory experiments, assess the impacts on spore aerosolization of certain experimental conditions, including spore type, spore loading, and decontamination simulated by spraying with deionized (DI) water instead of a decontamination agent.
- During the laboratory experiments, conduct a limited evaluation of DFUs and NAMs for use in AA sampling.

2 Field Sampling Exercise Approach

This section discusses the general approach for the field sampling exercise, including the facility description, sampling methods and materials, and the composite sampling plan.

2.1 Facility Description

The field sampling exercise was conducted at FAPH in Bowling Green, VA. FAPH is used to train active and reserve troops of the U.S. Armed Forces as well as for training personnel from other government agencies, including the Department of State, Department of the Interior, the U.S. Customs Service, and federal, state, and local security and law enforcement agencies. FAPH's Asymmetric Warfare Training Center (AWTC) is a 300-acre site consisting of a headquarters, barracks, administrative offices, training and maintenance facilities, several training ranges, and an "urban area." The urban area includes a subway station complete with subway cars, a train station with rail cars, and other urban buildings.

The Underground Transport Restoration (UTR)-Operational Technology Demonstration (OTD) project was conducted in September and October 2016 in the subway station in the urban area of the FAPH AWTC. The composite sampling was conducted in the subway tunnel on September 27 and 28, 2016. Figure 2-1 shows a schematic drawing of the portion of the subway tunnel where sampling was conducted.

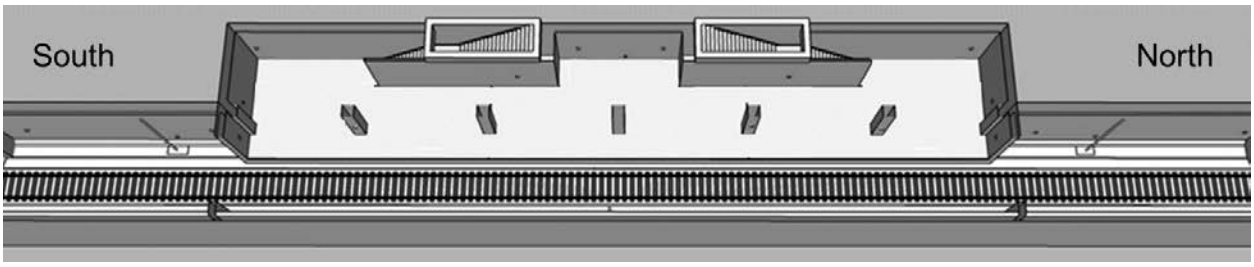

Figure 2-1. Portion of FAPH Subway Tunnel Used for Study

Table 2-1 summarizes the approximate dimensions (in feet [ft]) and volume (in cubic feet [ft^3]) of the subway tunnel.

Table 2-1. Approximate Dimensions and Volume of FAPH Subway Tunnel

Tunnel Section	Length (ft)	Width (ft)	Height (ft)	Approx. Volume (ft^3)
Track south of platform	53	22	19	22,000
Track center	162	16.5	19	51,000
Track north of platform	60	22	19	25,000
Platform	162	23	15	56,000
Kiosk (between staircases)	27	6.5	15	2,600
			Total volume	**157,000**

Figures 2-2 and 2-3 present photographs of the platform and track areas of the subway tunnel. Figure 2-4 is a still image from the closed-circuit television (CCTV) camera. No subway cars were used or present in the tunnel for the study. The entrances to the subway tunnel were sealed to make an enclosed space, and a non-pathogenic surrogate organism, *Bg*, was sprayed in the subway tunnel.

Figure 2-2. FAPH Subway Tunnel Platform from North End Looking South (Subway Car Not Present for Study)

Figure 2-3. FAPH Subway Tunnel Track (North of Platform)

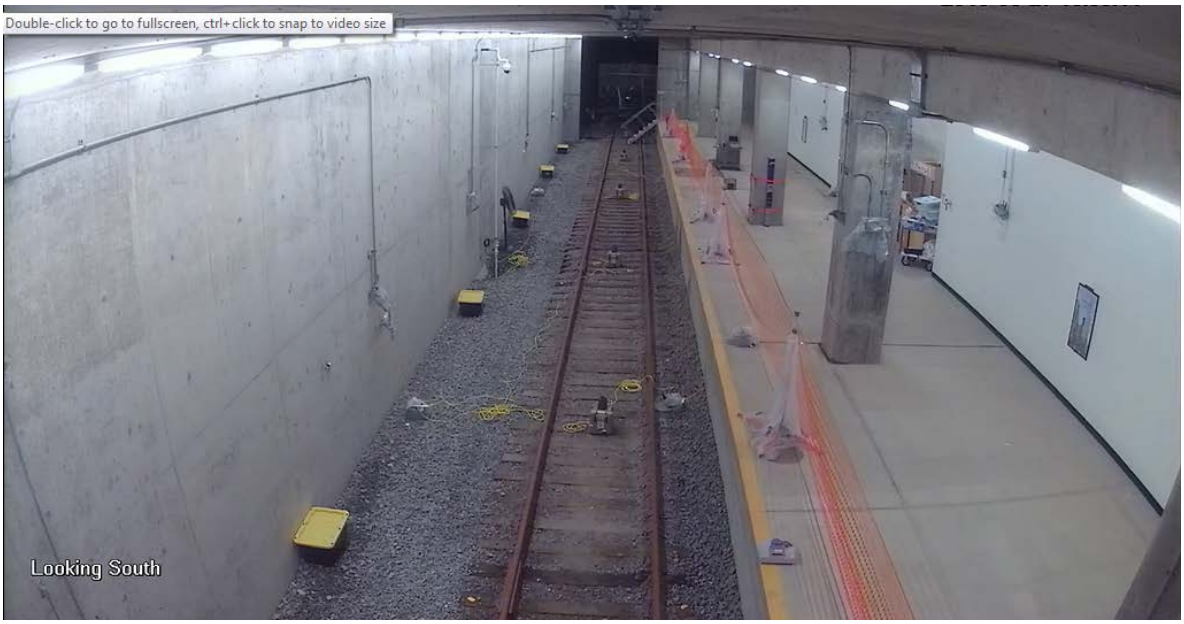

Figure 2-4. Image from CCTV Camera on Ceiling of Tunnel at North End of Platform Looking South

2.2 Sampling Methods and Materials

Equipment and materials for the field sampling exercise were prepared in laboratories at EPA's Research Triangle Park (RTP) campus in NC. Samples collected during the field sampling exercise were packed and transported to the EPA RTP Office of Research and Development (ORD) NHSRC Biocontaminant Laboratory (Biolab) for processing and analysis in accordance with culture-based microbiological assay techniques developed by EPA and the Centers for Disease Control and Prevention (CDC). Except for the settling plate samples, all sample results were quality checked by Biolab staff and results were delivered electronically to project personnel. The settling plates were incubated and enumerated on site at FAPH.

The sampling methods included AA sampling, RFC sampling, wet vacuum sampling, ballast coupon preparation and inoculation, and settling plate sampling, as discussed below.

2.2.1 Aggressive Air Sampling

The AA sampling procedure conducted in the subway tunnel used leaf blowers for forced aerosolization of particles, mixing fans to help maintain particle suspension, and two different types of filter-based samplers for particle collection, DFU samplers and an NAM duct with a prefilter. In brief, AA sampling in the subway tunnel was executed as detailed below.

- Seven mixing fans were placed in the subway tunnel at prescribed locations on both the track and platform areas.
- Nine DFU samplers were placed in the subway tunnel at prescribed locations on the track.
- One NAM outside the hot zone was fitted with flexible ductwork to locate the inlet on the platform floor and connect a 14-inch (in.) by 20-in. prefilter for sample collection. (Two NAM prefilters were planned, but the flange for connecting the NAM duct on the south stairway failed).
- Three leaf blowers were operated in a prescribed manner on all track and platform surfaces within reach of the operators.

The equipment and procedures for forced aerosolization and air sampling are described in detail below.

2.2.1.1 Forced Aerosolization

To provide forced particle aerosolization, corded electric leaf blowers (BV5600, Black and Decker Inc., Towson, MD) were used on the platform and track of the mock subway tunnel (Figure 2-5).

Figure 2-5. Black and Decker Inc. BV5600 Corded Electric Leaf Blower

For AA sampling during the field sampling exercise, the hot zone was divided into three areas: the platform, the track from the middle of the platform to the edge of the hot zone, and the track from the middle of the platform to the dead end. One operator was assigned to operate each of the three leaf blowers in the following sections of the subway tunnel (shown in Figure 2-6):

- Leaf blower 1: Subway track south of center line of platform
- Leaf blower 2: Subway platform
- Leaf blower 3: Subway track north of center line of platform.

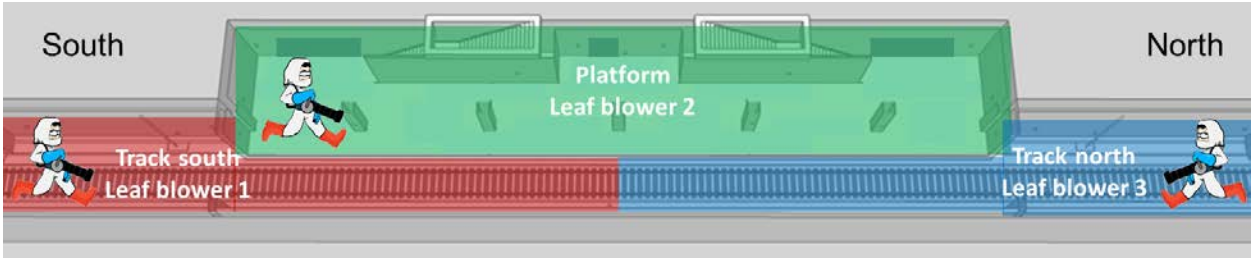

Figure 2-6. AA Sampling Leaf Blower Zones in Subway Tunnel

Table 2-2 summarizes the approximate dimensions in ft and square feet (ft^2) of the area covered by each leaf blower.

Table 2-2. Approximate Dimensions of Area Covered by Each Leaf Blower

Leaf Blower	Width 1 (ft)	Depth 1 (ft)	Width 2 (ft)	Depth 2 (ft)	Approx. Area (ft^2)	Description
1	53	22	81	16.5	2,500	Track south
2	162	16.5	81	6.5	3,200	Platform
3	60	22	81	16.5	2,700	Track north

The operators were given approximately 30 minutes (min) to cover each area. Using leaf blowers, the operators were required to sweep at a rate of 83 to 106 ft^2/min for a single pass.

The track level was divided into lanes to guide the leaf blower operators. The sections of the tunnel north and south of the platform are approximately 22 ft wide. These spaces were divided into five lanes as shown in Figure 2-7.

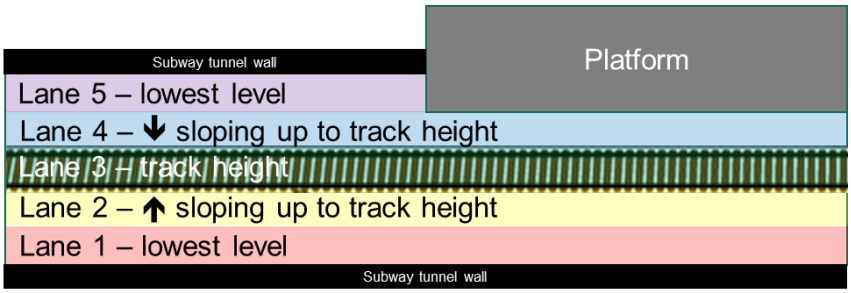

Figure 2-7. Lane Divisions for Leaf Blower Operators in Subway Tunnel

In the center section, the platform reduces the width of the track to 16.5 ft, and that space was divided into four lanes. The field operators did not actually mark the lanes in the tunnel because the topography of the subway tunnel provides clear delineations. The train tracks are approximately 2 ft higher than the ballast closest to the tunnel walls, creating the following divisions:

- Lane 1: First 4 ft from far tunnel wall; lowest part of subway tunnel
- Lane 2: From edge of Lane 1 to rail farther from platform; sloping
- Lane 3: Between rails
- Lane 4: From rail nearer to platform to edge of platform or edge of Lane 5; sloping
- Lane 5: First 4 ft from tunnel wall on platform side; lowest part of subway tunnel.

Leaf blower 1 and leaf blower 3 operated at opposite ends of the tunnel, starting from the far ends of the tunnel in Lane 1 and progressing toward the center of the platform. After completing Lane 1, leaf blowers 1 and 3 each returned to the end of the tunnel and completed a pass of Lane 2 and then each subsequent lane until all surfaces were covered. Leaf blower 2 covered the platform area starting from one side and progressing to the other. Each operator covered as much of his or her designated section as possible during each AA sampling period. The operators attempted to maintain a 45° angle and 1 centimeter (cm) of clearance between the leaf blower nozzle and surface, covering all horizontal and vertical surfaces within reach. Operators walked slowly forward through their designated areas, moving the nozzle of the leaf blower from side to side in an action similar to that of blowing leaves.

Seven mixing fans operated during AA sampling to assist with mixing and keeping particles airborne. The fans included oscillating pedestal fans (Model UP30BN-S, Airmaster Fan Company, Jackson, MI) and 42-in.-diameter barrel fans (Model HBPC4213, Triangle Engineering of Arkansas, Inc., Jacksonville, AR).

2.2.1.2 Air Sampling

DFU samplers (DFU-1000, Lockheed Martin Integrated Technology LLC, Gaithersburg, MD) were deployed to collect aerosol samples on the track level. DFUs are high-volume air samplers that use 47-millimeter (mm)-diameter polyester felt filters (DFU-P-24, Leidos, Reston, VA) with a 1-micrometer (µm) pore size for particle collection. The DHS BioWatch program and U.S. military use DFUs as samplers for bioaerosol detection. Nine DFU samplers were placed along the centerline of the track approximately 30 ft apart.

The UTR-OTD health and safety plan dictates that at least one high-efficiency particulate air (HEPA)-filtered NAM must be operating at all times to ventilate the subway tunnel. The nominal operating flow rates for the type of NAM deployed in the tunnel (Omni-Aire 2200C, Omnitec Design, Inc., Mukilteo, WA) is 1,000 cubic feet per minute (CFM) on the low setting and 1,800 CFM on the high setting. It was decided to take advantage of these high flow rates for aerosol sampling by attaching prefilters to the inlets of two NAMs. The initial plan for AA sampling included attaching 25-ft-long, 12-in.-diameter, non-insulated aluminum flexible ducts (Part 3XK08, Grainger Industrial Supply, Lake Forest, IL) to two NAMs, one at each stairwell landing, with a filter box (Part 62039, www.budgetheating.com) and prefilter installed on the inlet of each NAM duct for particle collection. However, only one NAM was deployed because the flange for connecting the NAM duct on the south stairway failed. The NAM prefilters were 14-in. by 20-in.

furnace filters (Filtrete™ MPR 2800, 3M, St. Paul, MN) rated to collect 97% of 1-µm particles. The prefilters were prepared by cutting away the metal mesh on the filter face and marking a 6-in. by 6-in. area in the center of the filter face.

An Aerodynamic Particle Sizer (APS, Model 3321, TSI, Inc., Shoreview, MN) was deployed on a cart located on the subway platform to measure the size distribution of particulates aerosolized during AA sampling. The particle size data were saved on a laptop in the field, downloaded to the EPA network, and summarized in a spreadsheet. The data also were used to produce graphs of the particle size distribution and total concentration over time during AA sampling.

Several Bioaerosol Button Samplers (SKC, Inc., Eighty Four, PA) were worn by personnel (personal samples) and mounted to a cart (area sample) to collect localized, task-specific filter samples of inhalable particles. Filters were retrieved from the button samplers, transported to the ORD NHSRC Biocontaminant Laboratory (Biolab) and analyzed for viable spores.

2.2.2 Robotic Floor Cleaner Sampling

Neato® XV-21 RFCs (Neato® Robotics, Newark, CA) were charged, tested, recharged, and packed for deployment in accordance with the procedure described in Appendix A. The Neato® XV-21 (Figure 2-8) is equipped with mapping and navigation technologies and is capable of returning to its starting position after covering the entire floor surface of an enclosed sampling area.

Figure 2-8. Neato® XV-21 RFC

An observer monitored the RFCs to ensure their proper operation and to notify sampling personnel if an RFC malfunctioned. If an RFC malfunctioned, it was immediately removed and replaced by a backup RFC. After sampling was complete, the RFC filter sample was retrieved from each deployed RFC in accordance with the RFC sample retrieval procedure described in Appendix B.

2.2.3 Wet Vacuum Sampling

Three areas on the subway platform were marked with magnetic strips for wet vacuum sampling. The wet vacuum model used was the Hoover® Max Extract Cleaner (F7425-900 SteamVac Dual V with SpinScrub Hand Tool, Hoover®, Glenwillow, OH). Appendix C describes the procedures used to prepare each wet vacuum for sampling. The Hoover® Max Extract Cleaner has a clean water tank and a dirty water tank (Figure 2-9). The clean tank was filled with 5 liters (L) of a sterile solution of 0.05% Tween® 20 surfactant in DI water.

Figure 2-9. Hoover® Max Extract Cleaner Wet Vacuum

Wet vacuum sampling was conducted concurrently with RFC sampling. The wet vacuums were operated on all accessible floor space in the designated sampling areas with both “Rinse” and “Power Scrub” modes turned on. The initial vacuuming stroke was made with the liquid dispensing trigger on, followed by two vacuum-only strokes (liquid dispensing trigger off) covering the same area. The wet vacuum then was moved over to cover an area consisting of 50% new area and 50% of the area just covered. A new initial vacuum stroke was made with the liquid dispensing trigger on, followed by two vacuum-only strokes covering the same area. Sampling proceeded in this manner (one wet stroke followed by two dry strokes) until the entire sampling area was covered.

An observer monitored the wet vacuum sampling to ensure that the operators were conducting sampling in accordance with the procedure described in Appendix C. If a wet vacuum malfunctioned, it was immediately replaced by a backup wet vacuum. For each wet vacuum, the entire dirty water tank was placed in a cooler and transported as one sample for microbiological analysis.

2.2.4 Ballast Coupon Preparation and Inoculation

During Stage 3, hot spots of contamination were brought into the subway tunnel in the form of ballast rocks inoculated with *Bg* spores. Each coupon consisted of 28 pounds (± 0.5 pound) of ballast rocks in a stainless-steel (SS) tray measuring approximately 12 in. by 12 in. by 3 in. deep, with a 1-in. lip around the perimeter suitable for clamping onto an aerosol deposition apparatus (ADA) as shown in Figure 2-10.

Figure 2-10. Ballast Coupons Showing Size and Weight in Pounds (left) and ADA Attachment (right)

The ballast coupons were fabricated at the EPA RTP campus using rocks similar in type and morphology to samples obtained from FAPH. The ballast used was 100% crushed granite meeting American Railway Engineering and Mining Association (AREMA) #4 ballast specifications. The ballast source was checked for microbiological contamination by agitating the rocks in a solution of sterile phosphate-buffered saline with 0.05% Tween® 20 (PBST) and plating samples of the solution in accordance with the procedure described in Section 4.2.1. No evidence of contamination (no growth) was observed. Therefore, the ballast coupons were not sterilized before spore inoculation. Before coupon assembly and inoculation, the coupon trays were autoclaved, and the SS ADAs were sanitized using bleach wipes (Dispatch® 69150, Clorox®, Oakland, CA).

The *Ba*-simulant spore used for hot spot coupon inoculation was a powdered spore preparation of *Bg* American Type Culture Collection (ATCC) 9372 mixed with silicon dioxide particles. *Bg* is a gram-positive, spore-forming, rod-shaped bacterium found in the environment, particularly in hay. This powdered spore preparation was obtained from the U.S. Army Dugway Proving Ground (DPG) Life Sciences Division (Dugway, UT). The procedure for preparing this preparation is described in Brown et al. 2007. Briefly, after 80 to 90% sporulation, the suspension was centrifuged to generate a preparation of approximately 20% solids. The dried spores were dry-blended and jet-milled with fumed silica particles (Degussa, Frankfurt am Main, Germany), resulting in a powdered matrix containing approximately 1×10^{11} viable spores per gram (g). The powdered *Bg* spore preparation was loaded into metered-dose inhalers (MDIs) in accordance with a proprietary protocol. Figure 2-11 shows an MDI and its actuator.

Figure 2-11. MDI and Actuator

Ballast coupons and 14-in.-square SS positive control coupons were inoculated with *Bg* spores using an MDI (Calfee et al. 2013). In brief, each coupon was inoculated in a separate ADA (Figure 2-10) designed to fit over a ballast coupon tray or one 14-in. by 14-in. coupon. A spore-loaded MDI was weighed, and then the MDI and actuator were inserted into the inoculation opening in the center top of the pyramidal SS ADA hood. The MDI was discharged once into the ADA and removed, and then the inoculation opening cover was closed. The MDI was weighed again, and the weight change was calculated to verify a successful inoculation (0.04-g to 0.07-g loss). This procedure was repeated until all the coupons were inoculated. The first, middle, and last coupons inoculated were SS positive control coupons that were left undisturbed for a minimum of 18 h and then sampled using a sponge stick (3M™ Sponge-Stick, St. Paul, Minnesota; catalog number SSL-10NB) in accordance with the CDC-published procedure (CDC 2012).

Twenty-four ballast coupons, each with 1-ft^2 ballast area, were prepared and inoculated with *Bg* spores. Each ballast coupon with its ADA still attached was double-bagged in polyethylene autoclave bags (01-829F, Fisher Scientific, Waltham, MA), sealed in a rugged plastic tote (44066, Centrex Plastics LLC, Findlay, OH), and transported to FAPH with the sampling equipment.

The total change in mass of the MDI over the inoculation of the 24 ballast coupons was 1.371 g. Recovery from SS positive control coupons using the sponge-stick swab method (CDC 2012) averaged 8.9×10^8 colony-forming units (CFU) per g of MDI weight change, with a standard deviation (SD) of 8.4×10^7 CFU/g and a coefficient of variation (CV) of 9.5%. Therefore, the best estimate of the total *Bg* spores (measured as CFU) contained in all of the 24 hot spot ballast coupons is 1.2×10^9 CFU total, or an average of 5.1×10^7 CFU/ft^2 of ballast coupon.

2.2.5 Settling Plate Sampling

During the overnight period after AA sampling with hot spots (Stage 3), significant settling of spores was expected, with hot spot contamination. To quantify the overnight settling of spores, agar plates were placed on the platform and track surfaces during Stage 4 after the completion Stage 3. Two types of agar plates measuring 100-mm in diameter each were used: tryptic soy agar (TSA) plates (Difco 236050, Becton Dickinson and Company, Franklin Lakes, NJ) and Brilliance™ *B. cereus* agar plates (Oxoid CM1036, Thermo Fisher, Waltham, MA). *Bg* is easily identified and enumerated when cultured on both of these types of agar plates. *Bg* forms orange-pigmented colonies on TSA plates and blue-pigmented colonies on Brilliance™ agar plates.

One TSA plate and one Brilliance™ agar plate were collocated at each of the 100 settling plate locations in the grid, for a total of 200 plates. Section 2.3.4 discusses the Stage 4 sampling locations and procedures in more detail.

2.3 Composite Sampling Plan

After UTR-OTD post-decontamination surface sampling, this project's field sampling exercise investigated the composite sampling methods of AA, RFC, and wet vacuum sampling. Briefly, the sampling plan consisted of collecting one set of RFC and wet vacuum samples (Stage 1), and then conducting the AA sampling procedure (Stage 2). Afterwards, hot spots of spore-contaminated ballast material were uncovered in the subway tunnel and the AA sampling procedure was repeated (Stage 3), followed by an overnight period of particle settling onto agar plates (Stage 4). The sampling concluded with collection of

another set of RFC and wet vacuum samples (Stage 5). Figure 2-12 shows a timeline of the field sampling exercise activities in the subway tunnel relevant to this project.

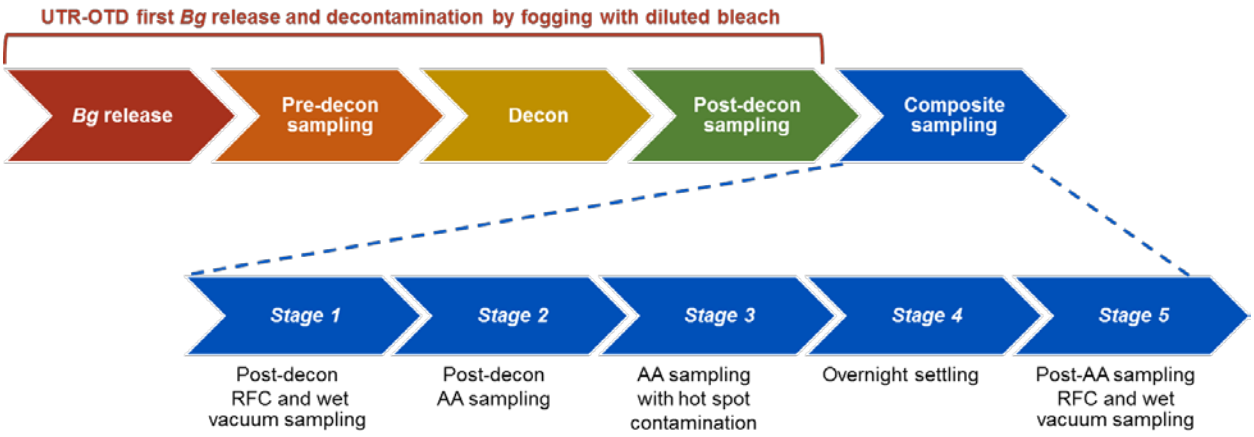

Figure 2-12. Timeline of Relevant Activities in Subway Tunnel

Possibly the greatest challenge in this study was the short period of time (less than 24 h) that the subway tunnel was available for the field sampling exercise after UTR-OTD post-decontamination surface sampling. The field sampling exercise was divided into the following five stages:

Stage 1: Post-decontamination RFC and wet vacuum sampling, Round 1

1. Delineation of areas on subway platform to be sampled using RFC and wet vacuum methods
2. RFC and wet vacuum sampling on the subway platform
3. Collection, labeling, and storage of RFC and wet vacuum samples

Stage 2: Post-decontamination AA sampling, Round 1

1. Setup of AA sampling mixing fans, DFU samplers, and NAM prefilters
2. Placement of sealed, spore-contaminated ballast coupons
3. AA sampling on platform and track levels
4. Collection, labeling, and storage of AA samples

Stage 3: AA sampling with hot spots of contamination, Round 2

1. Samplers loaded with clean filters
2. Unwrapping of pre-positioned, spore-contaminated ballast coupons in subway tunnel to create hot spots
3. AA sampling on platform and track levels
4. Collection, labeling, and storage of AA samples

Stage 4: Overnight settling

1. Placement of pre-labeled TSA and Brilliance™ agar settling plates in tunnel to collect spores during overnight settling period
2. Collection of settling plates and placement in incubator

Stage 5: Post-AA sampling RFC and wet vacuum sampling, Round 2

1. RFC and wet vacuum sampling on subway platform
2. Collection, labeling, and storage of RFC and wet vacuum samples
3. Enumeration of colonies on TSA and Brilliance™ agar settling plates
4. Packaging and transport of all samples to EPA RTP campus for processing and analysis

2.3.1 Stage 1: RFC and Wet Vacuum Sampling, Round 1

RFC sampling was conducted on the subway platform to assess post-decontamination residual spore levels on the platform. Magnetic boundary markers (Neato® Robotics, Newark, CA) were used to delineate the three areas for RFC sampling, RFC1, RFC2, and RFC3, shown in Figure 2-13.

