Designation: E3371 – 22

Standard Test Method for Measuring the Ability of a Synthetic Polymeric Material to Resist Bacterial Adherence[1]

This standard is issued under the fixed designation E3371; 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

Synthetic polymeric materials, used in packaging for food containers, personal care products and other items, may have inherent antimicrobial properties. Others may contain antimicrobial additives. Many methods, such as Test Method E2180 are used to determine quantitative bacterial reductions caused by these additives. Test Method E3151 specifically examines the ability of tubular, yarn and fiber specimens to resist bacterial colonization/adherence. However, these methods do not quantitatively measure the number of culturable bacteria adhering to the flat surfaces of these synthetic polymeric materials, as measured in this test method.

1. Scope

1.1 This method is designed to evaluate (quantitatively) the number of bacteria attached to the flat, two-dimensional surfaces of synthetic polymeric materials and polymeric coatings on various substrates that may or may not contain bound or incorporated anti-adherent agents. The method focuses on assessing the ability of the surface to reduce bacterial attachment. Other microorganisms such as yeast and fungal conidia may be tested using this method.

1.2 This test method quantitatively determines the differences in bacterial adherence seen between synthetic polymeric surfaces that allow bacterial adherence and those that do not, comparing the number of organisms recovered from the control surface to the number recovered from the test specimen surface after the contact time. Knowledge of microbiological techniques is required for these procedures.

1.3 This test method specifies proper methods for measuring the ability of a synthetic polymeric material to resist adherence against specified organism. Due to individual sensitivities, the result of one test organism might not be applicable for other organisms.

1.4 This test method is designed to measure the potential ability to resist bacterial adherence of a non-porous surface compared directly to a polyester control panel known to support bacterial adherence under specific testing conditions.

1.5 Antimicrobial treated non-porous surfaces may demonstrate ability to resist bacterial adherence in this method. This method does not purport to differentiate between anti-adherence and antimicrobial activity nor is it designed to reflect specific end-use or environmental conditions. Any product that demonstrates ability to resist bacterial adherence in this method should be measured for antimicrobial activity using a separate test technique such as Test Method E2180 or ISO 22196.

1.6 The method focuses on assessing the ability of synthetic polymeric materials and polymeric coatings on various substrates to reduce bacterial attachment. The specimen with absorbing or adhesive surfaces may be unable to be disinfected properly before testing, or may trap inoculated organism during recovery process and thus lead to a false result. This method does not apply to specimens with absorbent or adhesive surfaces.

1.7 The values stated in SI units are to be regarded as standard. The values given in parentheses after SI units are provided for information only and are not considered standard.

1.8 *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.*

1.9 *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. Referenced Documents

2.1 *ASTM Standards:*[2]

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E2180 Test Method for Determining the Activity of Incorporated Antimicrobial Agent(s) In Polymeric or Hydrophobic Materials

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E3151 Test Method for Determining Antimicrobial Activity and Biofilm Resistance Properties of Tube, Yarn, or Fiber Specimens

2.2 *ISO Standard:*[3]

ISO 22196 Measurement of Antibacterial Activity on Plastic and Non-Porous Surfaces

2.3 *FDA Manuals:*[4]

Bacteriological Analytical Manual (BAM)

3. Terminology

3.1 *Defintions:*

3.1.1 For definitions of terms used in this test method, refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *anti-adherence, n*—term describing a state where the attachment of bacteria to the surface of products is suppressed or describing the effect of an agent which reduces the ability of bacteria to attach to product surfaces.

3.2.2 *anti-adherent, adj*—term describing a state where the attachment of bacteria to the surface of products is suppressed or describing the effect of an agent which reduces the ability of bacteria to attach to product surfaces.

3.2.3 *ability to resist bacterial adherence, n*—percent reduction, calculated from the number of bacteria recovered from the surface of the test specimen and the reference control after inoculation with bacteria, incubation, saline wash, and quantification of attached organisms.

3.2.4 *antimicrobial, n*—difference in the logarithm of the culturable cell count found on supernatant of an antibacterial-treated product and an untreated product after inoculation with and incubation of bacteria.

3.2.5 *reference control material, n*—synthetic polymeric material, that does not inhibit the attachment of the test bacteria.

3.2.5.1 *Discussion*—This material is commonly referred to as the untreated control and is used for test validation calculations.

4. Summary of Test Method

4.1 A fixed inoculum volume (0.4 mL ± 0.02 mL) at a known concentration is pipetted onto the surface of the test specimens and controls and then incubated under appropriate conditions for the test organism.

