Designation: E3092 – 18

Standard Practice for Evaluating Efficacy of Vaporous Decontaminants on Materials Contaminated with *Bacillus* Spores and Contained Within 0.2µm Filter-Capped Tubes[1]

This standard is issued under the fixed designation E3092; 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 vaporous decontaminants on *Bacillus* spores dried on the surface of coupons made from porous and non-porous materials and contained within 0.2µm filter-capped tubes.

1.2 This practice should be performed only by those trained in microbiological techniques, are familiar with antimicrobial (sporicidal) agents and with the end use of such 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 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

E2111 Quantitative Carrier Test Method to Evaluate the Bactericidal, Fungicidal, Mycobactericidal, and Sporicidal Potencies of Liquid Chemicals

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals

E2414 Test Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Carrier Surface (Withdrawn 2014)[3]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

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

3.2 For inactivators and neutralizers of decontaminants see Test Methods E1054.

3.3 *Definitions of Terms Specific to This Standard:*

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

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

3.3.3 *exosporium, n*—the outermost layer of spores of *Bacillus anthracis* and its close relatives *Bacillus thuringiensis* and *Bacillus cereus*.

3.3.4 *macrobacillus, n*—a *Bacillus* endospore that possess an exosporium.

3.3.5 *microbacillus, n*—a *Bacillus* endospore that does not possess an exosporium.

3.3.6 *vapor, n*—a substance in the gas phase at a temperature lower than its critical temperature, such that it can be condensed back into a liquid by increasing the pressure on it without reducing the temperature.

3.3.7 *vaporous decontaminant, n—for the purpose of this practice,* a vaporous decontaminant can be interpreted to include gases, vapors, fogs, mists and thermal decontaminants.

[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] The last approved version of this historical standard is referenced on www.astm.org.

4. Summary of Practice

4.1 This practice quantitatively evaluates the efficacy of vaporous decontaminants on coupons contaminated with *Bacillus* spores (pathogenic and non-pathogenic strains). Spores are dried on coupon surfaces, and the coupons are then transferred to 0.2 µm filter-capped 50-ml conical tubes (**1-3**).[4]

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 × 2 cm) coupons are preferred; however non-flat coupons and smaller coupons have been tested using this practice.

4.3 Fifty-ml conical tubes are used for extraction vessels. This allows for greater flexibility in coupon material selection, accommodating materials that are difficult to manufacture in extremely small sizes, for example, concrete, asphalt, carpet and wallboard.

4.4 Contaminated test coupons are subjected to decontamination procedures. Control coupons are subjected to identical procedures without the decontaminant.

4.5 Solution controls (spores suspended in aqueous solution) will represent the 100 % recovery reference for calculating spore survival after decontamination treatment and analysis.

4.6 Spore extraction percentage will be calculated by dividing the number of spores recovered from each spore-inoculated control coupon by the number of spores recovered from the solution controls.

4.7 The number of surviving spores from decontamination tests will be divided by the extraction percentage to determine the number of surviving spores in CFU ml^{-1}. This spore concentration is then multiplied by 10 ml to give a total number of spores surviving (CFU) from each test sample. A log_{10} transformation of the total surviving spores will then be 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 prepared small enough to fit inside a 50-ml conical tube.

5.2 This practice defines procedures that are quantitative, scalable, rapid, sensitive, safe, reduces consumables, minimizes labor and addresses statistical confidence (**1**, **2**, **4**).

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 100 % of spores were assayed.

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

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

5.2.4 *Safety*—Inoculated coupons are contained within 0.2 µm filter-capped 50-ml conical tubes. The 0.2 µm filter allows vaporous decontaminants to pass through while preventing escape of spores, thereby providing an important level of containment when working with pathogenic strains.

5.2.5 *Simplicity of Testing*—Tests and extractions are performed in the same 50-ml conical tube to minimize 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 (**1-3**).

5.2.7 Wide application for numerous *Bacillus* species and strains.

NOTE 1—This practice differs from similar quantitative methods (E2111, E2197 and E2414) in the size and variety of coupon materials available for testing, in the practical confidence of the statistics, the application of the decontaminant, scalability and sensitivity.

6. Apparatus

6.1 *Autoclave.*

6.2 *Shaking Incubator.*

6.3 *Incubator.*

6.4 *Phase-Contrast Microscope.*

6.5 *Centrifuge.*

6.6 *Water Bath.*

6.7 *Single-tube Vortex Mixer.*

6.8 *Multi-tube Vortex Mixer.*

6.9 *Analytical Balance.*

6.10 *-80 °C Freezer.*

6.11 *Stopwatch or Electronic Timer.*

6.12 *Manual or Electronic Pipettes.*

6.13 *Bio-Safety Cabinet (BSC).*

6.14 *Environmental Chamber,* capable of maintaining temperature ±2 °C and relative humidity ±5% of target parameters; must be capable of maintaining vapor concentration.

