Designation: E2894 – 13

Standard Test Method for Applying Aerosolized *Bacillus* Spores as Dry Inocula to Inanimate Surfaces[1]

This standard is issued under the fixed designation E2894; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, poses a formidable threat as an infectious bioagent. A combination of significant environmental stability, ease of production,[2] and high mortality through the inhalational route[3] makes *B. anthracis* an ideal bioagent. Aerosolized powders are expected to be the primary mechanism for disseminating *B. anthracis* spores, likely delivered as an aerosol cloud from either a line-source, for example, low-flying aircraft, or point-source, for example, spray device. The characteristics of the aerosol (particle size and composition) will influence its behavior, thereby affecting its potency as a threat agent. This test method is designed to perform surface deposition of aerosolized spores with the primary objective of evaluating the utility of self-sanitizing antimicrobial materials (SSAMs). However, it could be used for other types of research, for example, decontamination, reaerosolization, fate and transport, contamination avoidance, collective protection, and individual protection, as well. We are aware of other consensus standards for applying spores to surfaces (Test Method E2149, AATCC Test Method 100, Test Method E2180), but these methods are based on liquid inocula. SSAMs are more active in the presence of moisture,[4] and thus standard test methods using water-based inocula may overestimate their effectiveness. Protocols for the preparation, aerosolization, extraction, viable enumeration, and calculation of spores for testing and determining the effectiveness of SSAMs are described.

1. Scope

1.1 This test method is designed to uniformly apply fluidized spores to surfaces as an aerosol under defined conditions.

1.2 This test method is specific to *B. anthracis* Delta (Δ)Sterne (BAΔS), but could be adapted for work with other types of *Bacillus* species.

1.3 This test method is suitable for working with any type of environmental surface.

1.4 This test method should be performed only by those trained in aerobiology, microbiology, or a combination thereof.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[5]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2149 Test Method for Determining the Antimicrobial Activity of Antimicrobial Agents Under Dynamic Contact Conditions

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] Woods, J. B., Darling, R. G., Dembek, Z. F., Carr, B. K., Cieslak, T. J., and Lawler, J. V., *et al.*,*USAMRIID's Medical Management of Biological Casualties Handbook*, 6th edition, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, 2005.

[3] LeClaire, R. D., and Pitt, M. L. M., "Biological Weapons Defense: Effect Levels," *Biological Weapons Defense: Infectious Diseases and Counterterrorism*, Humana Press Inc., Totowa, NJ, 2005, pp.41–61.

[4] Prugh, A., and Calomiris, J. J., "Inactivation of *Bacillus anthracis* Spores Delivered as Liquid Suspension or Aerosol to Self-Decontaminating Fabric," Defense Technical Information Center (DTIC), AFRL-HE-WP-TP-2006-0060, 2006.

[5] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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E2180 Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *AATCC Standards:*[6]

AATCC Test Method 100 Antimicrobial Finishes on Textile Materials: Assessment of

3. Terminology

3.1 *Definitions*—For definitions of general terms used in this test method, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *aerosol, n*—a suspension of solid or liquid particles in a gaseous medium.

3.2.2 *anthrax, n*—an infectious disease of warm-blooded animals caused by the gram-positive, endospore-forming bacterium, *Bacillus anthracis*.

3.2.3 *biological aerosol, n*—a suspension of particles containing biological agents which have been dispersed in a gaseous medium.

3.2.4 *carrier, n*—substrate onto which aerosolized spores are deposited.

3.2.5 *count median diameter (CMD), n*—the calculated diameter in a population of particles in a gas or liquid phase above which there are as many particles with larger diameters as there are particles below it with smaller diameters.

3.2.6 *fluidizing agent, n*—an additive used to reduce agglomeration of particles.

3.2.7 *line source, n*—a source of air, noise, water contamination, or electromagnetic radiation that emanates from a linear geometry.

3.2.8 *lyophilize, v*—to freeze-dry.

3.2.9 *point source, n*—a source of air, noise, water contamination, or electromagnetic radiation that emanates from a single point.