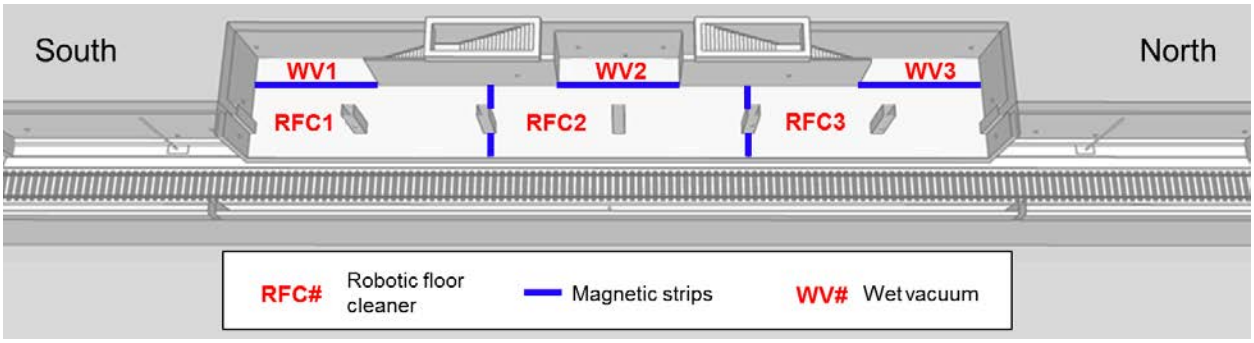

Figure 2-13. RFC and Wet Vacuum Sampling Areas

Three magnetic strips were used to continue the line formed by the walls enclosing the stairwells. These strips divided the three RFC sampling areas from the three wet vacuum sampling areas. Two additional magnetic strips divided the remaining area of the platform into thirds. One dividing line extended from the wall enclosing the south stairwell to the support column south of center, then from the opposite side of the column to the edge of the platform. The last dividing line was in the corresponding location on the north side of the platform. Each RFC sampling area measured approximately 900 ft^2 (54 by 16.5 ft). One RFC was placed and started in each of the three sampling areas.

Wet vacuum sampling was conducted on the subway platform at the same time as RFC sampling in the three areas marked for wet vacuum sampling on Figure 2-13, WV1, WV2, and WV3. Each wet vacuum sampling area measured approximately 175 ft^2 (27 by 6.5 ft).

After sampling was complete, the RFC filter sample was retrieved from each deployed RFC in accordance with the RFC sample retrieval procedure described in Appendix B. Wet vacuum samples were collected as detailed in Appendix C.

2.3.2 Stage 2: AA Sampling, Round 1

Aggressive air sampling was conducted in the subway tunnel to assess residual spore levels in the subway tunnel post-decontamination. As discussed in Section 2.2.1.1, three leaf blowers were used for forced aerosolization during the Stage 2 AA sampling. Figure 2-14 shows the locations of the seven mixing fans and the DFU and NAM samplers used during the AA sampling as discussed in Section 2.2.1.

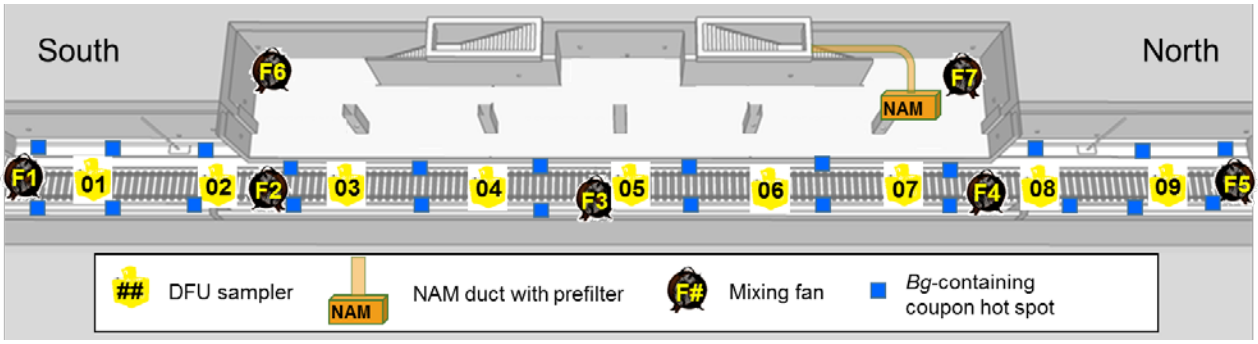

Figure 2-14. AA Sampling Mixing Fan, Sampling, and Coupon Locations

During Stage 2, sampling was conducted concurrently with the leaf blowers. Nine DFU samples and one NAM prefilter sample were collected from the locations shown in Figure 2-14. DFUs were placed on the tracks equidistant from each other. In addition, seven mixing fans were placed at the following locations: one at each end of the track facing the center of the tunnel (locations F1 and F5), two (F6 and F7) in the corners of the platform at the bottom of each stairway and oriented facing the center of the platform, one (F3) in the center of the tunnel wall facing the platform, and fans F2 and F4 along the tunnel wall at each end of the platform facing the center of the platform. All of the fans were angled 45° upward and faced the center of the zone. The fans operated for the entire time the operators were in the hot zone. Oscillating pedestal fans were placed at locations F2, F3, F4, F6, and F7, and barrel fans were placed at locations F1 and F5. After sampling was complete, the DFU and NAM filter samples were retrieved from each deployed sampler and placed in labeled plastic bags.

In addition, ballast coupon hot spots inoculated with *Bg* spores were placed in the tunnel along the walls at the locations shown in Figure 2-14 in preparation for Stage 3. The coupons remained sealed to prevent cross-contamination of Stage 2 AA samples.

2.3.3 Stage 3: AA Sampling with Hot Spots of Contamination, Round 2

During Stage 3, new DFU and NAM prefilters were loaded, and the ballast material coupons inoculated with *Bg* spores were uncovered. Figure 2-14 shows the coupon locations. Then, a second round of AA sampling was conducted using the same procedures and locations used during Stage 2 as discussed above.

2.3.4 Stage 4: Overnight Settling

After AA samples were collected, TSA and Brilliance™ agar plates were collocated at each location shown in Figure 2-15 (platform) and Figure 2-16 (tracks).

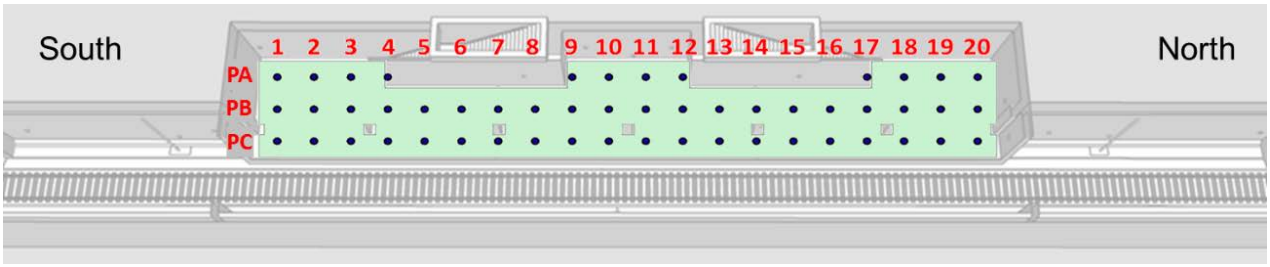

Figure 2-15. Settling Plate Placement Marked by Dots on Subway Platform

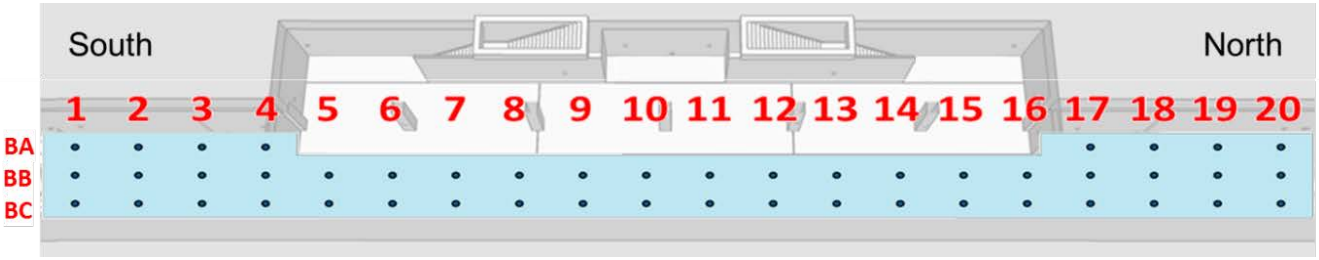

Figure 2-16. Settling Plate Placement Marked by Dots on Subway Tracks

The plates were labeled as follows: P (platform) or B (ballast), row (A, B, or C), and column number (for example, PC13). The first settling plate was placed in the tunnel at 00:16, on September 28, 2016, and the last was placed at 00:53 on the same day. The tunnel was left overnight (approximately 7.5 h) to allow aerosolized particles to settle. The plates then were collected and placed in an incubator between 08:00 and 09:00 on September 28, 2016, when field study personnel returned to the site. The settling plate sampling was conducted to test the hypothesis that using the AA sampling method in a large area will distribute hot spot contamination throughout the entire area so that the contamination is more likely to be detected by surface sampling methods.

2.3.5 Stage 5: RFC and Wet Vacuum Sampling, Round 2

After the settling plates were collected during Stage 4, the magnetic boundary markers were replaced and a second round of RFC and wet vacuum sampling was conducted using the same procedures and locations as during Stage 1. This second set of RFC and wet vacuum samples was collected to test the hypothesis that using the AA sampling method in a large area will distribute hot spot contamination throughout the entire area so that the contamination is more likely to be detected by surface sampling methods.

3 Laboratory Experimental Approach

This section discusses the approach used for the laboratory experiment component of the project. The laboratory experiment included a DFU filter loading evaluation, NAM prefilter comparison, and forced aerosol evaluation as discussed below.

3.1 DFU Filter Loading Evaluation

The DFU filter loading evaluation was conducted to gain an understanding of how the DFU sampler flow rate is affected by dust loading of the filters. The question of how DFU sampler flow is impacted by filter loading arose during the FAPH field exercise, when DFUs were exposed to very high particulate concentrations during Stage 3, drastically reducing the flow rates during AA sampling.

The laboratory experiment was designed to simulate similar dust loading and to model the approximate relationship between dust loading on the filter and flow rate. The experiments were conducted inside the aerosol wind tunnel in laboratory BB005 on the EPA RTP campus. The wind tunnel controlled the environmental conditions at 20 degrees Celsius (°C) ± 2 °C and relative humidity (RH) at 30% ± 2% during all testing and contained the dust exhausted from the DFU. Figure 3-1 shows the test setup.

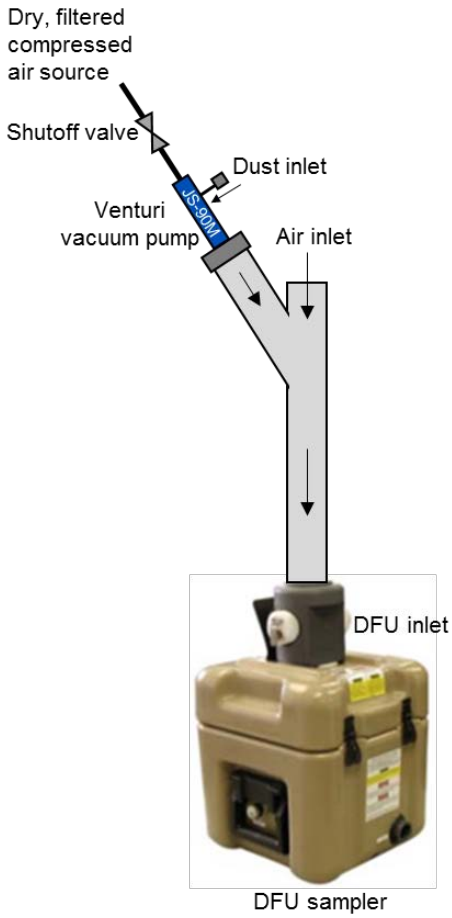

Figure 3-1. DFU Filter Loading Test Setup (Arrows Indicate Air Flow Direction)

The dust injection setup was constructed using a Venturi vacuum pump (Model JS-90M, Vaccon Company, Inc., Medway, MA). This pump uses compressed air and critical flow orifices to create a vacuum. Compression fittings were used to create a small funnel to hold the dust injected by the Venturi pump. The dust inlet was connected to a 2-in.-diameter Y-fitting and straight tubing to the inlet of the DFU sampler. The Y-fitting allowed make-up air to be pulled into the DFU inlet because the dust injection system can only operate at one pressure and therefore only one flow rate.

The DFU filter cassettes were preloaded with 47-mm polyester felt filters and weighed using an analytical balance (Model GA200D, Ohaus Corp., Parsippany, NJ). Arizona test dust A2 Fine Grade (Powder Technology, Inc., Arden Hills, MN) was selected for this experiment because this dust is a good match to the size distribution data for particles obtained during Stage 3 of the field sampling exercise. After a small amount of dust was injected into the DFU filter assembly, the DFU inlet velocity was measured, and then the filters were removed and weighed.

3.2 NAM Prefilter Comparison

Five types of 14-in. by 20-in. furnace filters were tested to compare the relative spore collection of each filter type under the same controlled circumstances. The chosen filters were all commercially available, four from Filtrete™ (3M, St. Paul, MN): Filtrete™ Basic Flat Panel, Filtrete™ 1500 Ultra Allergen, Filtrete™ 1900 Maximum Allergen, and Filtrete™ 2400 Elite Allergen Extra. The fifth filter was a WEB® Absorber Electrostatic Carbon Filter (WEB Products Inc., Creola, AL). These filters were chosen specifically either because the wire support on the filter face and back are easily separated from the pleated filter material or because the filter had no wire components, allowing filter pieces to be processed using a Stomacher® 400 circulator without puncturing the plastic bag.

Each filter was prepared for testing by removing the protective plastic film, placing the filter on a disinfected surface, and, if necessary, using disinfected diagonal wire snips to trim the wire support from both the face and the back of the filter as close to the filter frame as possible. The wire was carefully peeled off each face and discarded. A 6-in. by 6-in. square in the center of the filter face was then measured using a disinfected ruler and marked using permanent marker. To test each filter, the filter was fitted into a 14-in. by 20-in. filter box connected to the inlet of the NAM. A 12-in.-diameter, 30-in.-long duct with several mixing baffles installed inside was connected to the face of the filter box. Figure 3-2 shows the test setup.

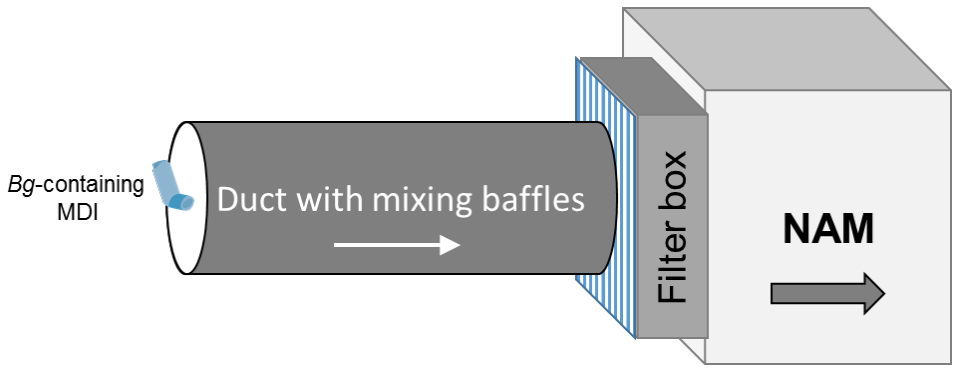

Figure 3-2. NAM Prefilter Comparison Test Setup (Arrows Indicate Air Flow Direction)

The NAM was switched on and set to high speed. An MDI containing *Bg* spores (described in Section 2.2.4) was used to dispense spores into the duct. The MDI was weighed, vortexed, placed in a one-piece medical MDI actuator, and actuated twice into the inlet of the duct. The first actuation seated the MDI in the actuator without any visible output, and the second actuation dispensed a visible puff of aerosol. The NAM was turned off 1 minute (min) after the second MDI puff, and the filter was removed, bagged, labeled, and transferred to the Biolab for processing. The filter was bagged in a 15-in. by 24-in. Twirl'em® plastic bag (Labplas, Sainte-Julie, QC, Canada).

Five replicate tests were completed for the Filtrete™ 1500 Ultra Allergen, Filtrete™ 1900 Maximum Allergen, and Filtrete™ 2400 Elite Allergen Extra filters. Two tests were conducted for the Filtrete™ Basic Flat Panel, and one test was conducted for the WEB® Absorber Electrostatic Carbon Filter. Section 4.1.2.2 discusses the procedures for filter sample processing, and Section 4.2.1 discusses filter sample analysis.

3.3 Forced Aerosolization Evaluation

The forced aerosolization evaluation tests were designed to evaluate the fraction of available spores that would be aerosolized from subway surfaces using a leaf blower as described in Section 2.2.1.1 during the field sampling exercise. Forced aerosolization testing was conducted in an environmentally controlled chamber in laboratory B155A on the EPA RTP campus. The chamber controlled the environmental conditions at 20 °C ± 2 °C and the RH at 30% ± 5% during all testing. Aerosolization experiments were conducted on approximately 12-in.-square trays of ballast rock material inoculated with either *Bg* or *Bacillus thuringiensis* var. *kurstaki* (*Btk*) spores. Table 3-1 summarizes the test matrix.

Table 3-1. Test Matrix for Bench-Scale Forced Aerosolization Experiments

Spore Type	Target Loading (CFU/ft^2)	Simulated Decontamination?	Temperature (°C)	Humidity (% RH)	Number of Replicates
Bg	10^4	No	22	30	5
	10^7	No	22	30	5
	10^7	Yes	22	30	5
Btk	10^4	No	22	30	5
	10^7	No	22	30	5
	10^7	Yes	22	30	5

Section 4.1.2.3 discusses the procedures for forced aerosolization filter processing, and Section 4.2.1 discusses sample analysis. The following sections discuss the forced aerosolization evaluation experimental setup, coupon inoculation, and simulated decontamination.

3.3.1 Experimental Setup

A small wind tunnel designed to conduct bench-scale AA sampling experiments was placed inside the environmentally controlled chamber in laboratory B155A on the EPA RTP campus. The AA sampling wind tunnel used for testing primarily was constructed of SS and used a blower to pull air through the tunnel. Figure 3-3 shows the AA sampling wind tunnel for the forced aerosolization evaluation.

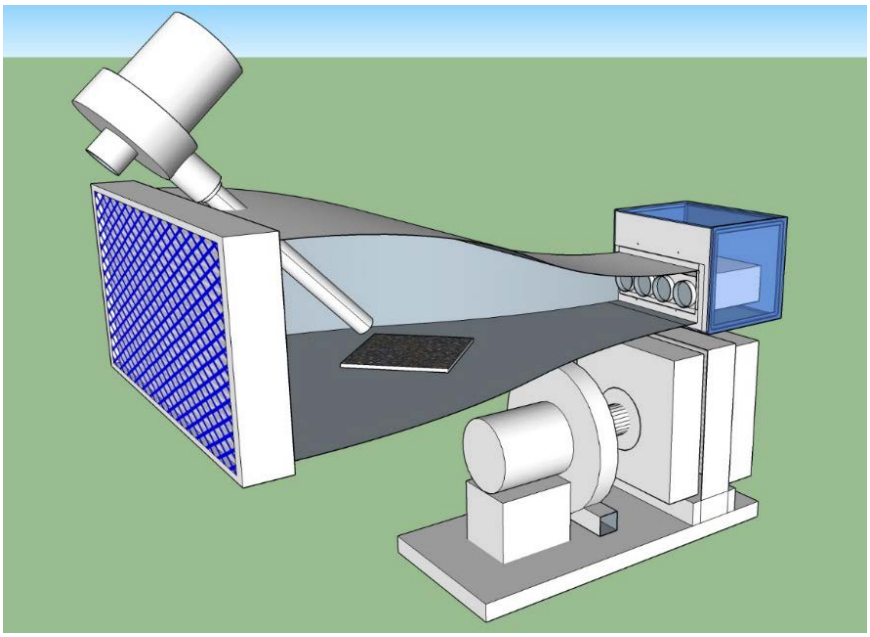

Figure 3-3. AA Sampling Wind Tunnel for Forced Aerosolization Evaluation

The tunnel was kept at a slightly negative pressure relative to the chamber to minimize spore contamination of the work space in the chamber. Pressure was monitored using a Magnehelic® series 2000 (Dwyer®, Michigan City, IN) differential pressure gauge. The wind tunnel included an upstream HEPA-filtered section where the ballast coupon was placed on a mechanical turntable in the center of the bottom surface of the wind tunnel. Figure 3-4 shows a ballast coupon and the leaf blower nozzle inside the AA sampling wind tunnel.

Figure 3-4. Leaf Blower Nozzle and Ballast Coupon Inside AA Sampling Wind Tunnel

The upstream section of the AA sampling wind tunnel allows placement of the electric leaf blower nozzle at a 45° angle from the vertical plane and at distances of 0 to 12 in. from the ballast coupon surface. The forced aerosolization evaluation tests used the same leaf blower used during the field sampling exercise. The leaf blower nozzle was placed approximately 2 in. from the ballast coupon surface. When the leaf blower was activated, particles were removed from the coupon and carried downstream to a 14-in. by 20-in. Filtrete™ 1500 filter connected to a NAM powered on high speed.

As for the NAM prefilter comparison, the wire support was removed from each filter but marked differently for post-processing. A disinfected ruler and permanent marker were used to mark across the top of the pleat closest to the middle of the filter. Then the 1/3 and 2/3 points along each long side were measured and marked, and the ruler was used to guide the permanent marker across the pleats, creating dotted lines across the filter face. This marking resulted in six boxes on the filter face as shown in Figure 3-5.

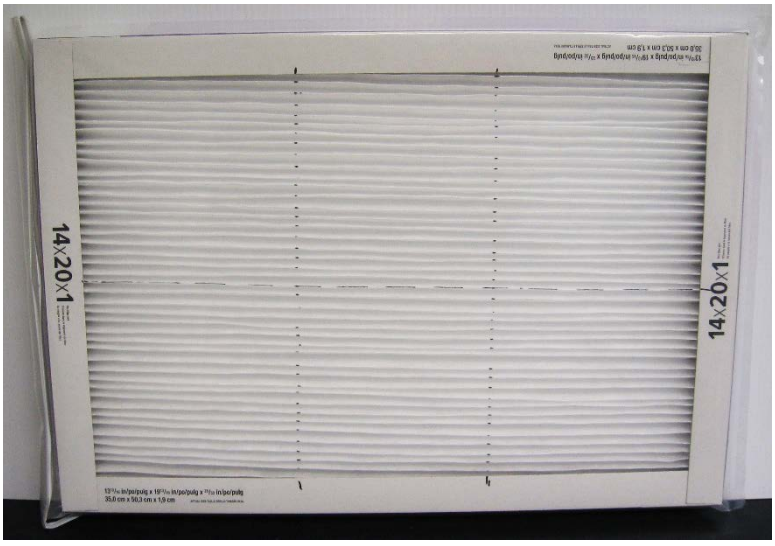

Figure 3-5. Filtrete™ 1500 Filter Marked for Forced Aerosolization Evaluation

The filter was bagged in a 15-in. by 24-in. Twirl'em® plastic bag and labeled. One filter was retained and processed as a blank.

3.3.2 Coupon Inoculation

Ballast coupons consisting of SS trays filled with rocks were prepared in the same manner as the coupons for the field sampling exercise described in Section 2.2.4. The coupons were inoculated with two types of *Bacillus* spores commonly used as surrogates for *Ba*, *Bg* and *Btk*. Dry powdered *Bg* spores were obtained from the same source specified in Section 2.2.4. Like *Bg*, *Btk* also is a gram-positive, spore-forming, rod-shaped bacterium found in soil. *Btk* produces an endotoxin protein during sporulation that is commonly used as a pesticide. The bar-coded *Btk* used for this project is a genetically modified strain developed by the Edgewood Chemical Biological Center (ECBC; Gunpowder, MD) that allows the spores to be distinguished from naturally occurring *Btk* through polymerase chain reaction analysis. The bar-coded *Btk* preparation was obtained from DPG.

The coupons were inoculated with *Bg* spores in the same manner discussed in Section 2.2.4. The bar-coded *Btk* cells were cultured by 10-L batch fermentation. After sporulation, the spores were concentrated into a wet pellet, washed three times, and lyophilized. The lyophilized spores were a dry aggregate, not a loose dry powder.

The Biolab prepared separate solutions of both *Bg* and *Btk* spore types. The spore solution required for spray-dry deposition is a suspension of the spores in a 90% ethanol solution. Table 3-2 summarizes the ingredients and measurements for preparing the spore solutions for spray-dry deposition.

Table 3-2. Ingredients and Measurements for Spore Solution for Spray-Dry Deposition

Ingredient	Amount Required to Prepare 30 mL Spore Stock Solution (approx. 1×10^8 CFU/mL)
Lyophilized spores	Approx. 0.05 g
Sterile DI water	0.85 mL
0.07% Tween® 20 in sterile DI water	2.13 mL
Ethanol	26.8 mL

The spore and ethanol solution was prepared as summarized below.

1. Measure the required amount of lyophilized spores into a sterile, 1.5-milliliter (mL) microcentrifuge tube (Thermo Scientific™ 3451, Waltham, MA).
2. Add sterile DI water to the microcentrifuge tube, and vortex the tube for 1 min.
3. After letting the tube rest at 15 min at ambient temperature, vortex the tube again for 1 min, then sonicate the tube for 1 min.
4. Repeat Step 3.
5. After 22 to 24 h, add 0.07% Tween® 20 in sterile DI water to a 50-mL conical tube. Transfer the contents of the microcentrifuge tube (spores and water) to the 50-mL conical tube using a pipette. Vortex the conical tube for 1 min, and then sonicate it for 1 min.
6. After 48 to 72 h, add 100% sterile ethanol to the conical tube.
7. Complete Steps 1 through 6 ten times.
8. Combine the contents of all 10 conical tubes in a glass jar, to yield 300 mL total.
9. Plate a sample of the final solution to perform a concentration check.

Spore solutions were plated to check the concentration. Solutions were diluted in 90% ethanol as needed to obtain the target concentrations of 5×10^8 CFU/mL for the high loading level and 5×10^6 CFU/mL for the low loading level.

