

Evaluation of Expedient Decontamination Options with Activated Peroxide-based Liquid Sporicides



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Assessment and Evaluation Report

National Homeland Security Research Center
Office of Research and Development
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Questions concerning this document or its application should be addressed to the principal investigator on this effort.

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Foreword

Following the events of September 11, 2001, addressing the critical needs related to homeland security became a clear requirement with respect to EPA's mission to protect human health and the environment. Presidential Directives further emphasized EPA as the primary federal agency responsible for the country's water supplies and for decontamination following a chemical, biological and/or radiological (CBR) attack. EPA's Homeland Security Research Program (HSRP) supports EPA's mission to assist in and lead response and recovery activities associated with CBR incidents of national significance. The National Homeland Security Research Center (NHSRC), within EPA's Office of Research and Development (ORD), leads the HSRP by conducting research and delivering products that improve the capability of the Agency and other federal, state and local agencies to carry out their homeland security responsibilities.

One goal of the HSRP is to provide information on decontamination methods and technologies that can be used in the response and recovery efforts resulting from a CBR release over a wide area. The complexity and heterogeneity of the wide-area decontamination challenge necessitates the understanding of the effectiveness of a range of decontamination options. In addition to effective fumigation approaches, rapidly deployable or readily available surface decontamination approaches have also been recognized as a tool to enhance the capabilities to respond to and recover from such an intentional CBR release.

Through working with ORD's program office partners (EPA's Office of Emergency Management and Office of Chemical Safety and Pollution Prevention) and Regional on-scene coordinators, the HSRP is attempting to understand and develop useful surface decontamination procedures for wide-area remediation. This report documents the results of a laboratory study designed to better understand the effectiveness of surface cleaning and decontamination methods in an attempt to develop a readily-deployable treatment procedure for surfaces contaminated with, for example, *Bacillus anthracis* spores.

These results, coupled with additional information in separate HSRP publications (available at www.epa.gov/nhsrc) can be used to determine whether a particular decontamination technology can be effective in a given scenario. The HSRP is pleased to make this publication available to assist the response community to prepare for and recover from disasters involving biological contamination. This research is intended to move EPA one step closer to achieving its homeland security goals and its overall mission of protecting human health and the environment while providing sustainable solutions to our environmental problems.

Jonathan Herrmann, Director
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List of Acronyms and Abbreviations

ADA	Aerosol Deposition Apparatus
AHP	Activated Hydrogen Peroxide
ATCC	American Type Culture Collection
<i>B.</i>	<i>Bacillus</i>
BSC	Biological Safety Cabinet
CBRN	Chemical, Biological, Radiological, and Nuclear
CFU	Colony Forming Unit(s)
cm	centimeters
CMAT	Consequence Management Advisory Team
COC	Chain of Custody
DF	decimal factor
DHS	Department of Homeland Security
DI	Deionized
DPG	Dugway Proving Ground
DQO	Data Quality Objective
DTRL	Decontamination Technologies Research Laboratory
ECBC	Edgewood Chemical Biological Center
ft	feet or foot
ft ²	square feet or foot
FAC	free available chlorine
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HEPA	High Efficiency Particulate Air
IBRD	Interagency Biological Restoration Demonstration
in	Inch(es)
INL	Idaho National Laboratory
LR	Log Reduction
MDI	Metered Dose Inhaler
MOP	Miscellaneous Operating Procedure
NHSRC	National Homeland Security Research Center
NIST	National Institute of Standards and Technology
OPP	Office of Pesticide Programs
ORD	Office of Research and Development
OSWER	Office of Solid Waste and Emergency Response
PAA	Peroxyacetic acid (or Peracetic acid)
pAB	pH-Adjusted Bleach
PARTNER	Program to Align Research and Technology with the Needs of Environmental Response
PBST	Phosphate Buffered Saline with 0.05% TWEEN® 20
PPE	Personal Protective Equipment
ppmv	parts per million by volume
psi	pounds per square inch
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
SD	Standard Deviation

SNL	Sandia National Laboratories
SAR	Supplied-Air Respirator
SI	International System of Units
STS	Sodium Thiosulfate
TSA	Tryptic Soy Agar
TSP	Trisodium Phosphate
UV	Ultraviolet (light)
VHP	Vaporized Hydrogen Peroxide
WA	Work Assignment
WARRP	Wide Area Recovery and Resiliency Program

Executive Summary

This project supports the mission of the U.S. Environmental Protection Agency's Office of Research and Development's Homeland Security Research Program (HSRP) by providing information relevant to the decontamination of areas contaminated with a biological agent.

This project evaluated "low tech" expedient decontamination options with an Activated Hydrogen Peroxide (AHP)-based liquid sporicide. The AHP decontamination solution was generated in an alkaline environment, but the final product reaches slightly alkaline to neutral pH rapidly, making it safer and easier to handle and substantially less corrosive than bleach-based sporicides.

The efficacy of liquid AHP was evaluated on common building materials (stainless steel, painted and unpainted plywood, concrete, carpet, linoleum, glass, and tile) experimentally inoculated with aerosolized spores of *Bacillus atrophaeus* (surrogate of *Bacillus anthracis*). Evaluation exercised two operational procedures: (Procedure 1) spray-apply AHP, maintain vertical position for 15 minutes (no rinse), and dry overnight before surface sampling, and (Procedure 2) spray-apply AHP, maintain vertical position for 15 minutes (no rinse), reapply AHP by spraying, maintain vertical position for 15 minutes (no rinse), and dry overnight before surface sampling. Estimates of the number of spores removed (e.g., aerosol or liquid runoff) from the material surface during the decontamination procedures were also performed.

For the AHP single application procedure (Procedure 1), the average log reduction (LR) values ranged from 1.6 to 8.4. Typically, nonporous materials were easier to decontaminate than were porous. The exception, carpet, was relatively easy to decontaminate. Of all the materials, concrete demonstrated the lowest LR (< 2 LR) with Procedure 1. Nonetheless, greater than 6 LR was achieved for 50% of materials decontaminated with the one step procedure (Procedure 1). The AHP dual-application procedure (Procedure 2) resulted in greater than 6 LR of seven on the eight materials tested (LR values from 6.8 to 8.4). High decontamination efficacy was not achieved for unpainted treated wood material, regardless of the application procedure (mean LR = 4.2). Complete kill (recovery of no viable spores after decontamination) was achieved on carpet and glass using Procedure 1, and on Tile, stainless steel, carpet, glass, and linoleum using Procedure 2.

These data suggest that this "low-tech" decontamination process, when using the two-application procedure described herein, can provide > 6 LR in the number of viable spores on the common building materials tested, with the exception of unpainted treated wood. The longer exposure duration and increased volume of AHP applied, associated with Procedure 2, afforded higher surface decontamination efficacy, especially for porous materials. Materials with high organic demand (e.g., unpainted wood) that are typically more difficult to decontaminate may present a challenge to the liquid AHP-based process. The preliminary fate of spores estimates suggest that the AHP liquid sporicide-based decontamination process leads to physical removal of spores from decontaminated surfaces, with a consequent transport of viable spores to air and to the post-decontamination liquid waste. In several tests, considerable amounts (up to 7 logs) of viable spores were recovered in the liquid runoff.

1. Introduction

The Department of Homeland Security (DHS) and other appropriate Federal departments and agencies have been tasked to develop comprehensive plans which “provide for seamless, coordinated Federal, state, local, and international responses to a biological attack.” As part of these plans, EPA, in a coordinated effort with DHS, is responsible for “developing strategies, guidelines, and plans for decontamination of persons, equipment, and facilities” to mitigate the risks of contamination following a biological weapons attack [1]. One goal of the EPA’s Homeland Security Research Program (HSRP) is to provide expertise and guidance on the selection and implementation of decontamination methods and provide the scientific basis for a significant reduction in the time required for recovery and the cost of decontamination events. To meet this goal, EPA’s National Homeland Security Research Center (NHSRC) provides expertise and products that can be widely used to help prevent, prepare for, and recover from public health and environmental emergencies arising from terrorist threats and incidents.

Decontamination can be defined as the process of inactivating or reducing a contaminant in or on humans, animals, plants, food, water, soil, air, areas, or items through physical, chemical, or other methods to meet a cleanup goal. In terms of the surface of a material, decontamination can be accomplished by physical removal of the contaminant or via inactivation of the contaminant with antimicrobial chemicals, heat, ultraviolet (UV) light, etc. Physical removal could be accomplished via *in situ* removal of the contaminant from the material or by physical removal of the material itself (i.e., disposal) [2-6]. Similarly, inactivation of the contaminant can be conducted *in situ* or after removal of the material for ultimate disposal [2, 3, 5, 6]. Following the 2001 anthrax incidents, a combination of removal and *in situ* decontamination was used [2, 3]. The balance between the two procedures was facility-dependent and factored in many issues (e.g., physical state of the facility). One factor was that such remediation was unprecedented for the United States Government, and no decontamination technologies had been proven for use against spores of *Bacillus anthracis* at the time. The cost of disposal proved to be significant and was complicated by the nature of the waste (e.g., finding an ultimate disposal site). Since 2001, a primary focus for facility remediation has been improving the effectiveness and practical application of *in situ* decontamination methods and evaluating waste treatment options to be able to provide information necessary to optimize the decontamination/disposal paradigm. This optimization has a significant impact on reducing the cost of and time for the remediation effort.

The HSRP’s decontamination-related efforts support the Office of Solid Waste and Emergency Response (OSWER), with the Office of Pesticide Programs (OPP) as a key stakeholder. OSWER, through its Special Teams which include the Chemical, Biological, Radiological, and Nuclear (CBRN) Consequence Management Advisory Team (CMAT), supports the emergency response functions carried out by the Regional Offices. OPP supports the decontamination effort by providing expertise on biological agent inactivation and ensuring that the use of pesticides in such efforts is done in accordance with the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Close collaboration between the different program offices having homeland security responsibilities is sought to rapidly increase the EPA’s capabilities to help the Nation recover from a terrorist event involving the intentional release of chemical, biological, and radiological (CBR) materials.

Quick, effective and economical decontamination methods that have the capacity to be employed over wide areas (outdoor and indoor) required to increase emergency preparedness are one specific focus of the

HSRP. Numerous decontamination methods have been and continue to be evaluated under the HSRP. These decontamination approaches span the spectrum from sophisticated technologies such as fumigations; to more readily-available approaches such combined mechanical and chemical procedures (vacuum, scrub/wash and bleach) for surface decontaminations. If proven effective, expedient approaches involving washing and cleaning with readily-available equipment and off the shelf sporicides would significantly increase EPA's readiness to respond to a wide-area contaminant release.

The research described in this report was conducted by EPA under the DHS-led Wide Area Recovery and Resiliency Program (WARRP), which holistically aims to increase our Nation's ability to respond to and recover from acts of chemical, biological, or radiological (CBR) terrorism. This work was a continuation of previous decontamination studies with pH amended bleach (pAB)- based liquid sporicides. The effectiveness of amended bleach has been demonstrated, yet several downsides of this technology have been documented [2, 3, 7]. Bleach, and especially amended bleach, is known to be corrosive to metals and its application often necessitates the use of specialized personal protective equipment (PPE) due to chlorine off-gassing. This project evaluated an alternative to bleach, activated hydrogen peroxide (AHP), for 'low-tech' decontamination applications. Peroxides (-O-O-) are strong oxidants that offer an environmentally friendly alternative to the toxic and corroding chlorine-based decontaminants [8, 9]. The sporicidal effectiveness of hydrogen peroxide (H_2O_2) solutions is due to the peroxy anion (OOH^-) and hydroxyl free radicals (OH^\bullet) [9, 10]. Another peroxy compound that is commonly used, and often added as a supplemental oxidizing agent in mixtures with H_2O_2 , is peracetic acid – PAA, $CH_3CO(OOH)$ [8]. PAA is effective against all microorganisms, including bacterial spores, due to its high oxidizing potential. The disinfectant composition mixtures of H_2O_2 and PAA have a synergistic sporicidal efficacy [8]. Mixed H_2O_2 and PAA liquid sporicides are most commonly prepared just prior to use by combining two or more components. Due to stringent shipping and handling requirements, PAA is often generated *in situ*, prior to decontamination, using activated H_2O_2 solutions. This method has many variations, but the foundation of the chemistry is the same; generation of PAA through the perhydrolysis of an acetyl donor, with a specifically formulated H_2O_2 solution as a peroxygen source [9, 10].

1.1 Process

The general process investigated in this project was decontamination of building surfaces contaminated with *Bacillus* spores (i.e., surrogates of *B. anthracis*). Test coupons were decontaminated with AHP sprayed using two different decontamination procedures. Positive control coupons (i.e., contaminated with spores but not decontaminated) were used to determine the pre-treatment (pre-decontamination) loading on each coupon type and were a reference for decontamination efficacy calculations (see Section 3).

The decontamination operational procedures were developed based on the previous EPA and Sandia National Laboratories (SNL)/Interagency Biological Restoration Demonstration (IBRD) efforts [11]. Decontamination procedures provided by SNL were refined and tested through pilot-scale evaluation in a decontamination test chamber in the NHSRC testing facilities.

After decontamination, surface sampling of coupons was conducted with wetted wipes or vacuum socks, depending upon material type. Liquid runoff samples and aerosol samples were also collected to determine potential routes of contamination spread by the procedures. Quality control (QC) samples such as procedural blank coupons (coupons that underwent the decontamination process but which were not

inoculated) and negative controls (which were not inoculated and did not undergo the decontamination process) were included in order to monitor for cross-contamination.

All samples were analyzed for the quantitative determination of viable spores recovered. Overall decontamination effectiveness of AHP was determined as a function of the procedures and material types. The fate of the spores (viable spores transferred to runoff or air) was also determined.

1.2 Project Objectives

The primary objectives of this study were:

1. To determine occurrence and potential reduction of viable bacterial spores (i.e., effectiveness) on porous and nonporous building materials decontaminated with AHP-based liquid sporicides;
2. To determine the effect of varying the application procedure on sporicidal efficacy (i.e., the effect of re-application and the increased contact time); and
3. To determine the fate of the spores during the decontamination process.

The operational parameters of decontamination (e.g., pH and temperature of decontamination solution within the pot life of the decontamination solution, effectiveness of AHP delivery [as a function of the spray apparatus flow rate and spray coverage/pattern]) were considered important to understand the sporicidal activity of an AHP-based decontamination process and were characterized in addition to sporicidal effectiveness.

2. Experimental Approach

This section describes the test materials, test facilities and equipment, general decontamination approach and test conditions, and the methods that were used to evaluate the data related to the project objectives. Testing was conducted by EPA's Decontamination Technologies Research Laboratory (DTRL), in a spray chamber described previously [5] and located at EPA's Research Triangle Park facility. Sampling and analytical procedures are described in Section 4.

2.1 Preparation of Coupons

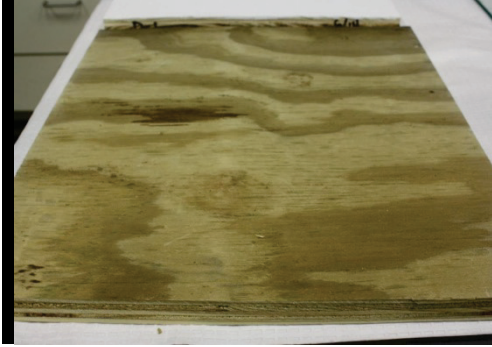
Coupons (14 inch by 14 inch) were prepared from eight types of porous and nonporous building materials (Table 2-1, Figure 2-1) that are typical of the materials found in residential dwellings and meet industry standards or specifications for indoor use. Documented procedures were established and followed for coupon preparation (see Appendix B, DTRL MOP #3150) to ensure uniformity across samples and experiments.

Table 2-1. Descriptions of Test Materials

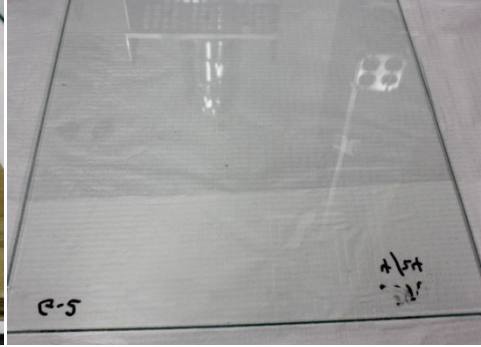
Materials	Source	Description	Part Number
Porous Materials			
Painted treated plywood	Georgia Pacific Atlanta, GA	23/32" Alkaline Copper Quaternary Treated Plywood	"Plytanium® Sheathing"
Unpainted treated plywood	Georgia Pacific Atlanta GA	23/32" Alkaline Copper Quaternary Treated Plywood, painted with BEHR Exterior Flat White	"Plytanium® Sheathing" BEHR Paint #4850 Home Depot #263-050
Carpet	Home Depot Durham, NC	Beaulieu Loop Carpet	409-921
Unsealed concrete	Home Depot Durham, NC	Quikrete Sand/Topping Mix, trowel-smoothed surface	929-522
Nonporous Materials			
Stainless steel	Dillon's Supply Co. Raleigh, NC	304 stainless steel, 16- gauge	n/a
Glass	Durham Glass, Inc. Durham, NC	1/4" (thickness) residential glass, seamed edges	n/a*
Linoleum	Home Depot Durham, NC	Armstrong Residential Sheet Vinyl Flooring	243-154
Tile	Home Depot Durham, NC	MARAZZI Ceramic Floor Tile; smooth, glazed finish	538-570

*n/a = not applicable

a. Unpainted treated plywood



b. Glass



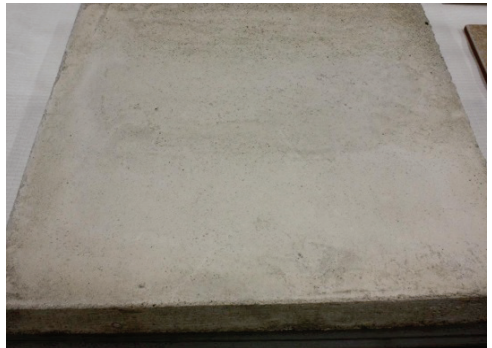
c. Painted treated plywood



d. Linoleum



e. Unsealed concrete



f. Carpet



g. Stainless steel



h. Tile



Figure 2-1. Test Coupons

The stainless steel and glass coupons were sterilized prior to use by steam autoclave utilizing a gravity cycle program (see Appendix B, BioLab MOP #6570). The remaining materials (painted and unpainted treated plywood, carpet, linoleum, tile, and unsealed concrete) were sterilized using VHP[®] (1000 parts per million volume * hours, ppm-hrs) generated using a STERIS VHP[®] 1000ED generator loaded with a 35% H₂O₂ Vaprox[®] cartridge. The sterility of the coupons was verified through the use of laboratory blank control samples.

2.2 Inoculation of Coupons

Inoculation of test and positive control coupons with spores of *B. atrophaeus* was performed via aerosol deposition using a metered dose inhaler (MDI).

The test organism for this work was a powdered spore preparation of *B. atrophaeus* American Type Culture Collection (ATCC) 9372 and silicon dioxide particles. This bacterial species was formerly known as *B. subtilis* var. *niger* and subsequently *B. globigii*. The preparation was obtained from the U.S. Army Dugway Proving Ground (DPG) Life Science Division. The preparation procedure is reported in Brown et al. [11] Briefly, after 80 – 90 percent sporulation, the suspension was centrifuged to generate a preparation of approximately 20 percent solids. A preparation resulting in a powdered matrix containing approximately 1×10^{11} viable spores per gram was prepared by dry blending and jet milling the dried spores with fumed silica particles (Deguss, Frankfurt am Main, Germany). The powdered preparation was loaded into MDIs by ECBC according to a proprietary protocol. QA documentation is provided by ECBC with each batch of MDIs. Control checks for each MDI were included in the batches of coupons dosed with a single MDI.

Coupons (test and positive controls) were inoculated with $\sim 2 \times 10^8$ spores of *B. atrophaeus* from an MDI using a modification to the procedure detailed in modified BioLab MOP #6561 (see Appendix B). Each coupon was inoculated (dosed) independently by being placed into a separate dosing chamber designed to fit one 14 in by 14 in coupon of any thickness. In accordance with modified BioLab MOP #6561, the MDI was discharged a single time into the dosing chamber. The MDIs are claimed to provide 200 discharges per MDI. The number of discharges per MDI was tracked so that use did not exceed this value. Additionally, in accordance with modified BioLab MOP #6561, the weight of each MDI was determined after completion of the contamination of each coupon.

Note: Due to problems with loading coupons observed during tests 1,6,8,9, and 14, an extra Quality Assurance (QA) step was temporarily introduced to the inoculation procedure. Before and after each stainless steel inoculation control coupon and every three test coupon samples, stainless stubs were dosed and immediately processed by the on-site Biocontaminant Laboratory. A new procedure for actuator cleaning between actuations was also developed (vertical actuator orifice was cleaned using compressed air between each of the actuations). The latter procedure was used in all subsequent inoculations.

The contamination control coupons (14 in by 14 in stainless steel coupons) were inoculated as the first, middle, and last coupons within a single group of coupons inoculated by any one MDI within a single test. Following dissemination, spores were allowed to settle onto the coupon surfaces for a minimum period of 18 hours.

A log was maintained for each set of coupons that were dosed via modified BioLab MOP #6561. Each record in this log consisted of the unique coupon identifier, the MDI unique identifier, the date, the operator,

the weight of the MDI before dissemination into the coupon dosing device, the weight of the MDI after dissemination, and the difference between these two weights. The coupon codes were pre-printed on the log sheet prior to the start of coupon inoculation (dosing).

After the minimum 18-hour period, the coupons were removed from the dosing chamber and moved to an isolated cabinet (Test Coupon Cabinet) which contained all inoculated coupons for a single test. The handling of the inoculated coupons, including movement to minimize or control spore dispersal, was done in accordance with the modified BioLab MOP #6561. Procedural blank coupons were stored in a separate isolated cabinet (Blank Coupon Cabinet).

2.3 Preparation of Decontamination Solution

The AHP decontamination formulation was developed by SNL under the WARRP research efforts. The original recipe for preparation of AHP provided by SNL is given in Table 2-2. For preparation of large batches of AHP, the original recipe was modified (Table 2-2) as follows:

- 15% H₂O₂ was used to prepare the decontamination solution; this change was due to facility-specific Health and Safety regulations regarding alkalization of 50% H₂O₂;
- the discontinued alkyldimethylbenzyl ammonium chlorides (C12-C16)/isopropyl alcohol –based surfactant (Variquat 80 MC) was replaced with a similar commercially available product; (alkyldimethylbenzyl ammonium chlorides (C12-16)/alkyldimethylamines(C12-C16)/ethanol- based surfactant; Maquat MC 1412-80%E; and
- the addition of potassium carbonate was slightly higher (160 g/5 L or 3.2 g/100 mL of final formulation) than in the original recipe (3 g/100 mL of final formulation); this amount was optimized for mixing of large batches of AHP and resulted in the pH values closest to the target pH of 9.5 immediately post-mixing.

Table 2-2. AHP Decontamination Solution Recipes

	Original recipe*		Modified recipe**	
Part A	DI water [mL]	76.9	DI water [mL]	2435
	K ₂ CO ₃ [g]	3	K ₂ CO ₃ [g]	160
	Variquat [mL]	0.1	Maquat [mL]	5
	Ethanol [mL]	5	Ethanol [mL]	250
Part B	50% H ₂ O ₂ [mL]	12	15% H ₂ O ₂ [mL]	2000
Part C	Triacetin [mL]	3	Triacetin [mL]	150

* original recipe as developed by SNL for small scale testing.

** large batch (5000 mL) modified recipe as used in EPA pilot-scale testing; details on stock solutions of chemicals used for preparation of decontamination solution are given in Appendix C.

The decontamination formulation was prepared according to DTRL MOP #3177 (see Appendix B) by mixing Part A with Part B, and then adding Part C (the Activator) to the Part A/B mixture. The solution was ready to use upon activation (combination of ingredients) and required no further dilution or manipulation of components.

The target concentration of H₂O₂ (6% immediately post mixing) was verified by potassium permanganate titration (see Appendix B, DTRL MOP #3177) and then monitored throughout testing (see Section 5.1.2). The temperature and pH measurements were performed for each batch of the AHP solution prepared and checked prior to each application of sporicide (see Section 5.1.1). Per communication with SNL, the pot life of the AHP formulation was assumed to be two hours.

2.4 Decontamination Procedures

Decontamination procedures were developed in collaboration with SNL and are described in detail in DTRL MOP #3177 (see Appendix B). An overview of the Decontamination Test Matrix is outlined in Table 2-3.

Briefly, sets of the building material coupons were inserted in vertical position in the test coupon holders of the small spray test chamber (Figure 2-2). The chamber dimensions were 4 ft high by 4 ft wide by 4 ft deep. The chamber was designed to accommodate three 14 x 14 inch coupons at a time in a horizontal or vertical orientation. In this study, only the vertical orientation assembly was utilized. The chamber was constructed of solid stainless steel with the exception of the front face and top which were both clear acrylic plastic. The acrylic door was fitted with ports to allow the insertion of the backpack delivery system into the chamber. Although the chamber is fitted with three ports (each port centered in front of one of the triplicate coupons), to administer a seamless and rapid (15 seconds) application of the decontaminant to the set of coupons, only the center-positioned port was used for this study. The other two ports remained in the closed position during testing.

Decontamination solution was applied using a SHURflo ProPack™ SR600 Rechargeable Electric Backpack Sprayer (SHURflo Inc., USA) equipped with a TeeJet spray wand with an adjustable tip (SHURflo Inc.,

USA). The sprayer wand could be moved to accomplish the desired spray pattern. The reverse-pyramid design of the chamber bottom allowed for collection of runoff from the coupons during the decontamination procedure through a central (3 in diameter) drain. The bottom of the chamber has a 189 L (50 gallon) collection capacity.



Figure 2-2. Decontamination Chamber for AHP-Based Decontamination Testing (Left) and Application of AHP Using Backpack Sprayer (Right)

Table 2-3. Decontamination Test Matrix

Test ID	Material	Decon Procedure
1R	Painted treated plywood	Procedure 1
2	Stainless steel	Procedure 1
3	Carpet	Procedure 1
4	Glass	Procedure 1
5	Linoleum	Procedure 1
6R	Painted treated plywood	Procedure 2
7	Stainless steel	Procedure 2
8R	Carpet	Procedure 2
9R	Glass	Procedure 2
10	Linoleum	Procedure 2
11	Concrete	Procedure 1
12	Tile	Procedure 1
13	Unpainted treated plywood	Procedure 1
14R	Concrete	Procedure 2
15	Tile	Procedure 2
16	Unpainted treated plywood	Procedure 2

Note: "R" tests indicate repeated tests, as initial attempt was aborted due to failed inoculation

The chamber was fitted with connections allowing filtered air to enter and filtered exhaust to exit via a readily accessible connection to the facility's air handling system. Aerosol samples were collected from the chamber using Via-Cell[®] BioAerosol Cassettes. The sampling point was approximately 5 cm above and 45 cm in front of the coupon surface (to approximate the head position of a decontamination worker).