3.2.10 *self-sanitizing antimicrobial material (SSAM), n*—a material containing an antimicrobial component that collectively acts as a microbicide.

4. Summary of Test Method

4.1 The test method describes the steps required to deposit spores onto surfaces and quantitatively assess loading distribution and recovery.

4.2 The test method describes the protocols for evaluating the antimicrobial activity of SSAMs.

4.2.1 Using an aerosol device capable of meeting the data quality objectives set forth in this test method, BAΔS spores are aerosolized and allowed to deposit onto surfaces.

4.2.2 Spores are recovered from the test and control carriers, and viability is assessed by dilution plating (CFU/mL).

4.2.3 Loading efficiency is determined as colony-forming units (CFU) per cm^2 obtained from the viability assay.

4.2.4 Loading variability is determined by calculating the coefficient of variation (CV) of the mean loading density from replicate samples.

4.2.5 Antimicrobial efficiency is calculated as the $\log_{10}$ reduction of viable spores between the test and control groups, and its significance is determined by a statistical comparison of the two populations.

5. Significance and Use

5.1 This test method provides defined procedures for creating fluidized spore aerosols with particular emphasis on particle size distribution and spore preparation.

5.2 The efficacy of disinfection technologies can be evaluated on finished items, as well as those under development.

5.3 This test method defines procedures for the validation of the aerosol generator, preparation of the test specimen, application of the aerosolized spores, enumeration of viable spores, assessing data quality, and determining effectiveness of SSAMs.

5.4 Safety concerns associated with aerosolizing microbial agents are not addressed as part of this test method. Individual users should consult their safety authority, and a detailed biological aerosol safety plan and risk assessment must be established prior to using this method. Users are strongly urged to consult *Biosafety in Microbiological and Biomedical Laboratories*.[7]

6. Apparatus

6.1 *Biological aerosol generator*—used to load microorganisms onto a substrate (see Annex A1 for detailed information).

6.2 *Autoclave*—capable of maintaining 121 to 123°C and 15 to 17 psig.

6.3 *Shaking Incubator*—capable of maintaining 36 ± 1°C and 300 r/min.

6.4 *Incubator*—capable of maintaining 36 ± 1°C.

6.5 *Phase Contrast Microscope*—capable of 400× and 1000× magnification.

6.6 *Centrifuge*—capable of maintaining 3000 × *g*.

6.7 *Water Bath*—capable of achieving 65°C.

6.8 *Single-tube Vortex Mixer.*

6.9 *Multi-tube Vortex Mixer.*

6.10 *Sonicating Water Bath*—50 to 60 Hz.

6.11 *Analytical Balance*—capable of weighing 0.001 g.

6.12 *Refrigerator*—capable of maintaining 2 to 8°C.

6.13 *Stopwatch or Electronic Timer.*

6.14 *Pipettor*—with a precision of 0.1 mL.

[6] Available from American Association of Textile Chemists and Colorists (AATCC), PO Box 12215, Research Triangle Park, NC 27709-2215. http://www.aatcc.org.

[7] CDC-NIH, *Biosafety in Microbiological and Biomedical Laboratories*, 5th Edition, U.S. Department of Health and Human Services, Washington, D.C., December 2009.

7. Reagents and Materials

7.1 *Reagents:*

7.1.1 *Bacillus anthracis*—ΔSterne strain spores, prepared according to procedures outlined in Annex A2.

7.1.2 *Trypticase Soy Agar (TSA).*

7.1.3 *Trypticase Soy Broth (TSB).*

7.1.4 *Sporulation Broth*—2.5 % nutrient broth, 200 mM glutamate, and salts, as defined in Table 1, pH 7 (see Annex A2.2.2 for preparation instructions).[8]

7.1.5 *Extraction Buffer*—pH 7, as defined in Table 2.

