Designation: E3178 – 18

Standard Practice for Evaluating Static and Cidal Chemical Decontaminants against *Bacillus* Spores using Centrifugal Filtration Tubes[1]

This standard is issued under the fixed designation E3178; 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.

1. Scope

1.1 This practice is used to quantify the efficacy of liquid or solid decontaminants on *Bacillus* spores dried on the surface of coupons made from porous and non-porous materials. This practice can distinguish between bactericidal and bacteriostatic chemicals within decontamination mixtures. This is important because many decontaminants contain both reactive compounds and high concentrations of bacteriostatic surfactants. All test samples are directly compared to pre-neutralized controls, un-inoculated negative growth controls, and solution controls on the same day as the test in order to increase practical confidence in the inactivation data.

1.2 This procedure should be performed only by those trained in microbiological techniques, are familiar with antimicrobial (sporicidal) agents and the application instructions of the antimicrobial products.

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

1.4 *This standard may involve hazardous materials, operations, and equipment. 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *CFR Standards:*[3,4]

Title 7 (Agriculture) CFR, Part 331 Possession, Use, and Transfer of Select Agents and Toxins

Title 9 (Animals and Animal Products) CFR, Part 121 Possession, Use, and Transfer of Select Agents and Toxins

Title 42 (Public Health) CFR, Part 73 Select Agents and Toxins

3. Terminology

3.1 For definitions of terms used in this Practice, see Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *decontaminant, n*—a physical or chemical agent or process that inactivates pathogenic or potentially pathogenic microorganisms in or on surfaces or objects.

3.2.2 *endospore, n*—a dormant, robust and non-metabolically active structure produced by certain types of bacteria from the Firmicutes phylum.

3.2.3 *exosporium, n*—the outermost structural layer of macrobacillus spores including *Bacillus anthracis*, *Bacillus thuringiensis* and *Bacillus cereus*.

3.2.4 *macrobacillus, n*—a *Bacillus* species that produces endospores that possess an exosporium including *Bacillus anthracis*, *Bacillus thuringiensis* and *Bacillus cereus*.

[1] This practice 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] 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.

[3] United States Select Agent Program administered by the Center for Disease Control (CDC) and/or the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS)https://www.selectagents.gov/

[4] https://www.access.gpo.gov

3.2.5 *microbacillus, n*—a *Bacillus* species that produces endospores that do not possess an exosporium including *Bacillus subtilis* and *Bacillus atrophaeus*.

4. Summary of Practice

4.1 For the purpose of this practice a decontaminant can be interpreted to include liquids and solids that inactivate microbes. This practice quantitatively evaluates the efficacy of decontaminants on coupons contaminated with *Bacillus* spores (pathogenic and non-pathogenic strains). Spores are dried on coupon surfaces. The spore-inoculated coupons are then transferred to Amicon[5] Ultra-15 Centrifugal Filter Units with Ultracel-100 membranes (filter units) contained in sterile 50-mL conical tubes (**1, 2**).[6]

4.2 Coupon material is selected according to the claims or intended use of the decontaminant. Coupons may be made of any material - hard, flexible, porous, non-porous, metallic, or non-metallic. Flat (2 cm × 2 cm) coupons are preferred; however, non-flat coupons and smaller coupons can be tested using this practice. Coupons that have been tested are 0.1-0.3 cm thick. Check that the coupons are thin enough to fit within the filter units mentioned in 4.1.

4.3 Filter units contained in sterile 50-mL conical tubes (**1, 2**) allows for greater flexibility in coupon material selection compared to microfuge tubes because these larger containers will accommodate materials that are difficult to manufacture in extremely small sizes including fabrics and painted surfaces.

4.4 Filter units contained in sterile 50-mL conical tubes (**1, 2**) allow the spore-inoculated coupons, which may contain both cidal reactive compounds and static compounds including surfactants, to be submerged and then be filtered away after the test. The membranes also allow for spores to be washed free from any residual chemicals that might have bound to the spore surfaces. Effectively, all the inoculated spores, including those bound to the coupons, can be recovered in the filter unit and assayed after filtering and washing the spore-inoculated coupons. Such data provide confidence that spores have been inactivated and the results are not merely the result of static activity.