4.2 After the specified contact time (24 h), the testing surface is gently washed with sterile saline with the aid of rocker to remove the unattached bacteria.

4.3 The remaining attached bacteria are collected by sonication and then counted.

4.4 Ability of a material to resist bacterial adherence is calculated from the numbers of bacteria recovered from the control and specimen surfaces.

5. Significance and Use

5.1 This method can be used to evaluate the effectiveness of incorporated or bound anti-adherent agents in synthetic polymeric materials and polymeric coatings intended to reduce the attachment of bacteria to the substrate surface.

5.2 The synthetic polymeric substrate surface may be tested repeatedly over time for assessment of persistent ability of a material to resist bacterial adherence.

5.3 This method is to quantify the degree of bacteria colonization of a surface to assess a materials ability to resist bacterial adherence because biofilm formation can contribute to material degradation and malfunction.

6. Apparatus

6.1 Autoclave, capable of producing 103 kPa (15 psi) of steam pressure at 121 °C and maintaining it for a minimum of 15 minutes.

6.2 Balance, capable of weighing to ± 0.01 g.

6.3 Binder clip, 15 mm to 25 mm width, or equivalent.

6.4 Cell counting chamber (optional).

6.5 Clear polyester panels (Leneta – Order #P300-7G or equivalent)

6.6 Colony counter (optional).

6.7 Cotton swabs, sterile.

6.8 Culture tubes, screw top, sterile, or equivalent.

6.9 Cuvettes.

6.10 Forceps, sterile.

6.11 Incubator, set at required temperature (35 °C ± 1 °C).

6.12 Inoculating loops, sterile, 4 mm ring diameter.

6.13 Petri dishes, (15 mm× 100 mm), sterile.

6.14 pH meter, any capable of measuring to 0.2 units.

6.15 Pipettes, (100 µL and 1000 µL) positive displacement.

6.16 Pipette tips, sterile.

6.17 Rocker, with rocking angle of 8° - 10°, and shaking speed of 30 - 50 rpm.

6.18 Round stick, 4 mm in diameter and min 30 mm, or equivalent.

[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] Available from International Organization for Standardization (ISO), ISO Central Secretariat, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, https://www.iso.org.

[4] Available from U.S. Food and Drug Administration (FDA), 10903 New Hampshire Ave., Silver Spring, MD 20993, http://www.fda.gov.

6.19 Sample bag, sterile, with a width of 76 mm and a minimal height of 127 mm, or equivalent.

6.20 Spectrophotometer, set at 600 nm.

6.21 Spiral plater (optional).

6.22 Synthetic polymeric cover film, 40 mm × 40 mm. Film shall not affect bacterial growth or absorb water. (Polyethylene film that is 0.05 mm – 0.1 mm thick is recommended. Stomacher bag or Whirl-Pac bag film or (equivalent) has been found to be acceptable).

6.23 Test materials, sterile if specified by interested parties.

6.24 Ultrasonic cleaner, any capable of a watt density output of 100 watts per gallon to 133 watts per gallon at a frequency of 42 kHz ± 6 %.

6.25 Vortex mixer.

7. Reagents and Materials

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall be understood to mean distilled water or water of equal purity.

7.3 *Media:*

7.3.1 Liquid Growth Medium, sterile (See Annex A1).

7.3.2 Solid Growth and Plating Medium, sterile (See Annex A1).

7.3.3 Inoculation Medium, 1/500 Nutrient Broth, (pH 7.0 ± 0.2), prepared as described in A1.2. Dilute the nutrient broth with distilled or deionized water to a 500- fold, dispense into suitable flasks or tubes prior to autoclaving.

NOTE 1—Alternate neutralizing agents may be required and dependent upon the active agents may be present in the specimens being tested.

NOTE 2—Media formulas are from the FDA's Bacteriological Analytical Manual (BAM). Other low-nutrient equivalent formulations may be used but should be stated in the test report.

8. Test Organism

8.1 *Bacteria*—Gram-positive bacteria *Staphylococcus aureus* ATCC 6538.[6]

8.2 Cultures of the test organism shall be maintained using appropriate microbiological practices.

NOTE 3—Other bacteria may be tested, as agreed upon by all parties involved. The suggested bacteria include *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538P and *Staphylococcus epidermidis* ATCC 12228.

NOTE 4—Other microorganisms such as yeast or fungal conidia may also be tested using this procedure. If other species are used, the species and the reason for their use should be documented in the test report. If the test specimens are to be challenged with a combination of organisms, no more than 3 organisms should be evaluated at one time.