Spore deposition for coupon inoculation was performed using the spray-dry deposition system and method described in Appendix D. Briefly, this procedure involves using an ultrasonic nozzle (model Q060-2-26-17-303-030, Sono-tek Corp., Milton, NY, USA) and a deposition stack (measuring 8 in. by 8 in. by 18 in. tall) to uniformly deposit dry spores from an alcohol-based suspension onto a surface. The deposition stacks were designed to deposit onto 7.75-in.-square material coupons. Therefore, modifications were necessary to accommodate deposition onto the ballast trays. For each deposition stack, a SS ballast tray was turned upside down, a 7.75-in.-square hole was cut in the bottom of the tray, and a spray-dry deposition stack was attached to the hole and sealed as shown in Figure 3-6.

Figure 3-6. Spray-Dry Deposition Stack Modified for Ballast Coupon Deposition

Using the same spore solutions, 14-in.-square SS positive control coupons also were prepared using the same equipment and methods as those used for the ballast coupons. Each SS coupon then was sampled using a sponge stick in accordance with the CDC-published procedure (CDC 2012). Results from the SS coupon swabs for each spore type and loading level were used as the measure of the number of spores deposited onto the ballast coupon for aerosolization fraction calculations.

3.3.3 Simulated Decontamination

Simulated decontamination involved spraying an inoculated coupon with DI water and allowing the ballast to dry before testing. For simulated decontamination, a sanitized, gravity-fed, high-velocity, low-pressure spray gun (ATD-6901, ATD tools inc. Wentzville, Missouri) was used to spray 180 mL of filter-sterilized DI water on the inoculated ballast tray. The water was applied uniformly to the ballast tray using an overlapping “S”-pattern (modeled on the CDC sponge stick swabbing protocol [CDC 2012]) until the entire 180-mL volume was dispensed. The uncovered tray then was left overnight to equilibrate in the test chamber at 20 °C ± 2 °C and 30% ± 5% RH. The loading rate of 180 mL/ ft² was chosen to mimic the UTR-OTD decontamination loading of 400 gallons of decontaminant released over the approximately 8,900-ft² site.

4 Testing and Measurements

This section discusses the testing and measurement protocols for this project, including sampling processing, analytical procedures, and flow measurement.

4.1 Sample Processing

All DFU, NAM, RFC, and wet vacuum samples collected during the field sampling exercise were packed on ice in coolers and transported to the EPA RTP Biolab on September 29, 2016. Samples from the laboratory experiment tests were delivered to the Biolab at the end of each day that the test was completed. Spores were extracted from the samples, and the spores in these extracts were assayed by growth on nutrient agar plates in the Biolab. The extraction processes for each sample type are described below.

4.1.1 DFU Filter Processing

Each DFU filter was aseptically transferred to a separate, 50-mL conical centrifuge tube for extraction. To each conical tube, 20 mL of sterile PBST was added, and then the tube was sonicated for 10 min and vortexed continuously for 2 min. The extracted liquid was analyzed for spores as described in Section 4.2.1.

4.1.2 NAM Prefilter Processing

The NAM prefilter samples included samples from the field sampling exercise, the laboratory experiment for the NAM prefilter comparison, and the laboratory experiment for the forced aerosolization evaluation as discussed below.

4.1.2.1 Field Sampling Exercise NAM Prefilters

The Biolab processed each NAM prefilter collected during the field sampling exercise in a sterile biosafety cabinet (BSC). The filter was removed from the bag, and the 6-in.-square area marked in the center was carefully excised using sterile scissors and cut in half. Each filter piece was placed in a 7-in. by 12-in. Stomacher® 400 circulator bag (BA6141/CLR, Seward Laboratory Systems Inc., Davie, FL), and 90 mL of sterile PBST was added. Each filter piece was processed with the Stomacher® 400 circulator speed set at 230 rotations per min (rpm) for 2 min. The extracted liquid was analyzed for spores as described in Section 4.2.1.

4.1.2.2 Laboratory Experiment NAM Prefilter Comparison

The Biolab processed the filters collected during the laboratory experiment NAM prefilter comparison study described in Section 3.2 in a sterile BSC. Filters were cut one at a time, and the BSC was sterilized between filters. Each filter was removed from the bag, and sterilized scissors were used to cut the 6-in.-square area marked in the center of the filter. For the Filtrete™ 1500 Ultra Allergen, Filtrete™ 1900 Maximum Allergen, and Filtrete™ 2400 Elite Allergen Extra filters, the 6-in. by 6-in. filter section was cut in half, and each filter piece was placed in a separate 7-in. by 12-in. Stomacher® circulator bag. For the WEB® Absorber Electrostatic Carbon Filter and the Filtrete™ Basic Flat Panel filter, each 6-in. by 6-in.

filter section was placed in a 7-in. by 12-in. Stomacher® 400 circulator bag. The second Filtrete™ Basic Flat Panel filter was excised completely from the frame and placed in a 7-in. by 12-in. Stomacher® 400 circulator bag. Table 4-1 summarizes the filter types and how they were processed.

Table 4-1. NAM Prefilter Comparison Testing and Processing

Filter Type	Filter Section Processed	Number of Replicates
Filtrete™ 1500 Ultra Allergen	Two 6-in. by 3-in. sections from center	5
Filtrete™ 1900 Maximum Allergen		
Filtrete™ 2400 Elite Allergen Extra		
WEB® Absorber Electrostatic Carbon Filter	6-in. by 6-in. center section	1
Filtrete™ Basic Flat Panel		
Filtrete™ Basic Flat Panel	Entire filter	1

Each Stomacher® 400 circulator bag contained 90 mL of sterile PBST and was processed with the circulator speed set at 230 rpm for 2 min. The extracted liquid was analyzed for spores as described in Section 4.2.1.

4.1.2.3 Laboratory Experiment Forced Aerosolization Evaluation NAM Prefilters

The Biolab processed and analyzed all filters collected from the forced aerosolization evaluation laboratory experiments in a sterile BSC. The filter was removed from the bag, and sterilized scissors were used to cut the filter into six pieces along the marked lines. Each of the six filter sections then was cut in half. Each of the 12 resulting filter pieces was placed in a separate, 7-in. by 12-in. Stomacher® 400 circulator bag, and 90 mL of sterile PBST was added to each bag. Each filter piece was processed with the Stomacher® 400 circulator speed set at 260 rpm for 1 min. All 12 samples were then combined in a 1-L container to make one sample that was sonicated for 10 min and analyzed for spores as described in Section 4.2.1.

4.1.3 RFC Sample Processing

Each RFC sample was transported to the Biolab in a Stomacher® 400 circulator bag containing the filter and dust collected from the dust bin. The filter was aseptically removed and placed in a separate Stomacher® 400 circulator bag, while the dust bin contents remained in the original bag. To each bag, 180 mL of sterile PBST added. The bag then was placed into a 10-in. by 15-in. secondary containment bag, which was put into an orbital shaker incubator (Model 3525, Barnstead, Melrose Park, IL) and agitated at 300 rpm for 30 min. The samples were then aseptically combined into a sterile 1-L bottle. Samples initially were plated in 1-mL aliquots, but background contamination prevented enumeration of *Bg* colonies. Therefore, separate procedures were developed for processing the samples from Stage 1 and Stage 5, as discussed below.

Stage 1 samples posed a challenge because of the low levels of spores and large amount of debris. The procedure developed to enumerate CFU per sample began by shaking the 1-L sample extract bottle, then withdrawing four 20-mL aliquots of “sludge” from the RFC sample extract and filtering each aliquot through a 70-µm cell strainer (352350, Thomas Scientific, Swedesboro, NJ) into a sterile conical tube to reduce the amount of debris. The conical tubes of filtered aliquots then were heat-treated at 80 °C for 20

min, vortexing every 5 min, to reduce background contamination. The samples were allowed to cool to room temperature and then centrifuged at 5,500 times gravity (× g) for 15 min at 4 °C to pellet the debris. The supernatant was filter-plated, and the pelleted debris was resuspended in 10 mL of sterile PBST and spread-plated on TSA plates in 1-mL aliquots. Figure 4-1 summarizes the Stage 1 RFC sample processing. The plates were incubated overnight at 35 °C ± 2 °C, and then the CFU were enumerated by visual inspection.

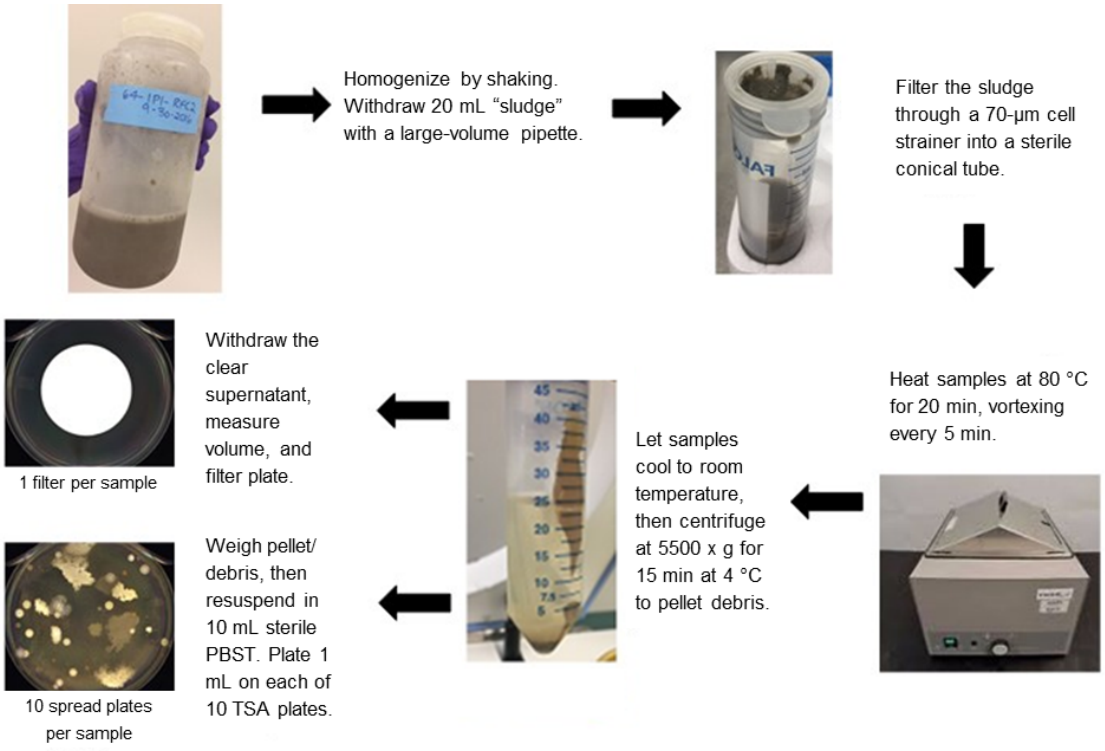

Figure 4-1. Summary of Stage 1 RFC Sample Processing

Stage 5 sample plates showed very high levels of background contamination, making enumeration impossible. From the RFC sample extract, 10-mL aliquots were transferred into 50-mL sterile conical tubes. The tubes were heat treated at 80 °C for 20 min, and then 0.01-mL, 0.02-mL, and 0.04-mL aliquots were plated in triplicate. The plates were incubated at 35 °C ± 2 °C overnight and enumerated by visual inspection. Contamination was present (large, white colonies) after heat treatment as shown in Figure 4-2, but estimated *Bg* counts were performed (small orange colonies).

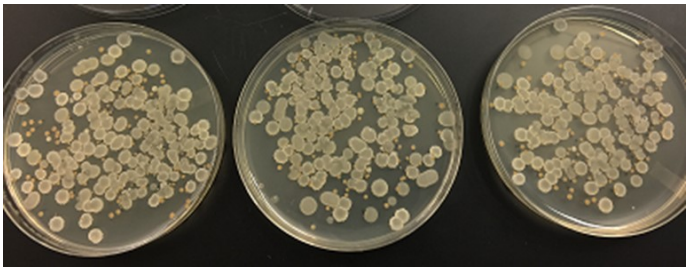

Figure 4-2. Example Plates from Stage 5 RFC Samples

4.1.4 Wet Vacuum Sample Processing

Wet vacuum samples were received in the original wet vacuum containers and aseptically transferred to sterile 1-gallon containers. A procedure was developed for processing the Stage 1 samples to address the challenges of a large sample volume containing large amounts of debris and background contamination while maintaining sensitivity to the low concentration of spores. The Stage 5 wet vacuum samples were spread-plated on TSA plates in 0.1-mL, 0.2-mL, and 0.4-mL aliquots. Because of the high levels of spores in the samples, the CFU were easy to enumerate compared to the Stage 1 samples.

To process the Stage 1 samples, 100 mL of the homogenized sample "sludge" was withdrawn and divided evenly into four 50-mL sterile conical tubes. The samples were heat treated at 80 °C for 20 min, vortexing every 5 min, to reduce background contamination. The samples were allowed to cool to room temperature, then centrifuged at 5,500 × g for 15 min at 4 °C to pellet the debris. The clear supernatant was filter-plated, and the pellet of debris was resuspended in 10 mL of PBST and spread-plated on TSA plates in 1-mL aliquots. Figure 4-3 summarizes the Stage 1 wet vacuum RFC sample processing. The plates were incubated overnight at 35 °C ± 2 °C and then the CFU were enumerated by visual inspection.

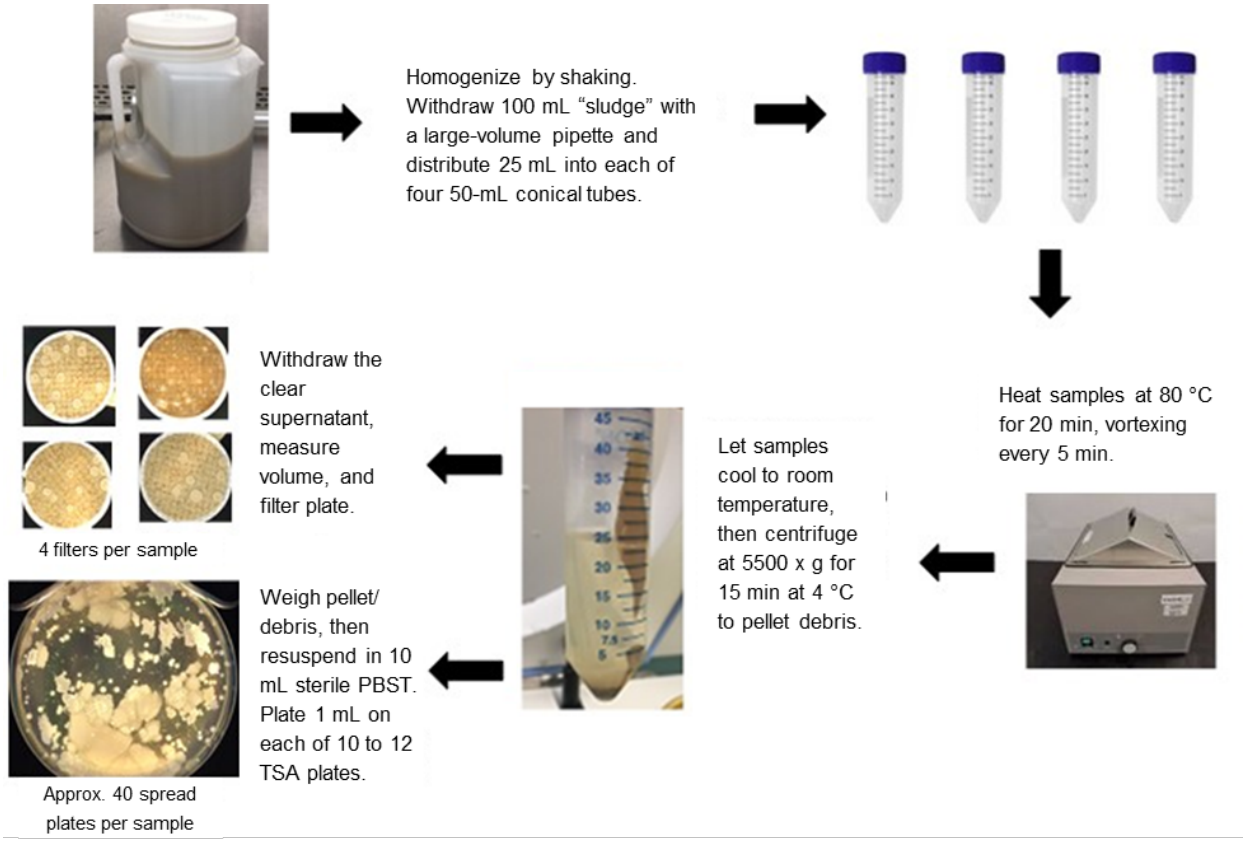

Figure 4-3. Summary of Stage 1 Wet Vacuum Sample Processing

To confirm that colonies observed among wet vacuum spread-plate background contamination were actually *Bg,* several colonies believed to be *Bg* were streak plated (T-streaked) for isolation onto fresh TSA plates and compared to the *Bg* control as shown in Figure 4-4. When the T-streaks were prepared,

occasionally cells from adjacent non-*Bg* colonies were inadvertently picked up in the loop as shown in Figure 4-4 in the lower middle and lower right plates. The small, orange colonies were counted as *Bg*, while the larger, white, doughnut-shaped colonies were not counted.

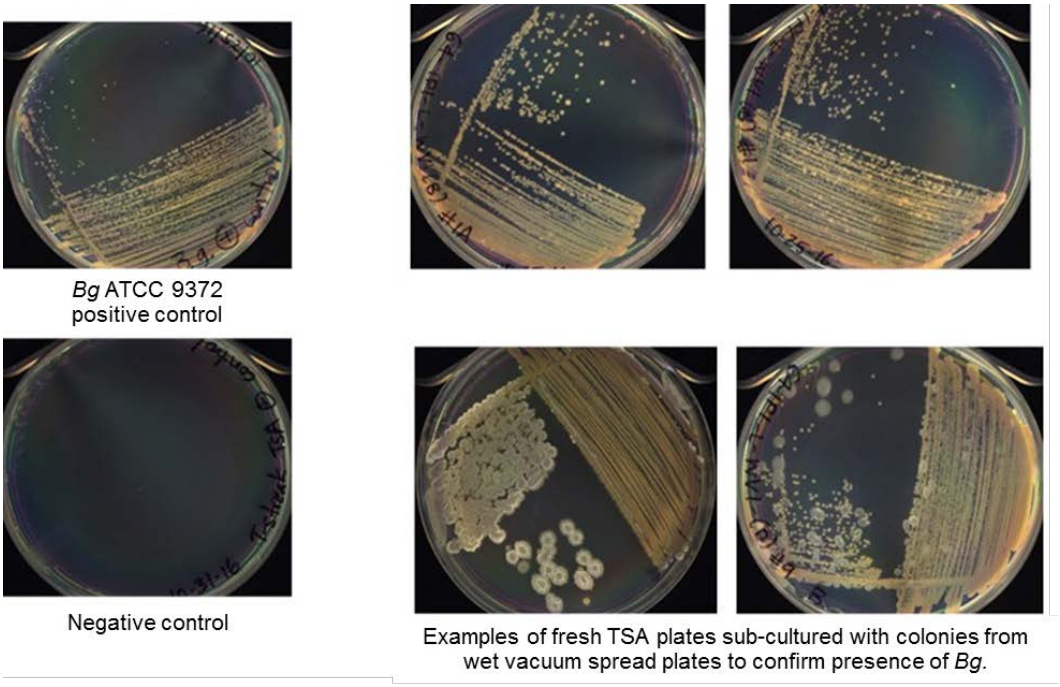

Figure 4-4. Verification of *Bg* Colonies in Wet Vacuum Samples

4.2 Analytical Procedures

Analytical procedures for this project included spore analysis and settling plate analysis as discussed below.

4.2.1 Spore Analysis

Spores were extracted from the samples as described in Section 4.1, and the spores in these extracts were assayed by growth on nutrient agar plates in the Biolab. The samples were analyzed quantitatively for the number of viable spores recovered per sample (CFU).

All sponge stick samples were extracted in Stomacher® 400 circulator bags in 90 mL of sterile PBST for 1 min at 260 rpm. The solution then was pipetted into sterile specimen cups and sonicated for 10 min before spiral plating. Reference tube samples collected during the spray-dry deposition process were vortexed for 2 min and sonicated for 10 min before spiral plating.

All sample types were plated in triplicate using a spiral plater (Autoplate® spiral plating system, Advanced Instruments Inc., Norwood, MA), which deposits a known volume of sample in three 10-fold serial dilutions on each plate. Plates were incubated at 35 ± 2 °C for 16 to 24 h for *Bg* and 27 ± 2 °C for 16 to 19 h for *Btk*. During incubation, colonies develop along the lines where the liquid was deposited on the rotating plate in decreasing amounts from the center to the edge of the rotating plate as shown in Figure

4-5). The number of CFU was determined using a QCount® colony counter (Advanced Instruments Inc., Norwood, MA).

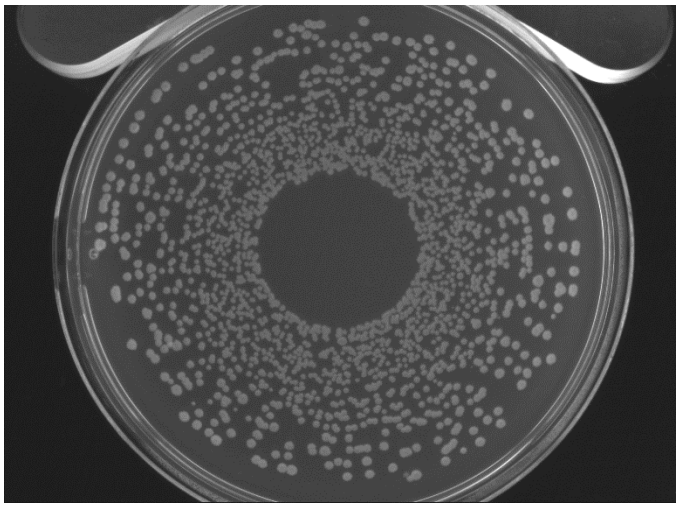

Figure 4-5. Bacterial Colonies on Spiral-Plated Agar Plate

Samples with unknown concentrations were plated with no dilution and with a 100-fold dilution. Samples with known low concentrations were plated with no dilution. The QCount® instrument automatically calculates the CFU/mL in a sample based on the dilution plated and the number of colonies that develop on the plate. This information was recorded in a spreadsheet.

Only plates meeting the threshold of at least 30 CFU were used for spore recovery estimates. After quantitation with the QCount® colony counter, samples with plate results below the 30-CFU threshold were either re-spiral plated with a more concentrated sample aliquot or filter plated to achieve a lower detection limit. The filter-plate volume was based on the CFU data from the QCount® results. The filters were placed onto TSA plates and incubated at 35 ± 2 °C for 20 to 24 hours before manual enumeration. Plates overgrown with indistinguishable colonies were re-spiral plated using a less concentrated aliquot.

When less than 30 CFU per plate were counted for a sample spiral plated with a neat (undiluted) aliquot, then one of the following two methods was used:

1. Spread plate an undiluted aliquot with a larger volume (0.1, 0.2, and 0.4 mL, each in triplicate)
2. Filter plate an undiluted aliquot with a larger volume (such as 1, 2, or 10 mL)

Filter plating was performed using the Pall MicroFunnel unit with 0.45-µm GN-6 Metricel white membrane (P/N 4804, Pall Corporation, Port Washington, NY). The sample aliquot was added to 10-mL of DI water, which then was poured over the filter. The vacuum system was opened and the liquid funneled through the filter, trapping the spores on the filter. The filter then was washed with another 10-mL aliquot of sterile DI water, removed from the plastic housing, and placed onto a TSA plate. Plates were incubated at 35 ± 2 °C for 16 to 24 h for *Bg* and 27 ± 2 °C for 16 to 19 h for *Btk* before manual enumeration.

4.2.2 Settling Plate Analysis

An incubator (Model 1555, VWR, Radnor, PA) was transported to a field trailer used by UTR-OTD personnel. The settling plates were incubated overnight at 37 °C ± 2 °C and enumerated on site. The number of CFU observed on half of each plate was counted and recorded. To estimate total growth on each plate, the recorded value was multiplied by 2. This approach was used to increase the efficiency of CFU counting, as plates had high CFU counts, and CFU were spatially homogeneous across the plate surface.

Settling plate counts, in CFU, were used to generate heat maps of spore settling after Stage 3 AA sampling with hot spot contamination. Settling plate results were interpolated using a Kriging method to estimate the distribution of contamination in the subway. Kriging is an interpolation technique used to predict values for locations that lack sample data. Specifically, this method assumes that the distance or direction between sampling points reflects a spatial correlation that can be used to explain variations in the surface. This approach has great potential in identifying hotspots and aiding in the understanding of wind flow patterns and decontamination efficacy for biological incidents.

4.3 Flow Measurement

DFU and NAM flow measurement procedures are discussed below.

4.3.1 DFU Flow Measurement

The flow rate of each DFU was measured in the laboratory as summarized below.

1. A tube with diameter identical to that of the DFU cartridge holder assembly was firmly attached to the top of the DFU inlet.
2. A thermal anemometer (Series 471, Dwyer, Michigan City, IN) was inserted into the side of the tube perpendicular to the flow direction.
3. The DFU was turned on, and velocity measurements were manually recorded.
4. The flow rate was calculated by multiplying the velocity by the cross-sectional area of the sampling tube.

The flow rate of each DFU was measured in the field at FAPH as summarized below.

1. A rotating-vane anemometer (Model DA 410, Pacer Instruments, Keene, NH) was placed on top of the DFU inlet.
2. The velocity measurement was recorded manually.
3. The flow rate was calculated by multiplying the velocity by the cross-sectional area of the DFU inlet.

4.3.2 NAM Flow Measurement

When a NAM and prefilter are used in the field for AA sampling, the pressure drop will increase due to particle loading of the filter, decreasing the sampling flow rate. Although it is not practical to measure the

flow rate in the field, the pressure drop of the filter assembly can easily be measured by attaching a pressure gauge to the filter holder assembly. Generating a pressure drop versus flow rate curve allows direct correlation of the pressure drop recorded in the field to the flow rate.

The NAM flow rate was measured in the laboratory with a prefilter attached to generate a pressure drop versus flow rate curve. The pressure drop of the filter assembly was measured by attaching a Magnehelic gauge (Model 2010, Dwyer, Michigan City, IN) to the filter holder assembly downstream of the prefilter. The velocity then was measured in the 12-in. round duct downstream of the filter using the Dwyer thermal anemometer. The prefilter size was reduced incrementally by masking symmetrical portions of the filter using corrugated plastic cutouts. The pressure drop and duct velocity were recorded for each size opening. The data were entered into a spreadsheet, the flow rate was calculated by multiplying the velocity by the duct cross-sectional diameter, and a curve was generated for the filter. The plan was to record the pressure drop across the NAM prefilter in the field and use the curve to estimate the NAM flow rate. However, this was not possible due to the failure of the plastic ductwork connecting the NAM to the subway tunnel.

5 Results

This section summarizes and discusses the field sampling exercise and laboratory experiment results.

5.1 Field Sampling Exercise Results

AA sampling was conducted in the mock subway tunnel during Stages 2 and 3 of the field sampling exercise at FAPH. Composite surface sampling was performed on the subway platform during Stages 1 and 5, including RFC and wet vacuum sampling. Stage 4 of the field sampling exercise was the overnight settling period when two types of agar plates (100 plates of each type) were placed on the platform and track areas to assess particle settling after Stage 3 AA sampling. The following sections discuss the results for each type of field sampling, followed by a discussion of the resources required to conduct the field sampling exercise.

