Designation: E2414 – 05

StandardTest Method for Quantitative Sporicidal Three-Step Method (TSM) to Determine Sporicidal Efficacy of Liquids, Liquid Sprays, and Vapor or Gases on Contaminated Carrier Surfaces[1]

This standard is issued under the fixed designation E2414; 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 test method determines the efficacy of sporicidal agents on microorganisms dried on the surface of solid carriers.

1.2 This test method can be used to evaluate sporicidal products (or decontaminant, disinfectant, and so forth), suspected, claimed, or assumed to have sporicidal activity. This test method allows:

1.2.1 Establishing the sporicidal efficacy of different disinfectants;

1.2.2 Identifying the effect, if any, of the surface materials on sporicidal efficacy; and

1.2.3 Comparing the relative resistance to disinfection of different microbial species or strains.

1.3 The values stated in SI units are to be regarded as the standard.

1.4 Strict adherence to the protocol is necessary for the validity of the test results.

1.5 *Follow all the safety guidance of the institution in which the testing is being conducted. 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:*[2]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

3. Summary of Test Method

3.1 This test method determines the efficacy of disinfectants on spores of *Bacillus subtilis* dried on carriers according to a general technique first described by J. L. Sagripanti and A. Bonifacino **(1, 2)**[3].

3.1.1 Spores of *B. subtilis* can be and have been replaced by similar amounts of spores of *Bacillus anthracis* to substantiate claims as may be needed in biodefense.

3.1.2 The material of the carrier is selected according to claims or intended use, or both, of the disinfectant product. General claims made to decontaminate metallic and polymeric materials are tested on carriers made of stainless steel and silicone medical rubber, respectively. Flat coupons (0.5 by 0.5 cm) are preferable. As may be required by claims or intended use, the test method can be accurately used also on a variety of carriers with diverse geometrical characteristics (additional examples of materials were reported by Sagripanti and Bonifacino **(1, 2)**.

3.1.3 Although the test method described herein refers specifically to liquid disinfectants, the same procedure can be used to assess sporicidal activity of vapors and gaseous sporicidal agents, provided adequate containment is accomplished (see 10.4.5).

4. Significance and Use

4.1 The quantitative micromethod described herein was designed to fulfill the following specifications:

4.1.1 To be quantitative (the number of viable spores loaded into carriers is determined by the spores quantitatively recovered in the controls),

4.1.2 Sensitive (sporicidal activity can be accurately measured up to 7 Log_{10} inactivation, see Section 12),

4.1.3 Reproducible (standard deviation of spore killing is smaller than 1 Log and usually closer to 1/10 of a Log, see Section 12),

4.1.4 Rapid (except for the overnight culture, it can be completed within 4 h),

[1] This test method is under the jurisdiction of ASTM Committee E54 on Homeland Security Applications and is the direct responsibility of Subcommittee E54.03 on Decontamination.

Current edition approved June 15, 2005. Published July 2005. DOI: 10.1520/E2414-05.

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

4.1.5 Economical (being a micromethod, it uses carriers, dishes, and plastic wares that are small, inexpensive, and disposable), and

4.1.6 Environmentally friendly (using a microlitre volume of disinfectant agent, the test method can be considered for all practical purposes as nondestructive).

5. Apparatus

5.1 *List of Equipment*—Make and models are provided as examples. Use the same or equivalents.

5.1.1 *Microcentrifuge,* rated to 12 000 g with rotor to hold 1.5-mL conical microcentrifuge tubes.

5.1.2 Autoclave.

5.1.3 Vortex mixer.

5.1.4 *Sonicator,* low-power water bath type, rated to 400 to 500 W (generally used for cleaning small objects by immersion).

5.1.5 Class II biosafety cabinet.

5.1.6 *Balance,* accurate to 1 mg.

5.1.7 *Refrigerator,* able to maintain 0 to 5°C.

5.1.8 *Freezer,* able to keep −80°C.

5.1.9 *Incubator,* able to maintain 37 ± 1°C (usually with a range from room temperature to 60°C).

5.1.10 Colony counter.

5.1.11 *Photomicroscope,* providing 1000× maximal magnification to control spore quality.

5.1.12 *Micropipets,* with corresponding sterile tips able to deliver volumes in the ranges of 10, 20, 100, 200, and 1000 µL.

5.1.13 *Timer,* any certified timer/watch/clock that can display time in seconds.

