Designation: E3151 – 18

Standard Test Method for Determining Antimicrobial Activity and Biofilm Resistance Properties of Tube, Yarn, or Fiber Specimens[1]

This standard is issued under the fixed designation E3151; 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 is designed as an *in vitro*, quantitative assay to evaluate the antimicrobial activity of specimens with tubular geometries or small segments of yarn or fibers that have been treated with an antimicrobial agent. Further, the method was designed to provide a quantitative assessment of a specimen's ability to resist microbial colonization and subsequent biofilm formation relative to an untreated control specimen.

1.1.1 The difference in number between the planktonic microbial population recovered from the treated test specimen and the population recovered from the control test specimen is the measure of the antimicrobial activity.

1.1.2 The measure of the ability of the treated test specimen to resist biofilm development is the difference between the adherent microbial population recovered from the treated test specimen and the adherent microbial population recovered from the control test specimen.

1.2 Testing is to be performed by individuals trained in microbiological techniques under appropriately controlled conditions to ensure the integrity of results and personnel safety.

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

1.4 *This standard may involve hazardous materials, operations, and equipment. 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.*

1.5 *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]

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

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

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

4. Summary of Test Method

4.1 The control and treated test specimens are placed into individual 6 × 50 mm culture tubes containing suspensions of a known biofilm-producing strain of *Staphylococcus epidermidis* (ATCC 35984[3]) at a specific titer and incubated at 35 ± 2 °C with mild agitation for 24 ± 2 h.

4.2 After the contact time, each specimen is transferred to an individual centrifuge tube containing a sterile buffered saline rinse solution, and the tube is sealed and carefully inverted several times to remove any non-adhered or loosely adhered bacteria.

4.3 The specimens are then transferred to new centrifuge tubes containing low concentrations of a surfactant dispersed in a neutralizing agent demonstrated to deactivate the antimicrobial agent with which the test specimen is treated.

4.4 These tubes are sealed, vortexed, and sonicated to suspend any bacteria adhered to the surface of the specimens and to disaggregate any biofilm clumps present.

4.5 The population of planktonic bacteria within the test inoculum exposed to each test specimen and the re-suspended

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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.

[3] ATCC is a registered trademark and ATCC 35984 is a trademark of American Type Culture Collection, Manassas, VA.

adherent bacteria harvested from the surface of each test specimen are enumerated using standard microbiological techniques.

4.6 The efficacy of the antimicrobial treatment versus the planktonic bacteria recovered in the neutralized inocula suspension and the adherent bacteria recovered from the surface of the specimens is the percent and $\log_{10}$ reductions calculated as the difference between populations from treated specimens and those from the controls.

5. Significance and Use

5.1 Although a number of standardized tests currently exist for assessing the antimicrobial activity of treated polymers and textiles, these are optimized for specimens that readily absorb the test inoculum or that have a flat surface on which the inoculum can be placed, and their use for specimens with tubular geometries or for small quantities (less than 0.5 g) of yarns or fibers requires significant manipulation of the specimen.

5.2 To adapt these methods for evaluating tubes, fiber, and yarn specimens requires distorting tubular specimens to create a flat surface or using unacceptably large quantities of fiber or yarn specimens. Rendering a test specimen having tubular geometry to a flat surface will limit its surface area available for exposure during the test and may require dissection of the specimen, which unacceptably alters it from its original state. Testing of treated fiber and yarn specimens using available standardized methods typically requires large quantities of material (greater than 0.5 g) that may not be available. In both cases, such manipulations may result in misleading results that do not reflect the antimicrobial efficacy of an unmodified specimen.

5.3 This method provides an environment in which the inoculum remains in intimate contact with the surfaces of these types of test specimens, exposing both the intra- and extraluminal surfaces of tubular specimens without significant modification, and requiring only small quantities of fibers or yarns to perform testing.

5.4 Classical antimicrobial test methods generally quantify the population or concentration of microorganisms that survive exposure to specimens treated with an antimicrobial agent without distinguishing whether the surviving microorganisms were in a planktonic or adhered/biofilm state.