7.1.6 *Hydrophobic Fumed Silica*—Aerosil (Trademarked) R 812 S[9]

7.1.7 *Nutrient Agar.*

7.1.8 *Phosphate-Buffered Saline (PBS).*

7.1.9 *Glycine.*

7.1.10 *Polysorbate 20.*

7.1.11 *Hydrochloric Acid (HCl)*—2.5N.

7.1.12 *Sodium Hydroxide (NaOH).*

7.1.13 *Deionized (DI) Water.*

7.1.14 *Ethanol*—laboratory grade.

7.1.15 *Sodium Hypochlorite*—10 %.

7.1.16 *Nitrogen Gas (N_2)*—dry.

7.2 *Materials:*

7.2.1 *Sterile Inoculating Loops.*

7.2.2 *Baffled Flasks*—1000 mL.

7.2.3 *Erlenmeyer Flasks*—250 mL.

7.2.4 *Glass Microscope Slides.*

7.2.5 *Petri Dishes.*

7.2.6 *Test Tube Racks.*

7.2.7 *Pipettes*—1, 5, 10, and 25 mL.

7.2.8 *Micropipettes*—capable of delivering 0.1 and 1 mL accurately and consistently.

7.2.9 *Sterile Centrifuge Tubes*—15 and 50 mL.

7.2.10 *Sterile Round-bottom Tubes*—15 mL.

7.2.11 *Borosilicate Solid Glass Beads*—3-mm diameter, sterile.

7.2.12 *Parafilm.*

7.2.13 *L-shaped Sterile Spreaders.*

7.2.14 *Test Specimens.*

[8] Buhr TL, McPherson DC, et al. Analysis of broth-cultured Bacillus atrophaeus and Bacillus cereus spores. *Journal of Applied Microbiology* 2008; 105:1604-1613.

[9] The sole source of supply of the apparatus known to the committee at this time is Evonik Industries, Hanau-Wolfgang, Germany. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

TABLE 1 Sporulation Broth (pH 7)

Reagent	Final Concentration
Nutrient broth	2.5%
Glutamate	200 mM
KH_2PO_4	13 mM
K_2HPO_4	28 mM
$CaCl_2 \cdot 2H_20$	1 mM
$MnCl_2 \cdot 4H_20$	0.1 mM
$MgCl_2 \cdot 6H_20$	1 mM
$ZnCl_2$	0.05 mM
$FeCl_3 \cdot 6H_20$	0.001 mM

TABLE 2 Extraction Buffer (pH 7)

Reagent	Defined Amount
Glycine	18.67 g
NaCl	8.5 g
KH_2PO_4	0.18 g
K_2HPO_4	1.5 g
Polysorbate 20	0.5 mL
Distilled water	1000 mL
Neutralizing agent, if necessary[A]	–

[A] Reference Test Method E1054 for determining neutralization efficiency.

8. Sampling, Test Specimens, and Test Units

8.1 Cut test specimens from finished products or those under development.

NOTE 1—The size dimensions of the test specimens can vary, but will need to fit into a 50-mL centrifuge tube for extraction.

8.2 The coupon size used to validate the method was a 2.6-cm diameter circle and is recommended for this method.

8.3 For SSAMs, place the specimens in the aerosol chamber with the reactive side facing upward.

8.4 For non-reactive materials (if used), the sample side facing upward needs to be consistent throughout the sample group.

9. Experimental Design

9.1 *Non-reactive Materials*—A minimum of three independent samples per specimen is required for statistical analysis of the data.

9.2 *SSAMs*—Generally, most SSAMs require incubation under specific environmental conditions (that is, temperature, relative humidity, time) for their antimicrobial activity to be effective. Following aerosol deposition, the samples will be incubated under the specific conditions as specified for a particular SSAM. This period is referred to as the incubation period. Aerosol deposition requires a significant period of time for the spores to settle onto surfaces and is exclusive from the incubation period. For this purpose, two sample sets will be included in the test plan—a conditioned (test) group and an unconditioned (control) group. A traditional control that does not include the antimicrobial treatment is not needed for this method. The unconditioned control samples will be incubated for the same duration of time as the conditioned test samples, but will be held at room temperature and < 50 % relative humidity, rather than at the test group's conditions, as identified by the user. Immediately following the incubation period, both the control and test samples will be extracted and enumerated. The log reduction of viable spores between the two groups will be the measurement used to determine antimicrobial efficacy.