5.1.14 *Rotator,* able to provide 15 to 20 rpm (of the type used in hematology chemistry) with a rack to hold 1.5-mL microcentrifuge tubes.

5.1.15 *Shaker,* able to control speed and inside temperature.

5.1.16 *Anaerobic Jar,* if testing gases.

5.1.17 *Cooler,* able to maintain temperature at 21 ± 3°C

6. Reagents and Materials

6.1 *Spores of B. subtillis (Strains ATCC 1031 or ATCC 9372), Bacillus globigii (Renamed B. atrophaeus SB 512), or B. anthracis (Pathogenic Strains Albia BA 1029, Ames, NCTC 1087, or Vollum or Nonpathogenic Strains Sterne, Delta-Sterne, or Pasteur BC3132)*—Stock suspensions are prepared under the appropriate biosafety containment and produced at a concentration between 1×10^9 and 5×10^9 colony-forming units/mL. A variety of media are available to grow spores of Bacillus species. It is recommended the use of sporulation media Media S whose formulation is described in Appendix X1. Preparations (made as suggested in Appendix X1) are accepted for use when consisting in more than 95 % spores as determined by both microscopic observation and acid resistance. Microscopic observation of spores stained with trypan blue should reveal less than 10 vegetative cells (shaped as rods) during the observation of 1000 spores (round shape). Testing spores for acid resistance is described in Appendix X2 and Appendix X3. Both tests for spore quality follow techniques published previously **(1, 2, 4)**.

6.2 *Sterile Luria*—Bertani broth (LB) (if in powder form, prepared as recommended by the manufacturer).

6.3 HPLC quality sterile water and sterile phosphate-buffered saline (PBS).

6.4 Nutrient agar plates.

6.5 Laboratory glassware, graduated cylinders, calibrated volumetric flasks.

6.6 Cupric chloride ($CuCl_2 \cdot 2H_2O$); *L*-ascorbic acid, and hydrogen peroxide if using cupric ascorbate as a positive control.

6.7 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[4]

6.8 *Carriers:*

6.8.1 *Spotting/Flat*—Preferable glass, but also rubber, stainless steel, polymeric plastics, or other materials cut in squares of 0.5 by 0.5 cm.

6.8.2 *Dipping*—Carriers that do not hold liquid and need to be contaminated by immersion, for example, screws, cylinders, and tubing. The size and shape of carriers should allow their introduction inside the 1.5-mL microcentrifuge tubes to be immersed in the 400 µL of liquid and have a total volume between 50 to 200 µL.

6.9 *Supplies:*

6.9.1 *Forceps/Tweezers,* to handle carriers.

6.9.2 *Sterile Disposable Petri Dishes,* 100 by 15 mm.

6.9.3 *Sterile Disposable Petri Dishes,* 47 mm in diameter (preferable to test sprays).

6.9.4 Sterile, disposable 1.5-mL polypropylene microcentrifuge tubes.

6.9.5 Sterile, disposable 15- and 50-mL conical-bottom centrifuge tubes.

6.9.6 Sterile, disposable spreaders.

6.9.7 Nonsterile, latex examination gloves.

7. Hazards

7.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the institutional biosafety regulations. Use equipment and facilities at the biosafety level indicated for the test microorganism. For recommendations on safe handling of microorganisms refer to Ref **(5)**.

7.2 Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, halogen-containing substances, quaternary ammonium compounds, or any other reagent suspected, claimed, or assumed to have sporicidal activity. Gloves and personal protective clothing or devices are worn during the handling of these

[4] *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 *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.

materials. A chemical fume hood or other containment equipment is used when performing tasks with hazardous chemical products.

8. Sample Handling and Storage

8.1 Disinfectants are stored following manufacturer's instructions or at room temperature (21 ± 3°C) if the product label does not indicate special storage conditions. Disinfectants requiring dilution or activation (or pH adjustment) before use are diluted or activated and tested within the time period specified by the manufacturer or within the shelf-life time after activation or dilution, if known for the particular disinfectant. If no information is available from the manufacturer or shelf-life after dilution or activation is unknown, then test within 4 h after dilution or activation.

8.2 Stocks of spores are stored refrigerated between 2 and 5°C.

9. Calibration and Standardization

9.1 Refer to the laboratory equipment calibration and maintenance standard operation procedures (SOPs) for details on test methods and frequency of calibration.