After the Via-Cell[®] BioAerosol Cassettes and coupons were assembled, the decontamination sequence continued as follows:

1. Verification of critical operational parameters (pH, concentration of H₂O₂ and temperature of AHP decontamination solution, check of the backpack sprayer flow and spray pattern; results are described in Section 4.3).
2. Application of a prescribed decontamination sequence. Two different decontamination procedures (Procedure 1 and Procedure 2) were evaluated for each type of test material. The Procedure 1 decontamination sequence was: "spray once-wait 15 min-no rinse-dry overnight", while the Procedure 2 sequence was "spray-wait 15 min-reapply-wait 15 min-no rinse-dry overnight".
3. After the exposure time (15 min or 2 x 15 min) was reached, test coupons, procedural blanks, and positive controls were moved to designated storage cabinets and allowed to dry overnight.
4. On the following day, coupons were sampled using sampling techniques described in Section 4.1 and transferred to the on-site Biocontaminant Laboratory for microbiological analysis. Samples were transferred in sterile primary independent packaging within sterile secondary containment containing logical groups of samples for analysis. All samples were accompanied by a completed chain of custody (COC) form.
5. After the on-site Biocontaminant Laboratory performed a quantitative assessment of viable spores for each type of samples, the determination of surface decontamination efficacy (comparison of viable spore concentrations from positive controls and test coupons) was performed (see Section 4.2).

2.5 Neutralization Tests

To test the effect of residual AHP on spore recovery, a set of preliminary tests was performed to investigate the need for neutralization of samples post-collection. The test matrix for these neutralization tests is shown in Table 2-4.

Table 2-4. Neutralization Test Matrix

Test	Decontaminated	Material Type	Total # of Coupons
A1	Yes	Plywood	5
A2	No	Plywood	5
B1	Yes	Stainless Steel	5
B2	No	Stainless Steel	5

Five replicate coupons of each type (sprayed and not sprayed) were sampled using wipes. Wipes were then extracted with the sample extraction solution, and the extract solution was then spiked with 1×10^7 spores of *B. atrophaeus*. Recoveries from the decontaminated coupons (sprayed) were compared to recoveries from blank (not sprayed) coupons. If a statistically significant difference existed between the two

populations (recovery from sprayed vs. recovery from not sprayed) for each coupon type, then neutralization of samples post-collection would be needed. No statistical difference was observed between recoveries from sprayed or not sprayed coupons, hence the use of neutralizer on coupons was not considered necessary (see Section 5.3.1).

The runoff samples were neutralized using a 30% sodium thiosulfate (STS) solution. The target concentration of STS in a single runoff sample needed to neutralize residual AHP without affecting recovery assays was adopted from the results of liquid-based testing performed by SNL. In the liquid-based experiments, 5 mL of 30% STS was added to 35 mL of total solution for each sample, resulting in the final STS concentration of 4.3%. The maximum volume of the AHP runoff per 15 second spray was estimated as approximately 200-250 mL depending on the backpack sprayer flow rate (in the preliminary testing, 210 mL of the AHP solution runoff was collected from stainless steel per spray; no water rinse included). Hence, 42 mL of STS solution per single spray application was added to each runoff collection container (carboy) prior to the runoff collection. Results for the concentration of H_2O_2 in the neutralized runoffs are given in Section 5.3.1.

3. Determination of Sporicidal Effectiveness

The sporicidal effectiveness (efficacy) of a decontamination technique is a measure of the ability of the method to inactivate and/or remove the spores from a contaminated material surface (i.e., represented by coupons in this study). The sporicidal effectiveness is evaluated by measuring the difference in the logarithm of the measured colony forming units (CFU) before decontamination (determined from sampling the positive control coupons) and after decontamination (determined from sampling the test coupons) for the same type of material. The number of viable spores recovered was measured as CFU and reported as a log reduction on the specific material surface as defined in Equation 1-1.

$$\eta_i = \frac{\sum_{k=1}^{N_C} \log(CFU_{C,k})}{N_C} - \frac{\sum_{k=1}^{N_s} \log(CFU_{S,k})}{N_s} \quad (1-1)$$

where:

η_i = Surface decontamination effectiveness; the average log reduction of spores on a specific material surface (surface material designated by i)

$\frac{\sum_{k=1}^{N_C} \log(CFU_{C,k})}{N_C}$ = The average of the logarithm (or geometric mean) of the number of viable spores (determined by CFU) recovered on the control coupons (C indicates control and N_C is the number of control coupons)

$\frac{\sum_{k=1}^{N_s} \log(CFU_{S,k})}{N_s}$ = The average of the logarithm (or geometric mean) of the number of viable spores (determined by CFU) remaining on the surface of a decontaminated coupon (S indicates a decontaminated coupon and N_s is the number of coupons tested).

When no viable spores were detected, a value of 0.5 CFU was assigned to the maximum plated volume to determine the detection limit for $CFU_{S,k}$, and the efficacy was reported as greater than or equal to the value calculated by Eqn. 1-1.

The standard deviation of the average log reduction of spores on a specific material (η_i) is calculated by Eqn. 1-2:

$$SD_{\eta_i} = \sqrt{\frac{\sum_{k=1}^{N_s} (x_k - \eta_i)^2}{N_s - 1}} \quad (1-2)$$

where:

SD_{η_i} = Standard deviation of η_i , the average log reduction of spores on a specific material surface

η_i = The average log reduction of spores on a specific material surface (surface material designated by i)

x_k = 1.1.1.1.1.1 The average of the log reduction from the surface of a decontaminated coupon (Equation 1-3)

N_s = 1.1.1.1.1.2 Number of test coupons of a material surface type.

and

$$x_k = \frac{\sum_{k=1}^{N_s} ((\log(CFU_c)) - \log(CFU_{s,k}))}{N_s} \quad (1-3)$$

where:

$\overline{\log(CFU_c)} = \frac{\sum_{k=1}^{N_c} \log(CFU_{c,k})}{N_c}$ = Represents the “mean of the logs” (geometric mean), the average of the logarithm-transformed number of viable spores (determined by CFU) recovered on the control coupons (C = control coupons, N_c = number of control coupons, k = test coupon number and N_s is the number of test coupons)

$CFU_{s,k}$ = Number of CFU on the surface of the k^{th} decontaminated coupon

N_s = 1.1.1.1.1.2.1.1 Total number (1,k) of decontaminated coupons of a material type.

4. Sampling and Analytical Procedures

4.1 Sampling Procedures

Within a single test, surface sampling of the materials was completed first for all procedural blank coupons before sampling of any test material. Surface sampling was done either by wipe sampling or vacuum sampling in accordance with the protocols documented below. Prior to the sampling event, all materials needed for sampling were prepared using aseptic techniques. The materials specific for each protocol are included in the relevant sections below. The general sampling supplies were purchased sterile or sterilized/disinfected prior to each sampling event.

4.1.1 Wipe Sampling

Wipe sampling is typically used for small sample areas of nonporous smooth surfaces such as ceramics, vinyl, metals, painted surfaces, and plastics [12]. Wipe sampling was done on all nonporous and some porous materials according to DTRL MOP #3144 (see Appendix B). The general approach is that a moistened sterile noncotton pad is used to wipe a specified area to recover bacteria, viruses, and biological toxins.

4.1.2 Vacuum Sock Sampling

Vacuum socks were used to sample plywood and carpet coupons due to the difficulty and inefficiency of implementing the wipe sampling procedure on these rough surfaces. Vacuum sock sampling was conducted according to DTRL MOP #3145 (see Appendix B).

4.1.3 Swab Sampling

DTRL MOP #3135 (see Appendix B) was used for collecting swab samples. The general approach was to use a moistened swab to wipe a specified area to recover bacterial spores. Swab samples were collected from all decontamination procedure equipment and test materials prior to their use in experimentation to confirm sterility.

4.1.4 Runoff Sampling

Prior to decontamination, the runoff collection vessel was charged with 30% STS neutralization solution to neutralize all AHP solutions immediately upon collection, as the AHP solution drips from the sprayed coupons. After all coupons from a single set were moved to the Decontaminated Coupon Cabinet or Procedural Blank Cabinet, the chamber was rinsed with deionized (DI) water. A pre-weighed, sterile runoff collection carboy was used to collect the runoff. The total mass of liquid collected was recorded for comparison of the final weight versus the initial weight value. For a given coupon set (material type or all blanks) all runoff was pooled, homogenized (by shaking the carboy), and analyzed as a composite sample. Triplicate 100 mL aliquots (subsamples) were collected from each carboy using aseptic technique. Following collection, the runoff aliquots were triple-contained in sterile bags and transported to the on-site Biocontaminant Laboratory for submission and analysis at the conclusion of the entire test. The runoff samples were stored at 4 ± 2 °C until processed, which occurred within 24 hours.

4.1.5 Aerosol Sampling

Aerosol samples were collected from the bulk environment of the spray chamber using Via-Cell® BioAerosol Cassettes. These samples were used to estimate the occurrence and magnitude of fugitive emissions of viable *B. atrophaeus* spores, during the decontamination process. The sampling point was approximately 5 cm above and 45 cm in front of the coupon surface (to approximate the head position of a decontamination worker). Aerosol samples were collected according to DTRL MOP #3155 (see Appendix B). Samples were not collected isokinetically, therefore the results should be used only to approximate the magnitude of *B. atrophaeus* spores aerosolized within the test chamber.

4.2 Microbiological Analysis

The on-site Biocontaminant Laboratory analyzed all samples for the presence (swab samples) of contamination or to quantify the number of viable spores recovered per sample (vacuum, air, and surface samples). For all sample types, phosphate buffered saline with 0.05% TWEEN® 20 (PBST) was used as the extraction buffer. The PBST was prepared according to BioLab MOP #6562 (see Appendix B). After the appropriate extraction procedure, as described in the sections to follow, the buffer was subjected to a five stage serial dilution (10^{-1} to 10^{-5}) in accordance with BioLab MOP #6535a (see Appendix B). The resulting samples were spread plated in triplicate onto tryptic soy agar (TSA) and incubated overnight (minimum of 18 hours) at 35 ± 2 °C. Following incubation, CFU were enumerated manually. Only data from plates with between 30 and 300 CFU were used for calculations of recovery. When fewer than 30 CFU were observed on plates from the least dilute samples, BioLab MOP #6565 (see Appendix B) was followed in an attempt to detect spores at the lowest level possible.

The extraction procedure used to recover spores varied by sample type (wipes, filter socks, liquid, filter cassette) and can be found in BioLab MOP #6572 for extraction of vacuum socks, BioLab MOP #6567 for extraction of wipe samples, BioLab MOP #6563 for analyses of swab samples, and BioLab MOP #6571 for air sample cassettes. These MOPs are included in Appendix B.

4.3 Activated Peroxide Characterization Measurements

4.3.1 Measurements of pH and Temperature of AHP

The pH and temperature measurements of the AHP solution were performed using an Oakton pH meter (Oakton PC 510; Eutech Instruments Pte. Ltd., Singapore, Singapore) equipped with a pH probe and a thermocouple probe. Calibration of the pH meter was performed daily.

4.3.2 Measurements of H₂O₂ Concentration in AHP

The concentration of H₂O₂ in the AHP solution was verified by analyzing with the potassium permanganate (KMnO₄) titration procedure described below.

4.3.2.1 KMnO₄ Titration Procedure:

Reagents:

- 5% Sulfuric acid (H₂SO₄)
1.0 N KMnO₄

Procedure:

1. Transfer approximately 20 g (~20 mL) of the H₂O₂ sample to a tared weighing container and weigh on an analytical balance. Record weight of the H₂O₂ sample.
2. Carefully wash the weighed sample into a 250 mL flask with distilled water, dilute to mark, and mix thoroughly.
3. Pipet a 25 mL aliquot into a 400 mL beaker containing 250 mL of DI water and 10 mL of H₂SO₄.
4. Titrate to the first permanent pink color with 1.0 N KMnO₄.

Calculation:

$$\% \text{ H}_2\text{O}_2 = [(\text{mL KMnO}_4) \times (\text{N}) \times (0.01701) \times (1000)] / (\text{grams of H}_2\text{O}_2 \text{ sample used})$$

where N = normality of the standard KMnO₄.

4.3.3 Measurements of the AHP Application Rate

The spray pattern was tested by spraying at the appropriate distance (1 ft) onto a piece of 1.17 ft by 1.17 ft blue construction paper mounted in the orientation of the material section. The spray was discharged (1 sec) into the center of the paper and the pattern was visually assessed for consistency, shape, and width.

The flow rate of the backpack sprayer was measured at the start and end of testing of each set of three coupons on which the sprayer was being used. The flow rate was determined by discharging the AHP from the backpack sprayer into a graduated cylinder for 10 sec. The volume of AHP dispensed was read from the graduated cylinder and recorded. The results are presented in Table 5-2.

The time for spray application and exposure time (15 seconds per set of three coupons and 15 minutes or 2 x 15 minutes, respectively) was measured using an National Institute of Standards and Technology (NIST)-traceable stopwatch. The exposure time, as defined here, was time at which the coupons were no longer dripping decontaminant (runoff). This time was determined during preliminary experiments using a single set of porous and nonporous material coupons and was utilized for all subsequent tests.

5. Results and Discussion

This section discusses results of the AHP solution characterization tests (Section 5.1), results of application characterization testing (Section 5.2), and results of the AHP-based decontamination of building materials using Procedure 1 and Procedure 2 (Section 5.3).

5.1 AHP Characterization

The pH, temperature and concentration of H₂O₂ measurements in the working decontamination solution were considered critical to understand dynamic changes of key decontamination agents (H₂O₂, PAA) in the AHP solution. These three parameters were monitored throughout the entire testing (immediately after mixing and before each application of decontamination solution). Test-specific results of the pH, temperature and H₂O₂ concentration measurements are shown in Appendix A (Table A-1 - Procedure 1 and Table A-2 - Procedure 2).

Table 5-1 shows cumulative results for the AHP critical characterization parameters. Sections 5.1.1 and 5.1.2 discuss these results in more detail.

Table 5-1. Activated Hydrogen Peroxide Characterization Parameters

Parameter measured	n	Average	SD	Min	Max
pH	30	8.5	0.7	7.4	9.6
H ₂ O ₂ [%]	24	6.0	0.3	5.3	6.4
T [°C]	30	28.4	2.1	27.1	32.8

n - number of measurements.

5.1.1 pH and Temperature

In the small scale liquid-based decontamination testing, the pH value acceptance criterion for the working AHP solution immediately post-mixing was set as 9.5 ± 0.1 pH units. The pH of decontamination solution post-mixing was considered to be a good indicator of proper preparation and sporicidal effectiveness of the AHP solution. This parameter was, therefore, also optimized for preparation of larger batches of AHP in the pilot-scale study.

The optimization of the decontamination solution initial pH was performed in a series of small-volume (100 mL) preliminary tests (Figure 5-1). The optimum starting pH values (in the 9.5-9.6 range) were achieved by a nominal increase of the ratio of potassium carbonate in the AHP recipe (see Section 2.3. for original recipe as developed by SNL and modified recipe used in the pilot-scale study). The pH profiles of test AHP solutions as a function of time were also investigated in these preliminary tests, with a drastic decline in pH values observed during the first ten minutes post-mixing (Figure 5-1).

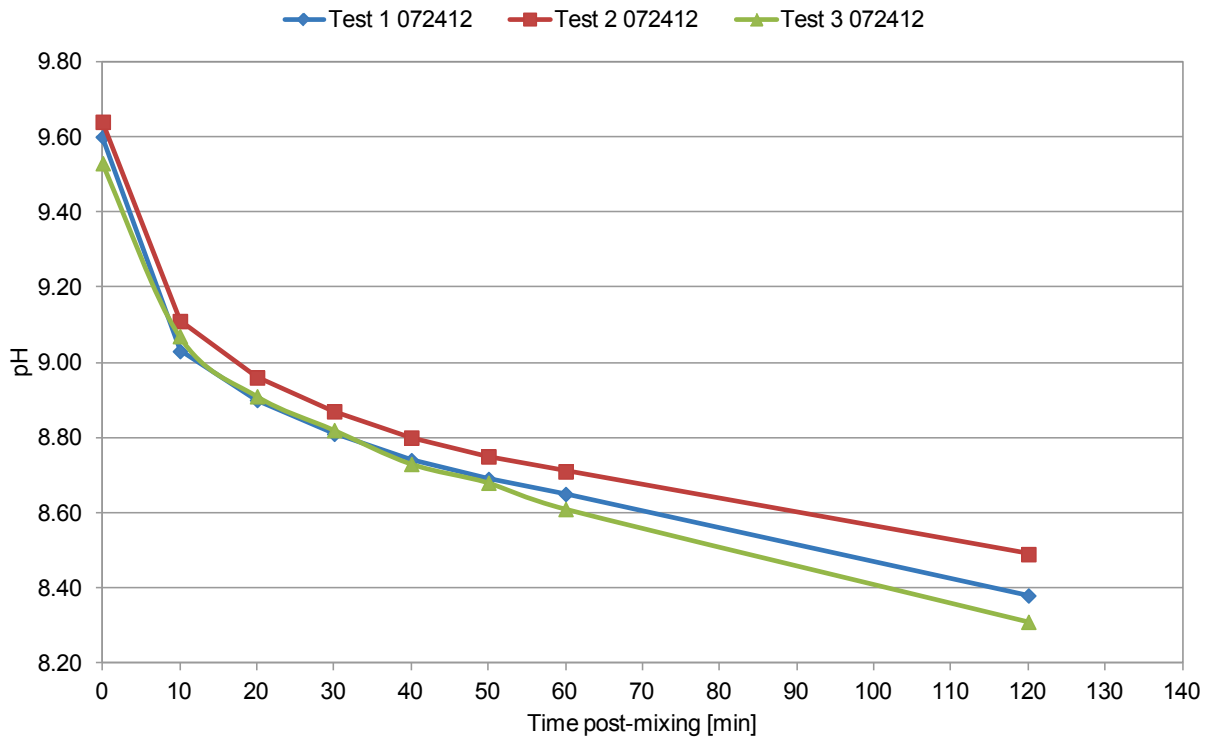


Figure 5-1. pH of the AHP Solution Over Time (0 to 120 min Post-Mixing); Results from Optimization Testing

Consequently, the strict pH acceptance criteria (9.5 ± 0.1 pH units) used by SNL for the small scale liquid-based testing was not considered practical for the pilot-scale testing decontamination experiments. However, as stated before, the pH measurements were performed post-mixing and before each application of AHP. Average pH of large batches of AHP (5000-10000 mL) prepared for pilot-scale decontamination testing had shown starting pH ranges of 9.2-9.6 (average pH 9.4 ± 0.1 standard deviation (SD)). The pH of the decontamination solution prior to application ranged from 7.4-9.0 (average pH 8.2 ± 0.6 SD). (see Tables A-1 and A-2 in Appendix A). The pH over time for all batches of AHP solutions used in the pilot scale testing are shown in Figure 5-2. The downward trend of pH versus time observed in the initial AHP testing (Figure 5-1) was also noted for all batches of decontamination solution used in the pilot-scale testing experiments (Figure 5-2). The average pH drop rate was 1.4 pH units per hour.

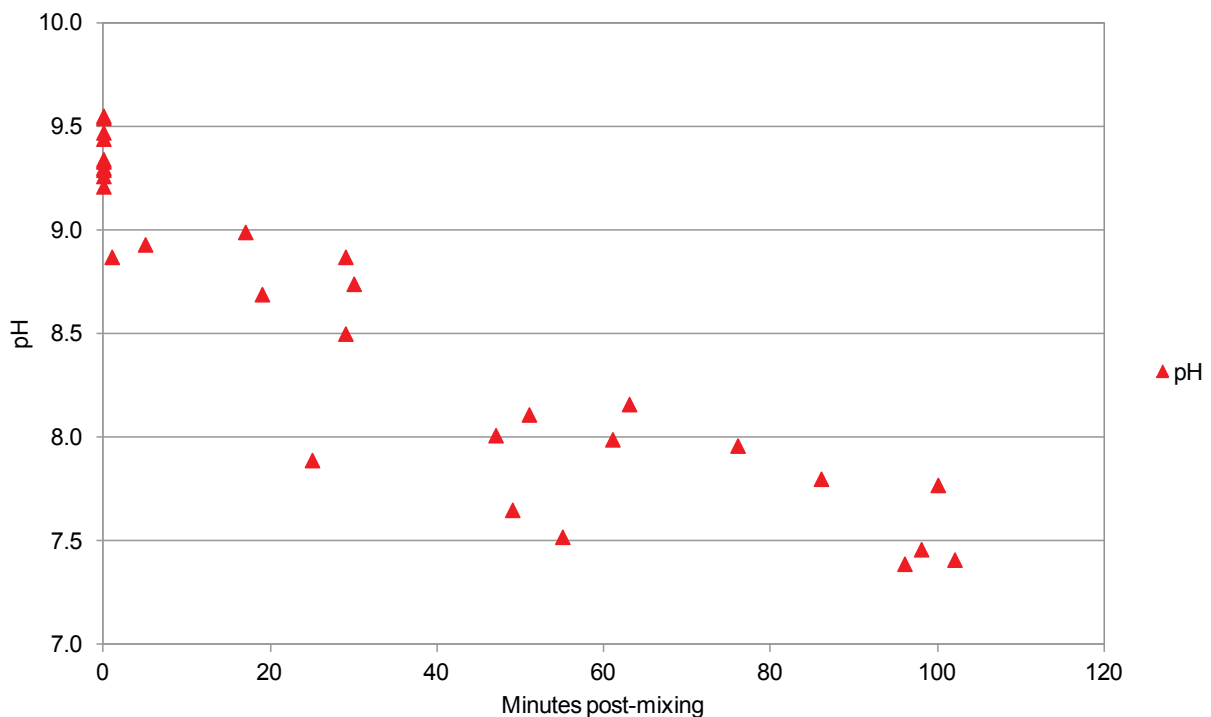


Figure 5-2. pH of AHP over Time (Combined Results for Ten Batches of Decontamination Solution)

Temperature was monitored for freshly-prepared batches of AHP and in samples taken from the backpack sprayer prior to each spray. Average temperature prior to application was $29.6\text{ }^{\circ}\text{C} \pm 1.8\text{ }^{\circ}\text{C}$ (range 27.1 to $32.8\text{ }^{\circ}\text{C}$) (see Tables A-1 and A-2 in Appendix A). The temperatures for all batches of AHP solutions used in the pilot scale testing are shown in Figure 5-3. A slight increase in temperature over time was observed for each batch of the AHP solution tested (average ΔT between preparation and last application of AHP was $3.4\text{ }^{\circ}\text{C}$, with a $2.9\text{ }^{\circ}\text{C}$ per hour average temperature increase rate).

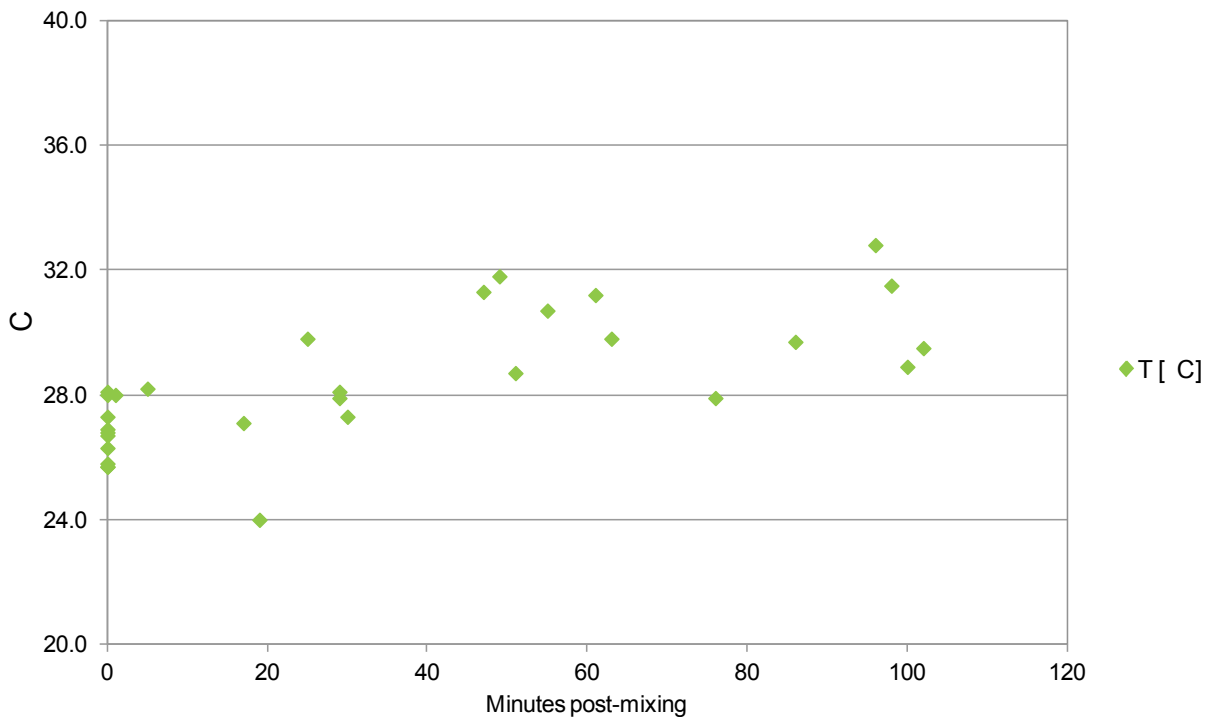


Figure 5-3. Temperature of AHP Over Time (Combined Results for Ten Batches of Decontamination Solution)

5.1.2 Hydrogen Peroxide Concentration

Concentration of H_2O_2 in the working decontamination solution was measured via permanganate titration in 24 samples of AHP taken from the backpack sprayer reservoir prior to each spray application and in selected freshly prepared batches of AHP.

The concentration of H_2O_2 in freshly prepared batches of AHP was $6.1\% \pm 0.2\%$. The average pre-spray concentration of H_2O_2 in the working AHP solution was $6.0 \pm 0.3\%$ (range 5.3% to 6.2%) (see Tables A-1 and A-2 in Appendix A). Cumulative results for H_2O_2 concentration over time measurements are shown in Figure 5-4. Unlike pH and temperature values, the H_2O_2 concentration in the AHP solution did not show any characteristic upward or downward trend ($\Delta\%\text{H}_2\text{O}_2$ in the AHP solution between preparation and last application ranged from -0.6% to +0.1%).