5.1.1 AA Sampling Results

Samples were collected by nine DFU samplers along the length of the track, one NAM on the north end of the platform, an APS on a cart on the platform, and several Bioaerosol Button Samplers worn by personnel (personal samples) and mounted to a cart on the platform (area sample). The following sections discuss the results for each type of sampler, followed by a summary of AA sampling results.

5.1.1.1 DFU Sampling Results

Analysis of data from the DFU samplers was the primary means of quantifying the amount of viable *Bg* spores aerosolized during each AA sampling stage. Table 5-1 summarizes the microbiological results and operating conditions of each DFU sampler deployed for post-decontamination AA sampling during Stage 2. Table 5-2 summarizes the results for the DFU samplers deployed during Stage 3, simulating an area with a large amount of contamination concentrated in distinct hot spots. The average air concentration of spores in CFU per cubic meter (CFU/m^3) was calculated by dividing the total CFU recovered from the two filters by the volume of air sampled (average flow rate multiplied by sampling time). This average concentration was calculated for each DFU unless there were missing data, and an overall average spore air concentration was calculated for each stage.

Comparison of the total CFU recovered from the DFU filters and the average calculated air concentration in CFU/m^3 between Stages 2 and 3 shows that the *Bg*-inoculated hot spots significantly impacted DFU spore recovery. The Stage 2 post-decontamination AA sampling overall spore air concentration was estimated at 200 CFU/m^3. The overall spore air concentration for Stage 3 AA sampling with hot spot contamination was estimated at 1.6×10^5 CFU/m^3, three orders of magnitude higher than the Stage 2 result. Based on the estimated spore load of the 24 inoculated hot spot coupons of 1.2×10^9 CFU and the subway tunnel volume of approximately 4,450 m^3 (157,000 ft^3), the theoretical spore air concentration if all spores in the hot spots were aerosolized and perfectly mixed in the tunnel would be 2.7×10^5 CFU/m^3. This result could lead one to estimate that the fraction of hot spot spores aerosolized during Stage 3 was 0.61. However, it is not realistic to assume that particles greater than 1 µm in diameter would be well mixed in such a large volume. Therefore, 0.61 is an upper limit of the fraction of spores aerosolized from the hot spots. A more reasonable assumption is that during AA sampling, spores are well mixed within 2 meters (m) of the floor. Then, the effective volume would be approximately 2,000 m^3. The theoretical

spore air concentration if all spores in the hot spots were aerosolized and perfectly mixed in the lower 2 m of the subway tunnel would be 6.1×10^5 CFU/m^3, resulting in a more reasonable estimate of the fraction of hot spot spores aerosolized in Stage 3 of 0.27.

Table 5-1. DFU Filter Results from Stage 2 AA Sampling (without Hot Spot Contamination)

Stage 2 DFU Location*	Filter 1 (CFU/mL)	Filter 2 (CFU/mL)	Filter 1+Filter 2 (CFU/mL)	Total Spores (CFU)	Average Flow Rate (m^3/min)	Sample Time (min)	Average Air Concentration (CFU/m^3)
DFU01	84	229	312	1.3×10^4	0.40	167	**190**
DFU02	194	110	304	1.2×10^4	0.43	164	**170**
DFU03	184	93	277	1.1×10^4	0.53	163	**130**
DFU04**	26	53	79	3.2×10^3	0.54	159	**37**
DFU05	10	6	16	6.6×10^2	0.49	156	**8.7**
DFU06	70	61	130	5.2×10^3	0.39	153	**88**
DFU07	174	81	255	1.0×10^4	0.54	150	**130**
DFU08	165	191	356	1.4×10^4	***	Not available	Not available
DFU09	899	851	1,750	7.0×10^4	0.57	143	**860**
					Overall Average	**157 min**	**200 CFU/m^3**

Notes:
* Locations listed south to north
**Unplugged for 1.5 min during sampling period
***Abnormality observed on video; total flow during sampling could not be determined

Table 5-2. DFU Filter Results from Stage 3 AA Sampling (with Hot Spot Contamination)

Stage 3 DFU Location*	Filter 1 (CFU/mL)	Filter 2 (CFU/mL)	Filter 1+Filter 2 (CFU/mL)	Total Spores (CFU)	Average Flow Rate (m^3/min)	Sample Time (min)	Average Air Concentration (CFU/m^3)
DFU01	1.3×10^3	1.2×10^3	2.5×10^3	1.0×10^5	**	15	Not available
DFU02	5.5×10^4	5.2×10^4	1.1×10^5	4.3×10^6	0.56	41	**1.9×10^5**
DFU03	3.7×10^4	4.2×10^4	7.9×10^4	3.1×10^6	0.52	42	**1.4×10^5**
DFU04	3.2×10^4	6.0×10^4	9.2×10^4	3.7×10^6	0.50	42	**1.8×10^5**
DFU05***	3.0×10^4	2.5×10^4	5.6×10^4	2.2×10^6	0.51	34	**1.3×10^5**
DFU06	3.1×10^4	4.0×10^4	7.1×10^4	2.8×10^6	0.48	43	**1.4×10^5**
DFU07	2.8×10^4	3.3×10^4	6.1×10^4	2.4×10^6	0.47	43	**1.2×10^5**
DFU08	5.8×10^4	5.9×10^4	1.2×10^5	4.7×10^6	0.55	44	**1.9×10^5**
DFU09	5.2×10^4	4.1×10^4	9.3×10^4	3.7×10^6	0.37	43	**2.3×10^5**
					Overall Average	**42 min**	**1.6×10^5 CFU/m^3**

Notes:
* Locations listed south to north
**Unit lost power after 15 min; no second flow measurement
***Original DFU failed; inlet swapped to new DFU unit

The inlet velocity of each DFU sampler was measured at the beginning and end of the sampling time, and during Stage 2, an additional measurement was taken for most DFUs. The volumetric flow rate was calculated by multiplying the inlet velocity measured in the field by the cross-sectional area of the DFU inlet. Figure 5-1 shows a plot of measured flow rate vs. sampling time. The average of the beginning and ending flow rates for each DFU (in Tables 5-1 and 5-2) was used to calculate the average air concentration for each DFU sample.

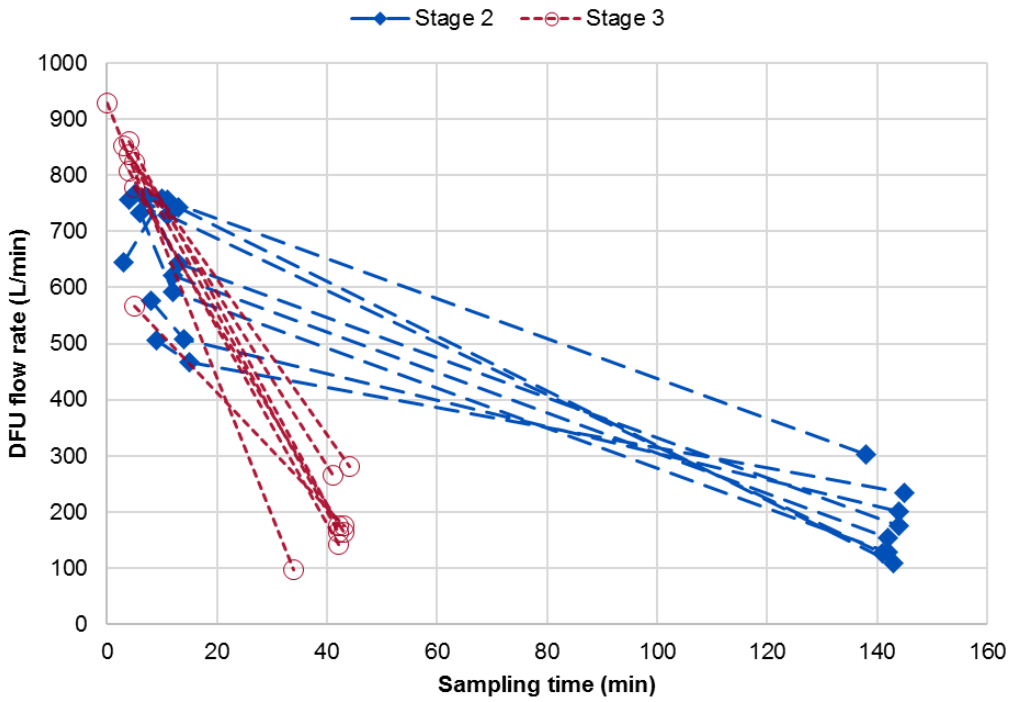

Figure 5-1. Starting and Ending DFU Flow Rates as Function of Sample Time for Stages 2 and 3

5.1.1.2 NAM Prefilter Sampling Results

Table 5-3 presents the limited amount of NAM prefilter data. The plastic duct connecting the subway tunnel to the NAM collapsed partially during both Stage 2 and Stage 3 sampling. Therefore, the sampling flow rate and time could not be determined for either stage.

Table 5-3. NAM Prefilter Results from Stage 2 AA Sampling (without Hot Spot Contamination) and Stage 3 AA Sampling (with Hot Spot Contamination)

Stage	Sampling Time (min)	Filter Piece 1 (CFU /mL)	Filter Piece 2 (CFU /mL)	Filter 1 + Filter 2 (CFU/mL)	Total Spores (CFU)
Stage 2	128	364	328	691	1.2×10^5
Stage 3	20	1.3×10^4	2.0×10^4	3.2×10^4	5.8×10^6

The NAMs used for the UTR-OTD were located on the upper level of the subway station, and a flexible plastic duct was used to connect the inlet of each NAM to an opening in one of the barriers constructed on the stairways to seal off the subway tunnel. However, the flexible plastic ducts are designed for use on

the outlet of the NAM rather than the inlet and are prone to collapse under even a modest pressure drop between the duct inlet and the NAM inlet. If the NAM is used for sampling in future work, the NAM unit should be placed as close as possible to the sampling area, and all connections to the inlet should be constructed of flexible aluminum ductwork. The Stage 3 NAM prefilter collection results are an order of magnitude higher than the Stage 2 results. However, without flow rate information, no real comparison can be made between the results for each stage.

5.1.1.3 APS Sampling Results

Figure 5-2 shows the Stage 2 and 3 results for the APS on a cart on the subway platform plotted as total particle concentration vs. time. The data are color-coded by activity, and the beginning and end of each AA sampling round are indicated.

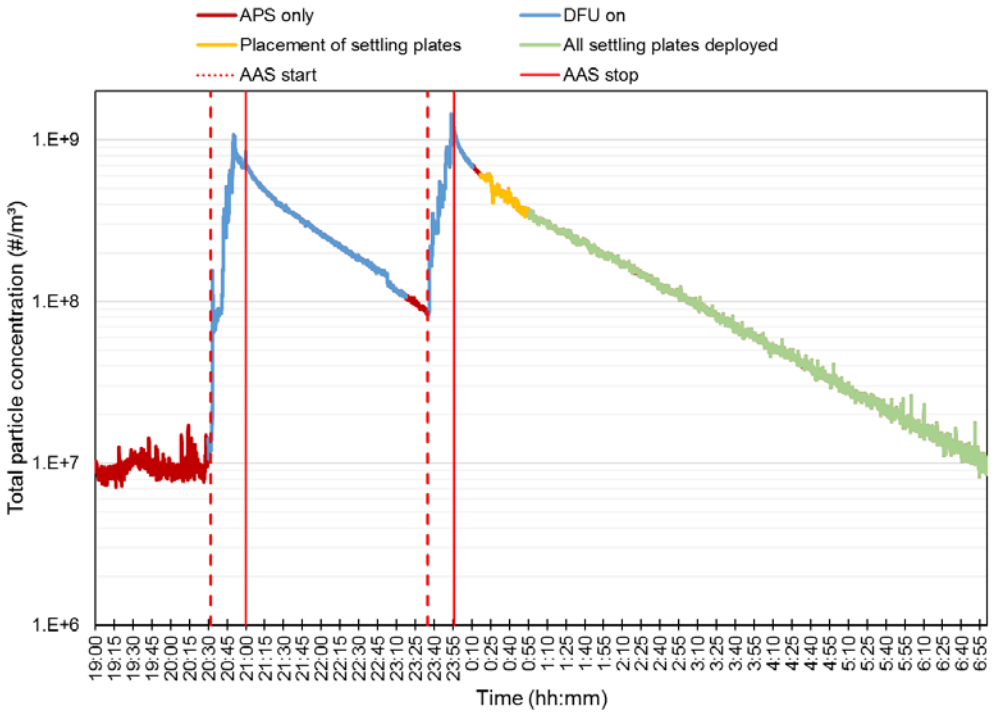

Figure 5-2. APS-Measured Total Particle Concentration vs. Time for Stages 2 and 3

The APS-measured concentration increased rapidly at the start of each AA sampling round, then decreased more slowly as particles were filtered out by samplers and ventilation NAMs and settled out of the air. Figure 5-3 shows the average measured particle size distribution at 20:45 during Stage 2 AA sampling, with a mass median aerodynamic diameter (MMAD) of 1.72 µm and geometric standard deviation (GSD) of 1.9. The average measured particle size distribution at 23:45 during Stage 3 AA sampling was nearly identical, with an MMAD of 1.68 µm and GSD of 1.9. The APS does not indicate the nature of the particulates, so no conclusions can be drawn from these data regarding the number of viable spores.

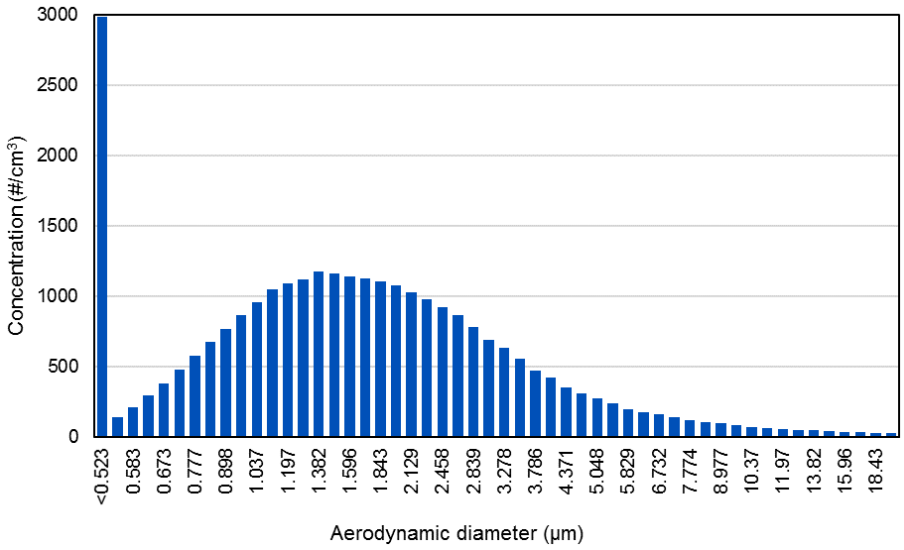

Figure 5-3. APS-Measured Particle Size Distribution at Time 20:45

5.1.1.4 Bioaerosol Button Sampling Results

Table 5-4 summarizes the Bioaerosol Button Sampler data from Stage 2 and Stage 3.

Table 5-4. Bioaerosol Button Sampler Results from Stages 2 and 3

Stage	Task	Spore Count (CFU)	Air Volume Sampled (m^3)	Spore Air Concentration (CFU/m^3)
2	Cart	82	1.37	60
	Leaf blower - platform	15	0.511	29
	Leaf blower - tracks north	1	0.508	2
	Leaf blower - tracks south	123	0.496	248
3	Cart	3.8×10^4	2.52	1.5×10^4
	Leaf blower - platform	2.3×10^3	0.324	7.0×10^3
	Leaf blower – tracks north	5.9×10^3	0.453	1.3×10^4
	Leaf blower – tracks south	2.1×10^5	0.460	4.6×10^5

There was large variability in the number of spores recovered from samplers worn by the different leaf blower operators and the sampler mounted on the cart. This variation is common for personal sampling and not surprising given that the operators were moving around the tunnel while operating the leaf blowers and wearing Level C personal protective equipment (PPE) (powered air-purifying respirators [PAPRs], hooded chemical-resistant coveralls, and two layers of chemical-resistant gloves). Even though the workers were conducting the same general task, the work practices, turbulent air movement, and potential clothing interference likely led to the high variability.

Table 5-5 summarizes the Bioaerosol Button Sampler data from Stage 5 during Stage 5 RFC and wet vacuum sampling. These results are comparable to the Stage 2 and 3 results presented in Table 5-4. The similarity in results demonstrates that most of the spores aerosolized during Stage 3 AA sampling settled out of the breathing zone during the Stage 4 overnight settling period.

Table 5-5. Bioaerosol Button Sampler Results from Stage 5

Stage	Task	Spore Count (CFU)	Air Volume Sampled (m^3)	Concentration Sampled (CFU/m^3)
5	RFC and wet vacuum	29	0.48	60
		135	0.48	281

5.1.1.5 Summary of AA Sampling Results for Stages 2 and 3

Table 5-6 summarizes the AA sampling results for Stages 2 and 3. The average spore collection and calculated air concentration results for each sampling method increased by orders of magnitude from Stage 2 to Stage 3 due to the presence of hot spot contamination. It is interesting that no Stage 2 post-decontamination AA sampling results are non-detect for *Bg* spores. Even after the tunnel was decontaminated by fogging with bleach, significant numbers of viable *Bg* spores remained on the surfaces. This Stage 2 AA sampling result is consistent with the RFC and wet vacuum sampling results in terms of detecting viable spores. However, there is a possibility that the prepositioned hotspots, even with multiple covers, may have introduced the spores during Stage 2 AA sampling. The hotspots were secured with three different layers and introduced right before Stage 2. Leaf blowing directly to the hotspot containers may have caused resuspension of spores. The results are inconclusive whether the spores were from the incomplete decontamination or the cross contamination from hotspots.

Table 5-6. Average CFU Collected and Average Calculated Spore Air Concentration for Stages 2 and 3

Sampler	Total Collection (CFU)				Spore Air Concentration (CFU/m^3)			
	Stage 2		Stage 3		Stage 2		Stage 3	
	Average	SD	Average	SD	Average	SD	Average	SD
DFU*	1.6×10^4	2.2×10^4	3.4×10^6	8.7×10^5	1.3	1.9	4.0×10^3	8.4×10^2
NAM**	1.2×10^5	5.8×10^6	Not available	Not available	NAM**	1.2×10^5	5.8×10^6	Not available
Button***	55	57	6.4×10^4	9.9×10^4	85	110	1.2×10^5	2.2×10^5

* DFU results are summarized from Tables 5-1 and 5-2. DFU02 in Stage 2 and DFU01 in Stage 3 experienced failures during sampling, and associated results are excluded from this data summary. The averages are for eight DFU samples per stage.
** The NAM duct collapsed partially during sampling. Therefore, flow rate and sampling time could not be determined. The averages are for one sample per stage.
*** Button sampler results are summarized from Table 5-4. The averages are for four samples per stage.

5.1.2 RFC and Wet Vacuum Sampling Results

To better understand the operational parameters of the RFCs in the field sampling exercise, operating times and surface area data were collected. Table 5-7 summarizes the operating times by unit and round.

Table 5-7. RFC Operating Time

Stage	RFC Location	Start Time	End Time	Sample Duration (min)
1	1	17:16	Not available*	Not available
	2	17:22	Not available*	Not available
	3	17:25	Not available*	Not available
5	1	19:57	20:19	22
	2	19:59	20:24	25
	3	20:01	20:50	49

*End time not recorded because personnel on required rest break and RFCs not in view of CCTV cameras

A geographic information system (GIS) was used to estimate the RFC travel distance and sampled surface area. The RFC path was traced by referencing on-site CCTV footage and was projected onto a two-dimensional diagram representing the subway platform. A buffer approximately 1 ft wide was applied to the path to determine the sampled surface area. Figure 5-4 shows the start and end location, path, and sampled surface area for RFC Location 2 during Stage 5. The unit sampled approximately half of the study area (460 of 1,000 ft^2) in 23 min, traveling 8,000 ft. Because of the complexity of this task, only the path and sampling area of the RFC at Location 2 during Stage 5 were simulated.

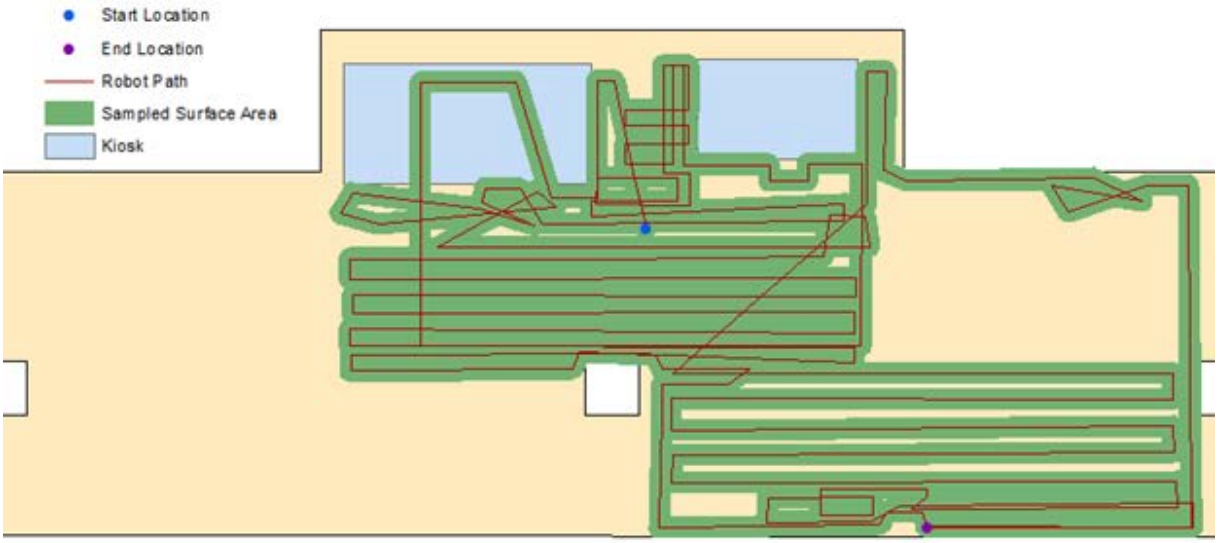

Figure 5-4. Stage 5 Sampling Path and Area for RFC Location 2

Recoveries from Stage 1 sampling were non-detect for spread plating but were re-evaluated through aliquot processing as described in Sections 4.1.3 and 4.1.4. *Bg* spores were detected from all 6 RFC and wet vacuum platform samples. The recoveries (Table 5-8) of these sample aliquots were 3 - 4 CFUs per ft^2 and 2 - 24 CFUs per ft^2 for RFC and wet vacuum samples, respectively. The total coverage of sampling using RFC and wet vacuum was approximately 1750 ft^2 with 6 samples. The traditional surface sampling using 37 mm cassettes was conducted on the platform prior to the Stage 1 composite sampling. A total of 49 samples were taken and 3 samples came back positive with 4 – 6 CUFs per ft^2 (EPA 2017). The total sampling coverage using 37 mm cassettes was approximately 49 ft^2 with 49 samples. This result shows how new composite sampling methods can improve the detection capability especially post decontamination sampling and reduce the number of samples, time, and labor compared to the traditional sampling methods during a wide area response.

The detected spore levels from Stage 1 post-decontamination composite sampling (Table 5-8) were negligible compared to levels from Stage 5 sampling after AA sampling with hot spot contamination (Table 5-9). This confirms that Stage 3 AA sampling distributed spores from hotspots throughout the test area. Spore recoveries from Stage 5 were three orders of magnitude greater than recoveries from post-decontamination sampling because of the aggressive agitation of the hot spots with leaf blowers and subsequent distribution of spores throughout the subway tunnel.

Table 5-8. RFC and Wet Vacuum Sampling Results from Stage 1 (Post-decontamination)

Stage	Sampling Technique	Location Sampled	CFU Count per Sampled Area	Location Area (ft^2)	Estimated Area Sampled (ft^2)	Sampling Time (min)	Recovery (CFU/ft^2)
1	RFC	1	1.9×10^3	900	450	Not available*	4
		2	1.4×10^3	900	450	Not available*	3
		3	1.8×10^3	900	450	Not available*	4
	Wet Vacuum	1	3.8×10^3	175	156	8	24
		2	2.2×10^2	175	90	5	2
		3	3.7×10^3	175	175	11	21
* Run time for Stage 1 RFCs could not be determined.							

Table 5-9. RFC and Wet Vacuum Sampling Results from Stage 5 (Post-AA Sampling with Hot Spot Contamination)

Stage	Sampling Technique	Location Sampled	CFU Count per Sampled Area	Location Area (ft^2)	Estimated Area Sampled (ft^2)	Sampling Time (min)	Recovery (CFU/ft^2)
5	RFC	1	2.1×10^6	900	450	29	4.7×10^3
		2	2.7×10^6	900	450	25	6.0×10^3
		3	2.9×10^6	900	450	48	6.4×10^3
	Wet Vacuum	1	4.2×10^6	175	156	12	2.7×10^4
		2	1.5×10^6	175	90	4	1.7×10^4
		3	3.2×10^6	175	175	10	1.8×10^4

As Table 5-10 shows, much more debris was collected by the RFCs during Stage 1 than Stage 5, most likely because the leaf blowers operating during Stages 2 and 3 had swept a large amount of the debris toward the walls of the platform and onto the track. In addition, vacuum-based devices such as RFCs are known to cause a small but detectable amount of dust resuspension, presumably due to the presence of surface agitation brushes, which also may have contributed to the RFCs collecting less debris during Stage 5.

Table 5-10. Debris Recovered from RFC Samples

Debris Weight	Stage 1			Stage 5		
	RFC1	RFC2	RFC3	RFC1	RFC2	RFC3
Debris weight* (g)	219.5	129.2	176.5	43.5	81.1	57.6
*Debris was saturated, and some debris may have been lost during sample processing and transfer.						

The same pattern of debris collection was not seen with the wet vacuums (Table 5-11).

Table 5-11. Sample Volume and Debris Recovered from Wet Vacuum Samples

Sample Volume and Debris Weight	Stage 1			Stage 5		
	WV1	WV2	WV3	WV1	WV2	WV3
Sample volume* (mL)	3,045	2,150	2,587	2,760	1,806	2,258
Debris weight (g)	467	308	319	311	183	332
*Sample was weighed; assumed 1 g = 1 mL.						

The amount of debris collected by the wet vacuums was nearly identical for Stages 1 and 5. The wet vacuum sampling area was at the back of the subway platform, and the leaf blowers may have swept part of the debris towards the back. Because the wet vacuums used a surfactant liquid to sample the floor, dust was more easily suppressed and vacuumed compared to the dry-vacuum based RFCs. This situation is evident in the recovery efficiencies summarized in Tables 5-12 and 5-13, which show that the wet-vacuum cleaners recovered, on average, 360% more spores than the RFCs.

