Designation: E2180 – 18

Standard Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials[1]

This standard is issued under the fixed designation E2180; 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.

INTRODUCTION

Polymeric materials such as vinyl pool liners, shower curtains, and various medical devices are treated frequently with incorporated or bound antimicrobial agents. Practices G21 is used to determine the ability of polymer materials to resist microbial attack or staining (see also Practice E1428); however, none of the methods permit quantitative evaluations of incorporated antimicrobial activity.[2] These antimicrobials typically require contact with the microbial cell for maximal activity. When aqueous based bacterial inoculum suspensions are applied onto a preservative-treated plastic or other hydrophobic material, the surface tension of the polymer often causes the inocula suspension to dome. Bacteria within the drops of inoculum may not contact the treated surface if the challenged surface does not dry, or upon drying, cells may become layered. This test standard involves an agar slurry inoculum vehicle that provides a relatively uniform contact of the inocula with antimicrobial-treated hydrophobic surfaces.

1. Scope

1.1 This test method is designed to evaluate (quantitatively) the antimicrobial effectiveness of agents incorporated or bound into or onto mainly flat (two dimensional) hydrophobic or polymeric surfaces. The method focuses primarily on assessing antibacterial activity; however, other microorganisms such as yeast and fungal conidia may be tested using this method.

1.2 The vehicle for the inoculum is an agar slurry which reduces the surface tension of the saline inoculum carrier and allows formation of a "pseudo-biofilm," providing more even contact of the inoculum with the test surface.

NOTE 1—This test method facilitates the testing of hydrophobic surfaces by utilizing cells held in an agar slurry matrix. This test method, as written, is inappropriate to determine efficacy against biofilm cells, which are different both genetically and metabolically than planktonic cells used in this test.

1.3 This method can confirm the presence of antimicrobial activity in plastics or hydrophobic surfaces and allows determination of quantitative differences in antimicrobial activity between untreated plastics or polymers and those with bound or incorporated low water-soluble antimicrobial agents. Comparisons between the numbers of survivors on preservative-treated and control hydrophobic surfaces may also be made.

1.4 The procedure also permits determination of "shelf-life" or long term durability of an antimicrobial treatment which may be achieved through testing both non-washed and washed samples over a time span.

1.5 Knowledge of microbiological techniques is required for these procedures.

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

1.7 *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.*

1.8 *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.*

[1] This test method 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] Price, D. L., Sawant, A. D., and Ahearn, D. G., "Assessment of the antimicrobial activity of an insoluble quaternary amine complex in plastics," *J. Industr. Microbiol*, Vol 8, No. 2, 1991, pp. 83–89.

2. Referenced Documents

2.1 *ASTM Standards:*[3]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E1428 Test Method for Evaluating the Performance of Antimicrobials in or on Polymeric Solids Against Staining by *Streptomyce species* (A Pink Stain Organism)

G21 Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi

3. Terminology

3.1 *Definitions:*

3.1.1 *agar slurry, n*—a semi-gelatinous liquid formed when 3 g/L agar-agar is added to a 0.85 % saline solution.

3.1.2 *inoculum, n*—in microbiology, a specimen comprised of living spores, bacteria, single celled organisms, or other live materials, yeast or the multicellular filamentous fungi, or combination of two or more types of microorganisms, that are introduced into a test medium or onto a specimen to be tested in order to investigate the lability of the medium or specimen to support microbial growth or to investigate its antimicrobial properties.

3.1.3 *inoculum vehicle, n*—the carrier solution used to transport the carrier solution used to transport the inoculum to a given sample or object.

3.1.4 *neutralizing recovery broth, n*—liquid growth media used to inactivate the effects of the test antimicrobial agent.

4. Summary of Test Method

4.1 This method involves inoculation of a molten (45 °C) agar slurry with a standardized culture of bacterial cells.

4.2 A thin layer of the inoculated agar slurry (0.5-1.0 mL) is pipetted onto the test and untreated control material (triplicate samples minimum).