9.2.1 *Neutralizer Effectiveness Test*—Determine the appropriate conditions for neutralizing the antimicrobial agent on the SSAM using Test Method E1054.

9.3 *Data Analysis*—Coefficient of variation (CV) analysis will determine if the loading distribution is uniform. For SSAMs, a student's *t*-test between the mean densities for the test and control populations will determine if the log reduction of spore viability is statistically significant.

10. Test Procedure

10.1 *Apparatus Operation*—Appendix X1 describes a device for aerosolizing lyophilized reagents and outlines its operation.

10.2 *Loading Samples with Spores*—The desired loading should produce no less than 10^5 CFU/cm^2 for the control samples. Appendix X1 describes the specific test conditions to obtain the required level of loading. In general, loading is carried out by first combining lyophilized spores (80 % by weight) and hydrophobic fumed silica (20 % by weight) with 3-µm diameter glass beads in a 15-mL sterile round-bottom tube, and subsequently grinding the mixture by using a vortex mixer for 2 min. The glass beads are discarded and the spore/silica mixture is dispensed onto the latex diaphragm of the fluidized bed aerosol generator. The spores are then aerosolized into the chamber and allowed to settle for ≥ 16 h.

10.2.1 *SSAMs*—Following the settling period, the SSAMs are aseptically removed from the chamber. The test samples are exposed to specific conditions (that is, humidity, temperature) for a specified time. The incubation conditions and time will be dependent on the specific SSAM, or application, or both, and will not be specified as part of this method. The control samples are incubated for the same length of time as the test samples, but are held at room temperature and < 50 % relative humidity.

10.3 *Spore Extraction*—Transfer each coupon to a 50-mL sterile centrifuge tube containing 10 mL of extraction buffer. Extract the samples for 5 min using a multi-tube vortex mixer set on high, followed by a 1-min sonication treatment. Prior testing must ensure the extraction buffer contains an effective neutralization agent using Test Method E1054. If necessary, the extraction buffer components should be supplemented with an additional neutralizer.

10.4 Determine the density of viable spores by performing dilution plating using TSA. Plates should be incubated for 16 to 24 h at 37 ± 2°C prior to enumeration.

11. Calculation or Interpretation of Results

11.1 *Loading Density (CFU/cm^2)*—Determine the loading density of viable spores recovered from each control and test sample. Verify that the surface loading on the control samples meets the specified requirements.

For determining surface loading (L) in CFU/cm^2:

$$L = \left(C \times \frac{1}{D_1} \times \frac{1}{D_2} \times V\right)/A \quad (1)$$

where:

C = colony count of a sample derived from viable plating,
D_1 = plating dilution (0.1 if 100 µl was plated),
D_2 = sample dilution (0.1 if the 10^{-1} dilution was plated),
V = volume of extraction buffer, and
A = surface area of samples (cm^2).

11.2 *Loading Variability*—Calculate the standard deviation and coefficient of variation (CV) of the loading densities for the control and test populations.

For determining standard deviation (σ):

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(L - \bar{L})^2} \quad (2)$$

where:

L = loading density of each sample,
$\bar{L}$ = mean loading density of the sample population, and
N = number of samples.

For determining coefficient of variation (CV):

$$CV = \frac{\sigma}{\bar{L}} \quad (3)$$

11.3 *Log Reduction*—Determine the antimicrobial efficacy by calculating the log reduction of spores between the test and control samples.