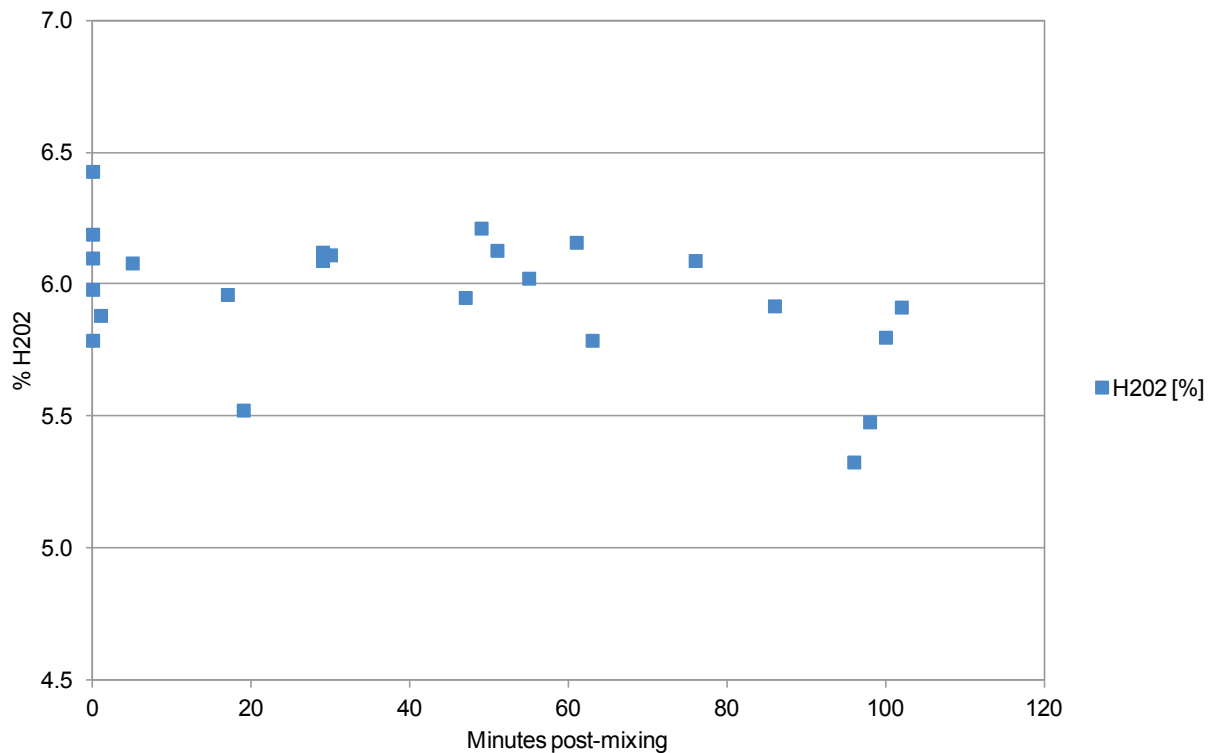


Figure 5-4. Concentration of H₂O₂ in AHP over Time (Combined Results for Ten Batches of Decontamination Solution)

5.1.3 PAA Concentration

Measurements of the PAA concentration in the AHP were outside the scope of this work. For screening purposes, a semi-quantitative test using The EM Quant[®] Peracetic Acid Test test-strips (EMD Millipore Chemicals, Billerica, MA, USA) was performed to determine PAA in the AHP working solution. This test is suited for the selective determination of PAA concentration in solutions, also in cases in which H₂O₂ is present. The PAA concentration measured in a 50x diluted aliquot of acidified AHP solution 1 h and 2.5 hours post-mixing was estimated as 10000 mg/L (1%) and 5000 mg/L (0.5%), respectively. A representative PAA concentration profile over time in the AHP was not tested further.

5.2 Application Characterization

The diameter of the AHP spray was determined to be within acceptable limits (12 in to 16 in diameter at 1 ft) for all decontamination tests.

Sprayer flow rates varied significantly between test days (Table 5-2). The reason for this variability is unknown, but the variability was suspected to have been caused by the foaming action of the decontaminant (the sprayer was not designed for foam applications). The flow rate used was always the maximum flow of the backpack sprayer, i.e., setting 4 (corresponding to 1 gallon/min flow rate according to factory specifications). The liquid volume delivered per each sprayer setting was confirmed in the initial testing with water (at 20 pounds per square inch (psi) and setting # 1,2,3 and 4, the sprayer delivered 800, 880, 960 and 1000 mL of water per minute, respectively).

There was no obvious correlation between flow rate and decontamination efficacy – samples with the highest log reduction values were sprayed on days where medium backpack sprayer flow rates were noted (080712 and 082212; see Tables A-1 and A-2 in Appendix A for details). A more systematic study would be needed to investigate whether or not the backpack sprayer flow rates can affect the decontamination efficacy directly.

Table 5-2. Sprayer Flow Rates

	Average	SD	Min	Max
	[mL/min]			
080112 test 11 12 13	521	102	372	696
080712 test 1,2,3,4	512	131	372	780
082212 test 5,7,10,15,16	587	110	408	780
091812 test 1R,6R,8R,9R,14R	970	114	780	1170

5.3 Decontamination Results

5.3.1 Neutralization Test Results

Table 5-3 shows results from neutralization tests for porous and nonporous materials.

Table 5-3. Neutralization Test Results for Porous and Non-Porous Materials

Test	Decontaminated	Material Type	n	Average CFU/per sample	RSD	T-test p value*
A1	Yes	Unpainted	5	3.71E+07	10%	0.004
A2	No	Unpainted	5	3.06E+07	11%	
B1	Yes	Stainless Steel	5	3.05E+07	11%	0.90
B2	No	Stainless Steel	5	3.11E+07	20%	

*Probability associated with a Student's paired t-Test, with a two-tailed distribution.

The Student's t-test was used to compare recoveries between the two treatments (sprayed and nonsprayed coupons). Analysis of the data with student's t-test suggested that the sprayed and unsprayed unpainted plywood results were significantly different ($p = 0.004$). However, an average recovery from the AHP-sprayed unpainted plywood coupons was higher than that from unsprayed samples. These data suggest that the presence of residual AHP does not have a negative bias on sample recovery. There was no

significant statistical difference ($p = 0.90$) between CFU recovery from sprayed and non-sprayed stainless steel coupons. Based on these results, the neutralization of coupons or samples after the AHP decontamination was determined to be unnecessary.

The H_2O_2 in runoff was neutralized using 30% STS. Neutralizer was added to the sterile runoff collection container immediately prior to each decontamination test (42 mL per each 15- second spray application). The average post-neutralization concentration of the residual H_2O_2 in the collected runoffs was 0.10% ($\pm 0.049\%$). The microbiological results from the runoff samples that required re-analysis (filter plating) were assessed for instabilities due to prolonged exposure to residual H_2O_2 in the solution. If such instabilities were noted, the filter plating results were considered invalid and excluded from further data analysis and processing. This phenomenon occurred during testing of Procedure 1 on concrete, tile, and unpainted wood. For these samples, the initial estimates of runoff spore concentrations were utilized.

5.3.2 Inoculation Results

In this study, test and positive control coupons of porous and nonporous building materials were inoculated with $\sim 2 \times 10^8$ aerosol-deposited *Bacillus* spores. Test specific results for positive controls (CFU/sample) are given in Table 5-4. The average CFU recovered from positive control coupon sets ($n = 3$) was 8.74×10^7 , with an average coefficient of variance of 33% (ranging from 9% RSD to 111% RSD; see Tables A-1 and A-2).

Reference control coupons were inoculated alongside test coupon sets. Control coupons were inoculated at the beginning, in the middle and the end of the inoculation procedures. The recoveries from inoculation control coupons are shown in Table 5-4 and demonstrate a repeatable inoculation procedure across all tests.

Table 5-4. Inoculation Control Results

	Average CFU/sample	% RSD
080112 test 11 12 13	1.53E+08	45%
080712 test 1,2,3,4*	8.63E+07	49%
082212 test 5,7,10,15,16	1.88E+08	51%
091812 test 1R,6R,8R,9R,14R	6.86E+07	25%

*One reference control rejected as non-representative of inoculation.

The positive controls and inoculation controls average recovery values (see Tables A-1 and A-2) indicate that the initial spore loading allowed evaluation of decontamination efficacies with a dynamic range of sporicidal effectiveness greater than 6 logs (i.e., ≥ 6 logs reduction (LR)). The high variability among the numbers of viable spores recovered from the positive control samples can be attributed to challenges related to reproducible surface-sampling procedures. Presumably, sampling of test coupons was analogously affected, hence the variability in the initial loading/CFU recovery of positive controls was not considered a source of methodical bias in calculations of the log reduction values.

5.3.3 Log Reduction Results

Tables A-1 and A-2 in Appendix A and Figure 5-5 summarize test-specific log reduction values. These data suggest that AHP-based liquid sporicide provides greater than 6 LR of spores on most of the common building materials tested, either with the one- or two-application procedure (Procedures 1 and 2, respectively). The exception was unpainted treated wood, where decontamination with Procedure 2 resulted in a 4.20 LR (3.24 LR with Procedure 1). The range in log reduction values for Procedure 1 was from 1.56 for concrete to 8.41 for glass (see Table A-1 of Appendix A for Procedure 1-specific results). The efficacy of Procedure 2 ranged from 6.84 LR for concrete to 8.36 LR for stainless steel (see Table A-2 of Appendix A for Procedure 2-specific results).

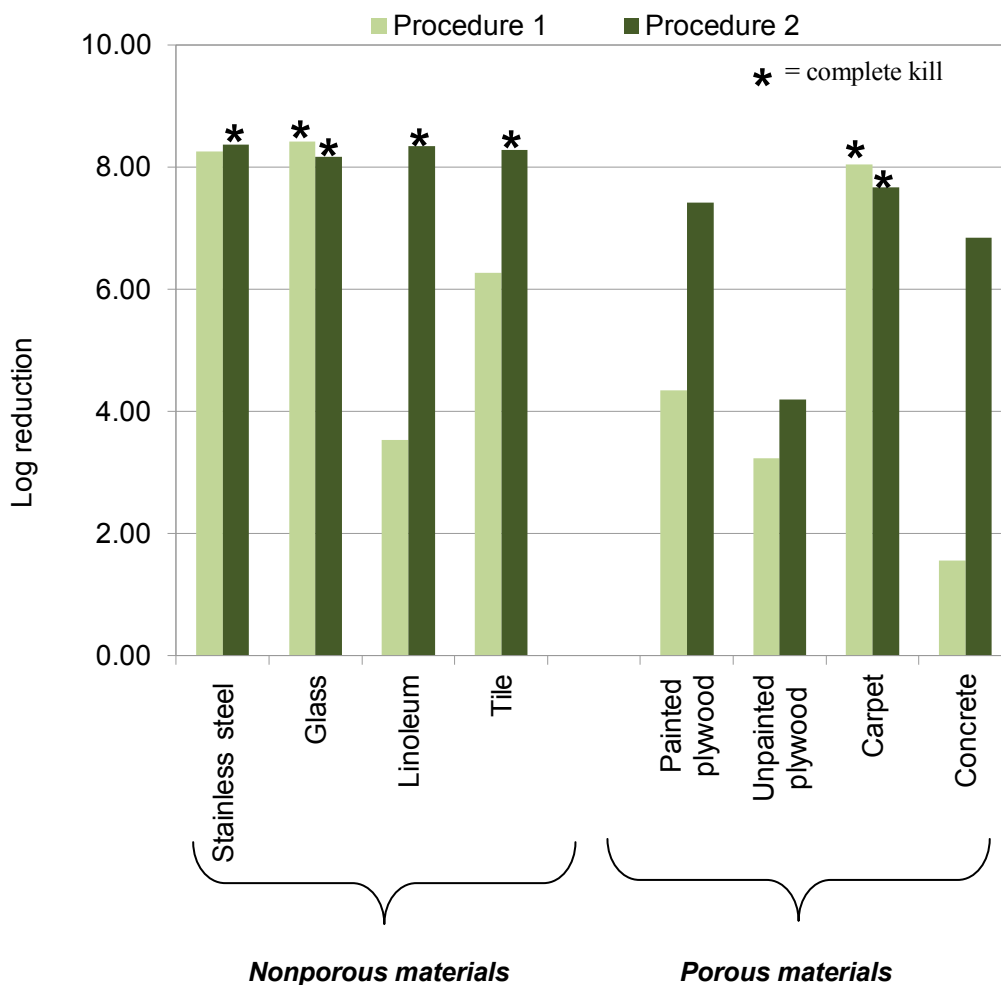


Figure 5-5. Material Specific Log Reductions for Procedure 1 and Procedure 2

Decontamination efficacy was dependent upon material type and operational conditions (Figure 5-6). The average LR for nonporous materials (stainless steel, glass, linoleum, and tile) was 6.62 ± 1.74 and $8.29 \pm$

0.09 for Procedure 1 and 2, respectively. Complete kill was observed for all nonporous materials using Procedure 2. The average LR for porous materials (painted treated plywood, unpainted treated plywood, carpet and concrete) was 4.29 ± 1.96 and 6.53 ± 1.59 for Procedure 1 and 2, correspondingly.

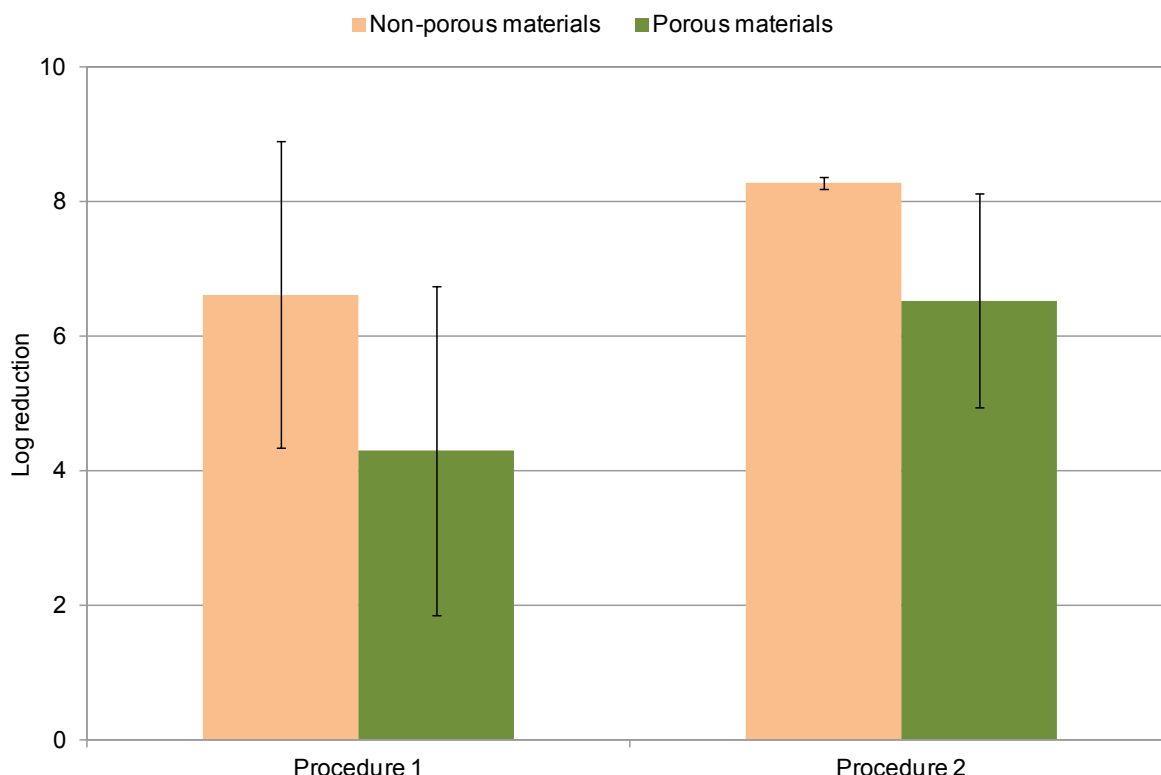


Figure 5-6. Average Log Reductions for Procedure 1 and Procedure 2 on Nonporous and Porous Building Materials

A comparison between 15-minute/no re-application (Procedure 1) and 2 x 15 minutes/re-application (Procedure 2) exposures suggest that longer exposure time and re-application may provide higher sporicidal efficacy, especially for porous materials. Lower variability in efficacies between materials was also observed when Procedure 2 was used.

5.3.4 Fate of Spores

The reaerosolization of *B. anthracis* during decontamination operations is a potential source of exposure for personnel performing cleaning, and a mechanism for spreading contamination to previously uncontaminated areas. The LD₅₀ for *B. anthracis* in humans is not definitively known but, based on animal data, is estimated to be in the range of 4,100 - 10,000 inhaled spores [13, 14]. The survival of spores in the post-decontamination runoff that contains viable spores brings into consideration other possible routes of accidental exposure of the personnel through cuts and scrapes, i.e., via cutaneous or gastrointestinal pathways [15].

Aerosol samples were collected using Via-Cell® BioAerosols cassettes (Zefon International, Ocala, FL) during application and exposure phases of each decontamination test run. Table 5-5 shows the recoveries from the material/procedure-specific aerosol samples. These results suggest that the multi-step decontaminations, like Procedure 2, may result in a greater spore reaerosolization. The highest number of viable spores detected in all air samples was 540 CFU (equivalent of about 1 CFU per liter, for samples collected during decontamination of concrete via Procedure 2). While this value may seem low (compared to an LD₅₀ of 4,100 to 10,000 spores), the value represents the reaerosolized spores from only three 14 x 14 inch coupons (total area of approximately 4.1 ft²). The decontamination cycle phase-related aerosolization rates were not systematically investigated in this study, but the spraying stage is presumably the phase when most of the spores were aerosolized.

Table 5-5. Air Samples Results

	Procedure 1	Procedure 2
	Total CFU/sample*	
Stainless steel	6.1E+00	5.3E-01
Glass	3.3E+00	1.2E+01
Linoleum	5.3E-01	5.3E-01
Tile	4.3E+00	2.0E+01
Painted treated plywood	6.1E+00	1.2E+01
Unpainted treated plywood	5.6E-01	5.3E-01
Carpet	5.3E-01	1.2E+01
Concrete	2.2E+00	5.4E+02

* Values in gray-shaded cells are based on detection limit.

Table 5-6 shows CFU recovered from the runoff samples, normalized per liter of runoff collected.

Table 5-6. Runoff Samples Results

	Procedure 1	Procedure 2
	Total CFU/L*	
Stainless steel	9.8E+00	5.2E+02
Glass	6.0E+00	7.1E+02
Linoleum	2.E+06	8.7E+04
Tile	8.0E+03	5.3E+02
Painted treated plywood	1.2E+06	2.8E+03
Unpainted treated plywood	1.0E+06	1.8E+07
Carpet	6.E+02	2.4E+04
Concrete	1.0E+04	3.4E+04

* Values in gray-shaded cells are based on detection limit.

The average number of spores detected in the Procedure 1 runoff samples was 5.5×10^5 CFU/L. The Procedure 2 runoff samples averaged 2.2×10^6 CFU/L, with a maximum of 1.8×10^7 CFU per liter (collected during decontamination of unpainted treated plywood via Procedure 2). The volumes of runoff collected varied drastically between the tests (2.0 to 6.6 L; the AHP runoff plus the post-exposure water rinse). Consequently, the CFU per liter estimates are influenced heavily by the post-decontamination chamber rinse step (see Appendix B, DTRL MOP# 3177, section 3.4.3) duration/rinse water volume-dependant variation. Procedure 2 appeared to result in a greater rate of physical removal of spores/higher rate of viable spore transfer to the runoff. Nonetheless, both procedures resulted in significant amounts of viable agent being transferred into the liquid runoff. While not directly comparable due to subtle differences in test procedures, the amount of viable spores being transferred to the runoff appears slightly higher than that achieved with similar pH-adjusted bleach procedures [16, 17]. Comparatively lower inactivation efficacies of AHP could account for this difference.

5.3.5 Summary of Results

Decontamination efficacy of AHP was evaluated using two application procedures (Procedures 1 and 2, respectively). The single application procedure (Procedure 1) resulted in surface LR values that ranged from 1.6 to 8.4. Typically, nonporous materials were easier to decontaminate than were porous. The exception, carpet, was relatively easy to decontaminate compared to other porous materials. Of all the materials, concrete demonstrated the lowest LR (< 2 LR) with Procedure 1. Nonetheless, greater than 6 LR was achieved for 50% of materials decontaminated with Procedure 1. The AHP two-application procedure (Procedure 2) resulted in > 6 LR for seven of the eight materials tested (LR values from 6.8 to 8.4). Greater than 6 LR was not achieved for unpainted treated wood material, regardless of the application procedure (mean LR = 4.2). Complete kill (recovery of no viable spores after decontamination) was achieved on carpet and glass using Procedure 1, and on Tile, stainless steel, carpet, glass, and linoleum using Procedure 2. For most materials, surface decontamination efficacy of AHP was similar to that observed previously for pH-adjusted bleach [16,17]. However, the data suggest that AHP may have lower efficacy than pH-adjusted bleach when applied to concrete surfaces.

The longer exposure duration and increased volume of AHP applied, associated with Procedure 2, afforded higher surface decontamination efficacy, especially for porous materials. Materials with high organic demand (e.g., unpainted wood) that are typically more difficult to decontaminate may present a challenge to the liquid AHP-based process. The preliminary fate of spores estimates suggest that the AHP liquid sporicide-based decontamination process leads to physical removal of spores from decontaminated surfaces, with a consequent transport of viable spores to air and to the post-decontamination liquid waste. In some instances, considerable amounts (up to 7 logs) of viable spores were recovered in the liquid runoff. Highest recoveries of spores in the runoff were observed during testing with painted and unpainted plywood. When compared to pH-adjusted bleach testing results, AHP-based decontaminations appear to result in substantially greater numbers of viable spores in the runoff fraction. Considering these findings, although surface reduction efficacies for AHP were similar to pH-adjusted bleach, overall decontamination efficacy may be lower for AHP.

6. Quality Assurance

This project was performed under an approved Category III Quality Assurance Project Plan titled Evaluation of Expedient Decontamination Options with Activated Peroxide-based Liquid Sporicides (June 2012).

All test activities were documented via narratives in laboratory notebooks and the use of digital photography. The documentation included, but was not limited to, a record of time required for each decontamination step or procedure, any deviations from the QAPP, and physical impacts on the materials. All the tests were conducted in accordance with developed DTRL and BioLab miscellaneous operating procedures (MOPs), listed in Appendix B, to ensure repeatability and adherence to the data quality validation criteria set for this project.

6.1 Sampling, Monitoring, and Analysis Equipment Calibration

Standard operating procedures for the maintenance and calibration of all laboratory equipment were used. All equipment was certified as being calibrated or having the calibration validated by EPA's on-site (RTP, NC) Metrology Laboratory at the time of use. Standard laboratory equipment such as weigh balances, pH meters, biological safety cabinets and incubators were routinely monitored for proper performance. Calibration of instruments was performed at the frequency shown in Table 6-1. Any deficiencies were noted in the laboratory notebooks. Instruments were adjusted to meet calibration tolerances and recalibrated within 24 hours. If tolerances were not met after recalibration, additional corrective action was taken, possibly including, recalibration and/or replacement of the equipment.

Table 6-1. Analysis Equipment Calibration Frequency

Equipment	Calibration/Certification	Expected Tolerance
pH meter	Perform a 2-point calibration with standard buffers that bracket the target pH before each use.	± 0.1 pH units
Stopwatch	Compare against NIST Official U.S. time at http://nist.time.gov/timezone.cgi?Eastern/d/-5/java once every 30 days.	±1 min/30 days
Clock	Compare to office U.S. Time @ time.gov every 30 days.	±1 min/30 days
Scale	Check calibration with Class 2 weights prior to weighing	± 0.1% weight
Micropipettes	Once a year, tested with standards traceable to SI through the NIST	±5%

6.2 Data Quality

This section discusses the Quality Assurance/Quality Control (QA/QC) checks and acceptance criteria for critical measurements considered critical to accomplishing the project objectives.

6.2.1 QA/QC Checks

Uniformity of the test materials was a critical attribute for assuring reliable test results. Uniformity was maintained by obtaining a sufficiently large quantity of material so that multiple material sections and coupons could be constructed with presumably uniform characteristics. Samples and test chemicals were maintained to ensure their integrity. Samples were stored away from standards or other samples which could cross-contaminate them.

Supplies and consumables were acquired from reputable sources and were NIST-traceable when possible. Supplies and consumables were examined for evidence of tampering or damage upon receipt and prior to use, as appropriate. Supplies and consumables showing evidence of tampering or damage were not used. All examinations were documented and supplies were appropriately labeled. Project personnel checked supplies and consumables prior to use to verify that they met specified task quality objectives and did not exceed expiration dates.

Quantitative standards do not exist for biological agents. Quantitative determinations of organisms in this investigation did not involve the use of analytical measurement devices. Rather, the CFU were enumerated manually and recorded. The QA/QC acceptance criteria were set at the most stringent level that could routinely be achieved (Table 6-2). Positive controls and procedural blanks were included with the test samples in the experiments so that well-controlled quantitative values were obtained. Background checks were also included as part of the standard protocol. Replicate coupons were included for each set of test conditions. MOPs executed by qualified, trained and experienced personnel were used to ensure data collection consistency. The confirmation procedure, control, blank, and method validation efforts are the basis of support for biological investigation results. When necessary, training sessions were conducted by knowledgeable parties, and in-house practice runs were used to gain expertise and proficiency prior to initiating the research.

Table 6-2. QA/QC Sample Acceptance Criteria

QC Sample	Information Provided	Frequency	Acceptance Criteria	Corrective Action
Procedural Blank (coupon not inoculated with biological agent)	Controls for sterility of materials and methods used in the procedure	1 per test from Table 2-1 or Table 2-2	No observed CFU	Reject results upon approval of WAM, otherwise analyze data with procedural blank results as test minimum,.Identify and remove source of contamination, if possible.