Table 5-12. RFC and Wet Vacuum Sampling Results from Stage 1 (Post-decontamination)

Stage	Sampling Technique	Location Sampled	CFU Count per Sampled Area	Location Area (ft^2)	Estimated Area Sampled (ft^2)	Sampling Time (min)	Recovery (CFU/ft^2)
1	RFC	1	1.9×10^3	900	450	Not available*	4
		2	1.4×10^3	900	450	Not available*	3
		3	1.8×10^3	900	450	Not available*	4
	Wet Vacuum	1	3.8×10^3	175	156	8	24
		2	2.2×10^2	175	90	5	2
		3	3.7×10^3	175	175	11	21
* Run time for Stage 1 RFCs could not be determined.							

Table 5-13. RFC and Wet Vacuum Sampling Results from Stage 5 (Post-AA Sampling with Hot Spot Contamination)

Stage	Sampling Technique	Location Sampled	CFU Count per Sampled Area	Location Area (ft^2)	Estimated Area Sampled (ft^2)	Sampling Time (min)	Recovery (CFU/ft^2)
5	RFC	1	2.1×10^6	900	450	29	4.7×10^3
		2	2.7×10^6	900	450	25	6.0×10^3
		3	2.9×10^6	900	450	48	6.4×10^3
	Wet Vacuum	1	4.2×10^6	175	156	12	2.7×10^4
		2	1.5×10^6	175	90	4	1.7×10^4
		3	3.2×10^6	175	175	10	1.8×10^4

The results for this study show that currently available wet vacuum cleaners and RFCs can systematically sample large contaminated areas. Two benefits of using wet vacuum cleaners for wide area sampling instead of the currently used sampling methods include (1) collection of fewer samples because one sample is generated per deployment and (2) less risk of personnel exposure to *Ba* spores because wetting reduces spore aerosolization. In addition to the advantage of wide area sampling, the wet vacuums have hand tools that can be deployed to sample areas where sampling is difficult, such as staircases and between furniture and other obstacles. However, for real-world application, wet vacuums require further evaluation with regard to various surfaces, spore concentrations, and environmental conditions (such as RH, exposure duration, high amounts of debris with animal remains, background contamination, etc.)

For the RFCs, the current test method focused only on the sampling mechanism of the individual RFCs by limiting the sampling surface area. Varying the area cleaning logistics or the algorithms of the RFCs was not part of this study. However, varying these parameters could increase the collection efficiency of RFCs for wide area sampling.

5.1.3 Settling Plate Results

Figures 2-15 and 2-16 show the sampling locations of the TSA and Brilliance™ agar settling plates for the platform and tracks, respectively. There were 52 settling plates of each type on the platform and 48 plates of each type on the tracks, arranged in a square grid pattern with an approximate spacing of 1 ft between locations. Separate heat maps of settled spores were generated for each plate type and sampling area combination using a simple kriging method. Prediction errors (Table 5-14) show a relatively high confidence in the performance of all four kriging models, evidenced by the root-mean-square standardized error (ratio of root-mean-square error to average standard error) being close to 1.

Table 5-14. Kriging Prediction Error by Agar Plate Type and Location

Location	Plate Type	Average Standard Error	Root-Mean-Square	Root-Mean-Square Standardized
Platform	TSA	85	63	0.7
	Brilliance™	91	74	0.8
Ballast	TSA	41	44	1
	Brilliance™	53	52	0.9

Figures 5-5 and 5-6 show the separate heat maps generated for the combined surface area (platform and track) from the TSA and Brilliance™ agar settling plate counts, respectively. A total of 10 data bins were manually defined, each representing 50 CFU. Although the kriging equations represent the best linear, unbiased predictor for unsampled locations, the resulting data points are not bound to minimum or maximum values, which can result in gradients that disagree with the input dataset. However, this is a widely accepted anomaly associated with kriging.

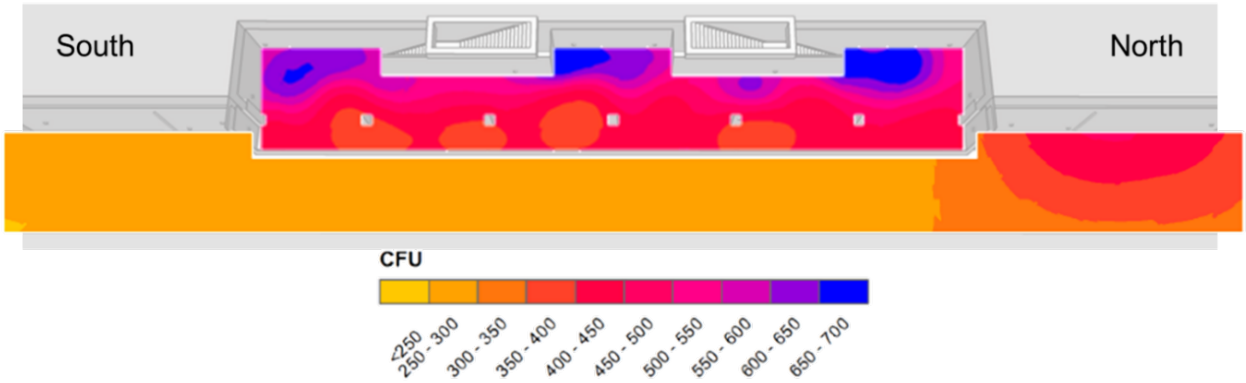

Figure 5-5. Heat Map of Spore Settling Generated from TSA Plate Counts

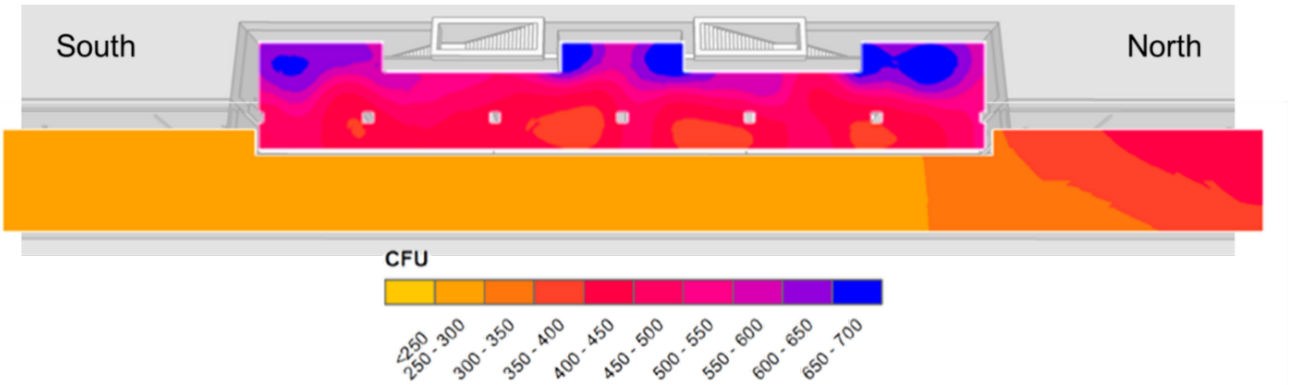

Figure 5-6. Heat Map of Spore Settling Generated from Brilliance™ Agar Plate Counts

A one-sample t-test of the difference between collocated agar plates (number of samples = 98) revealed no statistically significant difference between the TSA and Brilliance™ agar settling plates (p-value of 0.067). They show a pattern of higher spore settling on the platform than the tracks, particularly in the kiosk area and at the bottom of each staircase. The results also suggest a higher concentration of spores at the north end of the track (to the right in Figures 5-5 and 5-6).

The settling plate heat map results support the theory that the leaf blowers swept a large amount of debris towards the back of the platform where the wet vacuum cleaners were deployed. The wet vacuum cleaners suppress dust more easily than the RFCs by dispensing surfactant on the sampling floor. This sampling process is similar to the well-established wet-wipe surface sampling method because both methods use a wetting agent to recover spores. The wet vacuum spore recoveries were comparable to the extrapolated CFU/ft^2 results from the settling plates for the same section of the platform as the results summarized in Table 5-15. The spore counts from settling plates are lower than the ones from wet vacuum samples. This might be because the settling plates were distributed a couple of hours after the AA sampling as seen in Figure 5-2, which the significant portion of resuspended spores might have settled prior to the settling plate distribution.

Table 5-15. Settling Plate Comparison to Stage 5 Wet Vacuum Recovery.

Location	Settling Plate Type	Area (ft^2)	Spore Count (CFU)	Average Recovery (CFU/ft^2)	Notes
WV1	TSA	189	1.9×10^6	1.0×10^4	Extrapolated point data
		162	1.6×10^6	1.0×10^4	
	Brilliance™	189	1.9×10^6	1.0×10^4	
		162	1.7×10^6	1.0×10^4	
	Wet vacuum	156	4.2×10^6	2.7×10^4	Sampled area estimated
WV2	TSA	189	2.0×10^6	1.0×10^4	Extrapolated point data
		162	1.7×10^6	1.0×10^4	
	Brilliance™	189	2.0×10^6	1.1×10^4	
		162	1.7×10^6	1.1×10^4	
	Wet vacuum	175	3.2×10^6	1.8×10^4	Sampled area estimated

5.1.4 Resources Required for Field Sampling Exercise

To determine the resources required to conduct composite sampling during the field sampling exercise, each stage of the field exercise was broken up into activities, and then the time and number of people required for each activity was estimated. Table 5-16 summarizes the results, along with estimates of the area or volume sampled as appropriate. Because the flow rate of the NAM could not be determined during Stages 2 and 3, it is not included in the estimates of sampled volume for AA sampling.

Table 5-16. Time and Area Sampled by Field Sampling Exercise Activity

Stage	Description	Activity	Time (h)	No. of People	Man-hours (h)	Estimated Sampled Area (ft^2)	Estimated Sampled Volume* (ft^3)
1	RFC and wet vacuum sampling, Round 1	Setup	3	2	6	1,771	
		Sampling	2	2	4		
		Close-out	0.5	2	1		
2	AA sampling, Round 1	Initial setup	3	6	18		21,000
		Sample setup	0.5	4	2		
		Sampling	0.5	6	3		
		Extra sampling**	1.5	0	0		
		Close-out	0.5	4	2		
3	AA sampling, Round 2	Sample setup	0.5	4	2		5,800
		Sampling	0.5	6	3		
		Close-out	0.5	3	1.5		
4	Settling plate sampling	Setup	0.5	2	1	17	
		Sampling***	8	0	0		
		Close-out	0.5	2	1		
5	RFC and wet vacuum sampling, Round 2	Setup	1	2	2	1,771	
		Sampling	1	2	2		
		Close-out	1	2	2		
Rest breaks			3.5	6	21		
				Total	**72**	**3,559**	**27,200**

*Sampled volume does not include NAM sampling
**Samplers running while personnel took a rest break
***Settling plates left out overnight

5.2 Laboratory Experiment Results

This section discusses the DFU filter loading evaluation, NAM prefilter comparison, and forced aerosolization evaluation results for the laboratory experiments.

5.2.1 DFU Filter Loading Evaluation Results

Measurements of DFU inlet velocity and filter weight from tests with nine different DFU samplers were entered in a spreadsheet, and the DFU inlet cross-sectional area was used to convert the inlet velocity to volumetric flow rate. When the change in DFU flow rate was plotted as a function of dust loading, there appeared to be a linear relationship within the range of the data points. Therefore, regression analysis was performed using Microsoft Excel. The regression analyses for the individual data sets reported R^2 values above 0.90, p-values in the 10^{-8} range, and significance F-values in the 10^{-8} range, indicating that for the individual data sets collected, the regressions were linear over the range of the data. The data from the nine DFU samplers were combined, and the same regression analysis was performed. Figure 5-7 shows the results, which provided a slope coefficient value of -428, meaning for every gram of dust accumulated on the DFU filters, the flow rate reduced by 428 L/min. The R^2 value was 0.93, the p-v

alues were in the 10^{-26} and 10^{-19} range, and the significance F-values in the 10^{-19} range, indicating that the slope is non-zero and that there is confidence in the calculated regression values.

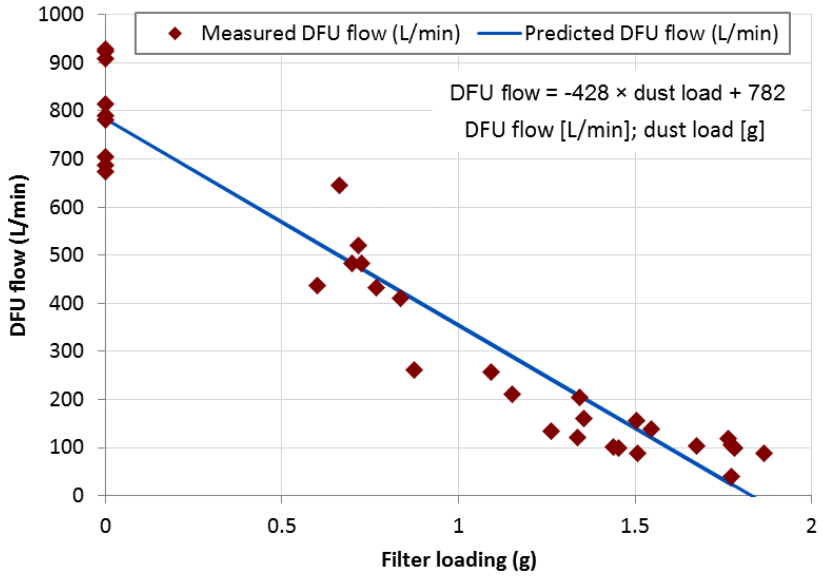

Figure 5-7. DFU Filter Dust Load vs. DFU Flow Rate

This relationship between DFU dust load and flow rate can be applied to the field sampling data to estimate the amount of dust collected by each DFU sampler during each stage of the field sampling exercise. Table 5-17 summarizes the sampling duration, measured end flow rate of each DFU sampler, and estimated dust load by stage.

Table 5-17. Estimated Field Sampling Exercise DFU Dust Load

Sampling Location	Stage 2			Stage 3		
	Sampling Duration (min)	End Flow Rate (L/min)	Estimated Dust Load (g)	Sample Duration (min)	End Flow Rate (L/min)	Estimated Dust Load (g)
DFU 1	167	233	1.3	15	N/A	N/A
DFU 2	164	201	1.4	41	265	1.2
DFU 3	163	176	1.4	42	176	1.4
DFU 4	159	109	1.6	42	165	1.4
DFU 5	156	129	1.5	34	97	1.6
DFU 6	153	154	1.5	43	143	1.5
DFU 7	150	126	1.5	43	165	1.4
DFU 8	Not available	Not available	Not available	44	280	1.2
DFU 9	143	302	1.1	43	176	1.4
Average	157	179	1.4	42	183	1.4

The average end flow rates and estimated dust loads for Stages 2 and 3 were not significantly different. However, the average sampling duration in Stage 3 was approximately one-quarter the sampling duration in Stage 2, indicating that site conditions should be considered when determining the duration of AA sampling. Under very dusty conditions, the samplers may require change-out part way through the AA sampling to prevent filters from becoming loaded to the point that the samplers do not function properly.

5.2.2 NAM Prefilter Comparison Results

Five types of 14-in. by 20-in. furnace filters were tested to compare their relative spore collection efficiencies. Table 5-18 summarizes the *Bg* spore collection results for each type of filter.

Table 5-18. NAM Prefilter *Bg* Spore Collection Results

Filter Type	Total Extracted (CFU)	Average (CFU)	SD (CFU)	Coefficient of Variation (%)
Filtrete™ 1500 Ultra Allergen	3.1×10^7	3.1×10^7	7.7×10^6	25%
	4.2×10^7			
	3.1×10^7			
	3.1×10^7			
	2.0×10^7			
Filtrete™ 1900 Maximum Allergen	2.1×10^7	2.6×10^7	6.6×10^6	25%
	2.3×10^7			
	2.3×10^7			
	2.6×10^7			
	3.7×10^7			
Filtrete™ 2400 Elite Allergen Extra	3.6×10^7	2.7×10^7	5.6×10^6	21%
	2.6×10^7			
	2.9×10^7			
	2.3×10^7			
	2.2×10^7			
Filtrete™ Basic Flat Panel	1.9×10^6	Entire 14-in. by 20-in. filter processed		
	9.3×10^6			
WEB® Absorber Electrostatic Carbon Filter	3.8×10^6			

The Filtrete™ 1500, 1900, and 2400 filters all performed comparably, with a one-way analysis of variance (ANOVA) p-value of 0.49. The spore recoveries for the Filtrete™ Basic Flat Panel and WEB® Absorber Electrostatic Carbon Filter were an order of magnitude lower than for the Filtrete™ 1500, 1900, and 2400 filters. Based on these data, Filtrete™ 14-in. by 20-in. furnace filters with a Filtrete™ rating of 1500 or higher should be used as NAM prefilters for AA sampling.

5.2.3 Forced Aerosolization Results

Laboratory tests were conducted to assess the fraction of spores aerosolized from ballast coupons by a leaf blower. These tests were conducted separately with *Bg* and *Btk* spores for three conditions: high spore loading (1×10^8 CFU/ft^2), low spore loading (1×10^6 CFU/ft^2), and high spore loading with simulated decontamination. Table 5-19 summarizes the results from forced aerosolization tests with *Bg* spores. The average fraction of spores aerosolized from ballast under high loading conditions was 0.34, which is comparable to the estimated 0.27 fraction aerosolized during Stage 3 of the field sampling exercise. The results from the forced aerosolization tests with (relatively) low spore loading showed noticeably more variability (43% coefficient of variation [CV]) than the high loading test results (12% CV). The same trend was observed with the simulated decontamination results (50% CV). As expected, the application of DI water to simulate a wet decontamination method decreased the fraction of *Bg* spores collected during the forced aerosolization tests. Although the fraction reaerosolized differed for the

different conditions, they were all within one order of magnitude. Smaller scale aerosolization tests from other surface types conducted in the same laboratory (EPA 2012b) have shown aerosolization fraction results that spanned several orders of magnitude.

Table 5-19. Forced Aerosolization Results for *Bg*-Inoculated Ballast Coupons

Test	Recovered (CFU)	Recovered – Background (CFU)	Average (CFU)	SD (CFU)	CV (%)	Amount Deposited (CFU)	Fraction Aerosolized
High Loading	5.8×10^7	5.8×10^7	5.9×10^7	7.4×10^6	12%	1.8×10^8	0.34
	5.4×10^7	5.4×10^7					
	5.1×10^7	5.1×10^7					
	6.6×10^7	6.6×10^7					
	6.8×10^7	6.7×10^7					
Low Loading	8.2×10^5	8.1×10^5	1.0×10^6	4.4×10^5	43%	1.6×10^6	0.65
	8.2×10^5	8.0×10^5					
	1.8×10^6	1.8×10^6					
	7.7×10^5	7.6×10^5					
	9.0×10^5	8.9×10^5					
Simulated Decontamination	4.8×10^6	4.8×10^6	1.2×10^7	6.1×10^6	50%	1.8×10^8	0.07
	1.1×10^7	1.1×10^7					
	1.5×10^7	1.5×10^7					
	2.1×10^7	2.1×10^7					
	1.0×10^7	1.0×10^7					

Table 5-20 summarizes the results from forced aerosolization tests with *Btk* spores.

Table 5-20. Forced Aerosolization Results for *Btk*-Inoculated Ballast Coupons

Test	Recovered (CFU)	Recovered – Background (CFU)	Average (CFU)	SD (CFU)	CV (%)	Amount Deposited (CFU)	Fraction Aerosolized
High Loading	3.1×10^7	3.0×10^7	2.9×10^7	1.4×10^7	48%	1.0×10^8	0.28
	4.7×10^7	4.7×10^7					
	1.4×10^7	1.3×10^7					
	2.6×10^7	2.6×10^7					
Low Loading*	8.7×10^5	8.5×10^5	8.8×10^5	5.0×10^4	6%	N/A*	N/A*
	9.5×10^5	9.3×10^5					
	8.8×10^5	8.6×10^5					
	9.6×10^5	9.4×10^5					
	8.5×10^5	8.3×10^5					
Simulated Decontamination	2.3×10^7	2.3×10^7	3.1×10^7	8.6×10^6	28%	1.0×10^8	0.30
	2.7×10^7	2.7×10^7					
	2.4×10^7	2.4×10^7					
	3.6×10^7	3.6×10^7					
	4.3×10^7	4.3×10^7					

The results from tests with high loading of *Btk* spores averaged 0.28 fraction aerosolized, which is not significantly different from the *Bg* result of 0.34 fraction aerosolized (unpaired t-test p-value of 0.40). The result from the aerosolization tests with *Btk* and simulated decontamination of 0.30 fraction aerosolized

was not significantly different from the *Btk* high spore loading results (unpaired t-test p-value of 0.86). Table 5-20 also presents the results from the low *Btk* spore loading tests. However, the amount deposited is not available. The SS positive control coupon swab results were one order of magnitude lower than expected from the positive control samples of the inoculum, although it is not known at this time what went wrong with these tests. The resulting reaerosolized fraction calculated from the low *Btk* spore loading data was an impossibly high 3.6.

6 Quality Assurance and Quality Control

The following sections discuss quality assurance (QA) and quality control (QC) for the project, including project documentation, the integrity of samples and supplies, instrument calibrations, critical measurements, and NHSRC Biolab quality checks.

6.1 Project Documentation

This project was performed under two separate Category B quality assurance project plans approved in August and September 2016. All test activities were documented through narratives in laboratory notebooks and using digital video and photographs. All tests were conducted in accordance with established operating procedures to ensure repeatability and adherence to the data quality validation criteria set for this project.

6.2 Integrity of Samples and Supplies

Samples were carefully maintained and preserved to ensure their integrity. Samples were stored away from standards or other samples that could cause cross-contamination. Supplies and consumables were acquired from reputable sources and were National Institute of Standards and Technology (NIST)-traceable when possible. Supplies and consumables were examined for evidence of tampering or damage before use. Supplies and consumables showing evidence of tampering or damage were discarded.

6.3 Instrument Calibrations

The project used established operating procedures for the maintenance and calibration of all laboratory equipment. All laboratory measurement devices used in this project were certified as having been recently calibrated or were calibrated by the on-site EPA Metrology Laboratory at the time of use. Table 6-1 summarizes the calibration frequency for instruments used during this project.

Table 6-1. Instrument Calibration Methods and Frequencies

Equipment	Calibration or Certification Method and Frequency	Expected Tolerance
Thermometer	Compare to independent NIST thermometer (a thermometer recertified annually by either NIST or an International Organization for Standardization (ISO)-17025 facility) value once per quarter	±1 °C
Temperature sensor (chamber)	Compare to independent NIST thermometer value once per year.	±1 °C
RH sensor (chamber)	Compare to calibration salts once per year	± 5 %
Thermal anemometer	Compare to NIST-traceable anemometer once per year	± 5 %
Stopwatch	Compare to official U.S. time at time.gov every 30 days	± 1 min/30 days
Micropipettes	Certified as calibrated at time of use; recalibrated by gravimetric evaluation of performance to manufacturer's specifications every year	± 5 %
Scale	Calibrate annually to Class 1 weights; compare reading to Class 2 weights every day	± 1 %

Any deficiencies were noted and the instrument replaced to meet calibration tolerances.

6.4 Critical Measurements

The following measurements were deemed critical to accomplish project objectives:

- Volume
- Counts of CFU per plate
- Plated volume
- Temperature of incubation chamber

Data quality indicators (DQIs) were used to determine if the collected data met the QA objectives. Decisions to accept or reject test results were based on engineering judgment used to assess the likely impact of the failed criterion on conclusions drawn from the data. The acceptance criteria were set at the most stringent levels routinely achievable. Table 6-2 lists the DQIs and acceptance criteria for the critical measurements.

Table 6-2. DQIs and Acceptance Criteria for Critical Measurements

Measurement Parameter	Analysis Method	Accuracy	Acceptance Criterion	Mean Value Pass/Fail
Volume	Serological pipette tips	0.1 mL	± 10% of target value	Pass
Counts of CFU per plate	QCount®	1.82×10^4 < QC Plate < 2.3×10^4	Within range of QC plate	Pass
Plated volume (liquid)	Pipette	2%	± 1%	Pass
Temperature of incubation chamber	NIST-traceable thermometer (daily)	± 2 °C	± 2 °C	Pass

Results for all the DQIs were within the target acceptance criteria set for this project.

Several QC checks were used for measurement instruments to ensure that the data collected met the criteria listed in Table 6-2. The integrity of the samples during collection and analysis was evaluated. Validated operating procedures conducted by qualified, trained, and experienced personnel ensured data collection consistency. When necessary, knowledgeable parties conducted training sessions, and in-house practice runs were conducted to gain expertise and proficiency before research began. The QC checks performed during this project are detailed in Section 6.5.

In addition to the measurement instrument checks, positive control samples and procedural blanks were included along with the test samples so that optimal spore recovery and unintentional contamination of test coupons could be assessed. Replicate coupons were included for each set of test conditions to assess the variability of each test procedure.

6.5 NHSRC Biolab Quality Checks

Quantitative standards do not exist for biological agents. An Advanced Instruments QCount® system was used to count viable spores. CFU counts greater than 300 or less than 30 were considered outside the targeted range. If the CFU count for bacterial growth did not fall within the target range, the sample was

either re-spiral plated at a different dilution, filter plated, or manually replated. Filter plate counts and manual replate counts were enumerated manually.

A QC plate was analyzed before each batch of plates was enumerated using the QCount®, and the result was verified to be within the range indicated on the back of the QC plate. As the plates were being counted, a visual inspection of colony counts made by the QCount® software was performed. Obvious count errors made by the software were corrected by adjusting the settings (such as colony size, light, and field of view) and recounting or by manually removing or adding colonies as needed.

The acceptance criteria for the critical CFU counts were set at the most stringent level routinely achievable. Positive controls were included along with the test samples so that spore recovery from the different surface types could be assessed. Background checks also were included as part of the standard protocol to check for unanticipated contamination. Replicate coupons were included for each set of test conditions to characterize the variability of the test procedures.

Further QC samples were collected and analyzed to check the ability of the NHSRC Biolab to culture the test organism as well as to demonstrate that materials used in this effort did not themselves contain spores. The checks included the following:

- **Field blank samples:** Filters and liquid samples transported to the field site but not used for sampling
- **Procedural blank samples:** filter samples collected in the same fashion as test samples but without a contaminated test coupon in place (laboratory tests only)
- **SS positive control coupons:** Coupons inoculated in tandem with the test coupons and meant to demonstrate the highest level of contamination recoverable from a particular inoculation event.

Table 6-3 summarizes the additional QC checks for NHSRC Biolab procedures. These checks provide assurances against cross-contamination and other biases in microbiological samples.

Table 6-3. Additional Quality Checks for Biological Measurements

Blank TSA sterility control: plate incubated but not inoculated	Each plate	No observed growth after incubation	Controls for sterility of plates	All plates incubated before use, so contaminated plates discarded before use
Replicate plating of diluted microbiological samples	Each sample	Reportable CFU of triplicate plates must be within 100%	Used to determine the precision of the replicate plating	Replate sample
		Reportable CFU between 30 and 300 CFU per plate		
Unexposed field blank samples	One per test	Non-detect	Level of contamination present during sampling	Clean up environment; sterilize sampling materials before use

The results from all QC sterility control and blank samples were non-detect for this project.