For determining log reduction of viable spores (ΔL):

$$\Delta L = \bar{L}_c - \bar{L}_t \quad (4)$$

where:

$\bar{L}_c$ = mean of the log values for the control samples:

$$\bar{L}_c = \frac{L_{c(1)} + L_{c(2)} + \ldots L_{c(n)}}{n} \quad (5)$$

$\bar{L}_t$ = mean of the log values for the test samples:

$$\bar{L}_t = \frac{L_{t(1)} + L_{t(2)} + \ldots L_{t(n)}}{n} \quad (6)$$

11.4 *Statistical Analysis*—An unpaired, two-tailed *t*-test at the 95 % confidence interval is performed to determine if the means of the test and control populations are significantly different. *P*-values ≤ 0.05 indicate that there is a 95 % probability that the differences in the means were not simply due to chance.

12. Report

12.1 State that the test was conducted as directed in Test Method E2894.

12.2 Sample identification, description of the material tested.

12.3 Microorganism used to conduct the testing.

12.4 Loading density of the microorganism (per gram for powders, per millilitre for liquids).

12.5 A description of the test device, including the device used to generate the aerosol.

12.6 Composition of the powder mixture used to aerosolize the microorganism.

12.7 Exposed surface area for each test specimen.

12.8 Flow rate in the aerosol system.

12.9 Composition of the buffer used to extract the spores.

12.10 Duration of the sample exposure to the aerosol.

12.11 Specifications for the SSAM incubation period.

12.12 Mean viable recoveries in CFU/cm^2 for the control and test populations.

12.13 Coefficient of variation for the control and test populations.

12.14 *P*-value comparing the control and test populations.

13. Precision and Bias

13.1 A precision and bias statement cannot be made for this test method at this time. Round-robin testing will be completed within five years following the publication date or the method will be withdrawn.

14. Keywords

14.1 aerosols; anthrax; antimicrobial; *Bacillus*; bioaerosol; chambers; deposition; extraction; infectious bioagents; spores

ANNEXES

(Mandatory Information)

A1. BIOLOGICAL AEROSOL GENERATOR

A1.1 The apparatus used to load microorganisms onto a substrate is composed of several commercially available components and can be readily assembled. The overall design of the apparatus can take various forms and can be fashioned in different dimensions while still meeting the validation requirements and data quality objectives listed below. Appendix X1 contains the description of a prototypical device that can be used to load lyophilized spores onto surfaces. However, it is the responsibility of the user of this test method to validate the performance of the device prior to use.

A1.2 Validation Requirements and Baseline Testing

A1.2.1 *Environmental Conditions*—Aerosolization procedure should be performed at room temperature, 20 to 25°C.

A1.2.2 *Sample-to-Sample Variation Objective*—The variability of spore loading for multiple samples loaded for a single test must have a CV ≤ 40 %.

A1.2.3 *Aerosolized Particle Characteristics*—The particles generated for this method will have a count median diameter (CMD) of 2.5 ± 1 µm. Standard particle measurement instruments are used to measure particle sizes. Spores will be aerosolized in a lyophilized form mixed with hydrophobic fumed silica as a fluidizing agent.

A2. SPORE PREPARATION FOR BAΔS

A2.1 Reagents

A2.1.1 *Trypticase Soy Agar (TSA).*

A2.1.2 *Trypticase Soy Broth (TSB).*

A2.1.3 *Sporulation Broth*—2.5 % nutrient broth, 200 mM glutamate, and salts, as defined in Table 1, pH 7.

A2.1.4 *Deionized (DI) water*—sterile.

A2.1.5 *Polysorbate 20.*

A2.2 Methods

A2.2.1 *Growth of BAΔS*—Remove and thaw a cryovial of spores stored at –80°C. Streak a loopful of the suspension onto a TSA plate and incubate overnight at 37°C. After incubation, collect a colony with a sterile loop and swirl it into 50 mL of TSB in a 250-mL flask. Incubate for 2.5 h at 37°C and 220 r/min to achieve a mid-log growth-phase culture.