6.15 *Appropriate PPE,* for example, gloves, safety glasses, lab coats, etc. (**5**).

7. Reagents and Materials

7.1 *Reagents*[5]:

7.1.1 *Bacillus anthracis*—Ames, Sterne, ΔSterne.

7.1.2 *Bacillus thuringiensis*—Al Hakam, cry^- HD-1.

7.1.3 *Tryptic Soy Broth (TSB).*

7.1.4 *Tryptic Soy Agar (TSA).*

7.1.5 *Nutrient Broth (NB).*

7.1.6 *Tween 80.*

7.1.7 *L-Alanine.*

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

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual 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.

7.1.8 *Inosine.*

7.1.9 *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 Annex A1 for preparation instructions) (**1, 6, 7**).

7.1.10 *Extraction Buffer*—pH 7, as defined in Table 2 (**2, 3**).

7.1.11 *pH-adjusted Bleach*—0.6% (v/v) hypochlorite, 0.2% (v/v) acetic acid, pH 7.

7.1.12 *90 % (v/v) Ethanol.*

7.2 *Materials:*

7.2.1 *Sterile 50-ml conical tube.*

7.2.2 *Sterile 50-ml conical tubes with 0.2µm filter cap,* for example, TPP TP87050; Techno Plastic Products[6].

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.*[7]

7.2.8 *L-shaped sterile spreaders.*

7.2.9 *1.5-ml sterile microcentrifuge tubes.*

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

7.2.11 *Sterile forceps.*

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* (**5**).

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 perform.

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

9.3 Acrystalliferous strains of *Bacillus thuringiensis* – Al Hakam, cry⁻ HD-1.

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

10. Preparation of Inoculum

10.1 Prepare five independent spore inocula from five independent spore preparations.

10.2 Transfer concentrated spores from -80 °C directly to a 50 °C water bath for at least 30 min. This temperature is maintained during spore inoculation to mitigate the risk of spore clumping prior to and during coupon inoculation.

10.3 Vortex concentrated spores for 15-30 s.

10.4 Transfer concentrated spores into pre-labeled 50-ml conical tubes containing preheated (50 °C) sterile 0.1 % (v/v) Tween 80. Spores from each independent spore preparation are used to prepare its corresponding independent spore inoculum. The volume of 0.1 % Tween 80 is set to achieve a target concentration of 1-2 × 10^8 spores ml^{-1}. Pipette tips should be rinsed by pipetting up and down twice in the 50-ml conical tube in order to rinse spores from the plastic tips.

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

10.6 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 on TSA plates. Invert plates and incubate at 35 ±2 °C for 16 ±2 h. Count and record data. 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 Optional: Spores may be mixed with inorganic debris prior to coupon inoculation. Kaolin ($Al_2Si_2O_5(OH)_4$) has been selected as a potential inorganic debris in published tests (**2**). Kaolin was suspended in 0.1 % Tween 80 at 100 g l^{-1} kaolin and autoclave-sterilized for 30 min on a wet cycle. Spores were suspended in kaolin at a final concentration of 1-2×10^8 spores ml^{-1}, 0.1 % Tween 80, 50 g l^{-1} kaolin. At a test concentration of 1-2×10^8 spores ml^{-1}, kaolin was 250-500× excess over spores by weight.

TABLE 1 Sporulation Broth (pH 7)

Reagent	Amount
Nutrient Broth	2.5 % (2.5 g l^{-1}) or 0.8 % (0.5 g l^{-1})
KH_2PO_4	2.15 g
K_2HPO_4	4.35 g
$CaCl_2 \cdot 2H_2O$	0.15 g
$MnCl_2 \cdot 2H_2O$	0.016 g
$ZnCl_2$	0.068 g
$FeCl_3 \cdot 6H_2O$	0.0003 g
Sterile, 18-megaohm water	Add water to 1000 ml final volume

TABLE 2 Extraction Buffer (pH 7)[A]

Reagent	Defined Amount
1× Extraction Buffer	
TSB	10 g
L-Alanine	100 mM
Inosine	1 mM
Tween 80	1 ml
Sterile, 18-megaohm water	Add water to 1000 ml final volume
2× Extraction Buffer	
TSB	20 g
L-Alanine	200 mM
Inosine	2 mM
Sterile, 18-megaohm water	Add water to 1000 ml final volume

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

[6] The sole source of manufacturing of the apparatus known to the committee at this time is Techno Plastic Products, Trasadingen, Switzerland. There are multiple sources for purchasing. If you are aware of alternative manufactures, 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.

[7] Trademarked by Bemis Corporate 2301 Industrial Drive Neenah, WI 54956.