4.5 Contaminated test coupons are subjected to decontamination procedures. Control coupons are treated in an identical manner to test coupons but with a pre-neutralized decontaminant. Recovery of viable spores from pre-neutralized decontaminant on the same day(s) as the test samples provides greater confidence in the test data by eliminating time as a test variable.

4.6 Spores suspended in an aqueous medium represent the spore recovery reference for calculating spore survival after decontamination treatment and analysis.

4.7 Spore extraction percentage is calculated by dividing the number of spores recovered from each spore-inoculated control coupon by the number of spores recovered from the aqueous medium controls.

4.8 The number of culturable surviving spores from decontamination tests is divided by the extraction percentage to determine the number of surviving spores in CFU mL^{-1} and account for any surviving spores not removed from the coupon during extraction. This spore concentration is then multiplied by 10 mL to give the total number of spores surviving (CFU) from each test sample. A $\log_{10}$ transformation of the total surviving spores is then performed ($\log_{10}$ (total CFU + 1)).

5. Significance and Use

5.1 The practice can be used to evaluate coupon materials of any composition, insofar as the coupon can be small enough to fit inside filter units mentioned in 4.1.

5.2 This practice defines procedures that are quantitative, scalable, rapid, sensitive, and safe, while minimizing labor and addressing statistical confidence (**1, 2**).

5.2.1 *Quantitative*—The total number of spores per coupon is determined by dilution-plating, and all spores remaining on the coupon are assayed for activity in the extraction tube to provide confidence that all the spores were accounted for.

5.2.2 *Statistical Confidence*—The use of five independent preparations of spore inocula for a statistical *n* of 5.

5.2.3 *Sensitivity*—Allows for complete detection of all culturable spores inoculated on a coupon, including the spores that remain attached to the coupon.

5.2.3.1 The limit of detection is dependent on the culturability of fully matured spores to germinate, outgrow and divide in the presence of the extraction medium (1% tryptic soy broth, 100 mM L-Alanine, 1 mM inosine, 0.05% Tween 80) and/or on tryptic soy agar.

5.2.3.2 Results presented in Refs (**1, 3**) (and currently unpublished results) indicate that these media, combined with the test temperatures and conditions described herein will generate results with a high level of practical confidence for detecting culturable *Bacillus* spores.

5.2.4 *Safety*—Inoculated coupons are contained within filter units.

5.2.5 *Simplicity of Testing*—Tests and extractions are performed in the same filter unit to minimize coupon handling steps.

5.2.6 *Scalable and Rapid*—A maximum of 36 samples can be processed in 1 h by two technicians; a total of 300 samples have been processed by six technicians in 5 h (**1, 2**).

5.2.7 Wide application for numerous *Bacillus* species and strains. The method has also been modified and used for vegetative bacteria and viruses as well (**1, 2**).

6. Apparatus

6.1 *Autoclave.*

6.2 *Shaking Incubator,* capable of maintaining temperature at ±2 °C within a minimum temperature range of 25-37 °C.

6.3 *Gneral Purpose Microbiological Incubator (±2 °C).*

6.4 *Phase-Contrast Microscope,* oil immersion with magnification ≥100×.

6.5 *Centrifuge,* capable of ≥3,100xg that can hold a swinging-rotor bucket for 50-mL conical tubes.

[5] Trademarked by Millipore, Billerica, MA, USA, UFC9010096

[6] The boldface numbers in parentheses refer to the list of references at the end of this standard.

6.6 *Water Bath,* capable of maintaining temperature at ±2 °C within a minimum temperature range of 50-65 °C.

6.7 *Single-tube Vortex Mixer.*

6.8 *Multi-tube Vortex Mixer.*

6.9 *Analytical Balance.*

6.10 *Ultra-Low Freezer,* set at ≤-60 °C.

6.11 *Stopwatch or Electronic Timer.*

6.12 *Manual or Electronic Pipettes.*

6.13 *Bio-Safety Cabinet (BSC).*

6.14 *Appropriate PPE,* for example, gloves, safety glasses, lab coats, etc. (**4**).

6.15 *Pipettes,* 200 µL and 1 mL.