QC Sample	Information Provided	Frequency	Acceptance Criteria	Corrective Action
Positive Control (Sample from material coupon contaminated with biological agent but not subjected to the test conditions)	Initial contamination level on the coupons; allows determination of log reduction; controls for confounds arising from history impacting bioactivity; controls for special causes. Shows viability of sampling technique and plate's ability to support growth.	3 or more replicates per test	Target loading of 1×10^7 CFU per sample with a standard deviation of < 0.5 log. ($5 \times 10^6 - 5 \times 10^7$ CFU/sample); Grubbs outlier test (or equivalent).	Outside target range: discuss potential impact on results with EPA WAM; correct loading procedure for next test and repeat depending on decided impact. Outlier: evaluate/exclude value.
Blank plating of microbiological supplies	Controls for sterility of supplies used in dilution plating.	3 of each supply per plating event	No observed growth following incubation	Sterilize or dispose of source of contamination. Replate samples.
Blank Tryptic Soy Agar Sterility Control (plate incubated, but not inoculated)	Controls for sterility of plates.	Each plate	No observed growth following incubation	All plates are incubated prior to use, so any contaminated plates will be discarded.
Decontamination method checks	Details on the materials and equipment used in the decontamination.	As outlined in MOP 3177	As outlined in MOP 3177	As outlined in MOP 3177
Spray pattern check	Distribution of decontamination solution or rinse water.	Per use	Decontamination solution sprayer: 12 in to 16 in diameter at 1 foot. DI water: 12 in to 16 in diameter at 3 feet.	Adjust nozzle
Exposed Field Blank Samples. A wipe kit will be handled, a vacuum sock kit will sample ambient air.	The level of contamination present during sampling.	1 per sampling event	Non-detect	Clean up environment. Sterilize sampling materials before use.
Unexposed Field Blank Samples. A wipe kit will be transferred without being handled, a vacuum sock kit will be transferred without switching on the vacuum.	The level of contamination present during sampling.	1 per sampling event	Non-detect	Clean up environment. Sterilize sampling materials before use.
Background swabs	Determine sterility of materials and equipment before use.	1 per material per use	Non-detect	Clean up environment. Sterilize sampling materials before use.

6.3 Acceptance Criteria for Critical Measurements

The Data Quality Objectives (DQOs) are used to determine the critical measurements (CMs) needed to address the stated objectives and specify tolerable levels of potential errors associated with simulating the prescribed decontamination environments.

The following measurements were deemed to be critical to accomplish part or all of the project objectives:

- Enumeration of spores on the surface coupon
- Plated volume
- Decontamination time
- Spray pattern.

After initial testing preparation of large batches of the AHP solutions, the H₂O₂ concentration and pH measurements were deemed to be non-critical measurements and were used to characterize the AHP liquid sporicide. The characterization of the AHP application was performed by measuring backpack sprayer flow rates, the spray pattern and the duration of application measurements.

The critical measurement acceptance criteria and completeness values achieved in testing are listed in Table 6-3.

Table 6-3. Critical Measurement Acceptance Criteria

Critical Measurement	Measurement Device	Accuracy	Precision	Detection Limit	Completeness
Plated volume	Pipet	± 2 %	±1 %	NA	100%
CFU/plate	Hand counting	±10% (between 2 counters)* ≤ 50% CV between triplicate plates	± 5	1 CFU	100%*
Decontamination Time (15 min, 2x 15 min)	Timer	±1 second	± 1 second	1 second	94%**
Spray pattern (14-16" from 1 ft)	Measuring tape	1/32" over the length of the measuring tape	NA	1/8"	100%

**25% of the plates within the quantification range (plates with 30 – 300 CFU) were counted by a second technician;*

*** For one test set of Procedure 1 (stainless steel, test ID # 2,) the spray application duration was 20 s instead of 15 seconds per set of three coupons.*

Plated volume critical measurement goals were met. All pipettes are calibrated yearly by an outside contractor (Calibrate, Inc.).

Plates were quantitatively analyzed (CFU/plate) using a manual counting method. For each set of results (per test), a second count was performed on 25 percent of the plates within the quantification range (plates with 30 - 300 CFU). All second counts were found to be within 10 percent of the original count.

Many QA/QC checks were used to validate spore recovery measurements. These QA/QC checks include samples that demonstrate the ability of the on-site Biocontaminant Laboratory to culture and grow spores as well as to demonstrate that materials used in this effort do not themselves contain spores. The checks included:

- Laboratory material coupons: includes all materials, individually, used by the on-site Biocontaminant Laboratory in sample analysis; materials have been confirmed sterile.
- Positive control coupons: coupons inoculated but not decontaminated; results are discussed in Section 5.3.2.
- Inoculation control coupons: stainless steel coupons dosed at beginning, middle, and end of each inoculation campaign, not decontaminated, to assess the variability of the inoculation operation; results are discussed in Section 5.3.2
- Procedural blank coupons; spores were detected on some blank and procedural blank coupons. Test - specific results for procedural controls are given in Tables A-1 and A-2; the potential impact on the decontamination efficacy results was considered negligible.

6.4 Data Quality Audits

This project was assigned QA Category III and did not require technical systems, performance evaluation or data quality audits by EPA or contractor QA personnel.

6.5 QA/QC Reporting

QA/QC procedures were performed in accordance with the QAPP for this investigation.

6.6 Amendment to Original QAPP

The DTRL MOP #3177 (see Appendix B) was prepared as an Amendment to the QAPP by the EPA WAM upon receipt of the final recipe for AHP from SNL.

7. References

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Appendix A: Process Parameters and Decontamination Efficiency Results

Table A-1. Process Parameters and Decontamination Efficiency Results for Procedure 1														
Test ID	Date	Material type	Log reduction	Positive Controls		Test Coupons		Procedural Blank Coupons (CFU)	Application frequency	Application duration (sec)	Decon duration (hh:mm)	AHP pH (pH units)	AHP Temp (°C)	H ₂ O ₂ conc (%)
				Avg. CFU recovered	RSD (%)	Avg. CFU recovered	RSD (%)							
1R	9/18/2012	Painted treated plywood	4.35	8.69E+07	28	3.90E+03	172	not performed	At 0 min, no rinse	15	0:16	7.39	32.8	5.3
2	8/7/2012	Stainless steel	8.26	1.49E+08	15	8.26E-01	45	1.81E+03	At 0 min, no rinse	20	0:15	8.74	27.3	6.1
3	8/7/2012	Carpet	≥8.04	6.24E+07	57	5.72E-01	3	2.05E+03	At 0 min, no rinse	15	0:15	8.16	29.8	5.8
4	8/7/2012	Glass	≥8.41	1.59E+08	17	6.20E-01	1	2.35E+02	At 0 min, no rinse	15	0:15	7.80	29.7	5.9
5	8/22/2012	Linoleum	3.53	1.46E+08	9	4.32E+04	162	6.45E-01	At 0 min, no rinse	15	0:15	7.52	30.7	6.0
11	8/1/2012	Concrete	1.56	3.49E+07	59	9.71E+05	117	2.00E+01	At 0 min, no rinse	15	0:15	7.41	29.50	5.9
12	8/1/2012	Tile	6.27	1.31E+07	111	7.05E+00	159	5.88E-01	At 0 min, no rinse	15	0:15	8.87	27.90	6.1
13	8/1/2012	Unpainted treated plywood	3.24	5.22E+07	73	3.03E+04	158	5.62E-01	At 0 min, no rinse	15	0:15	8.11	28.7	6.1

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Appendix A

Table A-2. Process Parameters and Decontamination Efficiency Results for Procedure 2														
Test ID	Date	Material type	Log reduction	Positive Controls		Test Coupons		Procedural Blank Coupons (CFU)	Application frequency	Application duration (sec)	Decon duration (hh:mm)	AHP pH (pH units)	AHP Temp (°C)	H ₂ O ₂ conc (%)
				Avg. CFU Recovered	RSD (%)	Avg. CFU Recovered	RSD (%)							
6R	9/18/2012	Painted treated plywood	7.42	8.69E+07	28	3.33E+00	141	7.14E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	8.87	28	5.9
7	9/18/2012	Stainless steel	≥8.36	1.51E+08	14	6.52E-01	2	6.29E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	8.93	28.2	6.1
8R	9/18/2012	Carpet	≥7.67	2.99E+07	15	6.40E-01	4	5.71E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	7.65	31.8	6.2
9R	9/18/2012	Glass	≥8.16	8.51E+07	28	5.83E-01	2	6.25E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	7.46	31.5	5.5
10	8/22/2012	Linoleum	≥8.34	1.46E+08	9	6.64E-01	1	6.06E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	7.99	31.2	6.2
14R	9/18/2012	Concrete	6.84	2.69E+07	22	3.87E+00	145	6.71E-01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	8.99	27.1	6.0
15	8/22/2012	Tile	≥8.28	1.24E+08	13	6.55E-01	3	1.25E+01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	8.92	28.5	NA
16	8/22/2012	Unpainted treated plywood	4.20	4.55E+07	25	2.90E+03	100	1.13E+01	At 0 min, re-apply after 15 min, no rinse	15	2 x 0:15	8.01	31.3	6.0

Appendix B: Miscellaneous Operating Procedures (MOPs)

Note: MOPs are dynamic documents. For the testing described in this report, the following MOPs were followed as presented.

DTRL Procedures

- MOP 3177 Activated Hydrogen Peroxide-based decontamination for WA 3-08: Procedure 1 and Procedure 2
- MOP 3135 Procedure for WA 1-25 Sample Collection using BactiSwab™ Collection and Transport Systems
- MOP 3144 Procedure for Wipe Sampling of Coupons
- MOP 3145 Procedure for Vacuum Sampling of Large and Small Coupons
- MOP 3150 Procedure for Fabrication of 14" x 14" Material Coupons
- MOP 3155 Procedure for Via-Cell® Air Sampling

BioLab Procedures

- MOP 6535a: Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spore
- MOP 6561: Aerosol Deposition of Spores onto Material Coupon Surfaces Using the Aerosol Deposition
- MOP 3128: Procedure for Preparing pH-Adjusted Bleach Solution with Trisodium Phosphate Substitute
- MOP 6562: Preparing Pre-Measured Tubes with Aliquoted Amounts of Phosphate Buffered Saline with Tween 20 (PBST)
- MOP 6563: Swab Streak Sampling and Analysis
- MOP 6565: Filtration and Plating of Bacteria from Liquid Extracts
- MOP 6567: Recovery of Bacillus Spores from Wipe Samples
- MOP 6570: Use of STERIS Amsco Century SV 120 Scientific Prevacuum Sterilizer
- MOP 6571: Recovery of Bacillus Spores from Via-Cell® Aerosol Sampling Cassettes
- MOP 6572: Recovery of Spores from Vacuum Sock Samples

MOP 3177

TITLE: ACTIVATED HYDROGEN PEROXIDE-BASED DECONTAMINATION FOR WA 3-08:
PROCEDURE 1 AND PROCEDURE 2

SCOPE: This MOP outlines the setup, operation, and timeline schedule for conducting decontamination testing in the H-130 small test chamber.

PURPOSE: To provide a standardized and repeatable procedure for all activated hydrogen peroxide-based decontamination tests to be conducted under WA 3-08 using Decontamination Procedure 1 and Procedure 2.

1.0 INTRODUCTION

Preparations for each test will be conducted according to the schedule listed in this procedure. Any deviations will be noted in the laboratory notebook, along with the reason for the deviation.

Section 2.0 lists the Day 1 preparation steps that need to be taken the day before decontamination testing. Section 3.0 details the Day 2 steps for the Decontamination Procedure 1 (spray-no rinse-dry). Section 4.0 discusses sampling and other post-decon procedures to be conducted on Day 3. Section 5.0 provides a summary of the tasks to be completed each day.

2.0 PREPARATION (Day 1)

NOTE: The test organism for this work will be a powdered spore preparation of *B. atrophaeus* (ATCC 9372) and silicon dioxide particles, pre-loaded in metered dose inhalers (MDI).

2.1 Materials for Inoculation Check

- Three (3) sets of sterilized 14 in by 14 in Material Coupons in VHP bags (8 coupons per set)
- Three (3) 14 in by 14 in reference coupons (stainless steel)
- Supplies for inoculation as listed in MOP 6561 (*Aerosol Deposition of Spores onto Material Coupon Surfaces using the Aerosol Deposition Apparatus*).

2.2 Inoculation of Coupons per MOP 6561

- a. Print the coupon codes prior to the start of coupon inoculation. These are unique identifiers described in Section 2.13 of the WA 3-08 QAPP. These coupon codes will be needed in Step c below.
- b. The inoculation team will assemble the aerosol deposition apparatus (ADAs) and clamp everything down before any inoculation begins (the day before inoculation, if possible). Pictures should be taken of the completed ADAs.
- c. After a coupon is dosed via MOP 6561, the coupon will be labeled with a unique identifier (printed out in Step a above). Write the sample ID code the label on the side of the coupon using a permanent marker (e.g., black or silver Sharpie®).
- d. Maintain a log for each set of coupons that are dosed via MOP 6561. Each record in this log will include the unique coupon identifier, the MDI unique identifier, the date, the operator, the weight of the MDI before dissemination into the coupon dosing device, the weight of the MDI after dissemination, and the difference between these two weights.

- e. After the minimum 18-hour settling period, remove coupons from the ADA and move to an isolated storage cabinet, which will contain all inoculated coupons for a single test. The handling of the contaminated coupons, including movement to minimize or control spore dispersal, will be done in accordance with MOP 6561. One person will be tasked with removing the clamps holding the dosing chamber to the coupon and the removal of the dosing chamber and gasket from the coupon. A second person, wearing new, sterile gloves for each coupon, will then be tasked with moving the coupon to the proper location (e.g., test and positive control coupons to the Test Coupon Cabinet and blank coupons to the Blank Coupon Cabinet).

NOTE: The contamination control coupons will be stainless steel coupons (14 in by 14 in) that will be contaminated in accordance with MOP 6561, then sampled and analyzed in accordance with Sections 2.6.1 and Section 3.1.1 of the WA 3-08 QAPP, respectively. The stainless steel coupons will be placed on a clean cookie sheet which will be labeled with the unique identifier described in Section 2.13 using a black Sharpie®. If the results from the contamination controls are outside the acceptance criteria, the results will be discussed with the EPA WAM immediately to determine the corrective action.

3.0 DECONTAMINATION

3.1 Preparation of the Decontamination Formula and Neutralizer

3.1.1 Decontamination Formula

Chemicals:

- Hydrogen peroxide (50%): Fisher Scientific, CAS: 7722-84-1
- Triacetin, Acros Organics CAS: 102-76-1
- Maquat® MC1412-80%, Mason Chemical Company, CAS: 68424-85-1
- Ethanol (99.5%), Acros Organics, CAS: 64-17-5
- Potassium Carbonate: Sigma Aldrich, CAS: 584-08-7

The decontamination formula (modification of formula # 10 developed by Sandia Laboratories) will be prepared in three parts (A, B, and C):

- Part A = water, buffer, Maquat 1412 and ethanol
- Part B = 15% hydrogen peroxide (H2O2) solution
- **Part C** = the activator, Triacetin

NOTE: Decontaminant should be prepared fresh each day (the formulation must be used within 2 hours after mixing).

△ Safety Requirements:

△ PPE Required: Safety glasses, lab coat, Nitrile gloves

△ All work performed in a chemical fume hood

To make 10 liters of decontamination solution:

1. Prepare **Part A**: In 4870 ml of water, mix in 320 g potassium carbonate, 10 ml of Maquat, and 500 ml of ethanol. Mix by hand in a carboy.
2. Prepare 5000 mL of **Part B**, 15% H₂O₂ solution, by adding 1500 mL of 50% H₂O₂ to 3500 mL of deionized (DI) water. Measure the pH and temperature of the Part B solution.
3. Prepare the decontamination solution as follows:
 - a. Add 4000 mL of **Part B** to all of **Part A** and mix by hand for 30 sec.
 - b. Add 300 mL of **Part C** (the activator, Triacetin) to the **Part A/B** mixture and stir for 1 min.
 - c. Record the time the decontamination solution was prepared in the notebook and on the carboy. Solution **shelf-life is two hours**.
 - d. Test the pH and temperature of each formulation, which should be around 9.5 ± 0.1 . This is the target pH **immediately** after Parts A, B, and C are first mixed. Record the pH and temperature in the logbook.

NOTE: The pH will drop rapidly over time and the temperature of the solution will increase; hence, both parameters must be recorded before and after each decontamination step.
 - e. Verify the H₂O₂ concentration by analyzing with the KMnO₄ titration procedure described below:

KMnO₄ Titration Procedure

Reagents:

- 5% Sulfuric acid (H₂SO₄)
 - 1.0N Potassium permanganate
1. Transfer approximately 20 g (~20 mL) of H₂O₂ sample to a tared weighing container and weigh on an analytical balance. Record weight of H₂O₂ sample.
 2. Carefully wash the weighed sample into a 250 ml flask with distilled water, dilute to mark, and mix thoroughly.
 3. Pipet a 25 mL aliquot into a 400 mL beaker containing 250 mL of distilled water and 10 mL of sulfuric acid.
 4. Titrate to the first permanent pink color with 1.0N potassium permanganate.

Calculation:

$$\% \text{H}_2\text{O}_2 \text{ (by weight)} = [(\text{ml KMnO}_4) \times (\text{N}) \times (0.01701) \times (1000)]/(\text{grams of H}_2\text{O}_2 \text{ sample used})$$

where N is the normality of the standard potassium permanganate.

3.1.2 Neutralizer

The neutralizer used to neutralize the runoff is 30% sodium thiosulfate (STS). Prior to decontamination, add 14 mL of the neutralizer per coupon to the carboy (42 mL per 3 coupons). This volume was based on the average volume of the runoff collected per test, as determined during the preliminary experiments.

3.2 Preparation of the Sprayer

NOTE: The ShurFlo 4 ProPack Rechargeable Electric Backpack Sprayer SRS-600 will be used for decontamination testing.

1. Rinse the sprayer with DI water.
2. Discard the DI water. Fill the sprayer and record the pH and temperature of the decontamination solution. Take a sample of solution to verify the concentration of H_2O_2 in the decon solution in the backpack via titration.
3. Check the flow rate and the spray pattern of the activated peroxide (decontamination) solution:
 - Verify the flow rate prior and post each test using a 1 L graduated cylinder and a stopwatch; time the spray for 10 seconds to verify the flow rate. Record in the logbook.
 - The spray pattern will be tested by spraying at the appropriate distance (1 ft) onto a piece of 14 in by 14 in blue construction paper mounted in the orientation of the material section. The spray shall be discharged into the center of the paper and the pattern will be visually assessed for consistency with that shown in Figure 1.

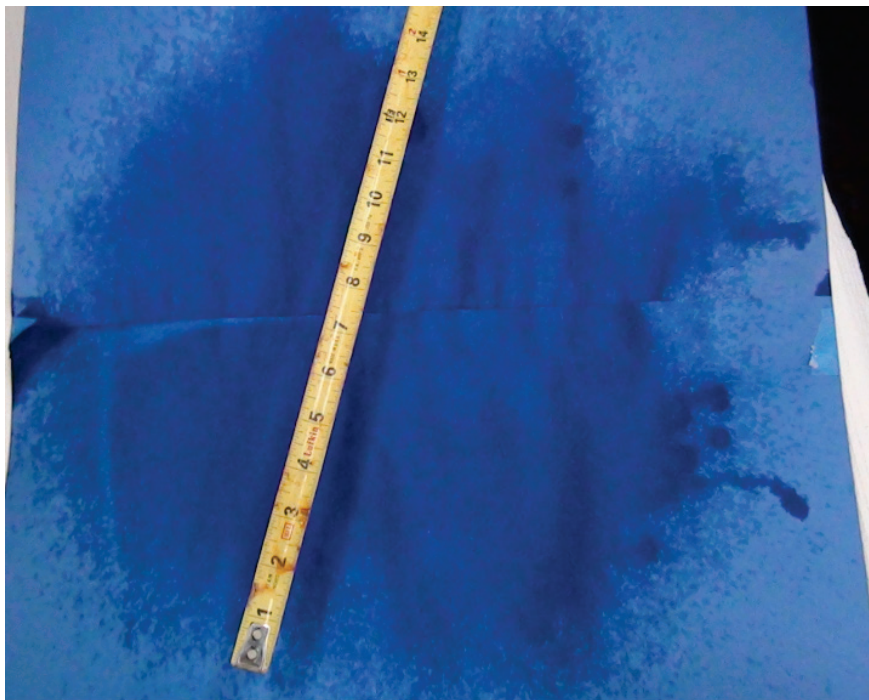


Figure 1. Spray Pattern from Nozzle 1 Foot Away

4. Determine the diameter of the spray to ensure that is within the acceptable limits (12 in to 16 in diameter at 1 foot). Record the spray pattern size.

3.3 Cleaning of the Decontamination Chamber

NOTE: The decontamination chamber is to be cleaned between each material type and before/after each test.

- △ A personal chlorine monitor must be worn by the employee cleaning the chamber. If the monitor alarms, all decon work will cease and additional ventilation brought in. If the alarm continues, leave the area and contact H&S.
- a. Using the backpack sprayer, spray the interior surfaces with adjusted bleach solution comprised of 10% germicidal bleach, 10% acetic acid (5% v/v) and, 80% germicidal bleach.
 - △ Leave the sprayer on a cart or table to prevent muscle strain.
 - b. After 15 minutes, rinse the surfaces with DI water; the runoff is to be collected in a carboy for proper disposal.

3.4 Decontamination Procedures using Procedure 1 (spray once-wait 15 min-no rinse-dry overnight) and Procedure 2 (spray-wait 15 min-reapply-wait 15 min-no rinse- dry overnight)

NOTE: The time for application of each procedural step and time between procedural steps on each coupon will be measured using an NIST-traceable stopwatch and recorded in the laboratory notebook.

NOTE: The time after the coupon is no longer dripping runoff (wait time post-application) was determined to be 15 minutes in preliminary experiments for a single set of porous and non-porous material coupons.

3.4.1 Sterility checks

Swab sampling, as described in MOP 6563 (*Swab Streak Sampling and Analysis*), will be used for sterility checks on coupons and equipment prior to use in the testing. Each item to be used in the decontamination process will be sampled before use. The randomly selected procedural blank coupon (from the same sterilization batch as the rest of the coupons of the same type) will also be swab sampled before the puffing operations begin. The multiple swab samples may be needed for the sterilization checks to be completed.

3.4.2 Procedural Blank Coupon Decontamination

1. Place a sterile pre-weighed carboy (with neutralizer – if deemed necessary as detailed in Section 3.1.2) under the drain of the chamber to collect the runoff from the coupons throughout the entire decontamination procedure.
2. Collect sterility check samples according to MOP 6563 (as detailed in Step 3.4.1 above).
3. Using the pH meter, record pH and temperature of the decontamination solution in the backpack sprayer. Take a sample of decon solution to verify the concentration of H_2O_2 via titration.
4. Move three procedural blank (not-inoculated) coupons to the decontamination chamber.
5. Start application of the decontamination solution; record the time.
6. Spray each set of three coupons for 15 seconds using the Z pattern specified for 14 in by 14 in coupons. Verify the post-decon flow rate of the backpack sprayer.
7. Using the pH meter, record pH and temperature of the decontamination solution in the backpack sprayer.
8. After 15 minutes record the time and carefully move the coupons to the Procedural Blank Cabinet for overnight drying.
9. After all coupons have been moved to the Procedural Blank Cabinet, rinse the chamber with DI water. Label the carboy and record the mass of liquid collected. Take a sample of runoff to verify the concentration of H_2O_2 in the runoff sample via titration. Sample three 100 mL aliquots of the runoff in 120mL sterile, prelabeled specimen cups and place into double sterile bags and send to the on-site Biocontaminant Laboratory for analysis.
10. Clean the decontamination chamber using the procedure described in Section 3.3.

3.4.3 Test Coupon Decontamination

3.4.3.1 Procedure 1

1. Place a sterile pre-weighed carboy with neutralizer under the drain of the chamber to collect the runoff from the coupons throughout the entire decontamination procedure.
2. Using the pH meter, record pH and temperature of the decontamination solution in the backpack sprayer. Take a sample of decon solution to verify the concentration of H_2O_2 via titration.
3. Move three test coupons from the Contaminated Coupons Cabinet to the decontamination chamber.
4. Start application of the decontamination solution.
5. Spray each set of three coupons for 15 seconds using the Z pattern specified for 14 in by 14 in coupons.
6. After 15 minutes, carefully move coupons to the Decontaminated Coupon Cabinet for overnight drying.
7. After all coupons have been moved to the Decontaminated Test Coupon Cabinet, rinse the chamber with DI water. Label the carboy and record the mass of liquid collected. Take a sample of runoff to verify the concentration of H_2O_2 in the runoff sample via titration. Sample three 100 mL aliquots of the runoff in 120mL sterile, prelabeled specimen cups and place into double sterile bags and send to the ON-SITE Biocontaminant Laboratory for analysis.

Clean the decontamination chamber using the procedure described in Section 3.3. Repeat the decontamination procedure for the remaining sets of inoculated coupons.

3.4.3.2 Procedure 2

1. Place a sterile pre-weighed carboy with neutralizer to collect the runoff from the coupons throughout the entire decontamination procedure.
2. Using the pH meter, record pH and temperature of the decontamination solution in the backpack sprayer. Take a sample of the decon solution to verify the concentration of H_2O_2 via titration.
3. Move three test coupons from the Contaminated Coupons Cabinet to the decontamination chamber.
4. Start application of the decontamination solution.
5. Spray each set of three coupons for 15 seconds using the Z pattern specified for 14 in by 14 in coupons.
6. After 15 minutes, re-apply the activated H_2O_2 solution - spray each set of three coupons for 15 seconds using the Z pattern specified for 14 in by 14 in coupons.
7. After 15 minutes, carefully move coupons to the Decontaminated Coupon Cabinet for overnight drying.
8. After all coupons have been moved to the Decontaminated Test Coupon Cabinet, rinse the chamber with DI water. Label the carboy and record the mass of liquid collected. Sample three 100 mL aliquots of the runoff in 120mL sterile, prelabeled specimen cups and place into double sterile bags and send to the ON-SITE Biocontaminant Laboratory for analysis.
9. Clean the decontamination chamber using the procedure described in Section 3.3. Repeat the decontamination procedure for the remaining sets of inoculated coupons.

3.5 Sample Load per Test

These decontamination tests should results in the following number and type of samples:

- Numerous swab samples to satisfy a sterility check (see Section 3.4.1 for description).

- 4 to 5 Procedural blanks (decontaminated procedural blanks stored in the Procedural Blanks Cabinet),
- 12 to 15 Test samples (decontaminated test coupons stored in the Decontaminated Test Coupons Cabinet),
- 12 to 15 Test controls (non-decontaminated positive control coupons stored in the Contaminated Coupons Cabinet),
- 5 to 6 Triplicate samples of runoff collected to a carboy with neutralizer for each decontamination sequence,

4.0 SAMPLING AND EXTRACTION

4.1 Wipe Sampling and Extraction

The procedure for wipe sampling of coupons is described in MOP 3144 (*Procedure for Wipe Sampling of Coupons*). The recovery of the spores from the wipe samples is adapted from the Idaho National Laboratory (INL) 2008 Evaluation Protocols and described in MOP 6567 (*Recovery of Bacillus Spores from Wipe Samples*). This procedure may be modified to include an extractive step before plating to eliminate extra debris. By eliminating the debris, filter plating using MOP 6565 (*Filtration and Plating of Bacteria from Liquid Extracts*) will be more effective. Dilution plating will follow MOP 6535a (*Serial Dilution: Spread Plate Procedure to Quantify Viable Bacterial Spores*).