4.3 After the specified contact time (24 h commonly used), surviving microorganisms are recovered via elution of the agar slurry inoculum from the test substrate into neutralizing broth and extracted via methods that provide complete removal of the inoculum from the test article (examples include sonication, vortexing, and/or manual extraction, that is, stomacher).

4.4 Serial dilutions are made, then pour or spread plates are made of each dilution. Agar plates and dilution broths are incubated for 48 ±2 h at a specified temperature dependent upon the optimal temperature for test organism.

4.5 Bacterial colonies from each dilution series are counted and recorded.

4.6 Calculation of percent reduction of bacteria from treated versus untreated samples is made.

5. Significance and Use

5.1 This method can be used to evaluate effectiveness of incorporated/bound antimicrobials in hydrophobic materials such as plastics, epoxy resins, as well as other hard surfaces.

5.2 The aqueous based bacterial inoculum remains in close, uniform contact in a “pseudo-biofilm” state with the treated material. The percent reduction in the surviving populations of challenge bacterial cells at 24 h versus those recovered from a non-treated control is determined.

5.3 The hydrophobic substrate may be repeatedly tested over time for assessment of persistent antimicrobial activity.

6. Apparatus

6.1 *Erlenmeyer Flask,* 250 mL.

6.2 *Petri Dishes,* (15 × 100 mm), sterile.

6.3 *Colony Counter.*

6.4 *Specimen Cups,* (120 mL), sterile or equivalent sterile equipment for extraction.

6.5 *Pipetters,* (1000 µL) positive displacement.

6.6 *Pipette Tips,* sterile.

6.7 *Test Tubes,* 16 × 100 mm.

6.8 *Incubator,* set at required temperature (25-35 ± 2 °C).

6.9 *Autoclave.*

6.10 *Water Bath,* capable of maintaining water at 45 ± 2 °C.

6.11 *Sterile Cotton Swabs.*

6.12 *Sonic Bath,* 47 Khz, cleaning non-cavitating.

6.13 *Vortex Mixer.*

6.14 *pH Meter.*

6.15 *Hot Plate,* with stirrer.

6.16 *Spectrophotometer,* set at 600 nm.

6.17 *Sterile Cuvettes.*

6.18 *Test Materials,* sterile if specified by interested parties.

6.19 *Cell Counting Chamber.*

7. Reagents

7.1 *Media:*

7.1.1 *Tryptic Soy Broth,* or appropriate broth.

7.1.2 *Tryptic Soy Agar,* or appropriate agar.

7.1.3 *Neutralizing Broth,* appropriate for the antimicrobial compound tested.(See Practice E1054.)

7.1.4 *Agar-agar.*

7.1.5 *NaCl.*

7.1.6 *Sterile Deionized Water.*

7.1.7 *Sterile 0.85 % Saline Dilution Blanks,* 9.0 mL in 16 × 100 mm test tubes or appropriate dilution buffer (such as phosphate buffer or Butterfield’s buffer).

7.2 *Test Organisms*—Specific organisms are recommended but choice of organism should be relevant to the environment in which the product will perform.

7.2.1 Gram-positive bacteria *Staphylococcus aureus* ATCC 6538.

[3] 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.

7.2.2 Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 15442 or *Klebsiella pneumoniae* ATCC 4352.

7.2.3 Other microorganisms such as yeast or fungal conidia may also be tested using this procedure. Exposure periods may be modified (up to 96 h) to address more resistant microorganisms.

8. Procedure

8.1 Grow 18 h bacterial cultures (three transfers) at a specified temperature dependent upon the optimal temperature for the test organism in tryptic soy or appropriate broth. These cultures should originate from 18-24 h growth coming from stock culture plates or growth on agar slants.

8.2 Prepare the agar slurry by dissolving 0.85 g NaCl and 0.3 g agar-agar in 100 mL of deionized water. Heat with stirring on a hot plate until the agar dissolves. One agar slurry should be prepared for each organism tested.

8.3 Sterilize the agar slurry by autoclaving for 15 min at 121 ± 2 °C, 15 psi., then equilibrate at 45 ± 2 °C.

8.4 Prepare 3.0 × 3.0 cm square samples of the treated and control test materials (sample minimum of triplicates for "0" h controls, incubation period treated samples and incubation period control samples for each test organism).