5.4.1 The phenotypic behavior of bacteria in the biofilm state differs substantially from when they are in the planktonic state, especially with respect to susceptibility to disinfectants, sanitizers, and antimicrobial agents. Therefore, evaluating the ability of a material's surface to resist bacterial colonization may be of equal or greater significance than its efficacy versus planktonic bacteria.

5.4.2 This method not only can assess the population of the challenge species that survives planktonic exposure to the test specimen, but also can then compare that to the population that survives in an adherent/biofilm state.

5.5 This test method is a batch-based system in which test specimens are exposed to a continuous, minimal fluid shear environment in the presence of the challenge inoculum. The appropriateness of this simulated environment relative to the intended end-use of the test material should be evaluated prior to testing.

5.6 Although this method is designed to provide an initial assessment of the antimicrobial activity exhibited by a material and its ability to resist microbial colonization under very specific test parameters, these conditions may not be representative of all environments to which the specimen may be exposed during its intended end-use. Various test parameters specified in this method can be modified to evaluate a material under conditions that may better simulate end-use environments, but such alterations of the method must be clearly described when reporting results.

6. Apparatus

6.1 *Autoclave (steam sterilizer),* any suitable for processing culture media, reagents, and labware.

6.2 *Biological safety cabinet*.

6.3 *Incubator,* any capable of maintaining a temperature of 35 ± 2 °C.

6.4 *pH meter,* any capable of measuring to 0.2 units.

6.5 *Vortex mixer*.

6.6 *Orbital shaker,* any capable of maintaining 100 rotations per minute (rpm).

6.7 *Refrigerator,* any capable of maintaining 4 ± 2 °C for storage of media, culture plates, and reagents.

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

6.9 *Timer (stopwatch),* one that displays hours, minutes, and seconds.

6.10 *Dissecting forceps,* fine tip.

6.11 *Scissors or Razor blade*.

6.12 *Pipette pumps,* 1- to 10-mL and 1- to 25-mL capacity.

6.13 *Serological pipettes,* sterile reusable or single-use pipettes of 10.0- and 25-mL capacity.

6.14 *Pipette and appropriate sterile pipette tips,* variable volume, positive displacement, 20- to 200-µL volume range.

6.15 *Pipette and appropriate sterile pipette tips,* variable volume, positive displacement, 100- to 1000-µL volume range.

6.16 *Petri dishes,* sterile, 15 × 100 mm.

6.17 *Microcentrifuge tubes,* sterile, 1.7 mL.

6.18 *Centrifuge tubes with caps,* sterile, 15 mL.

6.19 *Centrifuge tubes with caps,* sterile, 50 mL.

6.20 *Culture tubes,* sterile, 6 × 50 mm.

6.21 *Culture tubes and closures,* sterile, any with a minimum volume capacity of 10 mL and a minimum diameter of 16 mm. Recommended size is 16 × 125-mm borosilicate glass.

6.22 *Inoculating loops,* sterile, 4-mm ring diameter.

6.23 *Water absorbent laboratory wipe,* sterile.

6.24 *Sealing film,* paraffin or equivalent.

6.25 *Dilution vessels.*

7. Reagents and Materials

7.1 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.[4] Other grades may be used, provided it is pure enough to be used without lessening the accuracy of the determination.

7.2 *Growth Media:*

7.2.1 *Liquid Growth Medium,* Tryptic Soy Broth (TSB), sterile (See Annex A1).

7.2.2 *Solid Growth and Plating Medium,* Tryptic Soy Agar (TSA), sterile (See Annex A1).

7.2.3 *Inoculation Medium,* 1/500 Tryptic Soy Broth.

7.2.3.1 Aseptically add 1.0 mL of TSB (7.2.1) to 499 mL of distilled or deionized water (7.6).

7.2.3.2 Adjust the pH to a value between 6.8 and 7.2 using either sodium hydroxide or hydrochloric acid.

7.2.3.3 Sterilize by autoclaving.

7.2.3.4 If not used immediately after preparation, the inoculation medium can be stored at 4 ± 2 °C for no longer than 7 days.

7.3 *Phosphate Buffered Saline,* sterile.

7.4 *Neutralizing Solution,* appropriate for neutralizing the active antimicrobial agent in the treated test specimen (See Test Method E1054).