7. Reagents and Materials[7]

7.1 *Reagents:*

7.1.1 *Bacillus anthracis, cereus and thuringiensis*—acquisition, holding, preparations and/or testing of virulent strains require CDC or APHIS registration in the United States. Strains can be obtained from ATCC, the *Bacillus* Genetic Stock Center, BEI resources repository at National Institute of Health, or the Unified Culture Collection (UCC) at USA Medical Research Institute of Infectious Disease.[8,9]

7.1.1.1 *B. anthracis* strain examples include virulent Ames (UCC BACI387), attenuated Sterne (UCC BACI397), and attenuated ΔSterne (UCC BACI056).

7.1.1.2 *B. cereus* strain examples include a type strain (ATCC 10792), E33L (UCC BACI267), and 03BB102 (UCC BACI234).

7.1.1.3 *B. thuringiensis* strain examples include Al Hakam (UCC BACI229), cry- HD-1 (*Bacillus* Genetic Stock Center ID4A12).

7.1.2 *Tryptic Soy Broth (TSB).*

7.1.3 *Tryptic Soy Agar (TSA).*

7.1.4 *Nutrient Broth (NB).*

7.1.5 *Tween-80.* 0.1%, 3% and 20% stock solutions of Tween-80 suspended in deionized water.

7.1.6 *L-Alanine.*

7.1.7 *Inosine.*

7.1.8 *Sporulation Broth*—0.8% (w/v) Nutrient broth or 2.5% (w/v) Nutrient broth and salts, as defined in Table 1, pH 7 (see Appendix X1 for preparation instructions) (**3**).

7.1.9 *Extraction Buffer*—pH 7, as defined in Table 2 (**4, 5**).

7.1.10 *pH-adjusted Bleach*—0.6% (v/v) hypochlorite, ≥0.2% (v/v) glacial acetic acid, pH ≥6.5 and ≤7.0 within 1 h that it is mixed. The pH will gradually drop to pH ≥4.0 and ≤5.0 after storage for 7 d.

TABLE 1 Sporulation Broth (pH 7)

Reagent	Final Concentration
Nutrient Broth	25 g L^{-1} (2.5%) or 8 g L^{-1} (0.8%)
KH_2PO_4 (Molecular Wt 136.04)	2.15 g L^{-1} (15.8 mmol L^{-1})
K_2HPO_4 (Molecular Wt 174.18)	4.35 g L^{-1} (25 mmol L^{-1})
$CaCl_2 \cdot 2H_2O$ (Molecular Wt 147.01)	0.15 g L^{-1} (1 mmol L^{-1})
$MnCl_2 \cdot 2H_2O$ (Molecular Wt 197.9)	0.016 g L^{-1} (0.1 mmol L^{-1})
$MgCl_2$ (Molecular Wt 95.21)	0.095 g L^{-1} (1 mmol L^{-1})
$ZnCl_2$ (Molecular Wt 136.3)	0.068 g L^{-1} (0.05 mmol L^{-1})
$FeCl_3 \cdot 6H_2O$ (Molecular Wt 270.3)	0.0003 g L^{-1} (0.001 mmol L^{-1})
Sterile, 18 MΩ-cm water	Add water to 1 L final volume

TABLE 2 Extraction Buffer (pH 7)[A]

Reagent	Final Concentration
1X Extraction Buffer	
Tryptic Soy Broth	10 g L^{-1} (1%)
L-Alanine (Molecular Wt 89.09)	8.91 g L^{-1} (100 mmol L^{-1})
Inosine (Molecular Wt 268.23)	2.68 g L^{-1} (1 mmol L^{-1})
Tween® 80 (Polysorbate 80)	0.5 mL L^{-1} (.05%)
Sterile, 18-megaohm water	Add water to 1 L final volume
2X Extraction Buffer	
Tryptic Soy Broth	20 g L^{-1} (2%)
L-Alanine (Molecular Wt 89.09)	17.82 g L^{-1} (200 mmol L^{-1})
Inosine (Molecular Wt 268.23)	5.36 g L^{-1} (2 mmol L^{-1})
Sterile, 18 MΩ-cm water	Add water to 1 L final volume

[A] Can include a chemical neutralizer if necessary to neutralize any sporicidal activity of a chemical vapor.

7.1.11 *90% (v/v) Ethanol.*

7.1.12 *Sodium Thiosulfate (STS)*—

7.2 *Materials:*

7.2.1 *Amicon® Ultra-15 Centrifugal Filter Units with Ultracel,* 100K membranes (Millipore, Billerica, MA, USA, UFC9010096) (filter units).