7 Composite Sampling Method Conclusions and Recommendations

The current field and laboratory study results demonstrated the field application of innovative composite sampling methods using RFC, AA, and Wet Vacuum. All 6 RFC (3) and wet vacuum (3) samples from Stage 1 detected contamination on the platform after decontamination. However, the traditional sampling results using 37 mm cassettes on the platform showed 3 positives out of 49 samples. In addition, the recoveries (CFU per ft^2) of the innovative sampling methods were similar (with RFC) or better (~5 times more sensitive with wet vacuum) compared to the traditional sampling methods. The estimated total sampled area using 3 RFC and 3 wet vacuum for each stage (6 total samples in each of Stages 1 and 5) was approximately 1771 ft^2 out of approximately 3000 ft^2 total platform area (~59%). The traditional surface sampling method (37 mm cassettes) covered approximately 49 ft^2 with 49 samples (~1.6%). The total estimated person minutes to collect samples (preparation and collection) were 36 for 3 RFC samples (robot running time was ~30 minutes per robot), 84 minutes for 3 wet vacuum samples and 970 minutes for 49 cassette samples. The estimated costs per sample (labor, material, and waste) were $267 for RFC, $220 for Wet Vacuum, and $395 for 37 mm cassette. The estimated total costs were $19,355 ($395 per ft^2) for the traditional method and $1,461 ($0.82 per ft^2) for RFC and Wet Vacuum together. For AA sampling, the estimated total sampling time (preparation and collection) was approximately 25 hours for Stage 2 and 6.5 hours for Stage 3. The AA sampling required extensive set up time for air filtration, air mixing, and leaf blowing. In addition, the planning team spent most of time to plan out the AA sampling including safety, electricity requirement, NAM pre-filter preparation, etc.

The RFC and wet vacuum results clearly showed that these composite sampling methods provide the benefits of reduced sampling time during a response, fewer samples requiring processing, detection of spore presence at unknown hot spots of contamination, improved detection of widespread contamination when concentrations are close to (or potentially below) detection limits for traditional surface sampling methods, and shortened timeline to recovery. AA sampling results could not be compared to the surface sampling methods (both innovative and traditional methods) due to the uncertainty of spore contamination from pre-deployed hotspots during the Stage 2 AA sampling operation.

The laboratory and field study results confirmed the following cautions for using these methods:

1. The tested composite sampling methods generated a large quantity of debris/dust in the sampling media (water and filter). It is necessary to develop efficient sample processing procedures prior to analysis either at the site or at the laboratory.

2. Wet Vacuum sampling generates aqueous samples that necessitate a more secured approach to contain the samples during transport.

3. For RFC, it is difficult to assess the actual sampled area due to the unpredictable movement of robots. RFC may prematurely terminate sampling due to high filter pressure drop on dusty surfaces. It is recommended that magnetic strips be used to pre-define the discrete sampling area (~100-200 ft^2, dependent upon the amount of floor debris present).

4. The AA sampling method for dusty environments requires airtight containment of the site and improvements on the particle collection system such as the use of NAM pre-filter and/or cyclone before filter sampling for dusty environments.

5. AA sampling method has high electricity requirements for operation due to the use of air samplers, leaf blowers, and mixing fans. Careful site assessment will be necessary to determine whether AA sampling is a viable option for the given site. Battery powered blowers and collectors should be identified and evaluated to ease response operations.

6. Efficacy of AA sampling method may be impacted by the site conditions after the initial release such as high humidity, decontamination, precipitation, etc., which may decrease the spore resuspension potential and the overall AA sampling efficacy.

In summary, RFC and wet vacuum are likely useful composite sampling methods in addition to the traditional discrete surface sampling methods. The AA sampling method will need thorough site assessment for application and the current approach from asbestos abatement may need modifications to be applied for anthrax site sampling. The AA sampling plan should be developed depending on the site situations to properly address safety, containment, and effective sampling. Hence it is highly recommended that AA sampling be planned and executed at the sites with thorough planning incorporating input from a group of experts from industrial hygiene, aerosol science, and mechanical engineering.

The following section provides composite sampling method recommendations based on field test operator and observer comments during the field sampling exercise and recommended AA sampling procedures.

7.1 Field Test Operator and Observer Comments

The following sections provide input and suggestions for further improvement of field methods made by operators and observers involved in AA, RFC, wet vacuum, and settling plate sampling during the field sampling exercise.

7.1.1 PPE

- Operators preferred wearing PAPRs instead of full-face respirators.
- Operators preferred wearing Tyvek® suits instead of Tychem® suits.
- Because of the amount of physical activity and subsequent perspiration, outer gloves were slipping away from the suit-glove interface taped at the wrist. An improved taping method or waterproof adhesive may be needed.
- Because of the amount of physical activity and subsequent perspiration, operators should bring a dry change of clothes to change into during each rest period.
- Operators recommended wearing ice vests underneath Tyvek suits.
- Glove change-outs were inconsistent. A system should be used to remind operators when to change gloves.

7.1.2 Field Supplies

- Operators required backup PAPR batteries.
- Containers should be packed with a single type of item (for example, separate storage for gloves, data sheets, and plastic bags). Although all containers were labeled, access was not as easy as anticipated.
- More or larger carts should be used because the few on-site carts were overfilled and cluttered. Another option is a utility cart with more built-in storage.
- Clear containers should be used for easy identification of supplies.
- Data sheets and clipboards became contaminated because they were used in the hot zone. Instead, each data sheet could be placed in a bag, and the bag could be decontaminated.
- During future tests, laminated sheets and permanent markers for note-taking could be used as well as electronic tablets for data entry.
- Pictures of the completed data sheets should be taken for backup.
- In future tests, a portable refrigerator could be provided to store samples.
- Operators suggested wiping down all potentially contaminated equipment before it is brought on site.

7.1.3 AA Sampling Procedure

- Operators suggested shoulder straps for the leaf blowers.
- Operators mentioned trip hazards from long cords and suggested retractable cord reels.
- A planned deployment of all extension cords was suggested to minimize tangles and overlaps in the space.
- Hand signals should be devised because it was not possible to hear others when the leaf blowers were operating and because of the need for hearing protection.
- Operators suggested over-the-ear hearing protection worn over the PAPR hood.
- A guide mounted to the end of the leaf blower nozzle was suggested to ensure the appropriate distance from the surface.
- A time keeper or visible timer could be used to allow leaf blower operators to manage time more effectively.
- Portable, smaller, lighter equipment was suggested, including lithium battery-powered fans and blowers and a portable battery-powered wet/dry vacuum as an alternative to the DFU and NAM.
- Operators commented that the NAM filters required larger sample bags and that the wire backing had sharp edges.
- Operators commented that the bags used to store DFU filters were too large and reaching into the bags could create cross-contamination.
- Observers and operators recognized the need to constantly monitor the flow rates of the samplers to know when the filters were fully loaded.
- The NAM could be placed in the hot zone and the filter directly mounted to the NAM to prevent collapse of the flexible duct. Alternatively, only a metal duct should be used.
- All flanges for the NAMs should be mechanically secured. For this project, the flanges were secured using tape.

- Thicker, larger, or more layers of bags should be used for filter containment because some bags tore during transport.
- Operators suggested eye protection under PAPR hoods during active leaf blowing activities.
- Observers suggested investigating the possibility of using the leaf blower intake as a sampler in future tests by employing a filter or cyclone sampler.
- A two-handed, double leaf blower operation would shorten the application duration.

7.1.4 RFC Sampling Procedure

- Future work should use RFCs powered by lithium batteries.
- Larger Stomacher® 400 circulator bags were suggested for collection of the vacuum debris.
- Operators indicated difficulty emptying the dust bin into the Stomacher® 400 circulator bag and suggested placing the entire bin in the sample bag.
- Use of the filter as the sample was suggested, although it is not known if the filter would provide a representative sample of the dust collected.

7.1.5 Wet Vacuum Sampling Procedure

- Operators suggested transferring the wet vacuum liquid sample to a chemically resistant, airtight Nalgene bucket in the field.
- Large Twirl'em® bags should be used instead of zip lock bags as secondary containment for the dirty reservoirs because the zip lock bags had a tendency to tear when opened.
- A third person may be needed to take notes because the support person was occupied at all times in assisting the lead sampler.
- A third person may be needed for cord management with the wet vacuum because the cord drags on the contaminated surface and likely contaminates the support person. A third person, designated note taker, and clean handler would be beneficial.
- Observers and operators questioned the need for following the wet vacuum procedure of overlapping strokes and suggested that the effectiveness of this method be studied in the laboratory.
- A portable, battery-powered wet/dry vacuum was suggested as an alternative to the wet vacuum.

7.1.6 Settling Plate Sampling Procedure

- Operators suggested using more samplers and locating samples in a grid-like pattern if heat mapping is desired.
- Operators suggested an adequate number of personnel available to count plates.
- A large amount of waste was generated on site. Operators suggested consideration of disposal requirements and Department of Transportation regulations.

7.2 Recommended AA Sampling Procedures

This section discusses future AA sampling recommendations and a recommended AA sampling deployment based on the setup of the field sampling exercise. The recommended AA sampling

procedures can inform decisions related to AA sampling in a space comparable to the FAPH mock subway tunnel.

7.2.1 Future AA Sampling Recommendations

- The area designated for AA sampling should be airtight. Plastic sheeting should cover any opening to outside of the contaminated area. Pass-throughs with 12-in. flanges should be installed on the sheeting to allow NAMs to be installed inside the hot zone. The flanges allow venting of the NAM outside of the contained area.
- Determine the total surface area to be sampled to decide the number of operators, leaf blowers, mixing fans, and samplers required.
- Determine if electricity is available, the load capacity of circuits, and locations of the receptacles. If electrical load is an issue, determine the best ways to reduce load (such as using fewer samplers, fewer fans, or more battery-powered equipment [with backup batteries]).
- Limit the sampling area to 2,500 ft^2 per operator or less.
- Determine if NAMs can be deployed inside the sampling area. If so, they should be used as high-volume samplers. The installed HEPA filter can be used as the filter medium. However, the installed HEPA filter may be a deep-box filter that would require some disassembly of the NAM to replace. Additionally, HEPA filters are cumbersome to store and process. The recommended procedure for using a NAM as a sampler is outlined below. This method allows quick change-out of the filter and does not require cycling power on the NAM.
    - Acquire a piece of sheet metal the size of the NAM intake, a 12-in. diameter flange, 12-in.-diameter flexible metal duct, a 14-in. by 20-in. metal filter box, and 14-in. by 20-in. household filters with easily-removable support wire (such as Filtrete™ MPR 1500 or higher).
    - Cut a hole in the sheet metal, and fasten the flange to the hole. Insert the sheet metal with the flange onto the face of the NAM using the slots on the NAM.
    - Connect flexible metal duct to the flange. Position the open end near the location that will be sampled.
    - Connect the 14-in. by 20-in. metal filter box to the open end of the duct.
    - If needed, carefully cut away and remove the metal support mesh from the furnace filter.
    - Install the furnace filter into the filter housing.
- If the volume of the contaminated zone is large, the reaerosolized particles (especially large size) may not be suspended long enough to be collected by the centralized air filtration system. DFUs or other portable samplers may be beneficial to sample the localized resuspended particles before particle loss due to the gravitation settling. The samplers should be spaced at regular intervals or in a grid throughout the zone. The goal should be to maximize the volume of air sampled within the AA sampling period. During the field sampling exercise for this project, the combination of one NAM and nine DFUs yielded approximately 2,100 CFM of air sampled. Over the 160,000-ft^3 space, 76 minutes would be required to sample the entire contaminated zone volume (one air exchange). Use of more NAMs and DFUs will decrease the time required for AA sampling and increase the amount of air sampled.

- During the field sampling exercise for this project, passing the leaf blowers close to the surface was effective at removing much of the particulate from the surface in one or two passes. A typical leaf blowing motion may be sufficient to remove particulate in the path of the blower. However, known or suspected hotspot areas in the contained area should receive additional blowing time during AA sampling.
- Large mixing fans should be used. Seven were used during the field sampling exercise, but fewer may be acceptable in a smaller space.
- Supply one corded leaf blower per operator. Backup blowers should be on hand in case of failure.
- Supply heavy-duty power cords long enough to traverse the hot zone.
- Check flow rates frequently, and change out filters during AA sampling, especially in dusty environments. The furnace filters used for this project had a maximum rated dust load of 15 g, and the DFU filters were rated for 1 g of dust. In extremely dusty environments, filters may require change out before the end of AA sampling.
- In dusty environments, the addition of a cyclone separator upstream of the filter assembly may be useful to increase the life of the filter. A cyclone designed to operate efficiently at the flow rate of the sampler can be plumbed upstream of the sample filter. For example, the commercially-available Dust Deputy (Oneida Air Systems, Syracuse, NY) cyclone would be suitable for use with the DFU, and the Super Dust Deputy (Oneida Air Systems, Syracuse, NY) cyclone would be suitable for use with the NAM.

7.2.2 Recommended AA Sampling Deployment

- Set up mixing fans in the corners of the hot zone.
- Install the NAM sampling filter at or near the middle of the hot zone.
- Install DFU and other samplers in a gridded pattern, if possible.
- Decide how to subdivide the area and the route for sweeping the entire area.
- Run power cords and extension cords to the mixing fans, DFUs and other samplers, and leaf blowers.
- Turn on the fans.
- Load the filters for the DFUs and other samplers.
- Turn on the DFUs and other samplers.
- Load the NAM furnace filter. If the NAM is not already running, turn it on.
- Turn on the leaf blowers.
- Hold the leaf blower at or near 45° to the surface as close to the surface as possible without touching it.
- Progress through the zone, making sweeping motions that cover a 4-ft-wide path.
- When the area has been covered by AA sampling, turn off the leaf blower, the DFU samplers, and the fans.

References

Brown, G. S., ; R.G. Betty, J.E. Brockmann,D.A. Lucero, C.A. Souza, K.S. Walsh, R.M. Boucher,M. Tezak, M.C. Wilson, and T. Rudolph. 2007. “Evaluation of a wipe surface sample method for collection of *Bacillus* spores from nonporous surfaces.” *Appl Environ Microbiol.* 73 (3), 706 through 710.

Calfee, M.W.; S.D. Lee, and S.P. Ryan. 2013. “A rapid and repeatable method to deposit bioaerosols on material surfaces.” *J Microbiol Meth.* 92 (3), 375 through 380.

Centers for Disease Control and Prevention (CDC). 2012. “Surface sampling procedures for *Bacillus anthracis* spores from smooth, non-porous surfaces.” Accessed on March 3, 2017. On-line Address: https://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthracis.html

Lee, S.D; M.W. Calfee, L. Mickelsen, S. Wolfe, J. Griffin. M. Clayton, N. Griffin-Gatchalian, and A. Touati. 2013. “Evaluation of surface sampling for *Bacillus* spores using commercially available cleaning robots.” *Environ. Sci. Technol.* 47 (6), 2595-260, DOI:10.1021/es4000356.

EPA. 2012a. *Homeland Security: Strategic Research Action Plan 2012–2016.* EPA 601/R-12/008. Office of Research and Development. Washington, DC.

EPA. 2012b. “*Determination of the Difference in Reaerosolization of Spores off Outdoor Materials*.” Technical Report, EPA/600/R-14/259. Research Triangle Park, NC.

EPA. 2017. “*Underground Transport Restoration (UTR) Operational Technology Demonstration (OTD)*.” Technical Report, EPA/600/R-17/272. Research Triangle Park, NC.

Appendix A: Sampling Procedure Using Commercially Available Robotic Floor Cleaners for *Bacillus anthracis* Spores - Neato® XV-21

July 2017

Revision 0.0

National Homeland Security Research Center
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

A1. Scope and Applicability

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program within EPA's Office of Research and Development, and the Chemical, Biological, Radiological and Nuclear Consequence Management Advisory Division within EPA's Office of Land and Emergency Management, jointly developed this sampling procedure. This procedure is intended to provide a method for trained incident responders to collect environmental samples after a biological contamination incident. This procedure specifically applies to the collection of surface-bound particulates and microorganisms using off–the-shelf robotic floor cleaners (RFC). The purpose of this procedure is to guide the process of preparation, deployment, and collection using RFCs for sampling surfaces in a specified area. The results from the collected samples can be used to determine the presence or absence of contamination and the contamination level after natural outbreaks and after intentional or accidental releases of pathogenic microorganisms and biotoxins.

At the time of publication, this sampling procedure has not been validated. At the date of this publication, the RFC sampling procedure has been partially characterized for deployment feasibility and collection performance for bacterial spores. This procedure will be updated or replaced with a fully characterized and validated procedure upon availability. During emergencies, the use of non-validated methods may be warranted when validated methods are not available. EPA's use of non-validated methods must adhere to the EPA's Forum on Environmental Measurement (FEM) policy directive on method validation (EPA 2010). Further information on method validation is presented in *Validation of U.S. Environmental Protection Agency Environmental Sampling Techniques that Support the Detection and Recovery of Microorganisms* (EPA 2012).

A2. Summary

This sampling procedure is for the sampling of a horizontal surface (such as a floor) using an RFC. After sampling, the RFC is recovered and processed to determine the presence or absence of potential surface contamination. This procedure provides a step-by-step sampling procedure for the following RFC:

- **Neato® XV-21** (Neato Robotics, Inc., Newark, CA): vacuum cleaner used to sample both porous surfaces (such as carpet, wood, and bare concrete) and nonporous surfaces (such as vinyl, tile, laminate, coated wood, and coated concrete)

The sections below discuss the following:

- Definitions (Section 3)
- Health and safety (Section 4)
- Waste management (Section 5)
- Equipment and supplies (Section 6)
- Deployment procedure (Section 7)
- Sample collection (Section 8)
- Post-deployment sample handling (Section 9)
- Documentation (Section 10)

Section 11 lists the references used to prepare this procedure.

A3. Definitions

Biological agent contamination: contamination that can be attributed to natural outbreaks, and intentional or accidental releases, of pathogenic microorganisms and biotoxins.

Biotoxin: a poisonous substance either produced by or extracted from living or dead organisms

Conventional sampling method: a currently recommended surface sampling method (such as the use

of swabs, wipes, and vacuums fitted with filter-type collection media for biological agents) typically used on small, discrete areas; some conventional methods (such as the use of sponge wipes, swabs, and 37-millimeter [mm] vacuum cassettes) have associated multi-laboratory-verified or -validated analytical procedures

Enclosed facilities and objects: facilities and objects that typically have surface areas with clearly defined boundaries such as walls and that are isolated from exterior environments, including commercial and residential buildings and transportation vehicles

Method of dissemination: the means by which biological agents are dispersed; dispersal can occur over large areas in wet and dry forms through aerosol generation and spreading devices that contaminate indoor and outdoor sites or the food or supply chain

Outdoor areas and objects: building exteriors and other outdoor areas such as streets, parks, and other open spaces with no clearly defined boundaries

Pathogen: a disease-causing agent that invades a host and replicates, including viruses, bacteria, and fungi

Robotic floor cleaner (RFC): a commercially available, autonomous, floor-cleaning robot

Sampling area: area expected to be similar to the Neato® manufacturer's estimation of approximately 2,000 to 3,000 square feet (ft^2) on one cycle, depending on environment, flooring, furniture, and other factors

A4. Health and Safety

Laboratory testing of RFCs in contaminated areas indicates that the RFCs can cause resuspension of spores through physical surface agitation. Therefore, the site of an RFC sampling event could pose an exposure risk to the operator or support personnel. Operators should take precautions when setting up and deploying the RFCs, including the use of proper personal protective equipment (PPE), including (but not limited to) the use of a full-face powered air-purifying respirator (PAPR) with P100 cartridges and full body covering including built-in hood and foot covers. RFC deployment using delayed activation, if available, would reduce exposure risk.

Before exiting the exclusion zone (hot zone), sampling personnel should follow the standard operating guideline that provides guidance to EPA and its contractors on decontamination (decon) for personnel conducting long-term responses to biological contamination per EPA's CMAD decon line procedure. This standard operating guideline was developed specifically for biological responses using Level C PPE with a full-face PAPR or full-face air-purifying respirator (APR). Level C PPE is appropriate for most incidents involving biological agents and is required when the concentration and type of airborne substances is known and the Occupational Safety and Health Administration (OSHA) criteria for using APRs are met (OSHA 1999). Level C PPE includes a protective coverall with integral hood and booties, an APR (preferably a PAPR), inner and outer nitrile gloves, hard hat (optional), and disposable outer boot covers. All use of respirators must comply with the OSHA Respiratory Protection Standard (29 CFR 1910.134).

There may be additional health and safety concerns associated with these sampling procedures. If the RFCs are operated on a raised subway platform, the operator should be cognizant of a potential fall hazard because the platform will not have a guardrail in place. Operators should maintain a safe distance from the edge. Additionally, heat stress may be a factor depending on the ambient temperature and length of time spent in modified Level C PPE. Appropriate work/rest regimens should be adhered to based on the wet bulb globe temperature (WGBT) and other factors such as acclimatization, hydration and fitness.

A5. Waste Management

The maximum usage for an RFC is one sampling event. After a contaminated area is sampled, the sampling components of the RFC are collected for analysis. The rest of the RFC can be left in the hot zone for retrieval for sterilization and then can be either stored or disposed of.

A6. Equipment and Supplies

Vacuum-based RFCs consist of two components: (1) the robotic body and (2) the dirt collection bin with a particle filter. The Neato® XV-21 RFC is a common design consisting of a semi-circular autonomous body and a pop-out bin with filter. The filter is the RFC component that physically collects samples and therefore is the component that is isolated and secured for sample analysis.

The RFC is operational straight from the box and can be implemented for a field sampling event after its batteries are charged. The materials listed below are additional materials required to prepare the Neato® XV-21 RFC for sampling and then securing the RFC for shipment, storage, or analysis:

- Sterile, labeled, 10-in. (inch) by 15-in. sealable, transparent (if available) plastic bags (such as Twirl'em® [Labplas Inc., Quebec, Canada] bags) as primary, secondary, and tertiary containment
- Pre-printed labels
- Fourth containment container such as a large, clear, plastic bin
- Logbook
- Tychem® [Labplas Inc., Quebec, Canada] suits with hoods
- Boots and boot covers
- Sterile nitrile gloves
- Respirators (APRs or PAPRs)
- Sampling kit containers for pre- and post-sampling events
- Wetted bleach wipes (in a canister)
- Sampling cart
- National Institute of Standards and Technology (NIST)-calibrated timers (verify time at time.gov)
- Video camera (optional)
- Appropriate disposal bags
- Neato® XV-21 RFC with fully charged batteries
- Pens and permanent markers
- Checklist and chain-of-custody (COC) forms

A7. Deployment Procedure

The deployment procedures include pre-deployment preparation, the packaging of RFCs and supplies for deployment, and surface area assessment and RFC deployment.

7.1 Pre-deployment Preparation

Pre-deployment preparation should occur in the support zone (uncontaminated area where the sampling team and the sampling materials are not exposed to contaminants). The RFCs and the sampling equipment must be prepared before the sampling process. The steps below are required for an effective RFC sampling sequence before deployment.

1. Remove the RFCs from their boxes and place them on a table.
2. For each RFC, ensure that the sampling components (filter and dirt collection bin) are connected and installed in a functional manner and that the filter is in its proper position.
3. Label each RFC using a pre-set labelling scheme.
4. Using the supplied cable, charge the batteries of each RFC to full capacity. The RFC batteries should be fully charged before a cleaning cycle begins in accordance with the manufacturer's instructions. Refer to the user's manuals for battery installation and removal.

5. RFC is ready to use when the status light turns solid green. The manufacturer recommends charge the batteries overnight before use for the first time. After that, run through three complete cycles of charging and cleaning-until-recharge to get the most capacity from the batteries.
6. For each RFC, ensure that the RFC menu is in the right setting so that the sampler will have to press the start button once only to deploy the RFC.

7.2 Packaging of RFCs and Supplies for Deployment

The RFCs, sampling kits, and ancillary supplies should be placed in three separate bins. These bins can be large plastic bins with lids (such as Tupperware® [Tupperware Brands Corp., Orlando, FL] bins) that will be placed on a cart for easy transport between deployment zones. Attach a trash bag to the cart for disposal of used gloves and boot covers. The contents of each bin are summarized below.

1. **Container 1** holds a pre-charged Neato® XV-21 RFC and pre-labeled, post-deployment sampling kits that include (1) one pre-labeled 7-in. by 12-in. sterile, sealable, Stomacher® 400 (Seward Ltd., West Sussex, UK) circulator bag for primary containment and (2) two 10-in. by 15-in. Twirl'em® bags for secondary and tertiary containment).
2. **Container 2** holds supplies such as extra gloves and boot covers, extra sterile sampling bags, wetted bleach wipes, checklists, COC forms, pens, and timers.
3. **Container 3** is used to transport the collected samples.

7.3 Surface Area Assessment and RFC Deployment

The surface area requiring sampling should be determined. Based on this area, the sampling team should at a minimum consist of a lead sampler and a support person. The two-person team will wear the required site-specific level of PPE and work jointly to handle the RFCs and sampling supplies. A backup team should be prepared to relieve the sampling team at any time (two in, two out). At each predetermined sampling location, the team will deploy one RFC in accordance with the procedures specified in this sampling procedure. The duties of each person are described below.

The **lead sampler** shall perform the tasks below.

1. Check that the contaminated area is suitable for RFC deployment (no wires, liquid hazards, etc.). Set up physical boundary markers if needed.
2. Verify the RFC label with the support person, and then place the RFC near the entrance or exit of the sampling area floor.
3. Press the START button of the RFC, and check that the RFC begins the sampling process.
4. Visually verify that the RFC is functioning properly.
5. Don a new pair of gloves to deploy each additional RFC.

The **support person** shall perform the tasks below.

1. Verify that all items on the checklist are present on the cart.
2. Take notes in a logbook on the deployment procedure, such as RFC labels, start times, and other comments.
3. Don a new pair of gloves to deploy each additional RFC.

Notes: If the lead sampler or support person moves to another sampling area contiguous with the first sampling area, he or she does not need to don a new pair of gloves between RFC deployments. If the sampling areas are not contiguous, the team member must doff PPE (gloves and boot covers) outside

of the sampling area and don new gloves and boot covers before entering the new sampling area. Gloves must be changed after contact with any suspected contaminated surface or item.

Depending on the size of the area to be sampled, the two-person sampling team may leave the enclosed area and reenter (following appropriate requirements in the health and safety plan) it after autonomous sampling is completed. Many RFCs are equipped with mapping and navigation technologies and can return to their starting position after covering the entire floor surface of an enclosed sampling area.

A8. Sample Collection

After the RFC ceases operation (either completes sampling, ceases because of a dead battery, or ceases because it is immobilized or trapped), the lead sampler and support person will collect samples. Each team member's sample collection duties are summarized below.

The **lead sampler** will collect the sample (filter only) from the Neato® XV-21 RFC dirt bin using the steps below.

