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.

Designation: E3286 – 21

Standard Practice for Preparation Of Cell Monolayers on Glass Surfaces for Evaluation of Microbicidal Properties of Non-Chemical Based Antimicrobial Treatment Technologies[1]

This standard is issued under the fixed designation E3286; 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 provides a protocol for creating bacterial cell monolayers on a flat surface.

1.2 The cultures used and culture preparation steps in this Practice are similar to AOAC Method 961.02 and US EPA MB-06. However, test bacteria are applied to the carrier using an automated deposition device (6.2) rather than as a suspension droplet.

1.3 The carrier inspection protocol is similar to US EPA MB-03 except that carrier surfaces are inspected microscopically rather than visually, unaided.

1.4 A monolayer of cells eliminates the confounding effect caused by the shadowing effect of outer layers of bacteria stacked upon other bacteria on test specimens – thereby attenuating directed energy beams (that is, ultraviolet light, high-energy electron beams) before they can reach underlying cells.

1.5 An asperity-free surface eliminates the shadowing effect of specimen surface topology that can block direct exposure of target bacteria to non-chemical antimicrobial treatments.

1.6 This practice provides a reproducible target microbe and surface specimen to minimize specimen variability within and between testing facilities. This facilitates direct data comparisons among various non-chemical antimicrobial technologies.

1.6.1 Antimicrobial pesticides used in clinical and industrial applications are expected to overcome shadowing effects. However, this practice meets a need for a protocol that facilitates relative comparisons among non-chemical antimicrobial treatments.

1.6.2 This practice is not intended to satisfy or replace existing test requirements for liquid chemical antimicrobial treatments (for example Test Methods E1153 and E2197) or established regulatory agency performance standards such as US EPA MB-06.

1.7 This practice was validated using *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442) using a protocol based on AOAC Method 961.02. If other cultures are used, the suitability of this practice must be confirmed by inspecting prepared surfaces, by using scanning electron microscopy (SEM) or comparable high-resolution microscopy.

1.8 The specimens prepared in accordance with this practice are not meant to simulate end-use conditions.

1.8.1 Non-chemical technologies are only to be used on visibly clean, non-porous surfaces. Consequently, a soil load is not used.

1.9 *Units*—The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.10 *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.*

1.11 *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]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E1153 Test Method for Efficacy of Sanitizers Recommended for Inanimate, Hard, Nonporous Non-Food Contact Surfaces

E2197 Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal,

[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.

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and Sporicidal Activities of Chemicals
E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *AOAC Standard:*[3]
AOAC Method 961.02 Germicidal spray products as disinfectants

2.3 *U.S. EPA Standards:*[4]
MB-03 Standard Operating Procedure for Screening of Polished Stainless Steel Penicylinders, Porcelain Penicylinders, and Glass Slide Carriers Used in Disinfectant Efficacy Testing.[5]
MB-06 Standard Operating Procedure for Germicidal Spray Products as Disinfectants (GSPT): Testing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*[6]

2.4 *CDC Standard:*[7]
CDC 21-1112 Biosafety in Microbiological and Biomedical Laboratories, 5th Ed., 2009.[8]

3. Terminology

3.1 *Definitions:* For definitions of terms used in this practice, refer to Terminology E2756.

3.1.1 *asperity, n*—a protuberance in the small-scale topographical irregularities of a solid surface.

3.1.1.1 *Discussion*—In materials science, smooth surfaces are rough on a microscopic scale. The surface topology of even highly polished surfaces is similar to that of mountain ranges.

3.1.1.2 *Discussion*—Depending on the material and its surface finish, the distance between asperity peaks and valleys can range from <0.1 µm to >100 µm. When this distance is ≥1 µm, the peak can create a shadow between an irradiation source and microbes located on the peak's distal side. When a cavity is ≥1 µm deep, microbes can reside inside the cavity and be partly or fully protected from exposure.