4.2 Vacuum Sock Sampling and Extraction

The procedure for Vacuum Sock sampling of coupons is described in MOP 3145 (*Procedure for Vacuum Sampling of Large and Small Coupons*). The extraction from vacuum sock samples is described in MOP 6572 (*Recovery of Spores from Vacuum Sock Samples*). This procedure may be modified to include an extractive step before plating to eliminate extra debris. By eliminating the debris, filter plating using MOP 6565 will be more effective.

4.3 Swab Samples

Swab sampling will be used for sterility checks on coupons and equipment prior to use in the testing. The protocol that will be used in this project is described in MOP 6563. Each item to be used in the decontamination process will be sampled before use. The randomly selected procedural blank coupon (from the same sterilization batch as the rest of the coupons of the same type) will also be swab sampled before the puffing operations begin.

4.4 Runoff Collection

During application of the decontamination procedure for each set of coupons, a sterile pre-weighed carboy with neutralizer added (if needed) will be placed under the drain (described in Section 3.4). The neutralizer added will be sufficient to neutralize an amount of H₂O₂ equal to that to be sprayed during decontamination.

The runoff from the coupons throughout the entire decontamination procedure being tested will be collected for a given coupon set (material type or all blanks). Microbiological analysis of the aliquots will be done by dilution plating as described in MOP 6535a and by filtration of the liquid as described in MOP 6565.

4.5 Sampling Decontamination Equipment

The purpose of sampling the decontamination equipment after use is to confirm contamination of the unit with the target organism. The most logical place to sample to confirm contamination is the part of the sampling equipment that stays in contact with a decontamination solution or can potentially touch the coupon surface (such as outside of the nozzle). These parts will be sampled using the swab protocol described in MOP 3135 (*Procedure for Sample Collection using BactiSwab™ Collection and Transport Systems*). Swab analysis (growth/no growth) will be performed in accordance with MOP 6563.

4.6 Aerosol Samples

Aerosol samples collected with the Via-Cell® according to MOP 3155 (*Procedure for Via-Cell® Air Sampling*) will be processed according to MOP 6571 (*Recovery of Bacillus Spores from Via-Cell® Aerosol Sampling Cassettes*). The total flow in the duct will be measured using an S-type pitot tube as described in EPA Method 2. This measurement will be done at the beginning and end of each day of testing.

4.7 Activated Peroxide Measurement

The H₂O₂ concentration will be measured via KMnO₄ titration as described in MOP 3136.

4.8 pH Measurement

The pH of the solution will be measured with an Oakton pH5 pH meter. This meter will be calibrated daily.

5.0 TIMELINE SCHEDULE

The test cycle will start on Monday and end on Wednesday. Samples will be sent to the ON-SITE Biocontaminant Laboratory for analysis as they become available. Below is an outline of the tasks to be completed each day:

Day 1

- Inoculate coupons in H-130 (4 sets of coupons)
- Move test coupons to the blank and positive coupons storage cabinets

Day 2

- Prepare decontamination solution
- Check sprayer
- Clean the decontamination chamber before tests
- Perform sterility checks
- Perform decontamination in the H-130 small test chamber and move coupons to the appropriate storage cabinets for overnight drying
- Collect aerosol samples during decontamination
- Collect runoff samples during decontamination
- Clean the decontamination chamber post-last test

Day 3

- Sample blanks
- Sample test coupon and positives

Attachment A

MOP 3135

TITLE: Procedure for WA 1-25: Procedure for Sample Collection using BactiSwab™ Collection and Transport Systems

SCOPE: This MOP describes the procedure for collecting swab samples for Low Tech Decontamination Technique Testing

PURPOSE: The purpose of this MOP is to ensure all swab sampling is performed in a consistent manner.

Equipment/Reagents

- Disposable lab coat
- Nitrile examination gloves
- P95 Respirator
- Shoe covers
- Bouffant cap
- Safety glasses
- BactiSwab™ Collection and Transport System

1.0 PROCEDURE

1. Enter the COMMANDER airlock wearing appropriate, project-specific PPE (at a minimum gloves, lab coat, and safety glasses), making sure the airlock door is closed.
2. Through the sleeve, crush the BactiSwab™ ampule at midpoint.
3. Hold BactiSwab™ tip end up for at least five seconds to allow the medium to wet the swab.
4. Open the package and remove the BactiSwab™.
5. Label the plastic tube appropriately using the following scheme:

X-Y-N where,

X is the test number,

Y is the material abbreviation, and

N is the material number.

6. Remove the cap-swab from the plastic tube.
7. Swab the surface following the recommend guidelines for each material while spinning the cap-swab between the thumb and index fingers.

a. Brushes (B).

Pull the cap-swab through the brush bristles using one continuous stroke moving top to bottom and left to right.

b. Nozzles (N).

Swab around the squeegee, inside the divisions, and inside the opening for the hose attachment.

c. Buckets (P).

Swab the sides and the bottom surfaces in an “S” pattern.

d. Brush Handles (BH).

Swab the top quarter of the handle top the bottom then bottom to top turning the handle as you go.

e. Hoses (VH).

Swab inside and outside the hose opening that attaches to the nozzle.

f. Vacuums (V).

Randomly swab the folds of the High Efficiency Particulate Air (HEPA) filter, swab the bottom of the vacuum lid, them swab the walls and bottom of the canister. Swab the inside of the exhaust port.

8. Return cap-swab to tube.
9. Date and initial each sample tube. Enter this information into the lab notebook.
10. Complete the chain of custody form and relinquish the samples to the BioLab.

MOP-3144

TITLE: PROCEDURE FOR WIPE SAMPLING OF COUPONS

SCOPE: This MOP describes the procedure for wipe sampling both small and large coupons.

PURPOSE: The purpose of this MOP is to ensure consistent and representative sampling of such coupons.

EQUIPMENT (quantities are per sampling kit)

- Sterile sampling bag (10" x 14") – outer bag
- Sterile sampling bag (5.5" x 9") – inner "sample collection sterile sampling bag"
- Two sterile 50 mL Falcon Blue-Max™ Polypropylene Conical Tubes
- Sterile Kendall (ref. # 8402) 4-ply all-purpose sponge
- Sterile phosphate buffered saline with 0.005% TWEEN®-20, prepared according to MOP -6562
- Pipette or other method for aseptic dispensing of 5 mL liquid
- Sterile Posi-grip® forceps
- P-95 Particulate Respirators – to prevent contamination and for respiratory protection. (Specific projects may require additional respiratory protection and will be addressed in the project Quality Assurance Project Plan (QAPP), e.g., SAR)
- Powder-free Nitrile gloves (support person) and Kimtech Pure G3 Sterile Nitrile gloves (sampler)
- Dispatch® bleach wipes

1.0 PREPARATION

All materials needed for collection of each sample will be prepared in advance using aseptic technique. A sample kit for a single wipe sample will be prepared as follows:

1. Two sterile sampling bags (10" x 14", 5.5" x 9") and a 50 mL conical tube, capped, will be uniquely labeled as specified in the project QAPP. These bags and conical tube will have the same label. The 5.5" x 9" labeled sterile sampling bag will be referred to as the sample collection sampling bag.
2. A sterile all-purpose sponge will be placed in an unlabeled sterile 50 mL conical tube using sterile forceps and aseptic technique. The all-purpose sponge will be moistened by adding 2.5 mL of sterile phosphate buffered saline with 0.005% TWEEN®-20. The tube will then be capped.
3. The labeled 50 mL conical tube (capped), the unlabeled conical tube containing the pre-moistened all-purpose sponge, and the 5.5" x 9" labeled sampling bag will be placed into the 10" x 14" labeled sampling bag. Hence, each labeled sampling bag will contain a labeled 50 mL conical tube (capped), an unlabeled capped conical tube containing a pre-moistened all-purpose sponge, and an empty labeled

sampling bag.

4. Each prepared bag is one sampling kit.

2.0 SAMPLING PROCEDURE FOR SMALL 14"x14" COUPONS

1. A three person team will be used, employing aseptic technique throughout. The team will consist of a sampler, sample handler, and support person.
2. Throughout the procedure, the support person will log anything they deem to be significant into the laboratory notebook.
3. In general, the team works from the least contaminated sample set (i.e., control blanks) towards the most contaminated sample set (i.e., positive controls).
4. The sampling team will each don a pair of sampling gloves (a new pair per sample, non-sterile, as they will only be handling non-sterile items); the sampler's gloves shall be sterile sampling gloves as they are the only member of the team in contact with the sample. All members shall wear dust masks to further minimize potential contamination of the samples. Depending on the situation, respiratory protection beyond a dust mask may be required to protect the sampling team (e.g., SAR; this will be specified in the project QAPP). New disposable lab coats are required for the sample handler when changing between different types of materials or when direct contact between the coupon and lab coat occurs.
5. The sample handler will remove the coupon from the appropriate cabinet and place it on the sampling area, being careful to handle the coupon only around the edges.
6. The support person will record the coupon code on the sampling log sheet.
7. The support person will remove a template from the bag and aseptically unwrap it such that the sampler may grab it wearing sterile gloves.
8. The sampler will place the template onto the coupon surface and align it such that the edges of the coupon are visible through the holes on the template.
9. The support person will remove a sample kit from the sampling bin and record the sample tube number on the sampling log sheet next to the corresponding coupon code just recorded.
10. The sampler and support person will verify the sample code and ensure that the correct coupon and location are being sampled.
11. The support person will:
 - a. Open the outer sampling bag touching the outside of the bag.
 - b. Touching only the outside of the (10" x 14") bag, remove and open the unlabeled conical tube and pour the pre-moistened all-purpose sponge onto the sample or into the sampler's hands.
 - c. Discard the unlabeled conical tube.
 - d. Remove the sample collection sample bag (5.5" x 9"), being careful to not touch the inside of

the outer sampling bag, and open it touching only the outside.

- e. Maneuver the labeled 50 mL conical tube to the end of the outer sterile sampling bag and loosen the cap.
- f. Remove the cap from 50 mL conical tube immediately preceding the introduction of the sample into the tube.

12. The sampler will:

- a. Wipe the surface of the sample horizontally using S-strokes to cover the entire sample area of the coupon using a consistent amount of pressure.
- b. Fold the all-purpose sponge concealing the exposed side and then wipe the same surface vertically using the same technique.
- c. Fold the all-purpose sponge over again and roll up the folded sponge to fit into the conical tube.
- d. Carefully place the all-purpose sponge into the 50 mL conical tube that the support person is holding, being careful not to touch the surface of the 50 mL conical tube or plastic sterile sampling bag.

13. The support person will then immediately close and tighten the cap to the 50 mL conical tube and slide the tube back into the sample collection sampling bag and seal it.

14. The support person will then wipe the sample collection sampling bag with a Dispatch® bleach wipe and place it into the outer sampling bag.

15. The support person will then seal the outer sample collection bag now containing the capped 50 mL conical tube (containing the all-purpose sponge) inside a sealed 5.5" x 9" sample collection bag.

16. The support person will then decontaminate the outer sample bag by wiping it with a Dispatch® bleach wipe.

17. The support person will then place the triple contained sample into the sample collection bin.

18. All members of the sampling team will remove and discard their gloves.

19. Steps 2 – 18 will be repeated for each sample to be collected.

3.0 SAMPLING METHOD FOR LARGE (4'x4' or larger) COUPONS

3.1 Sample Layout

The sampling of large coupons is carried out using a sample grid to divide the large coupons into representative sections. These sections are then numbered and selected to be sampled at different times during the course of the experiment as a blank, a control, or an experimental group sample. This selection grid is pre-determined and the Project Quality Assurance Project Plan (QAPP) may overrule the template shown in Figure 1 if otherwise specified.

As in the example below, the first cell is sampled as a **Blank** before contamination. Starting in cell 3, every third cell is sampled as a positive **Control**. This sample is to be taken post-contamination and before decontamination. Every cell directly following a **Control** cell is sampled as **Experimental** and is taken following decontamination. The sample kit labeling will be based on this grid and the sampling team must ensure to correctly sample the coupons based on this template.

1 Blank	2	3 Control	4 Experimental
5	6 Control	7 Experimental	8
9 Control	10 Experimental	11	12 Control
13 Experimental	14	15 Control	16 Experimental

Figure 1. 4' x 4' Material Section Template and Sample Grid

3.2 Sampling Procedure

1. A two-person team will be used, employing aseptic technique throughout. The team will consist of a sampler and a support person.
2. Throughout the procedure, the support person will log anything they deem to be significant into the laboratory notebook.
3. The sampling team will each don a pair of sampling gloves (a new pair per sample, non-sterile, as they will only be handling non-sterile items); the sampler's gloves shall be sterile sampling gloves as they are the only member of the team in contact with the sample. All members shall wear dust masks to further minimize potential contamination of the samples. Depending on the situation, respiratory protection beyond a dust mask may be required to protect the sampling team (e.g., SAR; this will be specified in the project QAPP).
4. The support person will record the coupon code on the sampling log sheet.

5. The sampler will place the template onto the coupon surface (using clamps as necessary).
6. The support person will remove a sample kit from the sampling bin and record the sample tube number on the sampling log sheet next to the corresponding coupon code just recorded.
7. The sampler and support person will verify the sample code and ensure that the correct coupon and location (cell) is being sampled.
8. The support person will:
 - a. Open the outer sampling bag touching the outside of the bag.
 - b. Touching only the outside of the (10" x 14") bag, remove and open the unlabeled conical tube and pour the pre-moistened all-purpose sponge onto the sample or into the sampler's hands.
 - c. The unlabeled conical tube is retained for Step 9.
 - d. Remove the sample collection sample bag (5.5" x 9") being careful to not touch the inside of the outer sampling bag and open it touching only the outside.
 - e. Maneuver the labeled 50 mL conical tube to the end of the outer sterile sampling bag and loosen the cap.
 - f. Remove the cap from 50 mL conical tube immediately preceding the introduction of the sample into the tube.
9. The sampler will:
 - a. For a vertical coupon, the sampler will squeeze excess moisture from the sampling sponge to prevent dripping down the sampling surface. The excess moisture is caught in the unlabeled conical tube from Step 8c and is then discarded.
 - b. Wipe the surface of the sample using S-strokes to cover the entire sample area of the coupon (inside the grid) using a consistent amount of pressure.
 - c. Fold the all-purpose sponge concealing the exposed side and then wipe the same surface vertically using the same technique.
 - d. Fold the all-purpose sponge over again and roll up the folded sponge to fit into the conical tube.
 - e. Carefully place the all-purpose sponge into the 50 mL conical tube that the support person is holding being careful not to touch the surface of the 50 mL conical tube or plastic sterile sampling bag.
10. The support person will then immediately close and tighten the cap to the 50 mL conical tube and slide the tube into the sample collection sampling bag and seal it.
11. The support person will then wipe the sample collection sampling bag with a Dispatch[®] bleach wipe and place it into the outer sampling bag.
12. The support person will then seal the outer sample collection bag now containing the capped 50 mL conical tube (containing the all-purpose sponge) inside a sealed 5.5" x 9" sample collection bag.

13. The support person will then decontaminate the outer sample bag by wiping it with a Dispatch[®] bleach wipe.
14. The support person will then place the triple contained sample into the sample collection bin.
15. All members of the sampling team will remove and discard their gloves.
16. Steps 2 – 15 will be repeated for each sample to be collected.

MOP-3145

TITLE: PROCEDURE FOR VACUUM SAMPLING OF LARGE AND SMALL COUPONS

SCOPE: This MOP describes the procedure for vacuum sampling of porous areas.

PURPOSE: The purpose of this MOP is to ensure consistent and representative sampling of such areas.

EQUIPMENT (quantities are per sampling kit)

- 2 - Fisherbrand bags with round wire enclosure, 5.5" x 15" (Fisher Scientific, p/n 14-955-181)
- 1 - Fisherbrand bag with round wire enclosure, 10" x 14" (Fisher Scientific, p/n 01-002-53)
- 1 - Vacuum sock filtration kit (Midwest Filtration, p/n FAB-20-01-001A)
- Permanent marker
- Nitrile gloves

1.0 PREPARATION

All materials needed for each sample to be collected will be prepared in advance. A sample kit for a single vacuum sock sample will be prepared using the following procedure (as in MOP- 3141: Procedure for Assembling Vacuum Sock Sampling Kits):

1. Don nitrile gloves.
2. Remove the sock from the manufacturer's kit, place it in a 5.5" x 15" bag so that it is oriented tapered side down, and seal the bag by rolling the top down and folding the tabs. Discard the remains of the manufacturer's kit.
3. Label a 10" x 14" bag and a 5.5" x 15" bag with the sample ID as instructed by the project QAPP.
4. Open the labeled 10" x 14" bag and insert:
 - the labeled 5.5" x 15" bag,
 - an unlabeled 5.5" x 15" bag, and
 - the 5.5" x 15" bag containing the sock.
5. Close the bag and store in a clean dry location.

2.0 VACUUM SAMPLING OF SMALL (14" by 14") COUPONS

The following procedure will be used in this study for vacuum sampling of each coupon surface:

1. A two person team will be used, employing aseptic technique. The team will consist of a sampler and a support person.
2. Both members of the sampling team will each don a pair of sampling gloves (a new pair per sample); the sampler's gloves shall be sterile sampling gloves as they are the only member of the team in contact

with the sample. Both members shall wear dust masks to further minimize potential contamination of the samples. Further respiratory protection beyond a dust mask may be required to protect the sampling team (e.g., Supplied-Air Respirator (SAR); this will be specified in the project QAPP).

3. The sampler will plug in the vacuum power cord and then don his/her sterile gloves.
4. The vacuum will be maintained on a rolling cart for easy movement into place.
5. The support person will aseptically unwrap a template from the bag and present it to the sampler, taking care to not touch the template.
6. The sampler will place the template onto the coupon surface.
7. The sampler will hold the vacuum nozzle for the support person to place the vacuum sock assembly onto the nozzle.
8. The support person will open the sampling supply bin and remove the vacuum sock sample kit from the bin.
9. The support person will record the sample collection bag ID number on the sampling log sheet or in the laboratory notebook.
10. The sampler and support person will ensure that the correct sample coupon has been selected, referencing the coupon code on the sampling bag.
11. The support person will record the coupon code on the sampling log sheet next to the corresponding vacuum sock collection bag number that was just recorded.
12. The support person will:
 - a. Open the vacuum sock sample kit outer bag and remove the unlabelled vacuum sock assembly bag.
 - b. Open the small unlabelled sampling bag containing the vacuum sock assembly and, working from the outside of the bag, maneuver the assembly from the bottom to expose the cardboard applicator tube opening.
 - c. Firmly place the vacuum sock assembly onto the nozzle of the vacuum tube, using the bag to handle the sock assembly, while the sampler holds the vacuum nozzle.
13. The sampler will:
 - a. Ensure that the sock is correctly placed on the nozzle and adjust, if necessary. Care must be taken to not puncture or tear the sock.
 - b. Turn on the vacuum.
 - c. Vacuum "horizontally" using S-strokes to cover the entire area of the material surface not covered by the template, while keeping the vacuum nozzle angled so that the tapered opening of the vacuum sock is flush with the sample surface.
 - d. Vacuum the same area "vertically" using the same technique.
 - e. Turn off the vacuum when sampling is completed.

14. The support person will open the labeled 5.5" x 15" bag and remove the vacuum sock assembly from the nozzle with the inner sterile sampling bag, using care not to touch the sock.

NOTE: It is very important that the vacuum sock **not** be inverted after the sample is taken. During Steps 14 thru 17, keep the white "sock" that contains the sample in an upright position to ensure sample integrity.

15. The support person will then seal the inner sterile sampling bag, wipe it with a Dispatch[®] wipe, and place it into the outer sterile sampling bag.
16. The support person will then seal the outer sterile sampling bag and wipe it with a Dispatch[®] wipe.
17. The support person will then seal the 10" x 14" overpack sample bag now containing the outer and inner bags, the inner containing the vacuum sock assembly. The outermost bag will then be wiped with a Dispatch[®] wipe.
18. The sampler will wipe down the nozzle (inside and out) and end of the tubing with a Dispatch[®] wipe.
19. The support person will then place the triple contained sample into the sample collection bin.
20. All members of the sampling team will remove and discard their gloves.
21. Steps 2 – 20 will be repeated for each sample to be collected.

3.0 SAMPLING METHOD FOR LARGE (4'x4' or larger) COUPONS

3.1 Sample Layout

The sampling of large coupons is carried out using a sample grid to divide the large coupons into representative sections. These sections are then numbered and selected to be sampled at different times during the course of the experiment as a **Blank**, a **Control**, or an **Experimental** group sample. This selection grid is pre-determined and the Project QAPP may overrule the template shown in Figure 1 if otherwise specified. The first cell is sampled as a **Blank** (negative control) before contamination. Starting in cell 3, every third cell is sampled as a positive **Control**. This sample is to be taken post contamination and before decontamination. Every cell directly following a **Control** cell is sampled as **Experimental** and is taken following decontamination. The sample kit labeling will be based on this grid and the sampling team must ensure to correctly sample the coupons based on this template.

1 Blank	2	3 Control	4 Experimental
5	6 Control	7 Experimental	8
9 Control	10 Experimental	11	12 Control
13 Experimental	14	15 Control	16 Experimental

Figure 1. 4' x 4' Material Section Template and Sample Grid

3.2 Sampling Procedure

The procedure detailed below will be used in this study for vacuum sampling of each coupon surface. In addition to these collected samples, one vacuum sock would be left unopened and used as a [Trip Blank](#), or vacuum sock negative control. The purpose of this Trip blank is to verify that the vacuum socks – as received from the manufacturer – were not defective and/or contaminated, nor are the socks being contaminated simply by traveling through the collection process with the real samples. The vacuum sock negative control results should always come back negative.

1. A two person team will be used, employing aseptic technique. The team will consist of a sampler and a support person. Both members of the sampling team will wear task specific PPE as specified in the project QAPP. P95 respirators are the minimum requirement for sample protection.
2. The sampler and support person will don sterile gloves and position the sampling grid onto the coupon, using clamps if necessary.
3. The support person will plug in the vacuum power cord. The vacuum will be maintained on the floor with easy access to the sample. The exhaust of the vacuum should be directed directly into the air handling return duct to help prevent cross-contamination.

4. The sampler will don a new pair of sterile gloves and position the vacuum nozzle in anticipation of the support person.
5. The support person will don a new pair of nitrile gloves, open the sampling supply bin, and remove one vacuum sock sample kit from the bin.
6. The support person will record the sample collection bag number on the sampling log sheet.
7. The sampler and support person will confirm the correct sampling location has been selected by referencing the coupon code on the sampling bag.
8. The support person will:
 - a. Open the vacuum sock sample kit outer bag and remove the unlabelled vacuum sock assembly bag.
 - b. Open the small unlabelled sampling bag containing the vacuum sock assembly and, working from the outside of the bag, maneuver the assembly from the bottom to expose the cardboard applicator tube opening.
 - c. Firmly place the vacuum sock assembly onto the nozzle of the vacuum tube, using the bag to handle the sock assembly, while the sampler holds the vacuum nozzle.
9. The sampler will:
 - a. Turn on the vacuum.
 - b. Vacuum “horizontally” using S-strokes to cover the entire area of the material surface not covered by the template, while keeping the vacuum nozzle angled so that the tapered opening of the vacuum sock is flush with the sample surface.
 - c. Vacuum the same area “vertically” using the same technique.
 - d. Turn off the vacuum when sampling is completed.
10. The support person will open the labeled 5.5” x 15” bag and remove the vacuum sock assembly from the nozzle using the inner sterile sampling bag.
11. The support person will then seal the inner sterile sampling bag, wipe it with a Dispatch[®] wipe, and place it into the outer sterile sampling bag.
12. The support person will then seal the outer sterile sampling bag and wipe it with a Dispatch[®] wipe.
13. The support person will then seal the 10” x 14” overpack sample bag now containing the outer and inner bags, the inner containing the vacuum sock assembly. The outermost bag will then be wiped with a Dispatch[®] wipe.
14. The sampler will wipe down the nozzle (inside and out) and end of the tubing with a Dispatch[®] wipe.
15. The support person will then place the triple contained sample into the sample collection bin.
16. All members of the sampling team will remove and discard their gloves.
17. Steps 2–16 will be repeated for each sample to be collected.

MOP-3150

TITLE: PROCEDURE FOR FABRICATION OF 14" x 14" MATERIAL COUPONS

SCOPE: This MOP describes the procedure for construction of 14" x 14" material coupons.

PURPOSE: The purpose of this MOP is to ensure consistent manufacturing of these coupons.

INTRODUCTION

This MOP is split into five sections, each section being dedicated to one material type. Sections 1.0 to 5.0 present the methods for fabricating coupons from carpet, drywall, concrete, deck wood, and rough-cut barn wood, respectively.

1.0 CARPET

1.1 Equipment

- Beaulieu Solutions Walnut Ridge Loop Carpet
- 6 lb carpet padding
- 15/32" - Four Ply Plywood Sheathing
- 1/2" staples
- Safety Glasses
- Cut-Resistant Gloves
- Staple Gun
- Safety Razor Utility Knife
- Table saw
- Tape measure
- Straight edge
- 1/4" Duct Tape

1.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut a 14" x 14" square of 15/32" thick Four Ply Plywood Sheathing using a table saw.
3. Cut a 14" x 14" square of 6 lb carpet padding using a safety razor utility knife.
4. Cut a 14" x 14" square of Beaulieu Solutions Walnut Ridge Loop Carpet (or carpet type specified by the project QAPP) using a safety razor utility knife.
5. Align the carpet padding on top of plywood.
6. Align the Beaulieu Solutions Walnut Ridge Loop Carpet on top of carpet padding.

7. Carefully secure carpet and carpet padding to plywood sheathing using $\frac{1}{2}$ " staples and a staple gun around the outside edges (no more than $\frac{1}{8}$ " from coupon edge).
8. Seal the edges of the coupon with duct tape by taping the outer $\frac{1}{8}$ " edges of the front and stapling. Fold the tape over and staple to the back. This creates a sampling area of 13.5" square.