7.2.2 *Sterile 50-mL Conical Tube.*

7.2.3 *Baffled Flasks.*

7.2.4 *Sterile Petri Dishes.*

7.2.5 *50-mL Conical Tube and Microfuge Tube Racks.*

7.2.6 *Pipette Tips.*

7.2.7 *Parafilm*[10].

7.2.8 *L-shaped Sterile Spreaders.*

7.2.9 *1.5-mL Sterile Microfuge Tubes.*

7.2.10 *Coupon Materials*—All coupon materials must be a standardized surface area, preferably flat, 2 cm × 2 cm; however, it is understood that not all materials are easily adaptable to these size constraints.

7.2.11 *Sterile Forceps.*

7.2.12 *Sodium Thiosulfate (STS).*

8. Hazards

8.1 It is the responsibility of the individual user(s) of this practice to follow all safety guidelines and to be knowledgeable about these procedures. Individual users should consult their safety authority and establish detailed safety plans and risk assessments prior to using this practice. Users are strongly urged to consult the Biosafety in Microbiological and Biomedical Laboratories (**4**).

[7] *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

[8] Virulent strains require CDC (cross reference citation in 2.2) or APHIS (cross reference citation in 2.2) registration in the United States.

[9] 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.

[10] Trademarked by Bemis Company, Inc. Neenah, WI 54956

9. Test Organisms

9.1 Specific organisms are recommended, but the choice of organism(s) should be relevant to the environment in which the decontaminant is expected to be used.

9.2 Pathogenic and non-pathogenic stains of macrobacillus including *Bacillus anthracis* – for example, Ames, Sterne, ΔSterne.

9.3 Acrystalliferous strains of *Bacillus thuringiensis* – for example, Al Hakam, cry$^-$ HD-1.

9.4 Other macrobacillus and microbacillus strains, vegetative bacteria, bacteriophage and vertebrate viruses may also be tested using this practice.

10. Preparation of Inoculum

10.1 Prepare five independent spore inocula from five independent spore preparations. Spores from each independent spore preparation are used to prepare its corresponding independent spore inoculum.

10.2 Transfer 0.1% Tween 80 into a pre-labeled 50-mL conical tube using a volume of 0.1% Tween 80 that will achieve a target concentration of 1-2×10^8 spores mL^{-1}.

10.2.1 Preheat the 50-mL conical tubes with 0.1% Tween 80 at 50 ± 2 °C.

10.3 Transfer concentrated spores from an ultra-low freezer (6.10) set at ≤-60 °C directly to a 50 ± 2 °C water bath for at least 30 min.

10.3.1 Maintain spores at 50 ± 2 °C during spore inoculation to mitigate the risk of spore clumping prior to and during coupon inoculation.

10.4 Vortex concentrated spores for 15-30 s.

10.5 Transfer concentrated spores into pre-labeled 50-mL conical tubes containing preheated (50 ± 2 °C) sterile 0.1% (v/v) Tween 80 to achieve a target concentration of 1-2×10^8 spores mL^{-1}.

10.5.1 Rinse the pipette tips by pipetting up and down twice in the 50-mL conical tube in order to rinse spores from the plastic tips.

10.6 Hold the diluted spore inoculum at 50 ± 2 °C until coupon inoculation, which should occur within 24 h of preparing the inocula.

10.7 In order to titer the spore inoculum, transfer 0.1 mL of spore inoculum into 0.9 mL of 0.1% Tween 80, serially dilute and plate 0.1 mL on TSA plates.

10.7.1 Invert plates and incubate at 35 ± 2 °C for 16 ± 2 h. The time and temperature of plate incubation can be adjusted for strains, for example, *B. thuringiensis* HD-1 strains produce large colonies and this strain is incubated at 30 ± 2 °C for 16 ± 2 h in order to ensure countable plates.

10.7.2 Count and record data.

10.8 Optional: Spores may be mixed with an inorganic soil load prior to coupon inoculation. Kaolin ($Al_2Si_2O_5(OH)_4$) was selected as an inorganic debris in published tests (**3**).

10.8.1 Suspend kaolin in 0.1% Tween 80 at 100 g L^{-1} kaolin and autoclave-sterilize for 30 min on a wet cycle.

10.8.2 Suspend spores in kaolin at a final concentration of 1-2×10^8 spores mL^{-1}, 0.1% Tween 80 and 50 g L^{-1} kaolin.