3.1.2 *carrier, n*—inanimate surface or object inoculated with the test organism.

3.1.3 *electromagnetic spectrum, n*—the ordered array of known radiations, extending from the shortest wavelengths, gamma rays, through X rays, ultraviolet radiation, visible radiation, infrared and including microwave and all other wavelengths of radio energy.

3.1.4 *ultraviolet, adj*—invisible light radiation, adjacent to the violet end of the visible spectrum, with wavelengths from about 200 nm to 400 nm (nanometers).

3.1.4.1 *Discussion*—UV irradiation is commonly differentiated into three wavelength ranges: UVA (320 nm-400 nm), UVB (280 nm-320 nm) and UVC (200 nm-280 nm).

3.1.5 *visible light, n*—includes wavelengths from 400 nm-740 nm.

4. Summary of Practice

4.1 Bacterial cultures are grown in an appropriate trypticase soy broth (TSB) and harvested by centrifugation.

4.2 Cells in the resulting pellet are washed and resuspended in deionized water and transferred to the deposition device's reservoir.

4.3 A glass microscope slide (*carrier*) is placed onto the automated deposition device.

4.4 The cell suspension is nebulized and sprayed onto the microscope slide's surface. This process is repeated to prepare twelve specimens per treatment (three each: control and treated slides for bioburden testing and direct microscopic observation).

4.5 Once inoculated, carriers are air dried in a vacuum chamber for 2 h before being used as test specimens.

5. Significance and Use

5.1 There are no reproducible standardized protocols for preparing specimens used to evaluate the microbicidal efficacy of non-chemical treatments such as ultraviolet (UV), highenergy electron beam, or other forms of non-chemical antimicrobial technologies.

5.2 Conventional protocols for applying bioburdens to carriers (see Test Method E2197) cause cells to stack upon one another, thereby creating multiple cell layers in which cells in layers closer to the carrier are masked by cells in overlying layers, which makes relative comparison of different nonchemical antimicrobial treatments more difficult.

5.3 Steel and other metal carriers have asperities that can shield a percentage of the applied cells from direct exposure to electromagnetic irradiation.

5.4 The combined effects of 5.2 and 5.3 confound determination of the microbicidal effect of electromagnetic irradiation on test specimens.

5.5 The practice addresses these two confounding factors by:

5.5.1 Using glass microscope slides – the surfaces of which are asperity-free – as carriers.

5.5.2 Reliably depositing bacterial cells onto the carrier as a monolayer.

5.6 The resulting specimen ensures that all microbes deposited onto the carrier are exposed equally to the irradiation source thereby ensuring that the only variables are the controlled ones – starting inoculum concentration, wavelength (λ – in nm), exposure time(s), and resulting energy dose (J).

6. Apparatus

6.1 *Autoclave,* standard laboratory model capable of maintaining 121 °C for ≥45 min.

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[3] Available from AOAC International, 2275 Research Blvd., Suite 300, Rockville, MD 20850-3250, http://www.aoac.org.
[4] Available from United States Environmental Protection Agency (EPA), William Jefferson Clinton Bldg., 1200 Pennsylvania Ave., NW, Washington, DC 20460, http://www.epa.gov.
[5] https://www.epa.gov/sites/production/files/2018-01/documents/mb-03-07.pdf.
[6] https://www.epa.gov/sites/production/files/2018-01/documents/mb-06-09.pdf.
[7] Available from Centers for Disease Control and Prevention (CDC), 1600 Clifton Rd., Atlanta, GA 30329-4027, http://www.cdc.gov.
[8] https://www.cdc.gov/labs/BMBL.html

6.2 *Automated inoculum deposition system*[9]—as shown in Fig. 1, the device includes:

6.2.1 Aluminum framed enclosure with transparent, polymeric walls (spray chamber). Enclosure base has either surface-engraved grid axes or a mounted bracket.