A2.2.2 *Sporulation of BAΔS*—Autoclave 500 mL of 5% nutrient broth to sterilize. After autoclaving, add sterile-filtered components (glutamate and trace salts) to achieve the final concentrations (listed in Table 1) in 1 L of sporulation broth diluted to final volume with sterile, deionized water. Inoculate a loopful of mid-log growth phase culture into 100 mL of sporulation broth in a 1000-mL baffled flask. Place the flask on a 37°C shaking incubator and incubate at 300 r/min. After Day 3, observe the spores daily under a phase-contrast microscope until the culture demonstrates >80 % phase-bright spores.

A2.2.3 *Spore Harvesting and Purification*—Divide the spore solution into (2) 50-mL centrifuge tubes and centrifuge for 10 min at 3000 × *g* at room temperature. Discard the supernatant, resuspend the spore pellet in 20 mL refrigerated (4°C) sterile DI water, and centrifuge for 10 min at 3000 × *g* at room temperature. Discard, resuspend, and centrifuge in similar fashion twice more. After the third centrifuge cycle, discard the supernatant, and resuspend the pellet in 10 mL of sterile DI water. Using a water bath, heat-treat the spores for 30 min at 65°C. Centrifuge the solution again for 10 min at 3000 × *g* at room temperature. Discard the supernatant and resuspend the spore pellet in 20 mL 4°C sterile DI water. Repeat centrifugation, discard, and resuspension steps until the suspension contains > 85 % phase-bright spores when viewed under the phase-contrast microscope. Suspend the final spore

pellet in 10 mL sterile 0.05 % Polysorbate 20 solution. Sonicate the spore suspension for 1 min in a sonicating water bath. Vortex the sample for 30 s. Prepare microscope slides of the spore suspension and allow spores to settle so they will be in the same visual field. Take photos of the four quadrants and perform a manual count under phase-contrast microscopy to determine the ratio of phase-bright spores to phase-dark spores, vegetative cells, and debris.

A2.2.4 *Lyophilization of the Spores*—Centrifuge the spore stock suspensions at 3000 × *g* for 10 min. Discard the supernatant and resuspend the spore pellet in an equal volume of sterile DI water as the discarded supernatant. Lyophilize the spores according to the specifications provided by the manufacturer of the lyophilizing device. Aliquot the spores into 15-mL conical tubes and store at –80°C. Perform a viability assay on the lyophilized spore powder to determine the density (CFU/g). The spore concentration must be >-10^{10} viable spores per gram for suitable use.

A2.2.5 Final spore preparation shall be ≥ 80 % pure by phase-contrast microscopy with a concentration that exceeds 10^{10} CFU/gram of lyophilized spore powder.

APPENDIX

(Nonmandatory Information)

X1. EXAMPLE DEVICE: THE BIOLOGICAL DISPERSING SYSTEM (BDS)

X1.1 Diagram of the Biological Dispersing System (BDS)

X1.1.1 See Fig. X1.1.

X1.2 System Parameters

X1.2.1 The BDS consists of a glass-walled chamber, a fluidized bed aerosol generator,[10] an aerodynamic particle sizer (APS), and a computer (not shown) for data acquisition and control. The chamber consists of a 0.6-m^2 base by 1.2-m high enclosure with four primarily glass walls. Two doors are included for easy access. The left door opens the entire side, while the right door provides access to the lower portion of the chamber. A turntable for holding test samples is provided on the interior floor. The turntable has a variable speed control, providing rotation speeds up to 7 r/min. Rotation of the turntable is not used during spore loading, but is required for sample placement and retrieval. The top and bottom of the chamber have particle distribution manifolds that provide eight inlet and discharge ports for even distribution of the particles.