10.8.3 At a test concentration of 1-2×10^8 spores mL^{-1}, kaolin is 250-500 1-2×10^8 excess over spores by weight.

10.9 Optional: Spores may be mixed with organic soil load prior to coupon inoculation. Humic acid suspended in spent sporulation medium (SSM) was selected as an organic debris in published tests (**3**).

10.9.1 Collect the SSM after spore harvest and centrifugation (Ref (**3**) and Appendix X1).

10.9.2 Filter-sterilize the SSM through a 0.2µm filter. The SSM may be stored in an ultra-freezer at ≤-60 °C for long-term storage.

10.9.3 Suspend humic acid in SSM at 10 g L^{-1} humic acid and autoclave-sterilize for 30 min on a wet cycle.

10.9.4 Suspend spores in the humic acid + SSM at a final concentration of 1-2×10^8 spores mL^{-1}, 0.05% (v/v) Tween 80, 5 g L-1 humic acid, 0.5X SSM.

10.9.5 At a test concentration of 1-2×10^8 spores mL^{-1}, the humic acid is 25-50× excess over spores by weight.

11. Preparation of Coupon

11.1 Rinse coupons with 18-megaohm water.

11.1.1 Dry coupons on absorbent paper in an autoclave-safe container.

11.2 Autoclave temperature-insensitive coupons at 121 °C for 30 min on a wet cycle.

11.3 Soak materials that are temperature sensitive in pH-adjusted bleach for 10 min, followed by soaking in excess 90% ethanol for at least 15 min. Air dry overnight in BSC.

11.3.1 Store sterilized coupons in sterile containers under ambient (22 ± 3 °C) laboratory conditions until use.

11.3.2 Verify coupon sterility during testing when uninoculated coupons are taken through the entire testing procedure and checked for sterility.

NOTE 1—The pre-neutralized controls in the test matrix will confirm there is no residual hypochlorite activity.

12. Test Procedure (see Fig. 1)

12.1 *Carrier Inoculation*—Confirm inoculum titer on the day of coupon inoculation.

12.1.1 *Coupons (Fig. 1 a-c):*

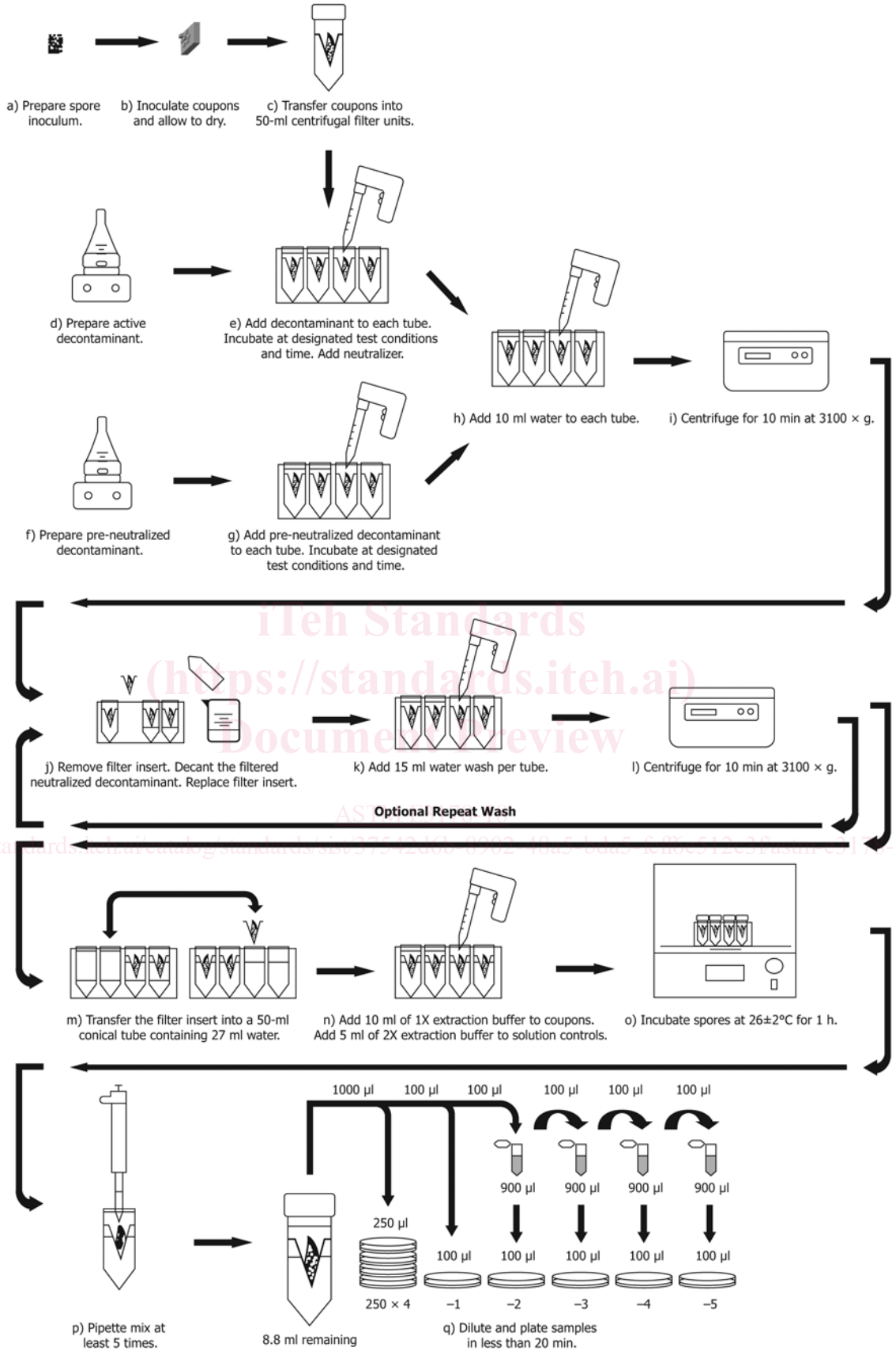
12.1.1.1 Vortex pre-warmed (50 ± 2 °C) inoculum for 15-30 s.