10. Procedure

10.1 *Brief Summary*—This method recovers spores by differential elution (in Fractions A, B, and C) by sequentially applying treatments of increasing dislodging strength. The forces to dislodge spores in each fraction are different and not interchangeable (loosely released by washing in A, sonication in B, and incipient germination in C). These steps were selected after trying many of possible combinations to maximize the recovery and accountability of spores.

NOTE 1—This procedure is to be performed by personnel trained in microbiology and with experience in the use of all laboratory equipment listed. No standard or description can replace necessary training and experience. Accordingly, the procedures in this test method are described at a level of detail that should allow their understanding and correct execution by anybody minimally proficient in microbiology and in the use of general laboratory equipment.

10.1.1 Each clean and sterile carrier (either flat 0.5 by 0.5 cm or dipping) receives 10 µL of a spore suspension containing between 1×10^9 and 5×10^9 organisms/mL (resulting in a microbial load between 1 to 5×10^7 spores per carrier), with or without organic load[5] and dried for 10 to 20 h at 20 to 25°C. The carrier loaded with spores is placed inside of a 1.5-mL microcentrifuge tube (labeled A). The sporicidal product being tested, is added to this tube, assuring that the inoculum in the carrier is completely submerged in the fluid (see 10.4.3). Exposure to different temperatures other than room temperature (21 ± 3°C) can be achieved by equilibrating the microcentrifuge tubes at the desired temperature within a laboratory cooler. Control carriers do not receive disinfectant, but instead receive an equal volume of sterile distilled water. The exposure time and temperature may vary according to manufacturer's specifications. After incubation with the disinfectant, ice-cold[6] LB medium with or without neutralizer is added. The carrier is immediately transferred to a new 1.5-mL microcentrifuge tube (labeled B) containing sterile distilled water at room temperature (21 ± 3°C) and sonicated for 5 min. Ice-cold LB medium is added and the carrier is transferred to a new 1.5-mL microcentrifuge tube (labeled C) with LB medium. The Tubes C are incubated in a rotator inside of an incubator at 37°C for 30 min. Ice-cold LB is added and the carrier discarded. The fluid contained in Tubes A, B, and C correspond to Fractions A, B, and C. The surviving spores in each fraction are enumerated by serial dilution and spread on petri dishes containing nutrient agar medium. Culture plates are incubated overnight (at least 12 h) at 37 ± 1°C and colonies are counted. Total spores surviving treatment with disinfectant are calculated by adding the spores counted in Fraction A, plus spores in Fraction B, plus spores in Fraction C. The sporicidal effect of a disinfectant is calculated by subtracting $\log_{10}$ of the total number of spores surviving the treatment with disinfectant from the $\log_{10}$ of the total number of spores in the controls incubated with sterile water. A summary of the method is shown in Figs. 1 and 2.

10.1.2 Use and change frequently nonsterile examination latex gloves for all handling during the procedure to avoid spore carryover.

10.2 *Carrier Inoculation:*

10.2.1 *Carrier Inoculation by Spotting:*

10.2.1.1 Prepare a suspension of spores in sterile distilled water at 5×10^9 spores/mL with or without organic burden.

10.2.1.2 The spores can be concentrated by centrifugation or diluted in water if required. Resuspend spores thoroughly before spotting on carrier.

10.2.1.3 For general sporicidal claims, use glass carriers in 0.5 by 0.5-cm squares (cut from microscope slides). For specific claims, carriers can be made of, for example, silicone medical rubber, light metal armor, building materials, or any material that reflects intended use of the tested disinfectant and that can be cut into 0.5 by 0.5-cm squares, or a combination thereof. Wash carriers three times with sterile distilled water and rinse once with 95 % ethanol. The carriers can be sterilized by autoclaving or other procedures which will not affect the properties of the carrier material.

10.2.1.4 Place the carriers flat inside the lower plate of a sterile plastic petri dish and load each carrier with 10 µL of the 5×10^9 spore/mL suspension as prepared in 10.2.1.1. The fluid must remain on the carrier or the carrier is discarded. Replace the cover of the petri dish and let the carriers dry 10 to 20 h (overnight) inside a biosafety hood.

10.2.2 *Carrier Preparation by Dipping*—If odd-shaped material is to be tested (screws, tubing, and so forth), each carrier device is immersed in a separate microtube with 50 µL of spore suspension, such that each carrier binds 1 to 5×10^7 spores (for experimental examples, see Refs **1, 2**). After immersing the carrier for 30 min in the spore suspension, remove each carrier with sterile forceps and let the carriers dry for 10 to 20 h at 20 to 25°C. Depending on the shape of the carrier, they are placed

[5] Organic load, as bovine serum albumen, horse serum at 5 % (v/v) final concentration (for details on the effect of organic burden refer to Ref (**3**)).