6.2.2 High-precision two-substance 230 V, 50 Hz Model 970 magnetic nozzle (Schlick, Untersiemau, Germany), sterile.

6.2.2.1 Nozzle must be designed to deliver a spray cone with inner spray zone diameter = 2.9 cm ± 0.2 cm, and outer spray zone area diameter = 4.4 cm ± 0.2 cm, to a carrier positioned 9.7 cm ± 0.2 cm from the nozzle's orifice (see Fig. 1 and Fig. 2).

6.2.2.2 Nozzle must be capable of generating a 0.1 s spray pulse; spray cone angle 17 ° (Fig. 1).

6.2.3 Liquid inlet receiver.

6.2.4 Microprocessor-based quartz counter (220-V A/C Gefran Model 550 timer or equivalent).

6.2.5 Mounting base and stand.

6.2.6 Power switch.

6.2.7 Pressure gauge and power-driven 3⁄2 magnetic valve, tubing and wiring.

6.3 Cabinet, biological safety, meeting BSL 2 requirements in accordance with CDC 21-1112, and having sufficient capacity to accommodate automated inoculum deposition system (6.2).

6.4 Centrifuge, with rotor capable of holding microcentrifuge tubes (7.12) and providing 5000 × g centrifugal force.

6.5 *Chamber, desiccator, vacuum,* sufficient capacity to hold at least three 100 cm diameter Petri dishes (7.7) and maintaining vacuum at 94 kPa ± 4 kPa.

6.6 Freezer, capable of maintaining -70 °C ± 2 °C.

6.7 Incubator, laboratory, capable of maintaining 36 °C ± 1 °C.

6.8 Liquid nitrogen (N_2) source (nitrogen tank).

6.9 N_2 tank pressure regulator, set to deliver N_2 at 200 kPa pressure.

6.10 *Mixer,* vortex.

6.11 *Pump, vacuum,* capable of drawing ≥ 98 kPa vacuum.

6.12 *Spectrophotometer,* capable of determining optical density (OD) at 650 nm.

6.13 *Sterilizer,* hot air, capable of maintaining temperature ≥ 230 °C

7. Reagents and Materials[10]

7.1 *Carriers*—glass microscope slides, sterile, 25 mm × 76 mm × 1 mm.

[9] The sole source of supply of the apparatus known to the committee at this time is PurpleSun, Long Island City, NY, www.purplesun.com. 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, which you may attend.

[10] *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.

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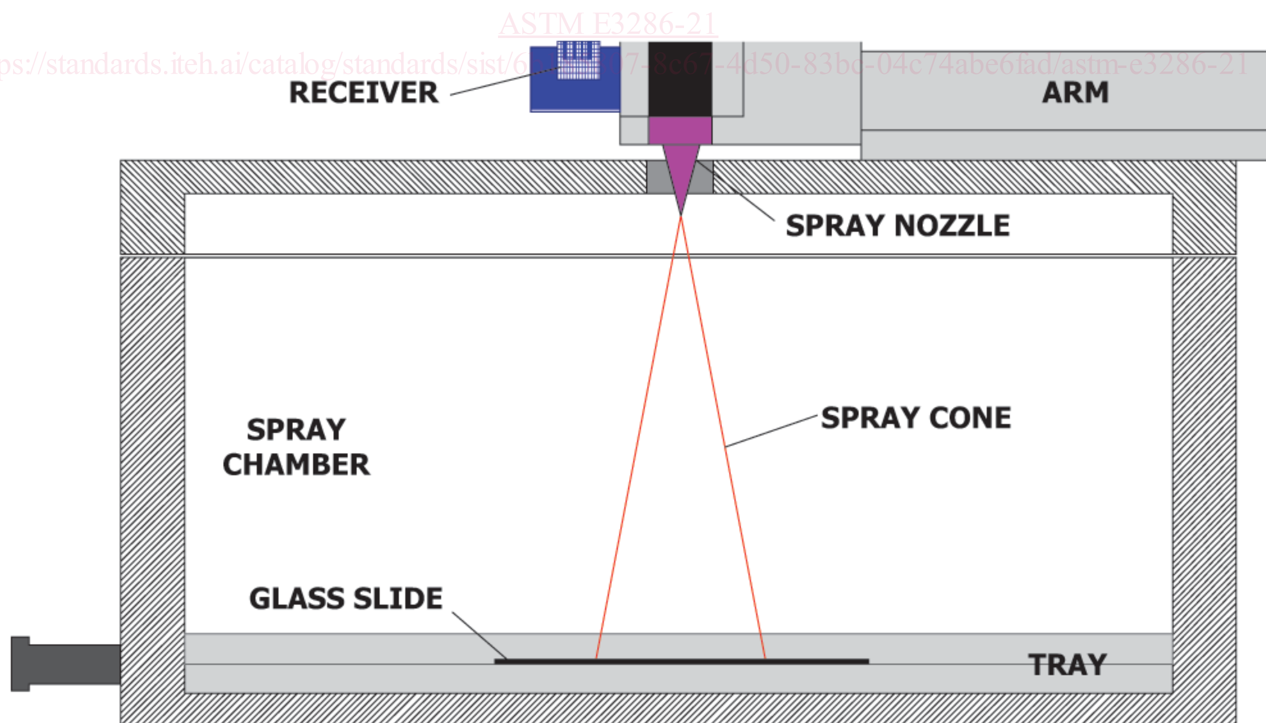