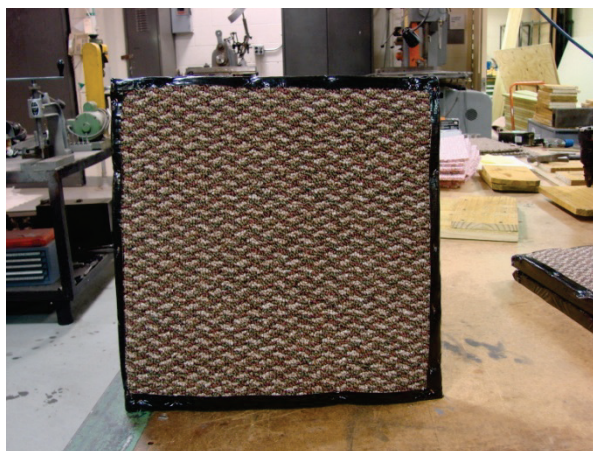


Figure 1. Carpet Coupon Front



Figure 2. Carpet Coupon Back

2.0 DRYWALL

2.1 Equipment

- Safety Glasses
- Cut-Resistant Gloves
- Table saw
- One sheet of $\frac{1}{2}$ " drywall
- Joint Compound
- Putty Knife
- Joint Tape
- Sanding Block
- KILZ latex primer
- Behr Interior Enamel Paint
- Paint brushes
- Tape measure

2.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut a 14" x 14" section of Drywall from a drywall sheet using a table saw.
3. Apply a skim coat of joint compound about 1.5" from each cut edge of the coupon to the cut edge on the front side using a putty knife.
4. Using two inch joint tape, apply one half of the tape (utilizing the factory fold) to the front side of the coupon over the joint compound.
5. Apply a second skim coat of joint compound over the tape using a putty knife.
6. Allow to dry.
7. After the compound has dried, apply a skim coat of joint compound to the back side of the coupon about 1" from the cut edge of the coupon to the cut edge using a putty knife.
8. Fold the joint tape over the cut edge to the backside of the coupon (it should extend about 1/2" over the back).
9. Apply a second skim coat of joint compound over the tape using a putty knife.
10. Allow to dry.
11. Remove any rough spots in the joint compound using a sanding block.
12. Allow to dry.
13. Smooth out all rough spots in the joint compound using a sanding block.
14. Apply one coat of KILZ latex primer to the front side of the coupon.
15. Allow to dry.
16. Apply one coat of Behr Premium Plus Interior Flat White Latex Paint to the front side of the coupon.
17. Allow to dry.
18. Seal the back side of the coupon with any non-white latex or enamel paint.



Figure 3. Drywall Coupon Front

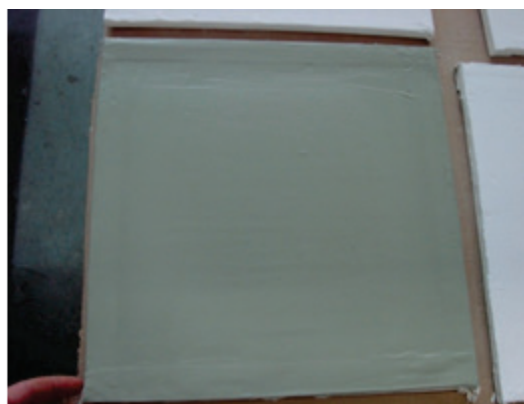


Figure 4. Drywall Coupon Back

3.0 CONCRETE

3.1 Equipment

- Quikrete Sand/Topping Mix
- Water Source
- Mixing trough
- Trowel
- Leveling board
- Plastic Covering for Curing Process

3.2 Procedure

1. Custom 14" x 14" forms have been manufactured for these coupons.
2. Prepare concrete mix according to indications on package using a trough and garden hose for the water supply.
3. Pour concrete mix into custom manufactured forms.
4. Use a trowel to smooth coupon surface and allow form to dry overnight.
5. Repeat for each coupon to be manufactured.
6. Lay plastic over the coupons and allow to cure for at least 5 days.



Figure 5. Concrete Curing

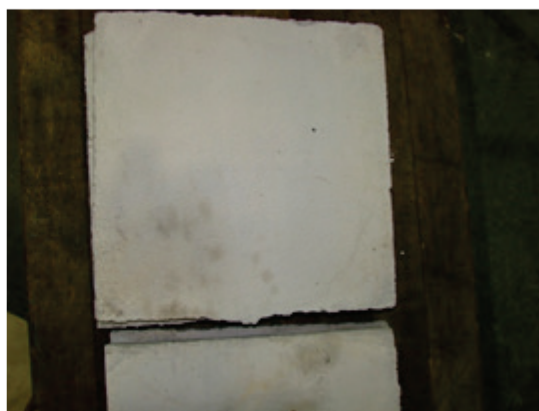


Figure 6. Finished Concrete Coupon

4.0 DECKWOOD

4.1 Equipment

- 5/4"x 6" Pressure treated decking board, allowed to dry at least 1 week after purchase.
- 2" Exterior Deck Screws
- 1-1/4" Common Nails
- Safety Glasses
- Cut resistant Gloves
- Circular Saw
- Impact wrench
- Behr Waterproofing Wood Protector
- Natural bristle brush
- Tape measure

4.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Using a table saw, cut enough of the decking wood into $\frac{3}{4}$ " wide strips to fabricate a 14" x 14" frame.
3. Cut an additional piece 1.5" wide to be used to simulate a floor joist.
4. Construct framing as shown in Figure 7, securing with 1-1/4" common nails.



Figure 7. Deck Wood Coupon Back



Figure 8. Deck Wood Coupon Front

5. Carefully cut 5/4" x 6" board into 14" sections using a table saw. Two full size boards will be used. A third board will be needed to be ripped to size to completely cover the frame, allowing a 1/8" gap in between.
6. Mount the boards to framing from left to right leaving a 1/8" gap between each pair of boards.
7. Mount each board using two deck screws in the center strut.
8. Cut the last board to approximately 2.5" or as needed to completely cover the framing.
9. Mount the last board using one deck screw.
10. Secure the outer coupon edges to the framing using 1-1/4" Common Nails.
11. Once the assembly is complete, seal the front of the coupon using one coat of Behr Waterproofing Wood Protector.

5.0 ROUGH CUT BARN WOOD

5.1 Equipment

- 1" X 6" pressure treated Brazilian Pine Dog Ear Picket Fence Lumber
- Safety Glasses
- Cut resistant Gloves
- 1" Staples
- Staple Gun
- Circular Saw
- Tape measure

5.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Using a radial arm saw, cut six 14" x 6" pieces of rough cut wood.
3. Lay three of the pieces, butted together, face down on a bench. Lay the other three pieces perpendicular to the first three to cover the butt joints of the wood. Use 1" staples to fasten the boards together from the back so that no staples show on the front.
4. Use a table saw to trim the coupon to 14" square.



6.0 PLYWOOD

6.1 Equipment

- 23/32" Alkaline Copper Quaternary Treated Plywood, allowed to dry at least 1 week after purchase.
- Safety Glasses
- Cut resistant Gloves
- Circular Saw
- Tape measure

6.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut a 14" x 14" square of 15/32" thick Four Ply Plywood Sheathing using a table saw.

7.0 PAINTED PLYWOOD

7.1 Equipment

- 23/32" Alkaline Copper Quaternary Treated Plywood, allowed to dry at least 1 week after purchase.
- Safety Glasses
- Cut resistant Gloves
- Circular Saw
- Tape measure
- Exterior Paint (i.e., BEHR Exterior Enamel, white)
- Paint brush and paint tray

7.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut a 14" x 14" square of 15/32" thick Four Ply Plywood Sheathing using a table saw.
3. Using a paint brush, apply two coats of exterior paint, allowing at least 2 hours for drying between coats (or per the paint manufacturer's label instructions)

8.0 GLASS

Ordered to size from Durham Glass, Inc.

9.0 LINOLEUM

9.1 Equipment

- Vinyl linoleum flooring, in 12'-wide roll
- Safety Glasses
- Cut resistant Gloves
- Sharp Knife / Box-blade
- Tape measure
- Construction adhesive (i.e., liquid nails)
- Heavy-duty staple gun, with staples

9.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut linoleum sheets into 14" x 14" squares
3. Cut 14" x 14" squares of 15/32" thick Four Ply Plywood Sheathing using a table saw, one for each coupon.
4. Apply a thin bead of construction adhesive around the perimeter of the rear of the sheet, and as a "z" within the middle of the rear of the sheet.
5. Place the linoleum square onto the plywood, rub the surface to distribute the adhesive. Fasten the vinyl to the plywood using heavy-duty staples, three staples along each side.

10.0 CERAMIC TILE

10.1 Equipment

- Ceramic tile (18" x 18")
- Safety Glasses
- Cut resistant Gloves
- Tape measure
- Tile saw

10.2 Procedure

1. Don safety glasses and cut-resistant gloves.
2. Cut tile into 14" x 14" squares by cutting 4" from each of two sides.

MOP-3155

TITLE: PROCEDURE FOR VIA-CELL[®] AIR SAMPLING

SCOPE: This MOP describes the procedure for air sampling using the Via-Cell[®] Bioaerosol Sampling Cassette.

PURPOSE: The purpose of this MOP is to ensure consistent and representative air sampling.

EQUIPMENT

- Zefon Via-Cell[®] Bioaerosol Sampling Cassette (p/n VIA010)
- EPA Method 5 Dry Gas meter box within annual calibration
- ½" I.D. vacuum tubing
- Labeled 5.5" x 15" sterile bag for tertiary containment
- Writing pen
- Project notebook

1.0 PREPARATION

1. Verify from the package that the Via-Cell[®] cassette is within its expiration date. If not, discard.
2. Label the Via-Cell[®] cassette with the appropriate sample ID according to the test plan or Quality Assurance Project Plan (QAPP).
3. In the project laboratory notebook, record the cassette sample ID, lot number, and expiration date.

2.0 AIR SAMPLING

There are two areas of operation for Via-Cell[®] air sampling: 1) at the cassette and 2) at the meter box. These two areas of operation may be performed by the same person or different people depending on the situation. When air sampling inside COMMANDER while occupied, for instance, a two-person team will be necessary; one person inside to connect the cassette and the other outside to start and stop the dry gas meter.

1. Wearing nitrile gloves, tear open the foil package using the tear strip on top. Use care when opening, as this package is re-sealable and is required to be used after sampling for transport to the laboratory for analysis.
2. Remove Via-Cell[®] from the package (see Figure 1).



Figure 1. Disassembled Via-Cell®, showing cap (far left), package, and cassette with outlet plug.

3. Remove the blue outlet plug from the cassette and place it into the foil for safe keeping (seen at the bottom of the green Via-Cell® cassette in Figure 1).
4. Connect the Via-Cell® sampler outlet to the dry gas meter using vacuum tubing and position the cassette in the desired location. The Via-Cell® sampler is capable of operating in any vertical or horizontal orientation and in confined spaces.
5. Perform a leak-check on the cassette by pulling a vacuum on the inlet cap. The meter box flow rate should be zero.
6. Remove the large blue inlet cap for the cassette and place into the foil package for safekeeping.
7. Record the dry gas meter's initial volume.
8. At the meter box, turn on the pump and set the sampling pump to a flow rate of 15 Lpm. Over the pressure drop of the Via-Cell® cassette, this is at a ΔH of 1.1" water as read on the front of the meter box. Pull a sample for the desired amount of time, monitoring the ΔH every ten minutes. When sampling is completed, and with new gloves, replace the blue plug in the outlet and the blue cap over the inlet.
9. Record the sampling time and final volume. Check to ensure flow rate was 15 Lpm.
10. Place the Via-Cell® cassette into the special foil bag and zip it closed. Apply the red safety seal label over the top of the foil bag opening to ensure sample integrity until analysis.
11. Place foil bag containing cassette inside a pre-labeled 5.5" x 15" sterile bag for tertiary containment.
12. Submit the cassette to the Biocontaminant Laboratory along with a Chain-of-Custody (COC); the cassette should be analyzed according to MOP 6571 within 24 hours of collection. The COC form should include the collection time and the analysis by time and date in the comments section.
13. Each sample should be associated with a laboratory blank (plain, unused Via-Cell® cassette) and a field blank of at least 150 liters of clean air in the same area as samples are collected.

MOP 6535a

Title: SERIAL DILUTION: SPREAD PLATE PROCEDURE TO QUANTIFY VIABLE BACTERIAL SPORES

Scope: Determine the abundance of bacterial spores in a liquid extract

Purpose: Determine quantitatively the number of viable bacterial spores in a liquid suspension using the spread plate procedure to count colony-forming units (CFU)

Materials:

- Liquid suspension of bacterial spores
- Sterile centrifuge tubes
- Diluent (sterile deionized water, buffered peptone water or phosphate buffered saline)
- Trypticase Soy Agar plates
- Microliter pipettes with sterile tips
- Sterile beads placed inside a test tube (will be used for spreading samples on the agar surface)
- Vortex mixer

Procedure: (This protocol is designed for 10-fold dilutions.)

1. For each bacterial spore suspension to be tested, label micro-centrifuge tubes as follows: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} ... (The number of dilution tubes will vary depending on the concentration of spores in the suspension. Aseptically, add 900 μ L of sterile diluent to each of the tubes.
2. Label three Trypticase Soy Agar plates for each dilution that will be plated. These dilutions will be plated in triplicate.
3. Mix original spore suspension by vortexing thoroughly for 30 seconds. Immediately after the cessation of vortexing, transfer 100 μ L of the stock suspension to the 10^{-1} tube. Mix the 10^{-1} tube by vortexing for 10 seconds, and immediately pipette 100 μ L to the 10^{-2} tube. Repeat this process until the final dilution is made. It is imperative that used pipette tips be exchanged for a sterile tip each time a new dilution is started.
4. To plate the dilutions, vortex the dilution to be plated 10 seconds, immediately pipette 100 μ L of the dilution onto the surface of a TSA plate, taking care to dispense all of the liquid from the pipette tip. If less than 10 seconds elapses between inoculation of all replicate plates, then the initial vortex mixing before the first replicate is sufficient for all replicates of the sample. Use a new pipette tip for each set of replicate dilutions.
5. Carefully pour the sterile glass beads onto the surface of the TSA plate with the sample and shake until the entire sample is distributed on the surface of the agar plate. Aseptically remove the glass beads. Repeat for all plates.
6. Incubate the plates overnight at 32 °C to 37 °C (incubation conditions will vary depending on the organism's optimum growth temperature and generation time.)

7. Enumerate the colony forming units (CFU) on the agar plates by manually counting with the aid of a plate counting lamp and a marker (place a mark on the surface of the Petri dish over each CFU when counting, so that no CFU is counted twice).

Since each dilution was tested in triplicate, determine the average of the triplicate plate abundances. Plates suitable for counting must contain between 30 - 300 colonies.

Calculations

Total abundance of spores (CFU) within extract:

(Avg CFU / volume (mL) plated) X (1 / tube dilution factor) X extract volume

For example:

<u>Tube Dilution</u>	<u>Volume Plated</u>	<u>Replicate</u>	<u>CFU</u>
10-3	100 µL (0.1 mL)	1	150
10-3	100 µL (0.1 mL)	2	250
10-3	100 µL (0.1 mL)	3	200

Extract total volume = 20 mL

$$(200 \text{ CFU} / 0.1 \text{ mL}) \times (1/10^{-3}) \times 20 \text{ mL} = \quad \times$$

$$(2000) \times (1000) \times 20 = 4.0 \times 10^7$$

Note: The volume plated (mL) and tube dilution can be multiplied to yield a 'decimal factor' (DF). DF can be used in the following manner to simplify the abundance calculation.

$$\text{Spore Abundance per mL} = (\text{Avg CFU}) \times (1 / \text{DF}) \times \text{extract volume}$$

MOP- 6555

TITLE: PETRI DISH MEDIA INOCULATION USING BEADS

SCOPE: This MOP outlines the procedure for cleaning, assembling, and using beads to inoculate agar plates.

PURPOSE: To provide an easily repeatable method for spreading liquid inoculation onto agar plates.

1.0 EQUIPMENT

1. #13 test tubes
2. 6 x 12 test tube racks (which hold 72 tubes)
3. Beads of various sizes (glass)
4. Glass autoclavable trays (stainless steel is eventually corroded by the bleach and autoclaving processes)
5. Bleach
6. DI water
7. Hot gloves
8. Amber bottle for collecting hazardous waste (with hazardous waste label)
9. Funnel
10. Aluminum foil
11. Label tape
12. Chemical hood
13. Autoclave
14. Oven
15. Labeled bead container or cup (All mold and bacteria beads must be kept separately)

2.0 CLEANING BEADS

1. When a sufficient number of beads have been collected, or at least once a day when beads are being used to spread colonies, place the used beads into a tray with a 1:5 ratio of bleach to deionized water solution.
Add the bleach to the beads first, under the protection of a chemical safety cabinet. Then add the deionized water. Cover the pan with aluminum foil and label it with the contents (for example: "bacteria beads in 1:5 bleach to DI water solution"). Soak the beads 12-24 hours (usually overnight) in a chemical fume hood.

2. After soaking, take a bottle brush and thoroughly scrub the beads.
3. Decant the bleach solution (collect the bleach for proper disposal) and rinse with deionized water 6 to 8 times, collecting the runoff after the first rinse for disposal (subsequent rinses can be discarded in the lab sink). Rinse until the decanted liquid is clear. Use a funnel to add the bleach waste to a labeled amber waste bottle. These liquids must be labeled "hazardous waste" and can then be stored, collected or disposed of properly.
4. Cover the beads with deionized water and autoclave for 1 hour on the liquid cycle.
5. Decant the deionized water and place the tray of beads in the Thelco lab oven at 121° C until dry (a minimum of 3 hours).
6. Remove the beads from the oven using proper safety equipment (heat gloves) and cover with clean aluminum foil to prevent contamination. Label each tray with the following information:
"Clean bacteria (or mold) beads," the date beads were cleaned, initials of the person who cleaned them.
7. These beads are then ready for use as described in "PLACING BEADS IN TUBES".

3.0 PLACING BEADS IN TUBES

1. Fill a 6 x 12 rack with tubes.
2. Place clean beads into a shallow pan, and then manually fill each tube with 7-15 beads/tube.
Note: Beads vary in size and will therefore fill the tubes to different heights.
3. Tightly attach a cap to each tube
4. Autoclave for using 1 hour gravity sterilization cycle (see MOP 6570). Autoclave tape must be placed on the top of each rack to provide evidence that the beads have been sterilized

4.0 SPREADING BEADS

1. To spread inoculum on the agar surface, one tube of beads should be used for each individual plate.
2. After the beads have been added, the plates can be stacked up to six plates high. The plates are then shaken 10 times from side-to-side. Turn the stack of plates ¼ turn, and again shake 10 times from side-to-side. Repeat this procedure (¼ turn and 10 shakes) two more times, so that the beads are shaken a total of forty times.
3. Turn the plates over (upside down), and tap the beads into the lid.
4. Aseptically dump the beads into a labeled bead container (mold and bacteria beads must be labeled and collected separately), which should be considered contaminated. This should be done one plate at a time, replacing the lid as quickly as possible to prevent contamination.

1. <http://serc.carleton.edu/microbelife/k12/LIMW/dilution.html>
2. <http://www.qbiogene.com/businessdivisions/platformnews/news0005-b.shtml>
3. <http://www.genlantis.com/objects/catalog/Product/extras/C400050.pdf>
4. EPA - SHEM Chemical Hygiene Plan.

MOP 6561

TITLE: AEROSOL DEPOSITION OF SPORES ONTO MATERIAL COUPON SURFACES USING THE AEROSOL DEPOSITION APPARATUS

SCOPE: This MOP outlines the procedure for assembly and usage of the Aerosol Deposition Apparatus (ADA).

PURPOSE: Precise and highly repeatable aerosol deposition of bacterial spores onto material surfaces for detection, sampling, and/or decontamination studies.

Materials:

- Aerosol Deposition Apparatus (ADA) (shown in Figure 1)
- Metered Dose Inhaler (MDI) preloaded with a bacterial spore suspension of known concentration (i.e., 1×10^9 spores per puff)
- Vertical MDI Actuator (shown in Figure 2)
- Material coupon (with dimensions at least that of the ADA)
- ADA-coupon gasket (1 per ADA) (see Figure 1)
- Clamping devices (i.e., medium-size steel binder clips, C-clamps (8 per ADA))
- Vortex mixer (shown in Figure 4)
- Aerosol trap (described in Appendix A and shown in Figure 4)
- Personal Protective Equipment (PPE) (gloves, lab coat, safety goggles)
- pH-adjusted bleach (pAB) (MOP 3128-A)
- 0.22 μ m pore-size syringe filters (shown in Figure 1)
- PVC tubing (3/8" OD, 1/4" ID)
- Mass balance (with 0.01 gram accuracy)
- Bench liner

1.0 STERILIZATION OF MATERIALS

Prior to the start of any experiment, all components must be sterilized and stored in a sterile environment until usage. Sterilization **is not necessary** for binder clips, MDI, vortex, or the aerosol trap.

ADAs can be sterilized by autoclave, VHP, or by wiping with pH-adjusted bleach (pAB) with subsequent deionized (DI) water and ethanol rinse/wipes. The ADA lid should be attached and in the closed position during the sterilization.

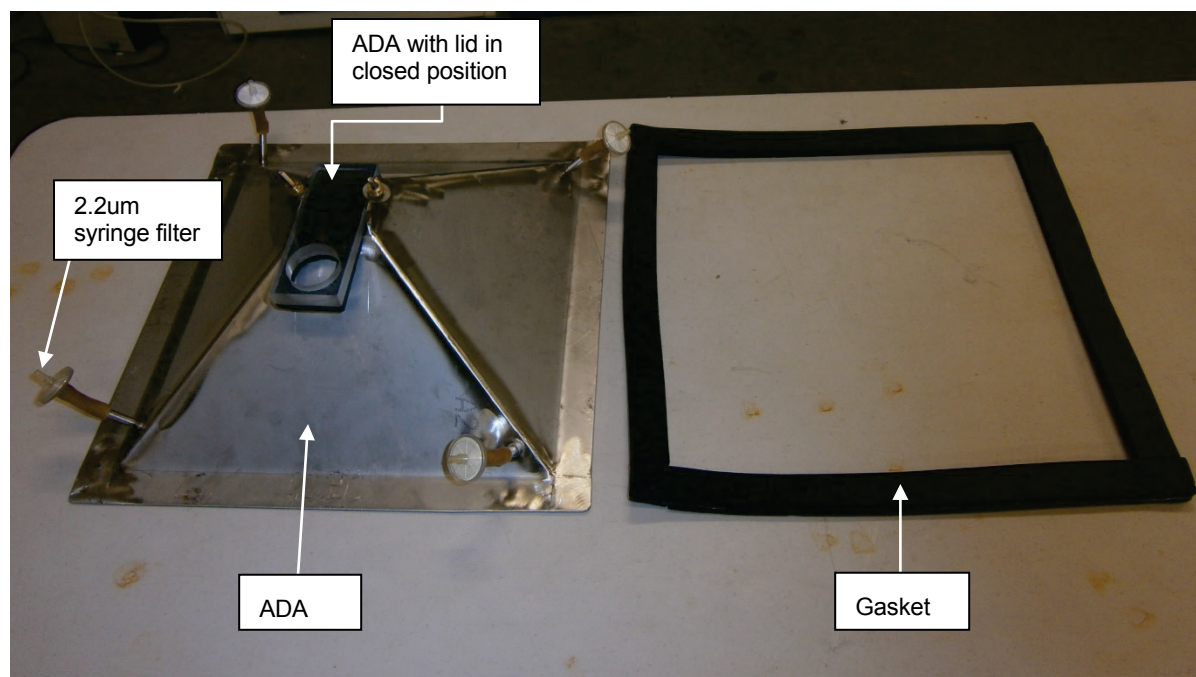


Figure 1. ADA apparatus

The MDI actuator, with attached MDI adaptor, can be wiped with pAB then rinsed with DI water.

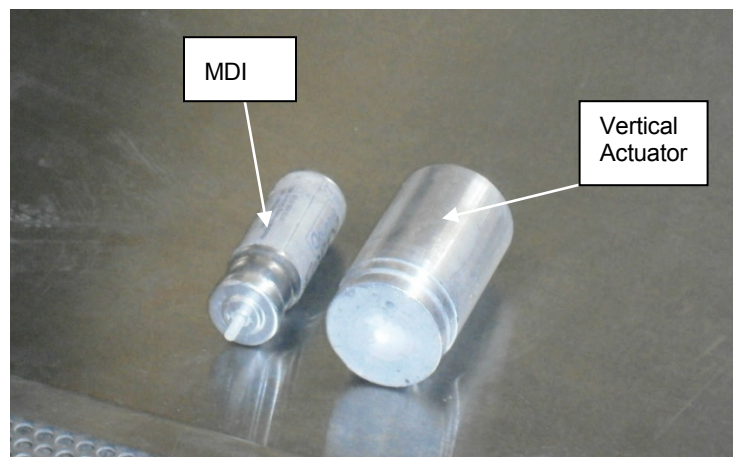


Figure 2. MDI and vertical actuator

Sterilization requirements for coupons vary by material. Regardless of the sterilization method, quality control (QC) checks (typically by collecting a swab sample per MOP 3135) should be administered to ensure the effectiveness of the sterilization method.

Gasket sterilization may also vary by material. Care should be taken to thoroughly degas gaskets if sterilized via fumigation.

2.0 PROCEDURE

1. Begin by donning PPE (gloves, lab coat, and protective eyewear).
2. Clean the workspace by wiping with pAB, next with DI water, and lastly with a 70-90% solution of denatured ethanol. Alternatively, new, clean bench liner may be placed on the work surfaces. Make sure the workspace is clean and free of debris.
3. Discard gloves and replace with fresh pair.
4. Using aseptic techniques (when possible) assemble the coupon/ADA by first placing the sterilized material coupon onto the clean lab bench or workspace, next place the sterilized gasket on top of the coupon, and lastly seat the ADA on the coupon + gasket. Orient each component so that it fits squarely with the previously placed item. Take care not to touch the inside of the ADA or the coupon surface. Secure these components by attaching medium-size binder clips, one at each corner, and one at the midpoint of each of the four sides of the ADA. The binders should firmly secure the coupon to the ADA, and apply sufficient pressure to the gasket to seal the union. If material coupons are too large to use binder clips other methods may be used to secure the coupon and gasket to the ADA (i.e., larger clamps, weight added to the ADA, etc.). Lastly, attach 0.2 μ m syringe filters to each vent tube on all ADAs (4 per ADA). Syringe filters can be attached using PVC tubing (3/8" OD, 1/4" ID).
5. Determine the weight of the MDI canister using a balance. Record the MDI ID number and the weight (to the nearest 0.01g) in lab notebook. In addition, keep a record of the total number of 'puffs' dispensed for each MDI canister.

NOTE: The MDI canister full is approximately 15 g, an empty canister is approx 9.5 g. To ensure the canister contains adequate spore suspension for dosing, canisters should be retired from use when their weight falls below 10.5 g.