[6] Ice-cold is 0°C. LB is equilibrated in an ice-containing waterbath.

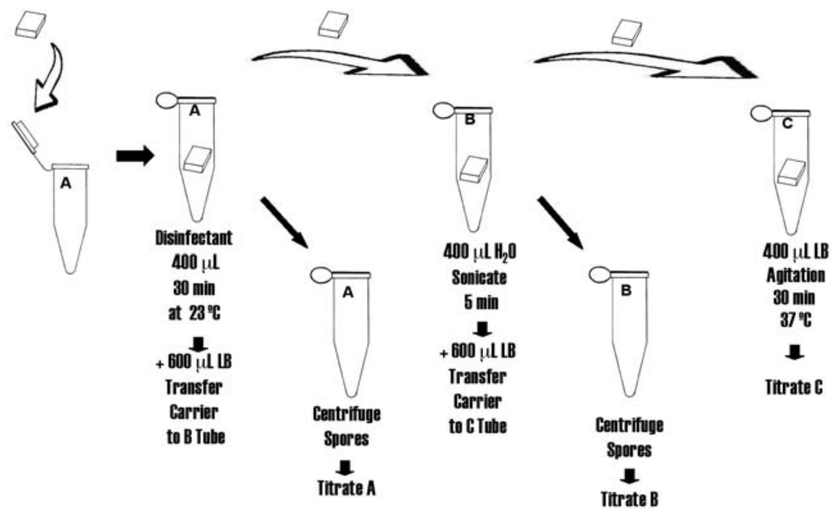

FIG. 1 Scheme of Test Using Carriers Loaded by Spotting

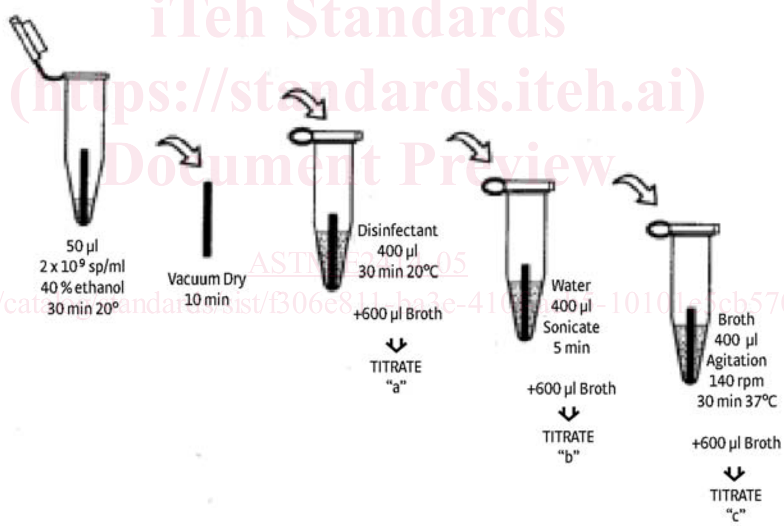

FIG. 2 Scheme of Test Using Carriers Loaded by Dipping

to dry in any holder that prevents contact with the contaminated end (holes drilled in a 6 to 13-mm thick acrylic plate work well to hold small cylindrical items with the contaminated end upward). The dried carriers are placed inside a 1.5-mL microcentrifuge tube until use as described in 10.4.

10.3 *Disinfectant Sample Preparation:*

10.3.1 Before opening the container, shake the container and clean the area around the cap with sterile 95 % ethanol. Remove the cap after the surface is dried, avoiding any touching of the inside of the cap. Remove any container seal attached with a sterile cutting instrument (that is, razor blade, scissor, or forceps).

10.3.2 Pour an appropriate aliquot of the sample into a 50-mL sterile conical bottom centrifuge tube. Do not introduce any instrument or pipet inside the container and close the cap tightly after use.

10.3.3 Ready-to-use products are tested without dilution.

10.3.4 For products requiring dilution, prepare all dilutions with the diluent recommended by the manufacturer or with sterile distilled or HPLC grade water using sterile standardized volumetric glassware.

NOTE 2—It is not mandatory, but highly recommended, to monitor the overall reproducibility of the test method by including cupric ascorbate as a positive control of (intermediate) sporicidal activity. Prepare fresh