10.8 Optional: Spores may be mixed with organic debris prior to coupon inoculation. Humic acid suspended in spent sporulation medium (SSM) has been selected as a potential organic debris in published tests (**2**). The SSM collected after spore harvest (see Refs (**1**, **6**, **7**) and Annex A1) was 0.2µm filter-sterilized and then stored at -75 ±5 °C. Humic acid was suspended in SSM at 10 g l^{-1} humic acid and autoclave-sterilized for 30 min on a wet cycle. Spores were then suspended 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, 0.5× SSM. At a test concentration of 1-2 × 10^8 spores ml^{-1}, the humic acid was 25-50× excess over spores by weight.

11. Preparation of Coupon

11.1 Rinse coupons with 18-megaohm water. Dry on absorbent paper in an autoclave-safe container.

11.2 Autoclave coupons at 121 °C for 30 min on a wet cycle. Materials that are temperature sensitive should be soaked in pH-adjusted bleach for 10 min, followed by a 90 % ethanol rinse. Store sterilized coupons in sterile containers at ambient (22 ±3 °C) laboratory conditions until use.

12. Test Procedure

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

12.1.1 *Coupons:*

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 Inoculate 12-18 coupons at one time. Then return inoculum back into the 50 °C water bath.

12.1.1.4 Select the next inoculum, working through all five independent 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, uncovered, overnight in BSC at ambient (22 ±3 °C).

12.1.1.6 Transfer inoculated coupons into pre-labeled filter-capped tubes. Store at ambient (22 ±3 °C) laboratory conditions until use.

12.1.2 *Solution Controls:*

12.1.2.1 Aseptically add 4.9 ml of sterile 0.1 % Tween 80 into 50-ml conical tubes with closed caps.

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

12.1.2.3 Use a P-1000 pipette to transfer a single 100 µl drop per inoculation. The pipette tip should be immersed half way into the inoculum when removing aliquots.

12.1.2.4 Inoculate solution controls in conjunction with coupons, up to 12-18 solution controls at one time. Then return inoculum back into the 50 ±2 °C water bath.

12.1.2.5 Select the next inoculum, working through all five independent preparations, performing Steps 12.1.2.2 through 12.1.2.4 until all solution control tubes have been inoculated.

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

12.2 *Test and Controls*—Coupons or controls, or both are incubated at the appropriate environmental test conditions with or without chemical vapor. Test and control coupons and solution controls contain ≥1 × 10^7 spores.

12.2.1 Incubate test coupons at the test environmental conditions with or without chemical vapor (5 test coupons per coupon material).

12.2.2 Incubate control coupons at ambient laboratory conditions (22 ±3 °C) (five (5) control coupons per coupon material).

12.2.3 Incubate negative controls (uninoculated coupons) at ambient laboratory conditions (22 ±3 °C) (1 negative control per coupon material).

12.2.4 Incubate test solution controls (4.9 ml of 0.1 % Tween 80 plus 0.1 ml of spore inoculum) at the test environmental conditions with or without chemical vapor (five (5) test solution controls).

12.2.5 Incubate control solution controls (4.9 ml of 0.1 % Tween 80 plus 0.1 ml of spore inoculum) at ambient laboratory conditions (22 ±3 °C) (five (5) solution controls).

12.2.6 There are a total of 21 samples per coupon material, for example, five (5) coupon materials = 125 samples.

12.3 *Extraction*—Samples are processed in sets of 10-12 samples at a time. See Fig. 1 (**1**).

12.3.1 *Coupons*—Add 10 ml of 1× extraction buffer to each sample.

12.3.2 *Solution Control*—Add 5 ml of 2× extraction buffer to each sample.

12.3.3 Incubate samples at 26 ±2 °C for 1 h.

12.3.4 Vortex samples for 2 min on a multi-tube vortexer set at 70 % full speed (for example, 70 out of 100 for a GlasCo vortexer) at ambient (22 ±3 °C) laboratory conditions.

12.3.5 Within 20 min following vortexing, serially dilute samples in 0.1 % Tween 80 and plate on TSA plates. Directly plate 1 ml across four plates (approximately 250 µl per plate), and 0.1 ml on one plate. Transfer 0.1 ml of sample into 0.9 ml of 0.1 % Tween 80, serially diluting and plating on TSA plates at ambient (22 ±3 °C) laboratory conditions.

12.3.6 Incubate all 50-ml conical tubes with the coupon and remaining 8.8 ml of extraction medium at 35 ±2 °C for 16 ±2 h. Invert TSA plates and incubate at 35 ±2 °C for 16 ±2 h.

12.3.7 Score tubes for growth/no growth, count plates and record data.

13. Results and Calculations

13.1 There should be no growth in the filter units with the negative control coupons. Positive growth indicates contamination and a flawed and questionable test result.

13.2 Determine titer of the spore inoculum, solution controls, test and control coupons.

13.2.1 The spore inoculum should be ≥ 1e8 spores ml^{-1}. Since 0.1 ml was inoculated per coupon this is equivalent to ≥ 1e7 spores $coupon^{-1}$, and ≥ 1e7 spores $test^{-1}$, and these titers would indicate a successful test.