12.1.1.2 Use a P-1000 pipette to transfer a single 100 µL drop of clean spores or spores mixed with inorganic debris or spores mixed with organic debris per inoculation. The pipette tip should be immersed half way into the inoculum when removing aliquots.

12.1.1.3 Since spores will settle in suspension, inoculate a maximum of 12-18 coupons at one time. Then return the spore suspension back into the 50 ± 2 °C water bath.

12.1.1.4 Select the next inoculum, working through all five independent spore preparations, performing Steps 12.1.1.1 through 12.1.1.3 until all coupons have been inoculated.

12.1.1.5 Allow coupons to dry overnight in an operating BSC under ambient conditions.



NOTE 1—Pre-neutralized decontaminant should be neutralized for at least 5 min prior to transferring to coupon. 5% sodium thiosulfate (STS) is the neutralizer in the figure.

FIG. 1 Illustration of Decontamination Test Method to Remove Static and Cidal Chemicals from Coupons (Source: Ref 2)

(1) Record the temperature inside the BSC.

12.1.1.6 Transfer inoculated coupons into pre-labeled filter units (4.1).

(1) Store coupons under ambient (22 ± 3 °C) laboratory conditions until use.

NOTE 2—Inoculated coupons can be stored indefinitely so long as there is no loss in spore titer. The recommended storage time is no longer than 12 months.

12.1.2 *Solution (Wet) Controls (not shown in* Fig. 1*):*

12.1.2.1 Aseptically add 27 mL of sterile 0.1% Tween 80 into 50-mL conical tubes, and then place the filter insert into the tube. The 27 mL of 0.1% Tween 80 prevents the solution in the filter unit from dripping through the membrane over an extended period of time, and the 100,000 molecular weight cutoff membrane keeps spores in the filter unit.

12.1.2.2 Aseptically add 4.9 mL of sterile 0.1% Tween 80 into the filter unit.

12.1.2.3 Vortex the pre-warmed (50 ± 2 °C) spore inoculum for 15-30 s.

12.1.2.4 Use a P-1000 pipette to transfer a single 100 µL drop of spores per solution control. The pipette tip should be immersed half way into the inoculum when removing aliquots.

12.1.2.5 Inoculate solution controls in conjunction with coupons, up to 12-18 solution controls at one time.

(1) Return inoculum back into the 50 ± 2 °C water bath.