7.5 *Modified Neutralizing Solution,* Neutralizing Solution (7.4) with 1 % by volume Polysorbate 80.

7.6 *Distilled or Deionized Water,* sterile.

7.7 *Dilution Fluid or Diluent,* sterile water, sterile saline, sterile buffered phosphate diluents or equivalent.

8. Test Organism

8.1 *Staphylococcus epidermidis*, American Type Culture Collection (ATCC) No. 35984.[3]

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

NOTE 1—To ensure the most consistent and accurate results, the purity of the cultures should be checked regularly using standard microbiological speciation techniques.

NOTE 2—Additional challenge species can be substituted to evaluate the breadth of a material's antimicrobial activity versus species to which it may be exposed. If an alternative challenge species is used in testing, it must be identified in the final report, along with any accommodative modifications made to the method (that is, changes to culture media, buffers, etc.). The test report also must indicate that ASTM Method E3151, modified, was used. The precision statistics reported in this method will not apply.

[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.

9. Preparation of Bacterial Inoculum

9.1 Grow a fresh 18- to 24-h shake culture of *S. epidermidis* (8.1) in sterile broth growth medium (7.2.1) at 35 ± 2 °C prior to performing the test.

9.1.1 Prepare these cultures from an 18- to 24-h growth from stock culture plates or agar slants.

9.2 Dilute this stock suspension of bacteria appropriately using inoculation medium (7.2.3) to achieve a final bacterial titer between 1.0×10^5 colony forming units (CFU)/mL and 5.0×10^5 CFU/mL with the target being 3.0×10^5 CFU/mL.

9.3 Centrifuge the diluted stock suspension to harvest the bacterial cells.

9.3.1 Centrifuge for 5 min at 15294 × g.

9.4 Remove at least 90 % of the resulting supernatant.

9.5 Reconstitute the pelletized cells in a volume of the inoculation medium (7.2.3) equal to the volume of supernatant removed.

9.5.1 This bacterial suspension is the test inoculum.

9.5.2 The test inoculum is to be used within 2 h of preparation.

9.6 Verify the titer of bacteria in the test inoculum by performing serial dilutions and utilizing a validated microbial enumeration technique (for example, pour plate, spread plate, spiral plate, or membrane filtration).

9.6.1 Report the bacterial titer in the test inoculum in the final report. If the bacterial titer of the test inoculum does not fall within the range of 1.0×10^5 CFU/mL to 5.0×10^5 CFU/mL, the test is considered invalid and the specimen must be retested.

NOTE 3—Alternative inoculum bacterial titers and inoculum media can be substituted to better simulate the end-use conditions. If so, all modifications must be documented in the test report and the test report must indicate that ASTM Method E3151, modified, was used. The precision statistics reported in this method will not apply.

NOTE 4—A nonionic surfactant may be added to the test inoculum to improve the wetting of hydrophobic specimens. Surfactants should be shown through prior testing not to cause a change in the bacterial population at the intended use-concentration. If used, the chemical name of the surfactant and its final concentration in the test inoculum must be documented in the test report. The test report also must indicate that ASTM Method E3151, modified, was used. The precision statistics reported in this method will not apply.

NOTE 5—In Steps 9.2 and 9.3, the stock suspension can be centrifuged prior to dilution to the target bacterial titer. An alternative validated approach for the harvesting of bacterial cells using centrifugation may be used, but the conditions of use (relative centrifugal force and duration of centrifugation) must be documented in the test report and the test report must indicate that ASTM Method E3151, modified, was used.

10. Test Specimen Preparation

10.1 A non-treated control specimen of composition and construction closely similar to the antimicrobially treated specimen(s) must be evaluated at the same time and under the same conditions as the treated specimen(s).

10.2 Cut test specimens of each sample being examined to create segments 40 mm in length.

10.2.1 Prepare three replicate specimens of each treated sample that is to be tested to help reduce variability in the test results.

10.2.2 Prepare three replicate specimens of the non-treated control sample for evaluation.

10.2.3 When preparing the test specimens, take care to avoid contamination with microorganisms or other contaminants that may impact the test results.