9. Procedure

9.1 *Preparation of Test Specimens:*

9.1.1 Testing shall be performed on at least three specimens from each untreated and treated test material. Include the three polyester panels for the reference control material. When testing a series of anti-adherent treatments for a single polymer, all anti-adherent specimens must be compared to the untreated reference control specimens. All tests are conducted at the same time using the same test inoculum.

NOTE 5—An untreated control of the same material is often not available, or if it is, may have some inherent properties that inhibit bacterial attachment. Therefore, the reference control listed in this method is required and used in the calculations. If calculations are performed against the submitted untreated control, this should be stated in the test report.

9.1.2 Prepare flat 50 mm ± 2 mm × 50 mm ± 2 mm specimens of the treated and untreated test materials. Specimens should be of uniform thickness. Unless otherwise specified, the standard size of the plastic cover film shall be a square of 40 mm × 40 mm for the 50 mm × 50 mm test specimen. If the test specimen is not of a standard size, then the size of the plastic cover film shall be reduced in direct proportion. Do not, however, reduce the size of film to less than 900 mm^2 and the edges of the cover film shall always be 2.5 mm to 5.0 mm inside the edge of the test specimen on all sides. If the size of the cover film differs from 40 mm × 40 mm, the actual size used shall be stated in the test report.

NOTE 6—If it is difficult or impossible to cut the product into a square of this size, then test specimens of other sizes and shapes may be used, as long as they can be covered with a plastic cover film of surface area between 900 mm^2 and 1600 mm^2. It is preferable to prepare test specimens from the final product itself. However, if the shape of the product prevents this, then the test specimens may be prepared in a format suitable for testing using the same raw materials and processing methods as are normally used for the product. If the test specimen differs from the 50 mm × 50 mm square dimensions, the actual dimensions used shall be stated in the test report.

NOTE 7—When preparing specimens, take care to avoid contamination with microorganisms or extraneous organic debris. Similarly, do not allow specimens to contact each other. Test specimens may be cleaned/disinfected/sterilized prior to testing (for example, by wiping with 70 % ethanol in water or exposure to 30 minutes of UV light) as agreed upon between all parties involved and stated in the test report.

NOTE 8—Cleaning of the test specimen can cause softening; dissolution of the surface coating or elution of components so should be avoided. If cleaning is required due to gross contamination, the cleaning method shall be stated in the test report.

9.2 *Preparation of test inoculum:*

9.2.1 Using a sterile inoculating loop, transfer bacteria from the stock culture to a sterile Petri dish with Nutrient Agar and incubate at 35 °C ± 1°C for 18 h to 24 h. From this culture, use a sterile inoculating loop to transfer bacteria onto fresh Nutrient Agar and incubate at 35 °C ± 1 °C for 18 h to 20 h. Using a sterile inoculating loop or cotton swab, suspend the

[5] *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.

[6] ATCC is a registered trademark and ATCC 6538 is a trademark of American Type Culture Collection, Manassas, VA.

grown organism in 1/500 NB to target 5×10^6 CFU/mL ± 0.5 log concentration of bacteria. A spectrophotometer and dilution in 1/500 NB may be used. The maximum number of sub-passages shall be no greater than 3 from freeze or stock plate.

NOTE 9—State in the report if different concentrations of inoculum are used.

9.2.2 Determine the exact concentration of the inoculum upon inoculation of test specimens by pour plate method using molten Plate Count Agar.

NOTE 10—If the test inoculum is not used immediately, then chill it on ice (0 °C) and use it within 2 h of preparation.

9.3 *Inoculation of Specimens:*

9.3.1 Place each prepared specimen into a separate sterile Petri dish with the test surface facing up. Pipette 0.4 mL of the test inoculum prepared in 9.2 onto the test surface. Cover the inoculum with a sterile 40 mm × 40 mm plastic cover film. Make sure that the test inoculum does not leak beyond the edges of the plastic cover film. After the specimen has been inoculated and the plastic cover film applied, replace the lid of the Petri dish.

9.3.2 The volume of inoculum used shall be adjusted to be proportional to the area of the cover film used and recorded in the test report. It is essential that the test inoculum does not leak beyond the edges of the cover film. For some surfaces (for example, those that are very hydrophilic), it might be difficult to prevent such leakage. When this occurs, reduce the volume of test inoculum applied to the test surface. Do not use less than 0.1 mL of test inoculum. When the volume of test inoculum is decreased, the concentration of the bacterial cells in the inoculum shall be increased to provide the same number of bacterial cells as when the normal volume of test inoculum is applied.