1. Don a new pair of sterile gloves.
2. Retrieve the RFC from its stopped place in the sampling area using the handle on top of the RFC.
3. Communicate relevant information to the support person, such as error messages, recovery location of the RFC, visible material in collection bin, and other information.
4. Dislodge the dirt collection bin from the body of the Neato® XV-21 RFC by lifting the lever.
5. Remove the dirt collection bin from the body of the Neato® XV-21 RFC.
6. Remove the filter from the dirt bin with the dirt bin turned upward by pulling the filter handle down and out to separate the filter from the dust bin (Figure 1). Do not disturb the filter surfaces.

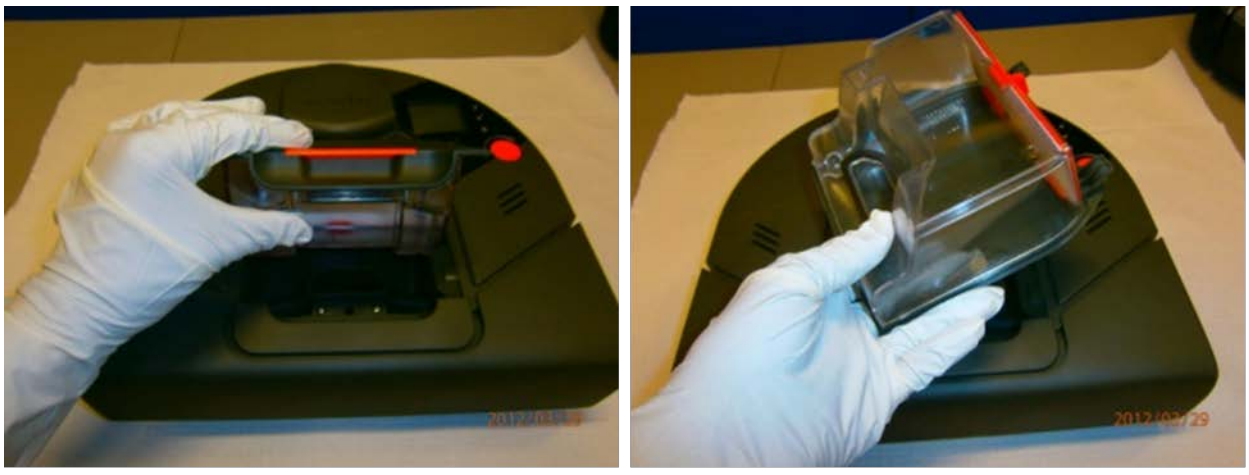

Figure 1: Filter Removal from Neato® XV-21 RFC

7. Put the filter in a pre-labeled, 7-in. by 12-in. Stomacher® 400 circulator bag (opened by the support person).

8. Turn the dirt bin upside down, and pour the dirt bin sample contents into the same Stomacher® 400 circulator bag used for the filter. Carefully empty the dust in the dirt bin to the bag. Place the dust bin on top of the RFC. Hold the bag upright, and wait for a few minutes for the dust to settle. Gently squeeze the bag to release the air and seal the bag.
9. Place this sample in its primary containment bag (Stomacher® 400 circulator bag) into a secondary pre-labeled containment bag measuring 10 in. by 15 in. (opened by the support person).
10. Doff gloves and don new gloves.
11. Disinfect the exterior of the secondary containment bag using wetted bleach wipes.
12. Place the secondary containment bag into a tertiary containment bag measuring 10 in. by 15-in., and disinfect the tertiary containment bag using wetted bleach wipes.
13. Place the tertiary containment bag in the transportation container (Container 3), and disinfect Container 3 using wetted bleach wipes.

The **support person** shall perform the tasks below.

1. When the lead sampler starts the RFC, record the start time, end time, start location, end location, any significant events that occur during the sampling event, and other important and relevant information.
2. Record the location where the RFC stopped, time of collection, and comments (such as error messages, description of the sampling area, RFC conditions, alerting sounds, and information from the lead sampler).
3. Assist the lead sampler as needed by opening sampling bags, managing power cords, and performing other required tasks.
4. Verify that the label on the RFC matches the labels on the sampling kits.
5. On the COC form, check the samples as complete.
6. Don new gloves to handle the sample bags.

A9. Post-Deployment Sample Handling

Post-deployment sample handling requires sample preservation, identification, COC, and archiving as discussed below.

9.1 Sample Preservation

Biological samples in the transportation container should be shipped in insulated containers with cold packs and appropriate biohazard label, refrigerated (2 to 8 degrees Celsius [°C]) until analysis, and be archived at 4 ± 2 °C. Samples should be processed and analyzed within 48 hours. Further sample transportation instructions are available at the Centers for Disease Control and Prevention website at http://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthracis.html.

9.2 Sample Identification

Each sample will be identified using descriptors of the sampled materials and unique sample numbers. The sampling team will maintain an explicit laboratory log that includes records of each unique sample number. Each sample will be identified by a material descriptor and a sampling location number. After transfer of the samples to the microbiology laboratory for analysis, each sample will be additionally identified (and photographed if available) by replicate number and dilution.

9.3 Sample Chain of Custody

Careful coordination with the microbiology laboratory is required for successful transfer of uncompromised samples in a timely manner for analysis. Test schedules will be confirmed with the laboratory before the start of each test. To ensure the integrity of samples and to maintain a timely and traceable transfer of samples, an established and proven COC or possession procedure is mandatory. Accurate records must be maintained whenever samples are created, transferred, stored, analyzed, or destroyed. The primary objective of these procedures is to create an accurate written record that can be used to trace the possession of the sample from the moment of its creation through the reporting of its results. A sample is in custody if it is in any one of the following states:

- In actual physical possession
- In view after being in physical possession
- In physical possession and locked up so it cannot be tampered with
- In a secured area that is restricted except to authorized personnel
- In transit

In the transfer of custody, each custodian will sign, record, and date the transfer. Sample transfer can be on a sample-by-sample basis or on a bulk basis. The protocol below will be followed for all samples as they are collected and prepared for distribution.

- A COC record will accompany the samples. When turning over possession of samples, the transferor and recipient will sign, date, and note the time on the COC record, which allows transfer of custody of a group of samples from the test site to the microbiology laboratory.

Samples will be carefully packed and shipped as hazardous material (hazmat) samples to the microbiology laboratory or will be hand carried between on-site laboratories.

9.4 Sample Archiving

Each sample will be archived by maintaining the primary extract at 4 ± 2 °C in a sealed extraction tube until the data set has undergone quality control checks and the sample has been released for disposal. Any deviations from sampling protocols must be documented in the laboratory logbook. Sampling duration, time of day, and observations also will be recorded in the laboratory logbook.

A10. Documentation

All observations and experimental details will be recorded in a scientific logbook. Entries must meet quality assurance requirements (such as the use of indelible ink, corrections made using lineout deletions, witnessed signatures, and other requirements). The logbook will include information for all deviations from project procedures, including spills, deviations from the aseptic technique, and faulty RFC function. In addition, if possible, the entire sampling procedure should be recorded on video. A video camera can be mounted above the sampling area. A team member should ensure that the entire sampling area is in the frame of the camera. Alternatively, a camera can be mounted to the operator if he or she remains in the enclosed area for the duration of the sampling event. Finally, the COC form ensures the integrity of samples and allows timely and traceable transfer of sample possession.

A11. References

Occupational Safety and Health Administration (OSHA). 1999. *OSHA's Respiratory Protection Standard.* Title 29 of the *Code of Federal Regulations* (CFR) 1910.134.

U.S. Environmental Protection Agency. 2010. Ensuring the validity of agency methods validation and peer review guidelines: Methods of analysis developed for emergency response situations. Washington DC: U.S. Environmental Protection Agency. Agency Policy Directive Number FEM-2010-01.

U.S. Environmental Protection Agency. 2012. Validation of U.S. Environmental Protection Agency environmental sampling techniques that support the detection and recovery of microorganisms. Prepared by: The FEM Method Validation Team, U.S. Environmental Protection Agency. Washington DC: U.S. Environmental Protection Agency. FEM 2012-01.

Appendix B: Sample Retrieval Procedure for Commercially Available Robotic Floor Cleaners for *Bacillus anthracis* Spores

July 2017

Revision 0.0

National Homeland Security Research Center
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

This sample retrieval procedure is for handling and analyzing samples contaminated with *Bacillus anthracis* (*Ba*) from robotic floor cleaners (RFC). The sections below discuss the following:

- Laboratory operations (Section B1)
- Sample processing (Section B2)
- Sample recovery (Section B3)
- Sample analysis (Section B4)

B1. Laboratory Operations

Ba is a National Institutes of Health (NIH) Risk Group 2 bacterial agent associated with serious or lethal human disease for which preventive or therapeutic interventions may be available (high individual risk but low community risk). It is also a select agent requiring registration with CDC and/or USDA for possession, use, storage and/or transfer. Samples contaminated with *Ba* should be handled in a Biosafety Level (BSL) 3 laboratory that requires special personal protective equipment (PPE). Risk Groups correlate with but do not equate to biosafety levels. Laboratory protective clothing must not be worn outside the laboratory. Facilities for washing and changing clothing after work should be available at the laboratory. All laboratory manipulations of samples must be performed in a Class IIor Class III Biological Safety Cabinet (BSC). Efforts should be made to avoid production of aerosols by working in a BSC. In addition, all centrifugation should be done using aerosol-tight rotors that are opened within the BSC after each run. Additional BSL 3 requirements can be found in CDC's Biosafety in Microbiological and Biomedical Laboratories (BMBL). The required PPE and other materials are listed below.

B1.1 Personal Protective Equipment

- Sterile, disposable, long-cuffed, nitrile gloves for outer and inner gloves
- Safety glasses
- Disposable coveralls with hood and solid front
- Disposable boot covers (booties)
- Respiratory protection from particulate hazards if necessary. PPE selection should be consistent with individual BSL3 facility guidance

B1.2 Other Required Materials

- Dispatch® hospital cleaner disinfectant towels with bleach (canister)
- 70% denatured ethanol wipes (canister)
- RFC samples (in a clear plastic container), accompanied by a relinquished chain-of-custody (COC) form
- National Institute of Standards and Technology (NIST)-certified timers
- Laboratory notebook
- Pre-labeled biohazard trash bags and bins
- Kimwipes® or equivalent low-lint paper wipes

- Seward™ Stomacher® 400 circulator bag racks or equivalent
- Sterile Seward™ Stomacher® 400 circulator bags
- Sonicator
- Orbital shaker incubator (OSI)
- Centrifuge
- 50- and 25-milliliter (mL) sterile serological pipettes
- Sterile 50-mL conical or centrifuge tubes
- Electronic serological pipetter, pre-charged
- 90-mL aliquots of phosphate-buffered saline with Tween® 20 (PBST) in sterile specimen cups
- Pens or pre-printed labels
- Tryptic soy agar (TSA) or equivalent agar plates
- Spiral plater
- Spiral plate spore counter
- 0.2- to 0.45-micron (µm) pore-size disposable analytical filter units
- Sterile 1,000-microliter (µL) pipette tips
- Sterile forceps
- Sterile deionized (DI) water (in about 10-mL aliquots)
- Calibrated top-loading balance (320 grams [g] x 0.001 g) and calibration weights
- 50-mL conical tube holders
- Vortexer
- Biological Safety Cabinet (BSC)

B2. Sample Processing

1. Verify that each COC form is complete and signed by authorized personnel at the package shipping/receiving dock.
2. Review each COC form to ensure that all of the samples are complete and that there is no notable variation in the sample identification (ID) labels compared to the IDs listed in the COC form. If variation has occurred, note it in the laboratory notebook.
3. Don appropriate PPE in accordance with BSL3 facility guidance..
4. Gather all necessary required materials (see Section B1.2), and place them on a clean cart beside the BSC within arm's reach so that sample processing may be performed without interruptions.
5. Clean the BSC using Dispatch® hospital cleaner disinfectant bleach towels. Wait for 5 minutes before spraying the cabinet with DI water. Wipe clean using Kimwipes®.
6. Wipe the workspace using the 70% denatured ethanol wipes. Dry the workspace using clean Kimwipes®. Discard outer gloves, and replace them with a new pair. Waste should be disposed of in accordance with BSL3 facility procedures.

7. Using Dispatch® bleach wipes, thoroughly disinfect the outside of the clear plastic container containing the RFC samples that may contain *Ba* spores. Discard outer gloves, and replace them with a new pair.
8. Inside the BSC, one at a time, remove the 10-inch (in.) by 15-in. Twirl'em® bags containing each RFC sample from the plastic container. Each bag contains an inner 10-in. by 15-in. Twirl'em® bag that in turn contains the 10-in. by 15-in. Stomacher® 400 circulator bag with the RFC filter.
9. Verify that the sample ID label on the outside of each Twirl'em® bag matches the samples listed on the COC form. Using Dispatch® bleach wipes, disinfect each outer bag, and then wipe the bag using clean Kimwipes® until it is dry. Discard the used bleach wipes and Kimwipes®.
10. Verify that the label on the inner Twirl'em® bag matches the label on the outer bag. If the inner bag sample ID does not match the outer bag ID, quarantine the sample for further analysis.
11. If no label discrepancies are observed, retrieve the inner Twirl'em® bag from the outer bag and discard the outer bag.
12. Disinfect the inner Twirl'em® bag with Dispatch® bleach wipes, and then wipe the bag using clean Kimwipes® until the bag is dry.
13. Retrieve the inner Stomacher® 400 circulator bag from the outer Twirl'em® bag, and discard the Twirl'em® bag. Take care not to let the outer Twirl'em® bag contact any surfaces that have not been disinfected until disposal.
14. Disinfect the Stomacher® 400 circulator bag with Dispatch® bleach wipes, and then wipe the bag using clean Kimwipes® until the bag is dry. Take care not to let the Stomacher® 400 circulator bag contact any surfaces that have not been disinfected.
15. Place each inner Stomacher® 400 circulator bag containing the RFC filter in a Seward™ Stomacher® 400 circulator bag rack or equivalent. Set each bag upright, and allow the filter dust to settle completely in the Stomacher® 400 circulator bag before opening the bag.

B3. Sample Recovery

1. Inside a BSC, aseptically add two pre-measured specimen cups containing 90 mL of sterile PBST to each Stomacher® 400 circulator bag containing a filter, resulting in a total of 180 mL of PBST added to ach sample. Place each sample in a new, secondary Stomacher® 400 circulator bag to prevent leakage.
2. Place each sample containing 180 mL of PBST lying flat into the OSI using flask clamps to hold each sample securely. Make certain that the sample bags will not tip over or become unsecured.
3. Agitate the samples in the OSI at 300 rotations per minute (rpm) for 30 minutes (min) at ambient room temperature. After agitation, remove the samples from the OSI, and transfer them to the BSC to be split before centrifugation.
4. Aseptically transfer the liquid from each sample bag to four individual, sterile, pre-labeled 50-mL conical tubes, making certain that the amount of liquid is evenly distributed. Once each sample has been split, measure each tube for weight using a calibrated balance. The weight of each tube should be ± 0.5 g of each other so that the centrifuge will be balanced.
5. Place the tubes from each sample into the centrifuge with sealed rotor and containment cups. Weights of tubes positioned opposite from one another should be ± 0.5 g. Centrifuge the tubes at

3,500 × gravity (× g) for 15 min. Do not use the brake option (if applicable) on the centrifuge to slow the rotor because as resuspension of pellets may occur.

6. After centrifugation, transfer the tubes to the BSC. From each tube, carefully remove all but 5 mL of supernatant using a 50-mL pipette and discard the removed supernatant. The pellet may be easily disturbed and not visible, so place the pipette tip away from the tube bottom or side.
7. Vortex and sonicate each tube using the steps below inside the BSC.
    a. Set the vortex mixer to the highest level.
    b. Turn on the sonicator water bath.
    c. Vortex each tube for 30 seconds (sec).
    d. Transfer tubes to sonicator bath, and sonicate them for 30 sec.
    e. Repeat the vortex and sonication cycle two additional times (three times per each sample tube).
8. Remove the suspension from one tube using a sterile 25-mL pipette and place it in one of the other tubes of the same sample. Repeat this process for the other two tubes of the same sample, resulting in one tube containing approximately 20 mL of the sample. This combined sample is the final elution suspension.
9. Measure the volume of the final suspension using a sterile 25-mL pipette, and record the volume in the laboratory notebook.

B4. Sample Analysis

1. Don appropriate PPE in accordance with BSL3 facility guidance.
2. In a BSC, Proceed to serially dilute and plate all elution suspension samples on TSA plates.
3. If the samples are turbid, wide-orifice pipette tips may be used to prevent clogging of pipette tips.
4. If dilution plating yields colony forming unit (CFU) counts below the quantification range, filter plate the samples.
5. Place all plates in an incubator set at 35 ± 2 degrees Celsius (ºC) for a maximum of 2 days. Plates should be examined within 18 to 24 hours after the start of incubation. Manually enumerate CFU counts for the target organism, and record the data. Re-examine the plates after 48 hours to check for additional CFU.

Appendix C: Sampling Procedure Using Commercially Available Wet Vacuum Cleaner for *Bacillus anthracis* Spores

July 2017

Revision 0.0

National Homeland Security Research Center
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

C1. Scope and Applicability

The U.S. Environmental Protection Agency's (EPA's) Homeland Security Research Program within EPA's Office of Research and Development, and the Chemical, Biological, Radiological and Nuclear Consequence Management Advisory Division within EPA's Office of Land and Emergency Management, jointly developed this sampling procedure. This procedure is intended to provide a method for trained incident responders to collect environmental samples after a biological contamination incident. This procedure specifically applies to the collection of surface-bound particulates and microorganisms using off–the-shelf robotic floor cleaners (RFC). The purpose of this procedure is to guide the process of preparation, deployment, and collection using RFCs for sampling surfaces in a specified area. The results from the collected samples can be used to determine the presence or absence of contamination and the contamination level after natural outbreaks and after intentional or accidental releases of pathogenic microorganisms and biotoxins.

At the time of publication, this sampling procedure has not been validated. At the date of this publication, the RFC sampling procedure has been partially characterized for deployment feasibility and collection performance for bacterial spores. This procedure will be updated or replaced with a fully characterized and validated procedure upon availability. During emergencies, the use of non-validated methods may be warranted when validated methods are not available. EPA's use of non-validated methods must adhere to the EPA's Forum on Environmental Measurement (FEM) policy directive on method validation (EPA 2010). Further information on method validation is presented in Validation of U.S. Environmental Protection Agency Environmental Sampling Techniques that Support the Detection and Recovery of Microorganisms (EPA 2012).

C2. Summary

This SP is for the sampling of a horizontal surface (such as a floor) using a wet vacuum cleaner. After sampling, the wet vacuum cleaner is recovered and processed to determine the presence or absence of potential surface contamination. This SP provides a step-by-step sampling procedure for the following wet vacuum cleaner:

- **Hoover Max Extract® Steam Vac Dual V® Cleaner (F7425-900 with SpinScrub Hand Tool; Hoover Company, North Canton, OH):** wet vacuum cleaner used to sample both porous surfaces (such as carpet, wood, and bare concrete) and nonporous surfaces (such as vinyl, tile, laminate, coated wood, and coated concrete)

The sections below discuss the following:

- Definitions (Section C3)
- Health and safety (Section C4)
- Waste management (Section C5)
- Equipment and supplies (Section C6)
- Deployment procedure (Section C7)
- Sample collection (Section C8)
- Post-deployment sample handling (Section C9)

- Documentation (Section C10)

Section C11 lists the references used to prepare this SP.

C3. Definitions

Biological agent contamination: contamination that can be attributed to both natural outbreaks and intentional or accidental releases of pathogenic microorganisms and biotoxins.

Biotoxin: a poisonous substance either produced by or extracted from living or dead organisms

Enclosed facilities and objects: facilities and objects that typically have surface areas with clearly defined boundaries such as walls and that are isolated from exterior environments, including commercial and residential buildings and transportation vehicles

Method of dissemination: the means by which biological agents are dispersed; dispersal can occur over large areas in wet and dry forms through aerosol generation and spreading devices that contaminate indoor and outdoor sites or the food or supply chain

Outdoor areas and objects: building exteriors and other outdoor areas such as streets, parks, and other open spaces with no clearly defined boundaries

Pathogen: a disease-causing agent that invades a host and replicates, including viruses, bacteria, and fungi

Sampling area: area expected to be similar to Hoover Company manufacturer's estimation of approximately 200 ft^2 per liquid container, depending on environment, flooring, furniture, and other site-specific factors

Wet vacuum cleaner: a commercially available, upright vacuum cleaner that dispenses and retrieves liquid cleaning agent

C4. Health and Safety

Laboratory testing of RFCs in contaminated areas indicates that the RFCs can cause resuspension of spores through physical surface agitation. Therefore, the site of an RFC sampling event could pose an exposure risk to the operator or support personnel. Operators should take precautions when setting up and deploying the RFCs, including the use of proper personal protective equipment (PPE), including (but not limited to) the use of a full-face powered air-purifying respirator (PAPR) with P100 cartridges and full body covering including built-in hood and foot covers. RFC deployment using delayed activation, if available, would reduce exposure risk.

Before exiting the exclusion zone (hot zone), sampling personnel should follow the standard operating guideline that provides guidance to EPA and its contractors on decontamination (decon) for personnel conducting long-term responses to biological contamination per EPA's CMAD decon line procedure. This standard operating guideline was developed specifically for biological responses using Level C PPE with a full-face PAPR or full-face air-purifying respirator (APR). Level C PPE is appropriate for most incidents involving biological agents and is required when the concentration and type of airborne substances is

known and the Occupational Safety and Health Administration (OSHA) criteria for using APRs are met (OSHA 1999). Level C PPE includes a protective coverall with integral hood and booties, an APR (preferably a PAPR), inner and outer nitrile gloves, hard hat (optional), and disposable outer boot covers. All use of respirators must comply with the OSHA Respiratory Protection Standard (29 CFR 1910.134).

There may be additional health and safety concerns associated with these sampling procedures. If the RFCs are operated on a raised subway platform, the operator should be cognizant of a potential fall hazard because the platform will not have a guardrail in place. Operators should maintain a safe distance from the edge. Additionally, heat stress may be a factor depending on the ambient temperature and length of time spent in modified Level C PPE. Appropriate work/rest regimens should be adhered to based on the wet bulb globe temperature (WGBT) and other factors such as acclimatization, hydration and fitness.

C5. Waste Management

The maximum usage for a wet vacuum cleaner is one sampling event. After a contaminated area is sampled, the sampling components of the wet vacuum cleaner are collected for analysis. The rest of the wet vacuum cleaner can be left in the hot zone for retrieval for sterilization and then can be either stored or disposed of.

C6. Equipment and Supplies

The Hoover F7452-900 cleaner has brushes and two nozzles to deliver equal suction power across the width of the nozzle. The cleaning nozzle is approximately 13 inches (in.) wide. The wet vacuum has separate clean and dirty liquid tanks as well as hand tools for cleaning hard-to-reach areas.

The dirty tank is isolated from the clean tank. A designated aliquot of about 100 milliliters (mL) is obtained from the dirty tank liquid for analysis. Before use during a field sampling event, the wet vacuum cleaner must be assembled from its store packaging. The user's manual provides assembly instructions. The materials listed below are additional materials required to prepare the Hoover F7452-900 cleaner for sampling and then securing the cleaner for shipment, storage, or analysis:

- Primary sample container, the dirty liquid tank
- Secondary sample containers consisting of extra-large food storage bags (such as Ziploc® XL HD Big Bags measuring 2 feet (ft) by 20 in.
- 0.05% Tween® 20 solution in a clean tank prepared using 5 liters (L) of deionized (DI) water with 2.5 mL Tween® 20, with 5-L level marked on the tank
- Sterilite 40-gallon wheeled industrial tote or equivalent (Item. No. 553504223, Walmart, Bentonville, AR)

  Note: The 0.05% Tween® 20 solution will be pre-loaded in the clean tank and stored in 40-gallon wheeled industrial totes until wet vacuum cleaner deployment.
- Pre-printed labels
- Logbook
- Sterile gloves

- Tychem suits with hood
- Boots and boot covers
- Chem tape
- PAPR or equivalent respirator
- Nitrile gloves
- Clear, labeled sampling kit containers for pre- and post-sampling events
- Wetted bleach wipes (in a canister)
- Sampling cart
- National Institute of Standards and Technology (NIST)-calibrated timers (verify time at time.gov)
- Appropriate disposal bags
- Pre-assembled Hoover F7452-900 cleaner stored in a sterile bag (such as a 42-in. by 42-in. Tyvek drawstring bag, General Econopak, Philadelphia, PA)
- Extension cords if needed
- 14-in. by 14-in., pre-cut sheets of Bond paper (in a sterile bag)
- Pens and permanent markers
- Tables and chairs
- Checklist and chain-of-custody (COC) forms
- Cooler (such as Igloo Model H-1353 industrial 5-gallon water cooler, Katy, TX)

C7. Deployment Procedure

The deployment procedures include pre-deployment preparation and the surface area assessment and wet vacuum cleaner deployment.

C7.1 Pre-deployment Preparation

Pre-deployment preparation should occur in the support zone (uncontaminated area where the sampling team and the sampling materials are not exposed to contaminants). The wet vacuum cleaners should each be labeled using a pre-set labelling scheme. The sampling kits and ancillary supplies should be placed in four separate bins. These bins can be large plastic bins with lids (such as Tupperware® bins) that will be placed on a cart for easy transport between deployment zones. Attach a trash bag to the cart for disposal of used gloves and boot covers. The contents of each bin are summarized below.

1. **Container 1** holds pre-labeled, extra-large food storage sampling bags containing the double-bagged dirty tank.
2. **Container 2** holds supplies such as extra gloves and boot covers, extra sampling bags, bond paper, wetted bleach wipes, boundary markers, serological pipettes, checklists, COC forms, pens, and timers.
3. **Container 3** contains the pre-loaded clean tank loaded with 0.05% Tween® 20.

C7.2 Surface Area Assessment and Wet Vacuum Cleaner Deployment

A sampling plan should be developed as part of the overall site decontamination plan and site safety plan. The sampling plan includes the following information:

- Surface area and layout of the sampling site
- Number of wet vacuum samplers required for each sampling site
- Sampling procedures

Based on this sampling site area, the sampling team should at a minimum consist of a lead sampler and a support person. The two-person team will wear the required site-specific level of PPE and work jointly to transport the wet vacuum cleaners and sampling supplies into the exclusion zone where the samples will be collected. A backup team should be prepared to relieve the sampling team at any time (two in, two out). At each predetermined sampling location, the team will deploy one wet vacuum cleaner in accordance with the procedures specified in this SP. The duties of each person are described below.

The **support person** shall perform the tasks below.

1. Verify that the items from the checklist are present on the cart.
2. Remove each Hoover F7452-900 wet vacuum cleaner from its bag, and place each wet vacuum cleaner individually just outside the sampling site.
3. Check and record pre-labeled information for each vacuum cleaner.
4. Attach the pre-loaded clean liquid tank to each wet vacuum cleaner (see Figure C-1).

Figure C-1. Hoover F7452-900 Clean Liquid Tank

5. Using the supplied cord (and an extension cord if necessary), plug in the vacuum cleaner.
6. Make notes of the deployment procedure in the logbook, including the start time, end time, start location, end location, label information, and any significant event during the sampling event. Take photographs if possible.
7. Don a new pair of outer gloves whenever deploying an additional wet vacuum cleaner.