FIG. 1 Schematic cutaway side view of the automated inoculum deposition assembly showing the nozzle, the spray chamber, the carrier, the inlet receiver (where the inoculant is loaded).

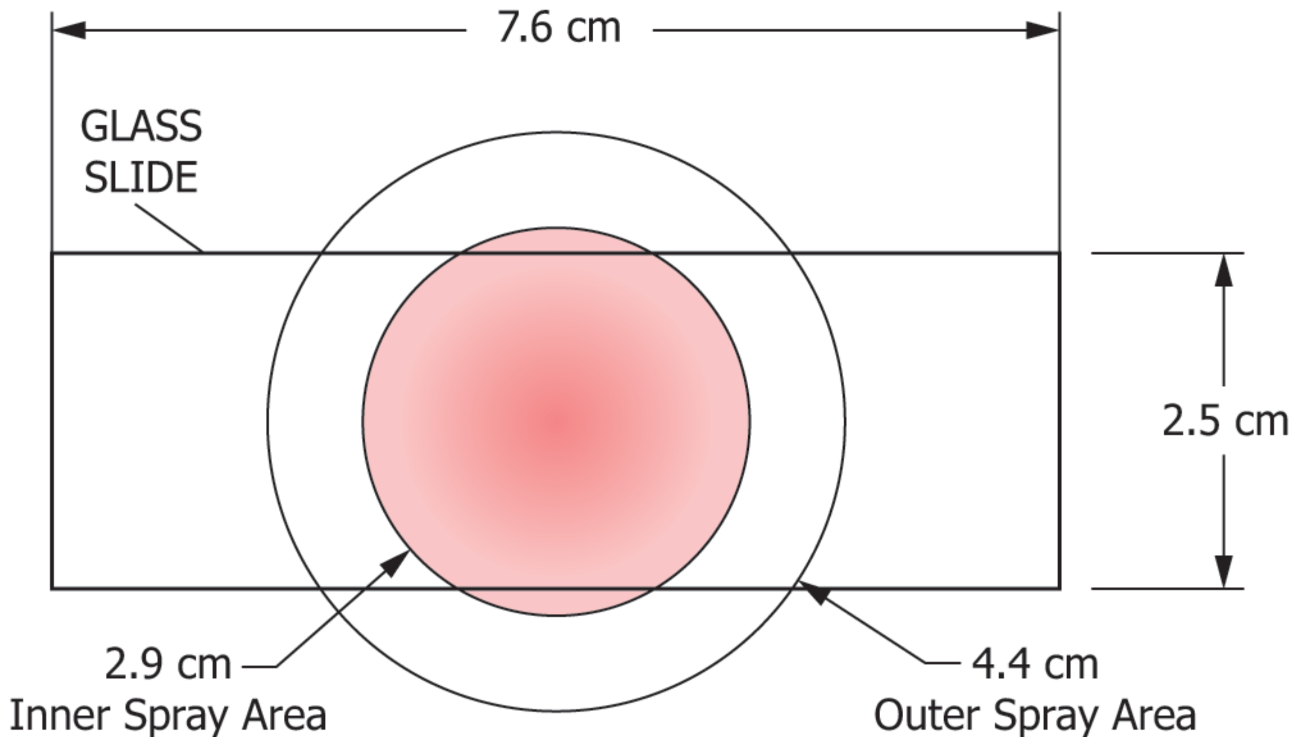

FIG. 2 Carrier schematic with approximate dimensions of the glass slide and the inner and outer spray area. Most of the spray was deposited within the inner spray area. Use mounting bracket or grid (6.2.1) to align specimen with nozzle orifice

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7.2 *Culture media:*

7.2.1 Culture media are available commercially or may be prepared in accordance with AOAC Method 961.02.

7.2.2 *Trypticase soy broth (TSB),* 10 mL, in 20 mm × 150 mm culture tubes.

7.3 *Ethanol,* volume fraction 95 %.

7.4 *Flask, Erlenmeyer,* 250 mL, sterile.

7.5 *Forceps,* flat head, *sterile*.

7.6 *Paper,* blotter.

7.7 *Petri dish,* glass, 100 mm dia.

7.8 *Pipettes,* sterile, 1 mL to 10 mL

7.9 *Pipettor,* micro, adjustable, 1 µL to 100 µL.

7.10 *Slides, microscope, glass,* 2.6 cm × 7.6 cm.

7.11 *Tips,* pipette, disposable, 1 µL to 100 µL, for use with micropipettor (7.9)

7.12 *Tubes,* microcentrifuge, polypropylene, screw capped, 2 mL, sterile, or equivalent.

7.13 *Water,* deionized, sterile (DIW).

8. Test Organisms

8.1 *Staphylococcus aureus* (ATCC 6538).