6. Next, assemble the MDI and actuator by inserting the MDI into the actuator, taking care not to activate the MDI.
7. Vortex the MDI/actuator assembly for 30 seconds (the MDI canister should be in direct contact with the vortex mixer).
8. Holding the MDI/actuator assembly upright (Figure 3), with a swift, firm motion, dispense three test 'puffs' into the aerosol trap to prime the MDI. It is important to vortex the assembly 10 seconds before every puff (the exception being 30 seconds prior to the initial puff of the experiment, as prescribed in Step 7).

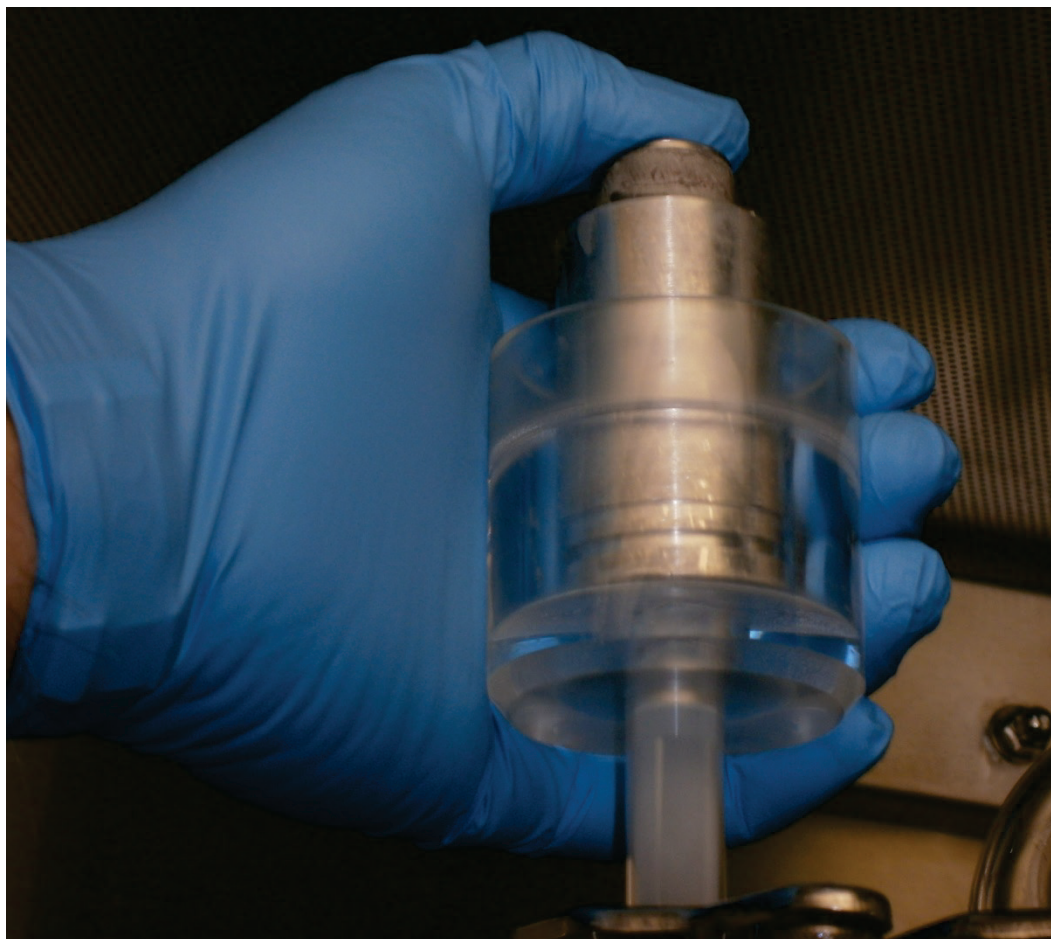


Figure 3. MDI orientation while dispensing test puffs into the aerosol trap.

9. Vortex the assembly for 10 seconds and then attach to the ADA lid by mating the ADA adaptor to the hole in the ADA lid. Loosen the lid screws enough to allow the lid to be slid into the 'open' position. Secure the lid in the open position by tightening the lid screws.

NOTE: The 'open' position is achieved when the hole in the lid aligns with the hole in the top of the ADA.

10. With a swift, firm motion, dispense the spores by activating the MDI. Hold the MDI in the activated position for 3 seconds before releasing. Activation is best achieved by grasping the MDI/actuator with two hands, and using a thumb to press the bottom of the MDI canister.
11. Follow the reverse order of the lid opening procedure to close the ADA lid.
12. Determine the weight of the actuator-MDI using a balance, and record the weight in lab notebook.

NOTE: If the dosing puff is faulty, return to Step 9 and attempt a second puff on the current coupon. Do not proceed to the next coupon until a 'successful' puff has been delivered. A 'successful'

puff is achieved when the weight of the actuator-MDI assembly has a 0.04 g to 0.07 g loss. Familiarity and professional judgment will be needed to determine the success of a puff.

13. Vortex the assembly for 10 seconds, then proceed to dosing the next coupon (Step 9).
14. Repeat Steps 9 through 13 until all coupons have been dosed.
15. Once all coupons have been puffed, remove the MDI from the actuator and weigh. Record the final weight and total number of puffs.
16. Allow spores to settle onto the coupon surface for at least 18 hours. Settling time should not exceed 26 hours.
17. Carefully remove binder clips (or other attachment device), and remove aerosol deposition apparatus (ADA) and gasket from coupon surface, taking care not to disturb the surface of the coupon.
18. Test coupon is now ready for use.
19. Decontaminate the ADA and associated components with the same procedures utilized during the initial sterilization.

APPENDIX A - Aerosol Trap

Purpose: This device allows test puffs of the MDI to be deployed without contamination of the surrounding area. Spores are pulled into the trap, contained, and inactivated.

This device consists of a suction source, a trap (containing pAB), and an inlet funnel. Aerosolized spores are pulled into the funnel, and forced into the trap. The spores are collected and inactivated as the aerosol flows through the pAB solution. The effluent air traveling toward the suction device is spore-free downstream of the trap. See Figure 4.

The aerosol trap should be assembled inside a biological safety cabinet (BSC) or chemical fume hood.

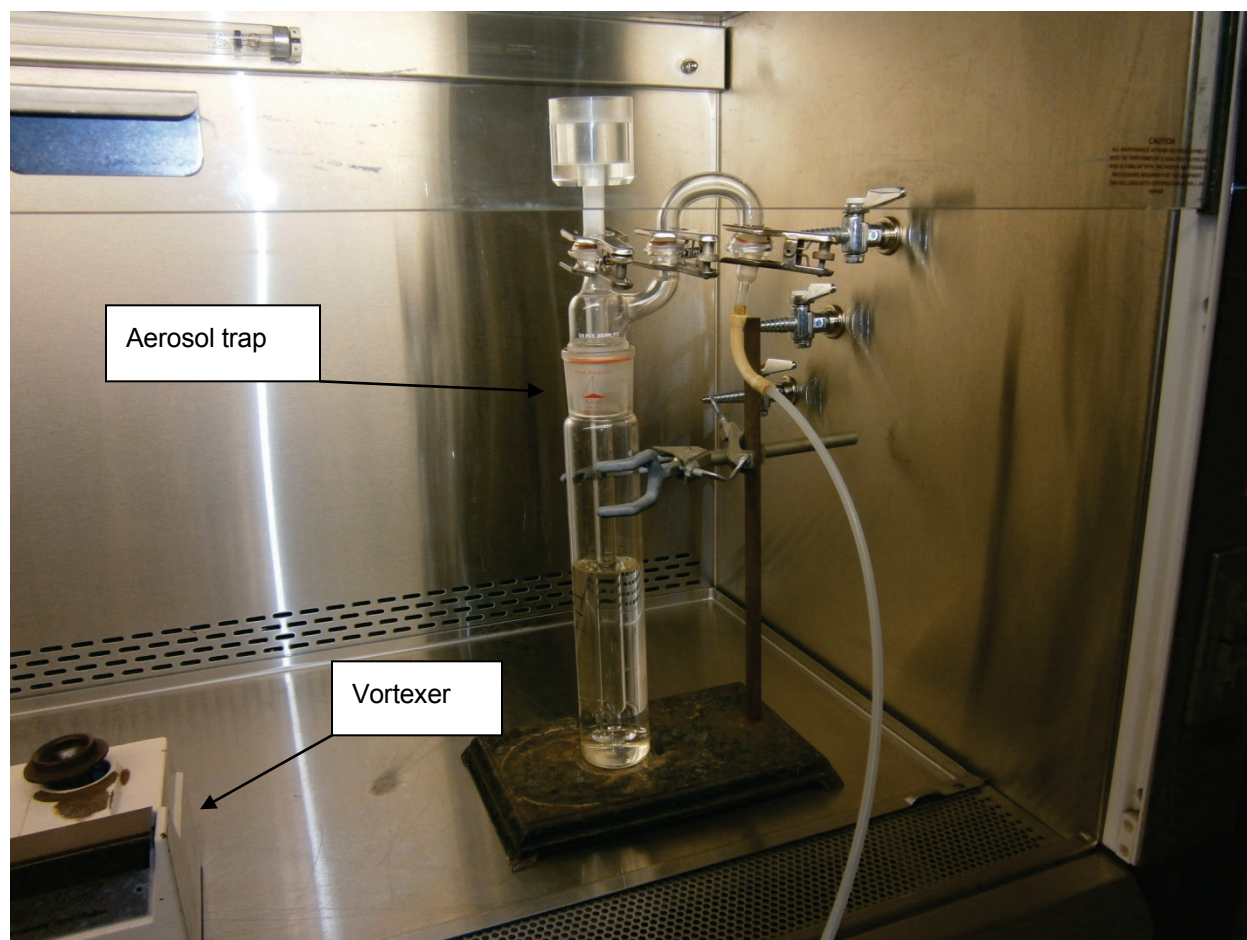


Figure 4. Aerosol trap

MOP-3128

TITLE: PROCEDURE FOR PREPARING pH-ADJUSTED BLEACH SOLUTION WITH TRI-SODIUM PHOSPHATE SUBSTITUTE

SCOPE: This MOP describes a procedure for reproducibly preparing the pH-adjusted bleach solution with trisodium phosphate (TSP) substitute.

PURPOSE: The purpose of this MOP is to ensure the solution meets QA specifications for each test.

Equipment/Reagents:

- Draeger or personal chlorine (Cl_2) monitor [Oakton Acorn Series pH 5 meter or equivalent]
- Funnel
- Triple-rinsed container suitable for transporting hazardous solutions
- Oakton pH 7 (pH = 7.00 ± 0.01 @ 25°C) buffer or equivalent
- DAP[®] Trisodium Phosphate (TSP) Substitute (Lowe's p/n 224908)
- Clorox Commercial Solutions Germicidal Bleach (Lowe's p/n 33692), less than 1 year old
- 5% v/v Acetic Acid (Ricca Chemical, p/n 7732-18-5 or equivalent)

1.0 PROCEDURE

1.1 Calibrate pH Meter

1. Turn meter on (Figure 1). Meter will automatically enter pH mode.
2. Rinse electrode thoroughly with DI water. DO NOT wipe the electrode.
3. Dip both the electrode and temperature sensor into pH 7.00 buffer solution. The glass bulb must be completely immersed into the sample. Stir gently, and wait for the reading to stabilize (about 40 seconds).
4. Press CAL key to enter the calibration mode. The display will momentarily flash "CA" to indicate Calibration. The display will show the current noncalibrated reading, blinking while in calibration mode.



Figure 1. Oakton pH meter

5. Allow the reading to stabilize. The meter will automatically recognize 7.00, 4.01, or 10.00 buffers.
6. Press Enter key once to confirm calibration. The LCD displays “CO” to indicate the calibration point has been confirmed. The meter exits calibration mode and returns to measurement mode.
7. Record the pH buffer measurement and temperature (Press MODE key to select parameter) in the appropriate lab notebook.

1.2 Bleach + TSP Preparation

1. Prepare TSP solution by dissolving ¼ cup per 1L of deionized water (DI H₂O).
2. The pH-adjusted bleach + TSP solution should consist of 9.8% germicidal bleach, 23.8% acetic acid, 26.4% TSP solution, and 40% DI H₂O. For example, to prepare 1 gallon (3.785 L) of solution, combine 370 mL of germicidal bleach, 1L of TSP solution, 900 mL of acetic acid, and 1515 mL of DI H₂O in that order. Record the total volume as V_{start} in the lab notebook.
3. Measure the pH of the solution (target pH = 6.8). If pH is above 7.0, add acetic acid. If below 6.5, add germicidal bleach. Record the volume required for adjustment as V_{add} . Calculate V_{total} as $V_{\text{start}} + V_{\text{add}}$ in the lab notebook.
4. Measure the free available chlorine (FAC) per MOP 3148. The target FAC is 6350 mg/L. The acceptable range is 6000 mg/L < FAC < 6700 mg/L.
 - a) If FAC exceeds the acceptable range, dilute the total volume with TSP solution by the percent difference between the target FAC and the actual FAC.

$$\text{Dilution volume} = [(\text{actual} - \text{target}) \div \text{target}] \times (V_{\text{total}})$$

- b) If the FAC is less than the acceptable range, add bleach and trisodium phosphate (TSP) solutions according to the following equations:

$$\text{Additional volume of bleach} = (\text{target} - \text{actual}) / \text{target} \times V_{\text{total}} \times 0.098$$

$$\text{Additional volume of TSP solution} = (\text{target} - \text{actual}) / \text{target} \times V_{\text{total}} \times 0.264$$

5. Recalculate V_{total} according to the all additions and repeat steps 3 and 4 until both parameters are met. Record the final FAC, pH, temperature, and time in the lab notebook.

MOP-6562

TITLE: PREPARING PRE-MEASURED TUBES WITH ALIQUOTED AMOUNTS OF PHOSPHATE BUFFERED SALINE WITH TWEEN® 20 (PBST)

SCOPE: This MOP provides the procedure for preparing PBST.

PURPOSE: This procedure will ensure that that the PBST is prepared correctly and that all measured tubes are filled aseptically.

1.0 PREPARING STERILE PHOSPHATE BUFFERED SALINE WITH TWEEN® 20 (PBST)

Phosphate Buffered Saline with Tween® 20 (PBST) is prepared 1 L at a time in a 1 L flask.

1. Add 1 packet of SIGMA Phosphate Buffered Saline with Tween® 20 (P-3563) to 1 L of deionized (DI) water.
2. Shake vigorously to mix until dissolved.
3. Label bottle as “non-sterile PBST” and include date and initials of person who made PBST.
4. Filter sterilize into two 500 mL reagent bottles using 150 mL bottle top filter (w/ 33 mm neck and .22 µm cellulose acetate filter) for sterilization. Complete this by pouring the liquid into the non-sterile PBST into the top portion of the filtration unit 150 mL at a time, while using the vacuum to suck the liquid through the filter. Continue to do this until 500 mL have been sterilized into a 500 mL bottle. Change bottle top filter units between each and every 500 mL bottle.
5. Change label to reflect that the PBST is now sterile. Include initials and date of sterilization. The label should now include information on when the PBST was initially made and when it was sterilized and by whom.
6. Each batch of PBST should be used within 90 days.

2.0 PREPARING 20 ML/5 ML PBST TUBES FOR USE DURING EXPERIMENTATION

Twenty (20) mL or five (5) mL of the prepared PBST will be added to each sterile 50-mL conical tube as detailed below. Each flat of conical tubes contains 25 tubes, so one 500 mL sterile bottle of PBST should fill approximately one flat when 20 mL tubes are needed and four flats when 5 mL tubes are needed.

1. Prepare the hood by wiping down with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or Techwipe. Then stock the hood with the following items if they are not already there:
 - The flats of sterile conical tubes you need to fill with PBST.
 - Sufficient bottles of sterile PBST to fill these tubes.
 - Ample 25 mL serological pipettes (at least 3 per flat) for 20 mL transfers and 10 mL

serological pipettes for the 5 mL transfers.

- Serological pipetter (automatic, hand-held pipette).
- Burner and striker.

2. Light the burner and adjust the flame for a width adequate to flame the lips of the PBST bottles.
3. Take one flat of sterile conical tubes and loosen each cap on the outside edges (about $\frac{1}{2}$ turn).
4. Open a serological pipette and insert into the serological pipetter, taking care to not touch the tip to any surface.
5. Hold the pipetter with the first three fingers of your right (or dominant) hand. With your left hand (or non-dominant hand), pick up a bottle of the PBST and use the bottom of your right hand to unscrew the lid. Place the lid upside down on the benchtop and quickly flame the lip of the bottle. Turn the bottle and repeat, taking care to thoroughly flame the lip without getting the glass so hot that it shatters.
6. Inset the tip of the pipette into the bottle and fill to the 20 mL line. Flame the bottle lip and place the bottle on the benchtop.

Note: If the tip of the pipette touches the outside of the bottle or any other surface in the hood, consider it contaminated. Discard the pipette and reload a new one.

7. Quickly pick up one of the tubes that you have loosened the cap on, and use the bottom of your right hand to remove the cap. Completely discharge the entire pipette into the tube, taking care not to touch anything with the tip of the pipette. Recap the tube and place back into the flat (the lid does not have to be tight – you will tighten the lids after you have completed filling the 10 outside tubes).

Note: If the tip touches the outside or rim of the tube (or any other surface in the hood), consider the tube and pipette contaminated. Discard both the tube and the pipette.

8. Pick up the PBST bottle and flame the lip. Repeat Steps 6 and 7 until all 10 of the tubes on the outside of the flat have been filled. Flame the lip of the PBST bottle and replace the cap. Slide the used pipette back into the plastic sleeve and put to the side of the hood for disposal. Then tighten the lid of each tube you just filled. But rather than placing it back into its original spot in the flat, switch it for the empty tube from the next row. When this has been completed, go around the outside of the flat again and loosen the lids of these 10 tubes. Repeat steps 4 through 7 to fill and cap these tubes.
9. This same procedure is used to fill the middle row of tubes from the flat, and if more than one flat of tubes is being filled, can be done at the same time as the outside rows of a second flat.
10. When all tubes have been filled, label each flat as follows, and place on the shelf in room E390B:
“PBST Tubes (20 mL or 5 mL)”
Date prepared
Your initials

11. These tubes should be made at least 14 days before they need to be used so that they can be verified as sterile. Any tubes that are cloudy or that have any floating matter/turbidity should be discarded. The tubes are stable for and should be used within 90 days.

3.0 CLEANUP FOR 20 ML/5 ML PBST TUBES

1. Dispose of the used pipettes in the nonregulated waste.
2. Plug in the serological pipetter so that it can recharge.
3. Replace any unused PBST in the liquid containment on the shelf. Make sure that the bottle is labeled as having been opened (date opened and initials of whoever used it).
4. Turn off the burner.
5. Wipe down the hood benchtop with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or TechWipe.

4.0 PREPARING 900 μ L PBST TUBES FOR USE DURING EXPERIMENTATION

1. Prepare the hood by wiping down with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or Techwipe. Then stock the hood with the following items if they are not already there:
 - A sterile beaker of microcentrifuge tubes.
 - Sufficient tubes of sterile PBST to fill these tubes (PBST may be aseptically transferred to 50 mL conical tubes for an easier aseptic transfer to the microcentrifuge tubes- it is easier than working from a 500 mL reagent bottle. Make certain that these 50 mL conical tubes are labeled to when the PBST was made, sterilized, etc.).
 - 1000 μ L micropipette.
 - 1000 μ L sterile pipette tips.
 - Microcentrifuge tube racks.
 - Labeled beaker or waste container used to hold non-regulated waste, such as tips, under the hood.
2. Carefully remove the microcentrifuge tubes one at a time from the beaker and close the top on each one before placing it in the tube rack. Place the tubes in the rack skipping every other row. Fill up two racks doing this.
3. Add 900 μ L of PBST to the microcentrifuge tubes by aseptically transferring the PBST from the sterile 50 mL conical tube containing the PBST. Do this by using the 1000 μ L micropipette and tips. Change tips whenever two rows of tubes are completed or whenever a contamination event (such as touching the outside of the 50 mL tube or the microcentrifuge tube) occurs. Put the dirty tips in the beaker or container used to contain waste (tips, tubes) in the hood. If any 900 μ L tubes are contaminated during the transfer, dispose of them in the waste container used to hold tips under the

hood. If a new box of tips has to be opened, make certain the date it was opened and initials of the person who opened it are clearly labeled on the box.

4. After both racks are full, carefully move all the tubes from one rack to fill in the empty rows on the other rack. In this manner, one rack should be completely filled with tubes at this point.
5. Label the rack of tubes as "Sterile 900 μ L PBST Tubes", along with the name of the person who completed the transfer, along with the date. Also, include the date that the original stock of PBST was made and the date it was sterilized, along with the initials of the person who completed those steps.

5.0 CLEANUP FOR 900 μ L PBST TUBES

1. Dispose of the waste that was put in the labeled beaker or waste container (micropipette tips and tubes) in the nonregulated waste. Then, place this beaker in the "To be decontaminated via sterilization- contaminated glassware" bin or if it is a disposable container, then it can be put in the non-regulated waste container.
2. Put the unused sterile tips and the micropipetter back in its original location.
3. Replace any unused 50 mL conicals of PBST in the liquid containment on the shelf. Make sure that the tube is labeled as having been opened (date opened and initials of whomever used it). If the tube could possibly be contaminated in any way, dispose of it in non-regulated waste.
4. Wipe down the hood benchtop with ethanol, followed by bleach, followed by DI water and a clean Kimwipe or TechWipe.

MOP- 6563

TITLE: SWAB STREAK SAMPLING AND ANALYSIS

SCOPE: This MOP provides the procedure for the process of completing a swab streak plate and subsequent qualitative analysis for samples being analyzed for *Bacillus* species.

PURPOSE: This procedure will ensure that the swab streak plate sampling and analysis methods are standardized and that the collection and plating of samples are free from contamination. These methods are specific to *Bacillus* species as the target organism.

1.0 PREPARING THE MATERIALS

There are two types of prepared swabs that can be used in this procedure:

Environmental Transport Swabs – purchased swabs that are individually packaged and pre-sterilized.

In-house Sterilized Swabs – swabs placed into autoclave pouches and sterilized using a 1-hour gravity cycle.

This procedure requires the following materials and equipment:

Tryptic soy agar (TSA) media plates

35 °C incubator

Nitrile (non-sterile) gloves

Sharpie for writing on plates

2.0 COLLECTING AND PLATING SAMPLES

The procedure for collecting and plating samples is dependent on the type of swab being used. Appropriate PPE should be worn at all times and include a lab coat, nitrile gloves and safety glasses.

2.1 Environmental Transport Swabs

2.1.1 Collection of Environmental Transport Swab Sample

1. Break the seal on the individually packaged and sterile swab. Remove the cap, collect the specimen with the swab applicator by touching the swab tip to the area in question, and then replace the cap on the swab.

2. Label the tube with the sample ID, the date, time, and initials of the person performing the procedure.
3. Place the swab into a secondary container, such as a sterile bag, and label the bag with the same information placed on the tube label.
4. Transport the sample(s) to the Microbiology Laboratory for processing.

2.1.2 *Plating of Environmental Transport Swab Sample*

1. When the sample is received in the Microbiology Laboratory, label three TSA plates with a Sharpie with the information from the swab packaging. Verify that the sample ID and date match.
2. Place label plates and swab samples under the biological safety cabinet. Remove the sample swab from the secondary container and the tube. Press onto the first plate in an S-stroke motion, turning the swab as it is plated to ensure that all of the surface area of the swab touches the plate. Press firmly, but not so hard that the surface of the media is broken.
3. Perform Step #2 on the remaining two plates.
4. Replace the swab into its tube and discard in the non-regulated waste container.
5. Repeat steps #1 through #4 for each sample.
6. Label three TSA plates as **Swab Blank A**, **Swab Blank B**, and **Swab Blank C**. These plates will serve as negative controls for the swabs.
7. Open a new/unused Environmental Transport Swab and use it to plate the three blank plates as detailed in Step #2.
8. Stack the triplicate plates media side up and place in a 35 °C ± 2 °C incubator for at least 18 hours. Note the time the plates were placed in the incubator.

2.2 In-house Sterilized Swabs

When In-house Sterilized Swabs are being used to collect samples, they need to be plated immediately (unlike the Environmental Transport Swabs that are transported to the Microbiology Laboratory for plating). Therefore, prior to travelling to the sample site, collect the following materials and supplies which will be needed:

- A minimum of three TSA media plates (in a media bag) per sample to be collected plus three additional plates to be used as negative controls for swab blanks.
- One In-house Sterilized Swab (in their autoclave pouches) per sample to be collected, one swab for the control plates, plus a few extras.

- Sharpie for labeling plates.

Use the following procedure to collect and plate samples.

1. Once at the sample collection site, take the TSA plates out of the media bag and label three plates for each sample with what is being swabbed (sample ID), date, time, and initials of the person performing the procedure.
2. As carefully and as aseptically as possible, remove the swab from the autoclave pouch by the stick end. Be sure not to touch the swab end to anything but the sample. If the swab's sterility is compromised, dispose of the swab and use one of the extras.
3. Collect the specimen with the swab applicator as detailed in the specific test protocol.
4. Press onto the first plate in an S-stroke motion, turning the swab as it is plated to ensure that all of the surface area of the swab touches the plate. Press firmly, but not so hard that the surface of the media is broken. Because these samples are being plated in the open air and not in a biological safety cabinet, be certain to limit the time that the lid is removed from the TSA plate.
5. Perform Step #2 on the remaining two plates.
6. Replace the swab into the autoclave pouch it came in and discard in the non-regulated waste container.
7. Repeat steps #1 through #6 for each sample.
8. Label three TSA plates as **Swab Blank A**, **Swab Blank B**, and **Swab Blank C**. These plates will serve as negative controls for both the swabs and the TSA.
9. Open another in-house sterilized swab from the autoclave pouch and use it to plate the three "Blank" plates as detailed in Step #2.
10. Put the TSA plates back into the media bag and transport to the Microbiology Laboratory.
11. When received by the laboratory, the triplicate plates will be stacked media side up and placed in a $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ incubator for at least 18 hours. Note the time the plates were placed in the incubator.

3.0 ANALYZING THE SAMPLES

The Swab Results Template, which follows this section, is used to record the results of the sampling. Some quantities of samples may require more than one form. Make certain that the data are filled in completely on each page. The analyst will use the information on the TSA plates to fill in the following blanks at the top of the form:

- Swab samples taken on: *(date)*

- Swabbed by: *(person)*
- Plating completed on: *(date)*
- Plated by: *(person)*

The following procedure is used to analyze the samples and complete the remainder of the Swab Results Template form.