Note: Change outer gloves after coming into contact with any suspected contaminated surface or item.

The **lead sampler** shall perform the tasks below.

1. Check that the contaminated area is suitable for vacuum cleaner deployment (no obstacles, trip hazards, etc.).
2. Verify the wet vacuum cleaner label with the support person, and then place the vacuum cleaner in a corner of the sampling area.
3. Ensure that the clean tank pre-loaded with 0.05% Tween® 20 and the dirty tank are properly seated in the vacuum cleaner.
4. Set the vacuum cleaner to “Wash Auto Rinse” mode, and slide the vacuum switch to “ON” position to start the wet vacuum sampling process (see Figure C-2).

Note: If moving between sampling areas, don new outer gloves at the new sampling location.

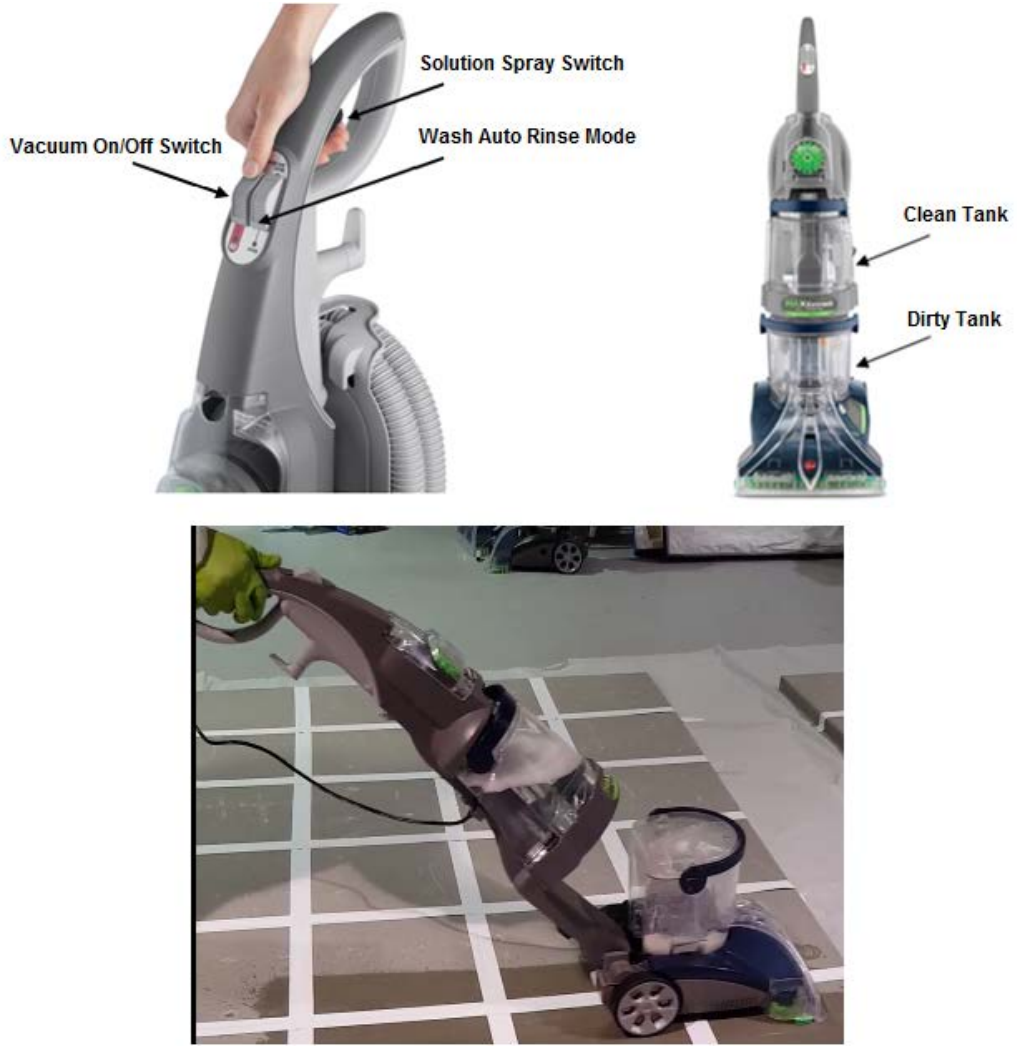

Figure C-2. Hoover F7452-900 Max Extract Cleaner

Each wet vacuum cleaner will be moved back and forth in a specified pattern on the designated sampling area using the steps summarized below.

1. Divide the width of the total sampling area by half of the nozzle width area ,and round the result to the nearest larger whole number, N. N will be the number of sampling strip passes. Each sampling strip will consist of half the nozzle width except for the last sampling strip, which will have a width of one nozzle (see Figure C-3).
2. Place the vacuum cleaner nozzle on the sampling area so that the front edge of the vacuum cleaner nozzle lip coincides with the line defining the beginning of the sampling area and the side of the nozzle coincides with the one side boundary of the second strip as shown in Figure C-3.
3. Complete Stroke 1, a backward stroke starting at the end of the sampling area.
4. Complete Stroke 2, a forward stroke.
5. Move the vacuum cleaner horizontally by half of the nozzle width area.
6. Repeat Steps 2 through 5 for each subsequent sampling strip to the end of the sampling area.

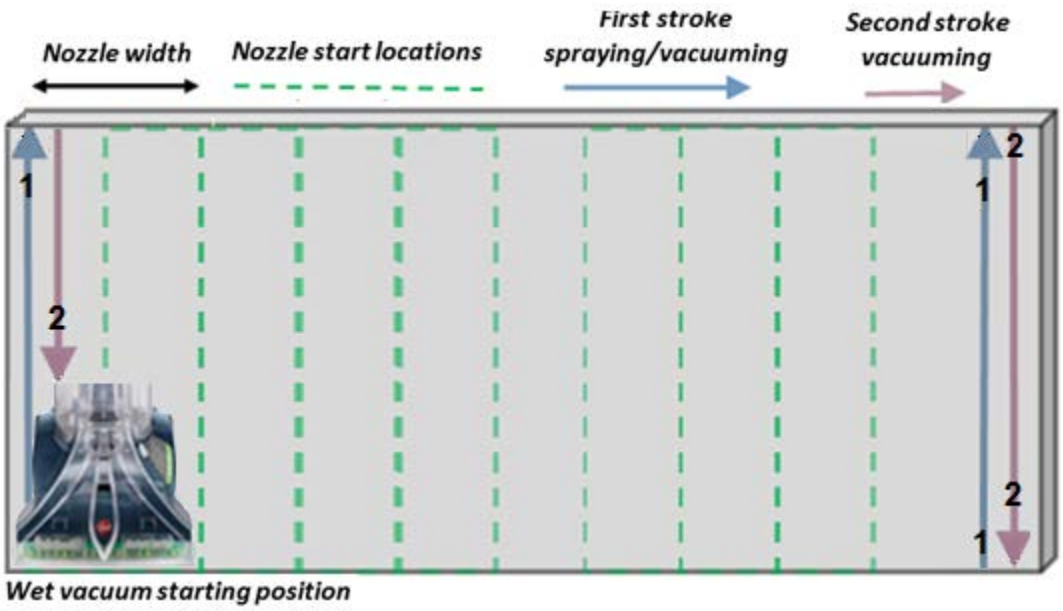

Figure C-3. Wet Vacuum Cleaner Sampling Pattern

C8. Sample Collection

After the vacuuming is completed, the samples will be collected from the wet vacuum cleaner. Each team member's sample collection duties are summarized below.

The **support person** shall perform the steps below.

1. Don a new pair of outer gloves.
2. Assist the lead sampler by handing out wetted bleach wipes as necessary.
3. Verify that the labels on the primary containers match the labels on the secondary containers and coolers, and that the lead sampler is handling samples correctly.
4. Check the samples on the COC form as complete.

5. Once all samples have been collected, assist the lead sampler in disinfecting Containers 1 through 3 and the sample cart.
6. Step out of the sampling area (exclusion zone) and proceed to the decon line (contamination reduction zone).

The **lead sampler** shall collect the liquid samples from the wet vacuum cleaner using the steps below.

1. Verify with the support person that the labels on the dirty tanks and the coolers match.
2. Retrieve the dirty tank from the wet vacuum cleaner (see Figure C-4), and disinfect the exterior of the tank using a wetted bleach wipe.

Figure C-4. Ziploc® Extra Large Bag and Hoover F7452-900 Dirty Tank

3. Double bag the dirty tank in pre-labeled, large food storage zipper bags (such as the Ziploc® bag shown in Figure C-4) for secondary sample containment. Disinfect the exterior of the outer bag using a wetted bleach wipe.
4. Place the bags with the dirty tank in a cooler such as the Igloo 5-gallon cooler shown in Figure C-5, and close the lid. Verify that the label on the dirty tank matches the cooler label. Add ice to the cooler.

Figure C-5. Igloo Industrial 5-Gallon Water Cooler

5. Disinfect the exterior of the cooler using a wetted bleach wipe, and place the entire cooler in Container 1. Disinfect the outside of Container 1 using a wetted bleach wipe.

6. Return the used wet vacuum cleaner to its bag, and disinfect the outside of the bag using a wetted bleach wipe.
7. Once all samples have been collected, assist the lead sampler in disinfecting Containers 1 through 3 and the sample cart.
8. Step out of the sampling area (exclusion zone) and proceed to the decon line (contamination reduction zone).

C9. Post-Deployment Sample Handling

Post-deployment sample handling requires sample preservation, identification, COC, and archiving as discussed below.

C9.1 Sample Preservation

Biological samples should be shipped ground in insulated containers with cold packs and appropriate biohazard label, refrigerated (2 to 8 degrees Celsius [°C]) until analysis, and be archived at 4 ± 2 °C. Samples should be processed and analyzed within 48 hours. Further sample transportation instructions are available at the Centers for Disease Control and Prevention website at http://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthracis.html.

C9.2 Sample Identification

Each sample will be identified using unique sample numbers. The sampling team will maintain an explicit laboratory log that includes records of each unique sample number. After transfer of the samples to the microbiology laboratory for analysis, each sample will be additionally identified (and photographed if available) by replicate number and dilution.

C9.3 Sample Chain of Custody

Careful coordination with the microbiology laboratory is required for successful transfer of uncompromised samples in a timely manner for analysis. Test schedules will be confirmed with the laboratory before the start of each test. To ensure the integrity of samples and to maintain a timely and traceable transfer of samples, an established and proven COC or possession procedure is mandatory. Accurate records must be maintained whenever samples are created, transferred, stored, analyzed, or destroyed. The primary objective of these procedures is to create an accurate written record that can be used to trace the possession of the sample from the moment of its creation through the reporting of its results. A sample is in custody if it is in any one of the following states:

- In actual physical possession
- In view after being in physical possession
- In physical possession and locked up so it cannot be tampered with
- In a secured area that is restricted except to authorized personnel
- In transit

The sampling team members will receive copies of test plans prior to each test. Pre-study briefings will then be held to apprise participants of the objectives, test protocols, and COC procedures to be followed. These protocols must mesh with any protocols established by EPA.

In the transfer of custody, each custodian will sign, record, and date the transfer. Sample transfer can be on a sample-by-sample basis or on a bulk basis. The protocol below will be followed for all samples as they are collected and prepared for distribution.

- A COC record will accompany the samples. When turning over possession of samples, the transferor and recipient will sign, date, and note the time on the COC record, which allows transfer of custody of a group of samples from the test site to the microbiology laboratory.

Samples will be carefully packed and shipped as hazardous material (hazmat) samples to the microbiology laboratory or will be hand carried between on-site laboratories.

C9.4 Sample Archiving Requirements

Each sample will be archived by maintaining each sample at 4 ± 2 °C in a sealed extraction tube until the data set has undergone quality control checks and the sample has been released for disposal. Any deviations from sampling protocols must be documented in the laboratory logbook. Sampling duration, time of day, and observations also will be recorded in the laboratory logbook.

C10. Documentation

All observations and experimental details will be recorded in a scientific logbook. Entries must meet quality assurance requirements (such as the use of indelible ink, corrections made using lineout deletions, witnessed signatures, and other requirements). The logbook will include information for all deviations from project procedures, including spills, deviations from the aseptic technique, and faulty wet vacuum function. In addition, if possible, the entire sampling procedure should be recorded on video. A video camera can be mounted above the sampling area. A team member should ensure that the entire sampling area is in the frame of the camera. Alternatively, a camera can be mounted to the operator if he or she remains in the enclosed area for the duration of the sampling event. Finally, the COC form ensures the integrity of samples and allows timely and traceable transfer of sample possession.

C11. References

Occupational Safety and Health Administration (OSHA). 1999. *OSHA's Respiratory Protection Standard.* Title 29 of the *Code of Federal Regulations* (CFR) 1910.134.

U.S. Environmental Protection Agency. 2010. Ensuring the validity of agency methods validation and peer review guidelines: Methods of analysis developed for emergency response situations. Washington DC: U.S. Environmental Protection Agency. Agency Policy Directive Number FEM-2010-01.

U.S. Environmental Protection Agency. 2012. Validation of U.S. Environmental Protection Agency environmental sampling techniques that support the detection and recovery of microorganisms. Prepared by: The FEM Method Validation Team, U.S. Environmental Protection Agency. Washington DC: U.S. Environmental Protection Agency. FEM 2012-01.

Appendix D: Coupon Inoculation Procedure for Spray-Dry Deposition

May 2017

National Homeland Security Research Center
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

SCOPE: Procedure for loading dry spores onto a test surface

PURPOSE: To provide consistent loading of spores onto test coupons for reaerosolization testing

MATERIALS:

- Deposition suspension in 50-milliliter (mL) conical tube
- 10% pH-amended household bleach solution
- 70% isopropanol solution
- Deionized (DI) water
- Coupon transfer case
- Disposable gloves
- Laboratory coat
- Safety glasses
- Test coupon
- New Era Pump Systems Inc. Multi-Phaser™ Model NE-1000 Syringe Pump
- 3-mL BD Luer lock syringe
- Deposition chamber
- Reference sample conical tube (sterile 50-mL conical tube with 10 mL of sterile phosphate-buffered saline with Tween® 20 [PBST])
- 50-mL conical tubes labeled for waste collection
- Vortex mixer
- Sonication bath
- Hospital-grade bleach disinfectant wipes (such as Dispatch®)
- Test coupons and medium-density fiberboard (MDF) coupon risers
- Spray adhesive

PROCEDURE:

Laboratory personnel should wear appropriate personal protective equipment (PPE), including a laboratory coat, safety glasses, and disposable gloves. All personnel handling the samples should be trained in the proper deposition procedure by an experienced staff member.

Spray-Dry Deposition

1. Ensure that all components of the deposition chamber are functional and correctly set up, as shown in Figure D-1 (with nozzle on deposition test stand) and Figure D-2 (with nozzle on spray-dry deposition chamber). **Note:** A clean, 50-mL conical tube labeled “waste” should be placed below the nozzle head when the nozzle is on the test stand to collect excess liquid.
2. Expel any sporistatic alcohol left in the deposition nozzle tubing and syringe from the decontamination process (see “Decontamination of the System” at the end of this procedure.
3. After expelling the alcohol, replace the syringe with a clean, 3-mL syringe.

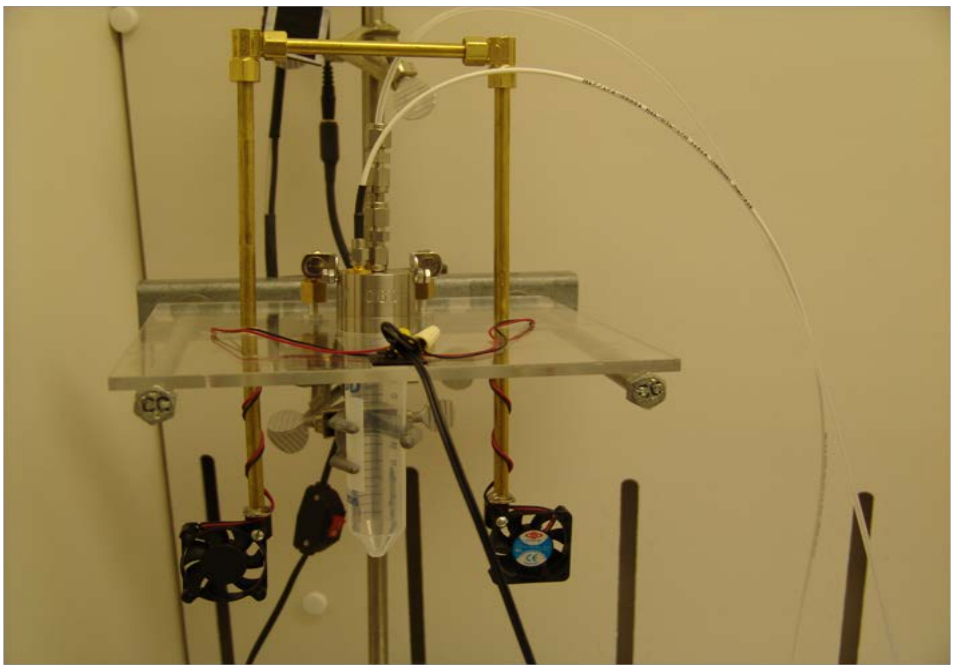

Figure D-1. Deposition Test Stand to Hold Nozzle for Collecting Reference Samples and between Depositions

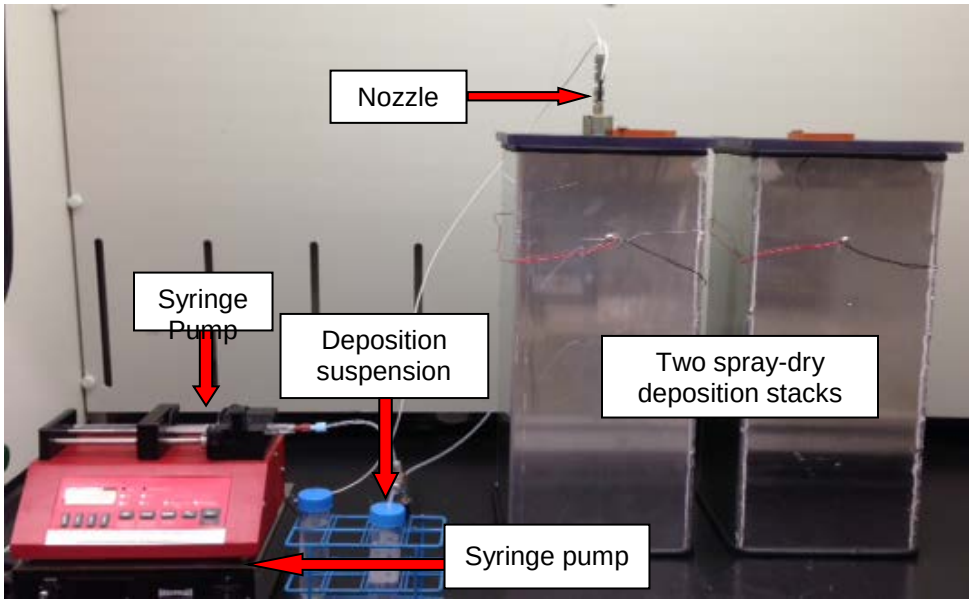

Figure D-2. Complete Spray-Dry Deposition Setup

4. Sonicate the deposition suspension for 30 seconds (sec), and then vortex the suspension for an additional 30 sec.
5. Place the deposition suspension tube in the tube rack, and screw on the cap with tube to connect the three-way valve.

Initial Programming of Syringe Pump

Refer to the user's manual if needed (New Era Pump Systems Inc. 2009).

1. Set the diameter, which is the internal diameter of the syringe to be used.
2. Set the rate to 200 microliters per minute (µL/min).
3. Set the dispensed volume to 200 µL.

Collection of a Positive Control Sample

1. Place the positive control tube in the test stand clamp.
2. Actuate the three-way valve to direct flow from the syringe to the deposition suspension, vigorously fill the syringe, and then expel its contents. Repeat this step 10 times.
3. Slowly fill the syringe, making sure there are no air bubbles.
4. Actuate the three-way valve to direct flow to the ultrasonic nozzle. Fully dispense the suspension from the syringe into the tubing leading to the nozzle.
5. Repeat Steps 2 through 4 until the desired volume is sampled.

Collection of a Reference Sample

1. Verify that a waste collection tube is in place under the ultrasonic nozzle, with the waste tube seated against the body of the nozzle.
2. Turn on lighting if needed.
3. Actuate the three-way valve to direct flow from the syringe to the deposition suspension, vigorously fill the syringe, and then expel its contents. Repeat this step 10 times.
4. Slowly fill the syringe, making sure there are no air bubbles.
5. Actuate the three-way valve to direct flow to the ultrasonic nozzle. Slowly dispense the suspension from the syringe into the tubing leading to the nozzle until the tubing is void of air.
6. Load the syringe into the syringe pump. Refer to the user's manual if needed (New Era Pump Systems Inc. 2009).
7. Turn on the syringe pump, and push any button to stop the LEDs from flashing.
8. Turn on the nozzle power generator.
9. Turn off the nozzle power generator.
10. Push the Start/Stop button on the syringe pump to dispense the deposition suspension at the programmed rate (200 µL/min) and volume (200 µL), and visually confirm that liquid is collecting on the nozzle.
11. Turn on the nozzle power generator, and verify that it is set to 2.5 watts (W).
12. Once a continuous mist is observed, continue dispensing another 10 µL.
13. Press the Start/Stop button on the syringe pump to stop the spray.
14. After approximately 3 sec, turn off the nozzle power generator and the syringe pump.
15. Remove, cap, and set aside the waste tube.

16. Place a reference sample collection tube under the nozzle, with the tube seated against the body of the nozzle.
17. Turn on the syringe pump, and push any button to stop the LEDs from flashing.
18. Turn on the nozzle power generator, and verify that it is set to 2.5 W.
19. Push the Start/Stop button on the syringe pump to dispense the suspension at the programmed rate (200 µL/min) and volume (200 µL).
20. When the pump has stopped dispensing, turn off the nozzle power generator and the syringe pump.
21. Allow the reference sample tube to settle for 2 min, and then carefully remove, cap, label, and vortex the sample for 30 sec.
22. Place the waste tube back into the tube clamp under the nozzle on the deposition test stand.
23. Remove the syringe from the pump and retract it slowly to remove all suspension from the tubing.
24. Dispense the suspension from the syringe into the tubing leading to the nozzle until the tubing is void of air.
25. Retract the syringe slowly to remove all suspension from the tubing.

Deposition onto a Test Coupon

1. Verify that a waste collection tube is in place under the ultrasonic nozzle, with the waste tube seated against the body of the nozzle.
2. Turn on the lighting if needed.
3. Load the coupon onto the transfer enclosure, place the deposition chamber over the coupon, and place the lid on the chamber.
4. Connect the mixing fans to the power supply.
5. Ground the chamber and lid.
6. Actuate the three-way valve to direct flow from the syringe to the deposition suspension, vigorously fill the syringe, and then expel its contents. Repeat this step 10 times.
7. Slowly fill the syringe, making sure there are no air bubbles.
8. Actuate the three-way valve to direct flow to the ultrasonic nozzle, and slowly dispense suspension from the syringe into the tubing leading to the nozzle until the tubing is void of air.
9. Load the syringe into the syringe pump. Refer to the user's manual if needed (New Era Pump Systems Inc. 2009).
10. Turn on the syringe pump, and push any button to stop the LEDs from flashing.
11. Turn on the nozzle power generator.
12. Turn off the nozzle power generator.
13. Push the Start/Stop button on the syringe pump to dispense the deposition suspension at the programmed rate (200 µL/min) and volume (200 µL), and visually confirm that liquid is collecting on the nozzle.
14. Turn on the nozzle power generator, and verify that it is set to 2.5 W.
15. Once a continuous mist is observed, continue dispensing another 10 µL.
16. Press the Start/Stop button on the syringe pump to stop the spray.

17. After approximately 3 sec, turn off the nozzle power generator and the syringe pump.
18. Turn on the syringe pump, and push any button to stop the LEDs from flashing.
19. Turn on the mixing fans.
20. Transfer the nozzle to the lid, and ground the lid.
21. Turn on the nozzle power generator, and verify that it is set to 2.5 W.
22. Push the Start/Stop button on the syringe pump to dispense the suspension at the programmed rate (200 µL/min) and volume (200 µL).
23. When the pump has stopped dispensing, turn off the nozzle power generator and the syringe pump.
24. Turn off the mixing fans.
25. Allow the deposition chamber to settle for 2 min.
26. Remove the nozzle, and slide the nozzle seat cover into place.
27. Disconnect all wires.
28. Remove the syringe from the pump and retract it slowly to remove all suspension from the tubing.
29. Dispense suspension from the syringe into the tubing leading to the nozzle until tubing is void of air.
30. Retract the syringe slowly to remove all suspension from the tubing.
31. Place the entire deposition chamber and transfer enclosure into the environmental chamber to dry and equilibrate. The minimum post-deposition equilibration time is 3 hours for all coupons.

Decontamination of the System

1. Retract all deposition suspension from the tubing, and dispense it back into the original container.
2. Actuate the three-way valve to direct flow from the syringe to the deposition suspension, vigorously fill the syringe, and then expel its contents. Repeat this step 10 times.
3. Remove the cap with tubing from the deposition suspension, and place the cap onto a 50-mL conical tube containing 10% pH-amended household bleach. Cap the deposition suspension tube with the original cap.
4. Fill the syringe with bleach solution, and dispense it into the nozzle tubing until approximately 1 mL has been ejected from the nozzle into the waste tube.
5. Refill the syringe.
6. Wait at least 2 min, and then expel all syringe and tubing contents into the waste tube.
7. Remove the cap with the tubing from the conical tube containing bleach solution, and place the cap into a conical tube containing DI water.
8. Fill the syringe with DI water, dispense the syringe into the nozzle tubing, and then fill the syringe with air and dispense it into the nozzle tubing to expel all liquid. Repeat this step four times.
9. Remove the cap with tubing from the conical tube containing DI water, and place the cap on the conical tube containing sporistatic alcohol.
10. Fill the syringe with sporistatic alcohol, dispense the syringe into the nozzle tubing, and then fill the syringe with air and dispense it into the nozzle tubing to expel all liquid. Repeat this step two times.

11. Fill the syringe with sporistatic alcohol, and dispense the syringe into the nozzle tubing until approximately 1 mL is expelled from the nozzle.
12. Refill the syringe with sporistatic alcohol.
13. Cap and dispose of the waste collection tube.
14. Place a new waste collection conical tube in the clamp under the nozzle.
15. Wipe all contaminated surfaces of the work area, the deposition chamber, and all experimental equipment using Dispatch® wipes or 10% pH-amended household bleach solution.
16. Wait at least 2 min.
17. Wipe all decontaminated surfaces of the work area, deposition chamber, and all experimental equipment with DI water.
18. Wipe all decontaminated surfaces of the work area, deposition chamber, and all experimental equipment with 70% isopropanol solution.

REFERENCE:

New Era Pump Systems Inc. (2009) *Multi-Phaser™ Model: NE-1000 Syringe Pump User Manual*, Publication #1200-01, Revision 15, V3.74, 02/10/09.

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