12.1.2.6 Select the next inoculum, working through all five independent spore preparations, performing Steps 12.1.2.3 through 12.1.2.5 until all solution control tubes have been inoculated.

12.1.2.7 Cap solution control tubes and store under ambient (22 ± 3 °C) laboratory conditions until use.

NOTE 3—Inoculated coupons can be stored indefinitely so long as there is no loss in spore titer. The recommended storage time is no longer than 12 months.

12.1.2.8 These solution (wet) controls represent the maximum number of spores that can be recovered since spores are maintained in 0.1% Tween 80 and never allowed to dry on a surface.

12.2 *Test and Controls (*Fig. 1 *d-g)*—Coupons and/or controls are incubated at the appropriate environmental test conditions with reactive or pre-neutralized decontaminant. Test and control coupons and solution controls contain $\geq 1\times10^7$ spores per sample.

NOTE 4—Refer to Test Methods E1054 to assess decontaminant neutralization.

12.2.1 Incubate test coupons with reactive decontaminant for the designated test conditions and time.

12.2.1.1 Add 2 mL of reactive decontaminant to each sample in filter units.

12.2.1.2 Incubate for designated contact time at ambient temperature (22 ± 3 °C).

12.2.1.3 After incubating for the designated contact time, 2 mL of the designated neutralizer (for example, 5% sodium thiosulfate (STS)) is added to the filter units to neutralize the reactive ingredients.

12.2.2 Incubate control coupons with pre-neutralized decontaminant for the designated test temperature and time.

12.2.2.1 Prepare the pre-neutralized decontaminant by mixing 2 mL of reactive decontaminant plus 2 mL of the designated neutralizer.

12.2.2.2 Add the pre-neutralized decontaminant solution to spore-inoculated coupons.

12.2.2.3 Incubated for the designated contact time at ambient temperature (22 ± 3 °C).

12.2.2.4 The pre-neutralized control provides confidence that chemical neutralization was successful. A comparison of this control and the solution controls with the test coupon isolates reactive-ingredient contact time as the sole test variable.

12.2.3 Incubate negative controls (uninoculated coupons) at ambient laboratory conditions (22 ± 3 °C).

12.2.4 Incubate solution controls (4.9 mL of 0.1% Tween 80 plus 0.1 mL of spore inoculum) at ambient laboratory conditions (22 ± 3 °C).

12.3 *Centrifugation and Washing (*Fig. 1 *h-m)*—All spore-inoculated coupons are centrifuged and washed. The solution controls and negative coupon controls are not centrifuged and washed.

12.3.1 Add 10 mL of sterile, autoclaved water to each filter unit to dilute the neutralized decontaminant solutions.

12.3.1.1 After the addition of water, centrifuge all tubes at 3100 xg for 10 min with maximum braking. The filter units retain all test substrates and spores while the neutralized decontaminant is collected in the conical tubes below the filter units during centrifugation.

12.3.2 After centrifugation, remove filter units using sterile forceps.

12.3.2.1 Decant the neutralized decontaminant filtrate.

(1) Place the filter units back into the now-empty conical tubes.

12.3.3 Add fifteen mL of autoclave-sterilized water to each filter unit.

12.3.3.1 Centrifuge at 3100 g for 10 min with maximum braking. The water is used to solubilize and remove potentially bacteriostatic, residual surfactants and salt precipitates. Vortexing after addition of water is not recommended since it adds a step and the caps can sometimes leak.

12.3.4 Optional second wash: After the wash step (second centrifugation), steps 12.3.2 and 12.3.3 can be repeated for a second wash in the event that strongly adhering bacteriostatic compounds such as cationic surfactants are being tested.

12.3.5 After centrifugation, transfer washed filter unit coupons into new 50-mL conical tubes containing 27 mL autoclave-sterilized water. The water below the filter units imparts hydrostatic pressure that inhibits extraction fluid from dripping through the membranes with minimal diffusion of the extraction buffer components through the membrane.

12.3.5.1 Discard the original 50-mL conical tubes containing the filtrate.

12.4 *Extraction (*Fig. 1 *n-q)*—All coupons and control samples are extracted. Samples are processed in sets of 10-12 samples at a time.

12.4.1 *Coupons*—Add 10 mL of 1× extraction buffer to each coupon sample.