1. Fill in the final two sections at the top of the form: **Plate results read on** and **Results read by**.
2. Take the first set of triplicate plates and note the sample IDs on the first three lines in the **Sample** column.
3. For each plate, check whether there was growth (**G**) or no growth (**NG**). No growth is indicative that the sample is sterile. Growth is indicative of an organism(s) being present, and should be described on the form. Be as detailed as possible, noting colony morphology (size, shape, color and any other distinctive things that can be seen concerning the growth).
4. The Swab Results Template form serves as the sample report and should be provided to the Project Manager.

Swab Results Template

Swab samples taken on:		Swabbed by:	
Plating completed on:		Plated by:	
Plate results read on:		Results read by:	
Sample Name	Result	If growth, describe	
	G <input type="checkbox"/> NG <input type="checkbox"/>		
	G <input type="checkbox"/> NG <input type="checkbox"/>		
	G <input type="checkbox"/> NG <input type="checkbox"/>		
	G <input type="checkbox"/> NG <input type="checkbox"/>		
	G <input type="checkbox"/> NG <input type="checkbox"/>		
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	G <input type="checkbox"/> NG <input type="checkbox"/>		
	G <input type="checkbox"/> NG <input type="checkbox"/>		
Controls	Result	If growth, describe	
Swab blank A	G <input type="checkbox"/> NG <input type="checkbox"/>		
Swab blank B	G <input type="checkbox"/> NG <input type="checkbox"/>		
Swab blank C	G <input type="checkbox"/> NG <input type="checkbox"/>		

Key G = Growth. NG = No Growth.

All plates are plated in triplicate resulting in sample identification of "A", "B", and "C".

MOP 6565

Title: FILTRATION AND PLATING OF BACTERIA FROM LIQUID EXTRACTS

Scope: This MOP outlines the procedure for filtration and subsequent cultivation of bacterial spores from a liquid extract.

Purpose: This method is deployed when results from spread-plate methods yield less than 30 colony-forming units (CFU) per plate. This method allows a lower limit of detection for bacterial recovery/survivorship assays.

Materials

- Petri dishes with appropriate agar
- 0.2 µm pore-size disposable analytical filter units (2 per sample)
- P1000 pipette and sterile tips
- Sterile forceps
- Pipettman and sterile serological pipettes

Procedure

1. For each liquid sample to be analyzed, gather two disposable analytical filter units and two Petri dishes containing the desired sterilized/QC'd media.
2. Label plates
3. Vortex liquid extract vigorously for 2 minutes, using 10 second bursts.
4. Using a P1000 sterile tip and aseptic techniques, immediately following vortexing, pipette 1 mL of the extract into one of the filter units.
5. Apply vacuum to the filter unit to pull the liquid through the filter and collect the spores on the surface of the filter.
6. Using a sterile serological pipette, rinse the filter unit by pipetting 10 mL of sterile DI H₂O along the inner sides of the unit while it is under vacuum.
7. Aseptically remove the filter from the filter apparatus using sterile forceps and lay the filter onto the agar surface within the Petri dish (spore side up).
8. Vortex the liquid extract vigorously for 10 seconds.
9. Using the appropriate volume serological pipette, collect the remainder of the liquid and dispense in the second filter unit.
10. Note and record the volume.

11. Apply vacuum to the filter unit, to pull the liquid through the filter and collect the spores on the surface of the filter.
12. Using a sterile serological pipette, rinse the filter unit by pipetting 10 mL of sterile DI H₂O along the inner sides of the unit while it is under vacuum.
13. Aseptically remove the filter from the filter apparatus using sterile forceps, and lay the filter onto the agar surface within the Petri dish (spore side up).
14. Incubate the plates at the optimal growth temperature for the organism used for 16 – 28 hours.
15. Enumerate and record the number of CFU on each plate

Data Calculations

Utilize the following equation to determine the total abundance of recovered spores:

$$N = CFU \times \frac{V_{Extract}}{V_{filtered}}$$

Where N is the total number of spores recovered in the extract, CFU is the abundance of colonies on the agar plate, $V_{Extract}$ is the total volume of the extract (before any aliquots were removed), $V_{Filtered}$ is the volume of the extract filtered.

MOP 6567

Title: RECOVERY OF *BACILLUS* SPORES FROM WIPE SAMPLES

Scope: This MOP outlines the procedure for recovering *Bacillus* spores from wipe samples

Purpose: To aseptically extract and quantify *Bacillus* spores from wipe samples in order to determine viability and obtain quantifiable data

1.0 MATERIALS

- PPE (gloves, lab coat, safety goggles)
- Biological Safety Cabinet (Class II)
- pH-Adjusted bleach
- Deionized water
- 70% solution of denatured ethanol
- Kimwipes
- Dispatch bleach wipes
- Non-regulated waste container
- 50 mL sterile conical tubes containing 20 mL of sterile phosphate buffered saline with Tween[®] 20 solution (PBST) (MOP 6562)
- Vortex mixer
- Cart
- Wire or foam rack for 50 mL conical tubes
- Tryptic soy agar plates
- 900 µL tubes of sterile PBST
- Pipettor and pipette tips for dilutions
- Incubator set to appropriate growth temperature for target organism (35 °C or 55 °C)
- Light box for counting colonies
- Lab notebook
- QAPP for project that is utilizing the wipe samples

2.0 Procedure

1. Begin by donning PPE (gloves, lab coat, and protective eyewear).
2. Obtain wipe samples that may contain *Bacillus* spores. Wipe samples should be received as one wipe/sponge in a sterile 50 mL conical tube delivered in secondary containment. Make certain that all of the samples are labeled. Review any chain of custody forms that may accompany the samples to ensure that all of the labels are consistent and that there is no notable variation in the samples. If variation has occurred, make a note of it in the notebook.
3. Clean the workspace (biological safety cabinet) by wiping surfaces with pH-adjusted bleach, next with deionized water, and lastly with a 70-90 % solution of denatured ethanol. Wipe with a kimwipe to remove any excess liquid. Make sure the workspace is clean and free of debris. Gather all necessary items to perform the task, place these items on a clean cart beside the biological safety cabinet, within arm's reach so that, once the procedure has begun, the task may be performed without interruptions.
4. Discard gloves and replace with fresh pair.
5. One at a time, under the biological safety cabinet, remove the sample tube containing the wipe sample from the secondary containment bag in which it arrived. Using the Dispatch bleach wipes, wipe each sample tube with one wipe, and then wipe it with a clean kimwipe. Discard the used bleach wipe and the used kimwipe in the secondary containment bag and place them in the non-regulated waste container. Remove gloves and don a fresh pair of gloves. Repeat this procedure for every sample. After each sample has been cleaned, place the tubes containing the wipe samples in an appropriate sized wire or foam rack to hold the tubes in an upright, vertical position.
6. Leaving the tubes in the rack underneath the biological safety cabinet, aseptically add 20 mL of PBST solution (this should be in a pre-measured, sterile conical tube, per MOP 6562) to each sample tube containing a wipe, one at a time. Remove the rack containing wipe samples from hood when all samples have had the PBST added. Place the rack with the samples on the cart.
7. Using the procedure to clean the biological safety cabinet, as found in Step 3, clean the biological safety cabinet again. Afterwards don a fresh pair of gloves.
8. Using a vortex mixer, agitate the wipe samples, four at a time, in a biological safety cabinet, for ten second bursts for two minutes total. Make certain to clean the biological safety cabinet after each set of four samples and change gloves between each set of samples.

Note: The reason that four samples are done at one time is to limit the time between agitation and plating. The samples need to be processed immediately after agitation, and agitation of more than four samples at a time leaves too much time between agitation and spread plating.

9. Using tryptic soy agar media plates that are appropriately labeled with the sample number, dilution set and date, complete dilution plating for the wipe samples immediately after the two minute agitation step is completed. The samples should also be agitated again for ten seconds directly prior to removing an aliquot from the sample tube. Each dilution tube should also be agitated for ten seconds prior to removal of aliquots. Dilutions should be completed using the techniques and methodology as described in MOP

6535a, and the 900 μ L tubes should be made with sterile PBST to stay consistent with materials/solutions. Plating in this manner should be repeated for all samples, with any changes in protocol noted in the lab notebook.

10. Once the dilution plating has been completed, the plates are to be placed in an incubator. For non-thermophilic *Bacillus* species, the plates should be placed at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 12-24 hours. For thermophilic *Bacillus* species, such as *Geobacillus stearothermophilis*, the plates should be incubated at $55^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 12-24 hours. The target *Bacillus* organism that will be used for the wipe samples will be specific to the project and noted in the QAPP.
11. After the plates have incubated for a sufficient amount of time (12-24 hours) and the growth from any *Bacillus* colonies is quantifiable, the colonies should be counted manually using the light box and the data should be properly recorded as dictated per project by the QAPP. All results will be checked for quality assurance and all data will be reported to the proper personnel as listed in the QAPP.

MOP NO. 6570

TITLE: Use of STERIS Amsco Century SV 120 Scientific Prevacuum Sterilizer

SCOPE: Basic instructions for use of the large STERIS autoclave

PURPOSE: To outline proper procedural use of the autoclave, using preprogrammed cycles, to effectively sterilize items, while complying with quality control standards.

Materials:

- Amsco Century SV 120 Scientific Prevacuum Sterilizer
- Items to be sterilized (liquids, solids, waste, etc)
- Pouches to contain materials during sterilization and maintain sterility until use
- Aluminum foil
- Autoclave indicator tape
- Sterilization verification ampoules (such as Raven ProSpore Ampoules)
- Thermally resistant gloves
- Deionized (DI) water

Procedure:

Start up:

1. Turn on the autoclave. The power switch is located behind the door in the top right corner. The digital touch screen on the front of the unit will power up and indicate that a memory test is in progress.
2. After the memory test is complete, the device will request that it be flushed. This should be conducted daily to minimize scaling inside the boiler. The flush valve is located behind the door on the bottom, left of the device (yellow handle). Move the valve to the open position and then press the "Start Timer" button on the touch screen. The flush will run for 5 minutes and will alert at completion with a single chime.
3. Once the flush is complete, close the flush valve and press the "Continue" button on the touch screen. The screen should then return to its default menu which has 2 choices "Cycle Menu" and "Options"

Basic Operation:

1. Prepare any items that need to be sterilized. The items must be carefully wrapped or sealed in sterilization pouches in order to maintain sterility when removed from the autoclave. Examples of

this include: wrapping any orifices with aluminum foil, placing whole items in autoclave pouches, loosely applying a cap on a bottle (to allow for the pressure changes inside).

2. Once prepared, each item should be outfitted with a sterility indicator such as a small piece of autoclave indicator tape; or by utilizing an autoclave pouch with a built-in sterility indicator strip. These indicators provide a visual verification that the sterilizing temperature (121 °C) was reached.
3. To add items to the autoclave, open the autoclave door by pressing down on the foot pedal on the bottom right corner on the front of the device.
4. Place items that need to be sterilized into the autoclave, adding or moving racks to accommodate the load. If liquids are being autoclaved, they must have secondary containment (usually a large plastic autoclave-safe tray) to contain any fluids in the event of a leak, spill or boil-over. Add an indicator ampoule to the first autoclave cycle of the day, regardless of the type of cycle.
5. Once the autoclave is loaded, press the foot pedal to close the autoclave door.
6. Once the door is sealed, a menu of the cycles can be seen by pressing the button on the touch screen labeled "Cycle Menu". Then choose the appropriate cycle by touching the corresponding button. If the cycle chosen is the one desired for the sterilization process, press the "Start Cycle" button. Otherwise, press "Back" to return to the prior menu screen.
7. After the cycle has started, the type of cycle, the number of the cycle, the items placed in the autoclave during the cycle, the time, whether or not an indicator ampoule was included in the load, and the initials of the person starting the cycle must be recorded in the autoclave log book, located in the drawer across from the unit labeled "Autoclave Supplies."
8. Quality control (QC) indicator ampoules, usually Raven ProSpore Ampoules with *Geobacillus stearothermophilus* (at a concentration of 10E6), are added to one cycle each day to ensure that the autoclave is functioning properly. These ampoules are used according to manufacturer's instructions. These ampoules must be properly labeled with the date when they were autoclaved and the initials of the individual who completed the cycle. At the beginning of each week, a positive control ampoule must be processed, where the ampoule is placed directly into the 55 °C water bath without being autoclaved. The positive control indicator ampoule should change from purple to yellow in color, indicating growth. All test ampoules should be placed in a water bath following the end of the cycle in which they are run. These ampoules should not change color (from purple to yellow but instead should remain a purple to purple-brown color). Ampoules should be checked at both 24 and 48 hour intervals for growth and then finally recorded and disposed of after 48 hours. All QC information concerning ampoules should be recorded in the autoclave notebook.
9. Upon completion of any cycle, the autoclave will alarm with a repeating beep for approximately one minute. Any time after this alarm starts, it is safe to open the main door (take caution because the steam escaping the chamber will be very hot when the door is opened). The contents from the autoclave will be very hot; use protection to remove items from the autoclave (thermally resistant gloves).

10. Place the contents of the autoclave in an appropriate place to cool, and close the autoclave door using the foot pedal.

Cycles:

Gravity Cycles:

Gravity cycles are used to sterilize glassware and other utensils, which are not submerged in nor contain any volume of liquid. These cycles are typically used for “dry” materials.

Currently there are two different gravity cycles programmed for daily operations: a 1-hour cycle and a 30-minute cycle. The time that the chamber is held at the sterilization temperature (121 °C) is the only difference between these two cycles. The different sterilization times allow for the compensation of the various sizes of materials and more resilient organisms. The 30-minute cycle is primarily used for a small quantity of material. The 1 hour cycle is used for large loads or items containing a large amount of contamination. The 1 hour cycle is recommended for inactivation of Gram-positive spore-forming bacteria.

Liquid Cycles:

Liquid cycles are used to sterilize a variety of liquids and solutions. The solutions are typically mixed prior to sterilization. It is important to have secondary containment to contain any fluids in the event of a leak, spill or boil-over. The 30-minute liquid cycle is used to sterilize small volumes of liquid (usually less than 2 L total). When attempting to sterilize any volume larger than 2 L, the 1-hour liquid cycle should be used to ensure complete sterilization. The 1-hour liquid cycle is the preferential cycle used as the destruction cycle for waste. In the event of materials (liquid or otherwise) being contaminated/exposed to microorganisms, the 1-hour liquid cycle will be used as the initial means of decontamination. When completing a decontamination cycle, if there is no liquid inside a container, then deionized water must be added to the container or the item must be submerge prior to the start of the cycle. Only items that are being decontaminated can go in destruction cycles. Decontamination cycles cannot be mixed with sterilization cycles.

MOP 6571

Title: RECOVERY OF *BACILLUS* SPORES FROM VIA-CELL® AEROSOL SAMPLING CASSETTES

Scope: This MOP outlines the procedure for recovering *Bacillus* spores from Via-Cell® aerosol sampling cassettes

Purpose: To aseptically extract and quantify *Bacillus* spores from Via-Cell® samples in order to determine viability and obtain quantifiable data

1.0 MATERIALS

- Via-Cell® Bioaerosol Sampling Cassettes (Zefon International, Ocala, FL, Part# VIA010)
- PPE (gloves, lab coat, safety goggles)
- Biological Safety Cabinet (Class II)
- pH-Amended bleach
- Deionized water
- 70% solution of denatured ethanol
- Kimwipes
- Dispatch bleach wipes
- Non-regulated waste container
- 50 mL sterile conical tubes containing appropriate volume of buffer
- Vortex mixer
- Cart
- Wire or foam rack for 50 mL conical tubes
- Sterile blade
- Sterile, disposable forceps
- Tryptic soy agar plates
- 900 µL tubes of sterile PBST
- Pipettor and pipette tips for dilutions
- Incubator set to appropriate growth temperature for target organism (35 °C or 55 °C)
- Light box for counting colonies
- Lab notebook
- QAPP for project that is utilizing the wipe samples

2.0 PROCEDURE

1. Begin by donning fresh PPE (gloves, lab coat, and protective eyewear).
2. Obtain Via-Cell[®] samples that may contain *Bacillus* spores. Via-Cell[®] samples should be received as one Via-Cell[®] cassette delivered in secondary containment. Make certain that all of the samples are labeled. Review any chain of custody forms that may accompany the samples to ensure that all of the labels are consistent and that there is no notable variation in the samples. If variation has occurred, make a note of it in the notebook.
3. Clean the workspace (biological safety cabinet) by wiping surfaces with pH-amended bleach, next with deionized water, and lastly with a 70-90 % solution of denatured ethanol. Wipe with a kimwipe to remove any excess liquid. Make sure the workspace is clean and free of debris. Gather all necessary items to perform the task, place these items on a clean cart beside the biological safety cabinet, within arm's reach so that, once the procedure has begun, the task may be performed without interruptions.
4. Discard gloves and replace with fresh pair.
5. One at a time, under the biological safety cabinet, remove the sample cassette. Using a sterile blade, cut through the tape around the outside of the cassette. Twist apart the cassette and discard of the top portion not containing the sample slide (portion of the cassette where the sample is collected). Using sterile, disposable forceps, remove the slide and place into the appropriate amount of buffer solution. Repeat this procedure for every sample.
6. Using the procedure to clean the biological safety cabinet, as found in Step 3, clean the biological safety cabinet again. Afterwards don a fresh pair of gloves.
7. Using a vortex mixer, agitate the Via-Cell[®] samples, four at a time, in a biological safety cabinet, for ten second bursts for two minutes total. Make certain to clean the biological safety cabinet after each set of four samples and change gloves between each set of samples.

Note: The reason that four samples are done at one time is to limit the time between agitation and plating. The samples need to be processed immediately after agitation, and agitation of more than four samples at a time results in excessive lag-time between agitation and plating.

8. Using tryptic soy agar (or other appropriate growth media) media plates that are appropriately labeled with the sample number, dilution set and date; conduct dilution plating for the Via-Cell[®] samples immediately after the two minute agitation step is completed. The samples should also be agitated again for ten seconds directly prior to removing an aliquot from the sample tube. Each dilution tube should also be agitated for ten seconds prior to removal of aliquots. Dilutions should be completed using the techniques and methodology as described in MOP 6535a, and the 900 μ L tubes should be made with the appropriate buffer to stay consistent with materials/solutions. Plating in this manner should be repeated for all samples, with any changes in protocol noted in the lab notebook.
9. Once the dilution plating has been completed, the plates are to be placed in an incubator. For non-thermophilic *Bacillus* species, the plates should be placed at $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 18-24 hours. For

thermophilic *Bacillus* species, such as *Geobacillus stearothermophilis*, the plates should be incubated at $55\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 18-24 hours. The target *Bacillus* organism that will be used for the wipe samples will be specific to the project and noted in the QAPP.

10. After the plates have incubated for a sufficient amount of time (18-24 hours) and the growth from any *Bacillus* colonies is quantifiable, the colonies should be manually counted using the light box and the data should be properly recorded as dictated per project by the QAPP. All results will be checked for quality assurance and all data will be reported to the proper personnel as outlined in the QAPP.

MOP 6572

Title: **RECOVERY OF SPORES FROM VACUUM SOCK SAMPLES**

Scope: This MOP outlines the procedure for recovering spores from vacuum sock samples

Purpose: To aseptically extract and quantify spores from vacuum sock samples in order to determine viability and obtain quantifiable data

1.0 MATERIALS

- PPE (gloves, lab coat, safety goggles)
- Biological Safety Cabinet (Class II)
- pH-Amended bleach
- Deionized water
- 70% solution of denatured ethanol
- Kimwipes
- Dispatch bleach wipes
- Non-regulated waste container
- 3 oz. sterile specimen cup containing 20 mL of sterile phosphate buffered saline with Tween[®] 20 solution (PBST) (MOP 6562)
- Sterile scissors
- Vortex mixer
- Cart
- Tryptic soy agar plates
- 900 µL tubes of sterile PBST
- Pipettor and pipette tips for dilutions
- Incubator set to appropriate growth temperature for target organism (35 °C or 55 °C)
- Light box for counting colonies
- Lab notebook
- QAPP for project that is utilizing the vacuum sock samples

2.0 PROCEDURE

1. Begin by donning PPE (gloves, lab coat, and protective eyewear).

2. Obtain vacuum sock samples that may contain *Bacillus* spores. Vacuum sock samples should be received as one vacuum sock in a sterile 5.5' x 9 bag secondarily contained in a 10' x 15' bag. Make certain that all of the samples are labeled. Review any chain of custody forms that may accompany the samples to ensure that all of the labels are consistent and that there is no notable variation in the samples. If variation has occurred, make a note of it in the notebook.
Clean the workspace (biological safety cabinet) by wiping surfaces with pH-amended bleach, next with deionized water, and lastly with a 70-90% solution of denatured ethanol. Wipe with a kimwipe to remove any excess liquid. Make sure the workspace is clean and free of debris. Gather all necessary items to perform the task, place these items on a clean cart beside the biological safety cabinet, within arm's reach so that once the procedure has begun the task may be performed without interruptions.
3. Discard gloves and replace with fresh pair.
4. Label a 3 oz. specimen cup to match the vacuum sock sample ID. The specimen cup contains 20 mL of sterile PBST.
5. When extracting samples, handle one sample at a time from start to finish. Begin by removing the inner bag from the outer bag. Discard the outer bag in the non-regulated waste container. Place the inner bag containing the vacuum sock under the hood. Loosen the cap on the 3 oz. specimen cup and open a pack of sterile scissors. Open the bag and remove the sock, careful not to touch the white part. Roll the non-sterile blue portion of the vacuum sock onto the smaller cardboard ring. Dispose of the larger cardboard ring. Wet the vacuum sock by holding the upper blue portion of the vacuum sock (around the smaller cardboard ring) and dipping the lower 1-inch of the vacuum sock into the PBST. The vacuum sock will be allowed to absorb the PBST for a few seconds. After wetting, the vacuum sock will be lifted up just above the opening of the specimen bottle, and a 1-inch vertical slit will be cut up the center from the bottom of the sock using sterile scissors (a new pair of scissors should be used for each sample). The vacuum sock is then cut horizontally from side to side about 1 inch from the bottom, allowing the two pieces to fall into the specimen bottle. The vacuum sock should be cut only where the sock has been wetted. Repeat the dip/cutting procedure until the entire collection portion of the sock has been excised. The upper top blue portion of the vacuum sock will then be discarded. Place used scissors in a discard pan. After samples are all extracted, scissors will immediately be autoclaved using a one hour gravity destruction cycle in preparation for use with the next sample batch. Remove gloves and don a fresh pair of gloves. Repeat the extraction procedure for every sample, while maintaining aseptic technique.
6. After cutting all vacuum sock samples, all specimen cups (up to sixty samples at a time) should be loaded into the sample cup holder of the orbital shaker-incubator. The samples are then agitated in the shaker incubator at 300 rpm for 30 minutes at room temperature. The samples are then removed from the shaker incubator and brought to the Biological Safety Cabinet for dilution plating.
7. Using the procedure to clean the biological safety cabinet, as found in Step 3, clean the biological safety cabinet again. Afterwards don a fresh pair of gloves.
8. Using tryptic soy agar media plates that are appropriately labeled with the sample number, dilution set and date, complete dilution plating for the vacuum sock samples immediately after the thirty minute agitation step is completed. The samples should also be agitated again for ten seconds directly prior to removing an aliquot from the specimen cup. Each specimen cup should also be agitated for ten

seconds prior to removal of aliquots. Dilution-plating should be carried out according to MOP 6535a. Dilution tubes used in MOP 6535a should contain PBST to stay consistent with materials/solutions. Repeat procedure for all samples.

9. Once the dilution plating has been completed, the plates should be incubated. For non-thermophilic *Bacillus* species, the plates should be placed at $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ for 18-24 hours. For thermophilic *Bacillus* species, such as *Geobacillus stearothermophilis*, the plates should be incubated at $55\text{ }^{\circ}\text{C}$

Appendix C: Stock Chemicals for Preparation of AHP Solution

- 1) Hydrogen peroxide (50%): Fisher Scientific, CAS: 7722-84-1
- 2) Triacetin, ACROS Organics, , CAS 102-76-1
- 3) Ethanol (99.5%), ACROS Organics, CAS 64-17-5
- 4) Potassium Carbonate, Sigma Aldrich, CAS 584-08-7
- 5) MAQUAT[®] MC1412-80%E, Mason Chemical Company, no CAS available

Appendix D

Appendix D: Coupon, Test Chamber and Equipment Cleaning and Sterilization Procedures

The pH-adjusted bleach solution to be used for cleaning surfaces in equipment in both the decontamination and microbiology laboratories is prepared as a 1:10 dilution of bleach in DI water, pH-adjusted to ~6.8 using glacial acetic acid.

The following steps are followed for cleaning the decontamination chamber between each material type and before/after each test:

1. Using the back sprayer, the interior surfaces are kept wet with solution for 10 minutes.
2. With the drain open, the surfaces will then be rinsed with DI water. The runoff is collected in a carboy and ultimately discarded.
3. After ensuring all runoff is removed from the chamber, the valve is closed in preparation for the next test.
4. A mop assembly with a disposable pad is used to wipe down the interior of the chamber with isopropyl alcohol or ethanol.
5. The pad is then removed and placed in a bucket of pH-adjusted bleach solution for decontamination prior to disposal.

The following steps are followed for cleaning the buckets after use in a test:

1. Fill the buckets with pH-adjusted bleach and leave them covered for at least 60 minutes.
2. Rinse all buckets five times with DI water.
3. Air dry prior to re-use.

The following steps are followed for cleaning the work surfaces before and after use:

1. Wet all surfaces with pH-adjusted bleach solution or using Dispatch[®] bleach wipes.
2. Rinse with DI water.
3. Wet and wipe surfaces with isopropyl alcohol or ethanol.
4. Air dry prior to re-use.
5. Alternatively, cover paper can be used and replaced before/after each use.

The sampling templates are autoclaved before/after each use.

The following steps are followed for cleaning the coupon cabinets before and after use:

1. Wet and wipe all surfaces with pH-adjusted bleach solution or using Dispatch[®] bleach wipes.
2. Rinse with DI water.
3. Wet and wipe surfaces with isopropyl alcohol or ethanol.
4. Air dry prior to re-use.

Appendix D

The gaskets used in MOP 6561 during the contamination procedure will be cleaned via fumigation with the STERIS VHP® sterilization cycle. This cycle entails the use of a STERIS VHP® ARD hydrogen peroxide (H₂O₂) generator at 250 parts per million by volume (ppmv) for 4 hours by maintaining this constant concentration in a decontamination chamber.

The carboys will be autoclaved according to MOP 6570.

Cement/concrete, stainless steel, and glass coupons will be autoclaved according to MOP 6570. Other types of coupons will be cleaned via fumigation with the STERIS VHP® sterilization cycle. This cycle entails the use of a STERIS VHP® ARD hydrogen peroxide (H₂O₂) generator at 250 ppmv for 4 hours by maintaining this constant concentration in a decontamination chamber.

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