8.2 *Pseudomonas aeruginosa* (ATCC 15442).

9. Hazards

9.1 Test organisms are both Risk Group 2 cultures.

9.1.1 Only laboratories that meet or exceed the Biosafety Level 2 (BSL 2) criteria stipulated in CDC 21-1112 should use this practice.

10. Procedure

10.1 Except for 10.3.2, use aseptic technique for all steps in this procedure.

10.2 *Test Culture Preparation:*

10.2.1 Rehydrate *S. aureus* (8.1) or *P. aeruginosa* (8.2) in accordance with supplier's instructions.

10.2.2 Add 10 µL of the rehydrated culture stock (8.1 or 8.2) stock (single use) to a tube containing 10 mL of TSB (7.2.2).

10.2.3 Vortex and incubate at 36 °C ± 1 °C for 24 h ± 2 h.

10.2.4 For *S. aureus*, after 24 h ±2 h incubation, vortex culture.

10.2.5 For *P. aeruginosa*, after 24 h ± 2 h incubation:

10.2.5.1 Remove the pellicle either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipette, or by vacuum removal.

10.2.5.2 Avoid harvesting pellicle from the bottom of the tube.

10.2.5.3 Visually inspect for pellicle fragments.

NOTE 1—Presence of pellicle in the final culture makes it unusable for testing.

10.2.6 Transfer 10 µL of culture from 10.2.4 or 10.2.5 to fresh TSB tube (7.2.2).

10.2.7 Vortex and incubate at 36 °C ± 1 °C for 24 h ± 2 h.

NOTE 2—Daily cultures may be subcultured for up to 5 days and each