[10] The sole source of supply of the apparatus known to the committee at this time is PITT-3, AlburtyLab Inc, Drexel, MO. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

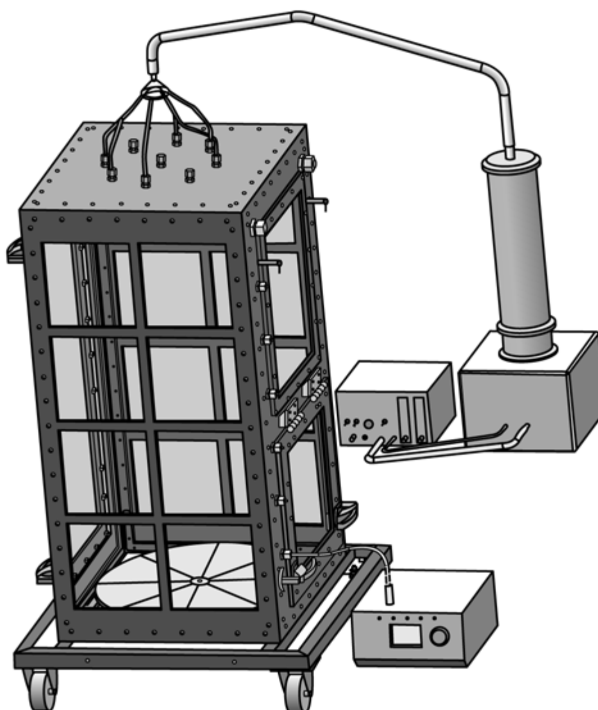

FIG. X1.1 Diagram of the Biological Dispersing System (BDS)

The PITT-3 delivers a uniform distribution of particle sizes. Particles are supported on a latex diaphragm and are aerosolized using an audio speaker mounted below the diaphragm. The speaker is excited using a signal generator. Adjustments in the audio signal frequency, amplitude, and wave form provide different aerosol characteristics. The aerosol generator is supplied with compressed air that lifts the suspended particles vertically into the tubing leading to the chamber distribution manifold. The system contains HEPA filters on the inlet and exit ports to ensure the air is purified prior to entering or exiting the system. The system was designed to fit into a biological safety cabinet, measuring at least 6.5 ft high by 4 ft wide by 2.5 ft deep, to allow secondary containment.

X1.3 Test Procedure

X1.3.1 In a certified biological safety cabinet (BSC), weigh out approximately 0.08 g lyophilized spore powder, 0.02 g hydrophobic fumed silica, and 15 (~0.5 g) 3-mm borosilicate glass beads into one (1) 15-mL sterile round-bottom tube, using Parafilm around the tube's cap to create an air tight seal.

NOTE X1.1—Once opened, the silica must be kept under dry nitrogen gas to avoid water absorption from air humidity.

X1.3.2 Using a single-tube vortex mixer, vortex the mixture for 2 min at high speed. At 30-s intervals, tap the tube on the counter to settle the spores to the bottom of the tube.

X1.3.3 In a certified BSC, remove the Parafilm and angle the tube so that the beads roll out of the tube. Count the beads to ensure they have all been removed. Reseal the tube with Parafilm and keep mixture in a 4°C refrigerator until aerosolization.

X1.3.4 Open the sample door of the BDS and load the test samples onto the turntable using sterile technique, rotating slowly as needed. Close the sample door when finished.

X1.3.5 Open the chamber of the PITT-3 and pour the lyophilized spore/silica mixture directly onto the center of the latex diaphragm.

NOTE X1.2—Some of the powder will remain in the tube. Refasten the chamber securely to prevent leakage.

X1.3.6 Turn on the air compressor and aerosol generator controller, adjusting the air flow to 20 L/min (~15 psi) and 80 % amplitude. Start the speaker software and set to a 100-Hz sine wave. Turn on the APS and associated software. Close all doors of the biological safety enclosure.

X1.3.7 Evaluate particle concentrations inside the chamber using the APS for 10 min. Power down the APS following the measurement.

X1.3.8 Allow the spores to settle overnight (≥ 16 h).

X1.3.9 Monitor the particle concentration in the chamber before retrieving the test samples, ensuring a background level of ≤ 5 particles/cm^3 has been reached.

X1.3.10 Open the sampling door of the BDS and aseptically remove the test samples, directly placing them into 50-mL centrifuge tubes containing 10 mL extraction buffer or sterile petri dishes.

X1.3.11 Close the sample door securely, re-plumb the air flow to bypass the PITT-3, and flush the chamber at 20 L/min for > 120 min.

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