



Designation: E3286 – 21

Standard Practice for Preparation Of Cell Monolayers on Glass Surfaces for Evaluation of Microbicidal Properties of Non-Chemical Based Antimicrobial Treatment Technologies¹

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.

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 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>

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), high-energy 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 non-chemical 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.