8.5 Place each sample into a sterile 15 × 100 mm petri dish.

8.6 Adjust bacterial broth cultures to 1-5×10^8 cells/mL with a spectrophotometer or cell counting chamber.

8.7 Dip a cotton swab into sterile 0.85 % saline (with or without a non-inhibitory levels of a surfactant) and pre-wet the test sample. This will aid in dispersing the agar slurry evenly on the sample.

8.8 Place 1.0 mL of standardized culture (1-5×10^8 cells/mL) into the 100 mL agar slurry equilibrated at 45 ± 2 °C. The final concentration should be 1-5×10^6 cells/mL in the molten agar slurry.

8.9 Pipet 0.5-1.0 mL of inoculated agar slurry onto the test and control samples. Slow, gentle application at a low angle of incidence relative to the sample will aid in formation of a film no more than 1 mm in depth. If the inoculum volume and/or surface area of the sample are modified, the inoculum volume should be adjusted to provide an agar slurry 1 mm in depth over the entire sample surface. The inoculum volume and surface area tested should be reported.

NOTE 2—For substrates found to be extremely hydrophobic, addition of non-inhibitory levels of a surfactant to the agar slurry may also aid in surface wetting properties. Alternatively, the slurry may be cooled to 25 ± 2 °C then applied to the polymer surface. Application of pre-cooled agar slurry has also been shown to aid in the dispersion of the inoculum over an extremely hydrophobic test surface.

8.10 Allow the agar slurry inoculum to gel and then place the samples in an incubator at a specified temperature dependent upon the optimal temperature for the test organism or one which mimics the temperature in which the test substrate will be utilized for the specified exposure period (usually 24 ± 2 h). Low humidity in the incubator can cause drying of the agar slurry inoculum on the samples, therefore relative humidity within the incubator should be at or above 75 %. This can be accomplished with open reservoirs of certain saturated salt in water solutions.[4]

8.11 Make serial dilutions (as described in 8.12 – 8.16) of the agar slurry recovered immediately from "0" h control samples and spread or pour plate each dilution to determine cfu/mL recoverable at time "0 h."

8.12 Following the specified contact time, aseptically remove the incubation period control samples and incubation period treated samples from the petri dishes to 120 mL specimen cups or other suitable container containing a sufficient volume of neutralizing broth to form an initial 1:10 or 1:100 dilution of the original inoculum.

8.13 Place the specimen cups containing the recovered test samples into a non-cavitating sonic bath and sonicate for 1 min.

8.14 Sonication should be followed by 1 min of vigorous mechanical vortexing. This should facilitate the complete release of the agar slurry from the sample. Note that the test surface may be imprint cultured onto tryptic soy agar following sonication and vortexing in order to determine release efficiency of the inoculum from the treated surface.

8.15 Perform serial dilutions of the initial neutralizing broth sufficient to include one dilution beyond the original inoculum (as determined in 8.11).

8.16 Spread or pour plate each dilution into tryptic soy agar or other appropriate agar (for example, Sabourauds agar for fungi) and incubate plates at an optimal temperature for the test organism for 48 ± 2 h.

8.17 Count and record colony numbers for each dilution plate.

9. Calculation

9.1 Determine the geometric mean of the number of organisms recovered from the triplicate incubation period control and incubation period treated samples by the following equation:

$$\text{geometric mean} = \frac{(\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3)}{3} \quad (1)$$

where:

X = number of organisms recovered from the incubation period control or incubation period treated samples.

9.2 *Percent Reduction*—Use the following equation to calculate the percent reduction:

$$\%\ \text{reduction} = \frac{(a - b) \times 100}{a} \quad (2)$$

where:

a = the antilog of the geometric mean of organisms recovered from the incubation period control samples (as determined in 9.1), and

[4] Corey, J. In Beuchat, L.R. ed., *Food and Beverage Mycology*, AVI Publishing Co., Inc., Westport, CT, p. 52.