9.4 *Incubation of the inoculated specimens:*

9.4.1 Unless otherwise specified, incubate the Petri dishes containing the inoculated test specimens at a temperature of 35 °C ± 1 °C and a relative humidity of not less than 85 % for 24 h ± 1 h. Other temperatures may be used if agreed upon by all parties. If a temperature other than 35 °C ± 1 °C is used, it shall be included in the test report.

NOTE 11—If incubation temperatures of less than 35 °C are used, the total count of the culturable bacteria may be reduced and affect the ability of a material to resist bacterial adherence compared to measurements conducted using a 35 °C incubation temperature.

9.5 *Recovery of bacteria from specimens after contact time:*

9.5.1 Immediately after the contact time, remove the cover films from all specimens with sterile forceps. All specimens should be washed as follows: *(1)* hold them up and fill 20 mL of 0.9 % saline into their original Petri dish, then place them back into the pre-filled Petri dish with their testing surfaces downward; *(2)* Transfer the covered Petri dishes onto a rocker with a shaking speed of 40 rpm for 60 s to wash off the nonadherent bacteria; *(3)* Use sterile forceps to hold up each specimen, gently tap the specimen against the edge of the Petri dish to remove any droplets, then discard the liquid. Repeat this washing process two additional times.

NOTE 12—A specimen whose density is close or higher than that of 0.9 % saline may sink to the bottom of the Petri dish, in which case the specimen should be placed with the testing surface upward in the Petri dish.

9.5.2 Right after the process mentioned in 9.5.1, transfer specimens into sterile sample bags and fill in 40 mL 0.9 % saline each. The sample bags should be then sealed properly to ensure the entire specimen to be fully immersed into the filled saline. The properly sealed sample bags should be then transferred to an ultrasonic cleaner, followed by sonication for 5 mins, then vigorous agitation for another 1 min. Carefully remove the test specimens from the sample bags using sterilized forceps. The process is shown in Fig. 1. Determine the titers of bacteria within the recovered 0.9 % saline solution using the pour plating or spiral plating technique. Following incubation, count the colonies and convert the total count into culturable bacteria per cm^2 (of testing area).

NOTE 13— If an alternative dimension of sterile sample bag is used and a different volume of 0.9% saline is applied, the volume of saline should be enough to ensure the entire specimensample is fully immersed into the filled saline. Same volume of saline should be applied to each sample bags and should be stated in the testing report.

NOTE 14—If there is concern regarding whether adherent bacteria were adequately removed from the surface of the specimens after completion of steps in 9.5.2, several techniques can be used prior to disposing of the test specimens. One technique to assess the adequacy of removal of adherent bacteria is to wash the test specimen with saline, similar to what was performed in step 9.5.1, place the specimen to the surface of tryptic soy agar in a Petri dish and lightly press the specimen for 10 s, then carefully take out the specimen, incubate the plates at 35 °C ± 1 °C for 24 h, and assess the number of culturable organisms. The number of colonies present on the plate provides a semiquantitative assessment of whether adherent bacteria were harvested adequately. Another technique is to view surfaces of the test specimen microscopically for biofilm removal. Extra

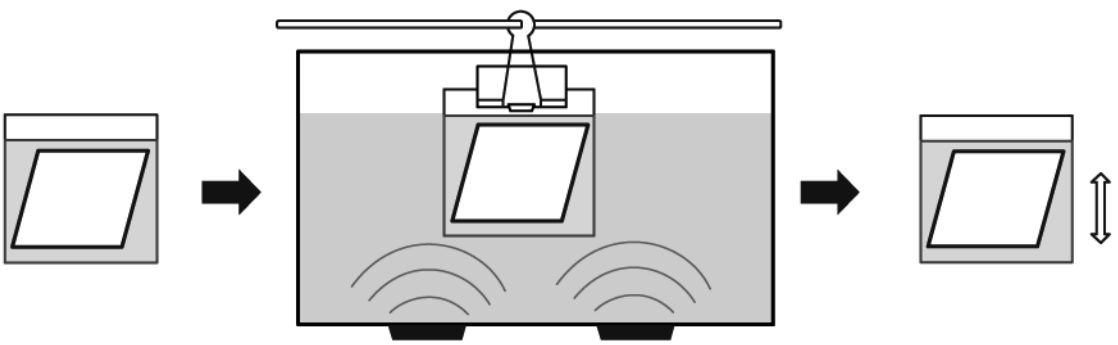

FIG. 1 Process Flow Chart for Collection of Bacteria