10.2.4 When testing a specimen composed of absorbent materials, the specimen must not absorb more than 0.2 mL of the challenge inoculum.

NOTE 6—This is to ensure that a volume of inoculum remains in the culture tube after the test specimen has been removed sufficient to determining the planktonic population that survived exposure.

10.2.5 Test specimens with a diameter greater than 3 mm should not be examined using this test.

NOTE 7—This is to ensure the sample is of a size that can be easily placed into and removed from the 6 × 50 mm culture tube used to expose the test specimen to the inoculum.

NOTE 8—If necessary, specimens can be sterilized after being cut to reduce the potential for contaminative microorganisms impacting the results of the test. A sterilization technique that does not affect the material composition of the specimens must be used. If test specimens are sterilized, the sterilization technique used must be documented in the test report.

11. Inoculation and Incubation of Test Specimen

11.1 *For Tubular Specimens:*

11.1.1 Transfer a 0.3-mL aliquot of test inoculum (9.5) to a sterile glass culture tube (6.20). Prepare a separate tube of inoculum for each specimen being examined.

11.1.2 Carefully place test specimens into the culture tubes of test inoculum.

11.1.3 Pipette additional 0.2-mL aliquots of the test inoculum (9.5) directly into the lumina of the tube specimens to ensure that both the outer surfaces and the luminal cavities are exposed to the test inoculum for the time of exposure.

11.1.4 The total volume of inoculum to which each specimen is exposed during the test is 0.5 mL.

11.1.4.1 If lumina cannot be filled due to the hydrophobic nature of the material, testing must be restarted using an inoculum prepared with a nonionic surfactant (Note 4).

11.1.5 Stretch a piece of paraffin sealing film (6.24) approximately 50 × 50 mm over the top of each culture tube to seal the tube for the duration of the exposure period.

11.2 *For Yarn and Fiber Specimens:*

11.2.1 Transfer a 0.5-ml aliquot of test inoculum (9.5) to a sterile glass culture tube (6.20). Prepare a separate tube of inoculum for each test specimen being examined.

11.2.2 Carefully place test specimens into the culture tubes of test inoculum.

11.2.3 Stretch a piece of paraffin sealing film (6.24) approximately 50 × 50 mm over the top of each culture tube to seal the tube for the time of exposure.

11.3 Incubate the inoculated test specimens at 35 ± 2 °C for 24 ± 1 h with mild agitation provided by an orbital shaker set to 100 rpm.

NOTE 9—Cases in which the outer diameter of the specimen is very small relative to the inner diameter of the culture tube may require that additional 40 mm segments of the sample be used to achieve an inoculum volume to sample mass, or sample surface area, ratio that approximates the environment in which the end product may be used. In such cases, the same number of segments of the non-treated control specimen and the antimicrobially treated specimen must be tested. The number of segments evaluated during testing must be documented in the test report.

NOTE 10—Various exposure periods may be used to evaluate the activity of the treated test specimen at time points relevant to use patterns of products in which the treated test specimen may be used. The exposure period used during testing must be documented in the test report. The test report also must indicate that ASTM Method E3151, modified, was used. The precision statistics reported in this method will not apply.

12. Recovery of Planktonic Bacteria from Test Inocula

12.1 Following the exposure period, use sterilized forceps to transfer each test specimen into a separate 50-mL centrifuge tube (6.19) containing 40 mL of sterile phosphate buffered saline (7.3).

12.1.1 Immediately after removing tubular specimens from the culture tubes, orient them above the culture tube such that the end of the specimen is above the mouth of the tube, and use a pipettor with a sterile tip to blow any liquid within the lumen into the culture tube. This step is not necessary for yarn and fiber specimens.

12.1.2 Carefully tap the end of the test specimen onto a sterile laboratory wipe (6.23), or other sterile absorbent material, to remove excess inoculum before transferring test specimens to the 50-mL centrifuge tubes containing 40 mL of sterile phosphate buffered saline. Set aside the 50-mL centrifuge tubes containing the test specimens in preparation for step 13.1.

12.2 Use sterile Pasteur pipettes with bulbs to transfer the entire volume of inocula remaining in the culture tubes into separate sterile microcentrifuge tubes, one microcentrifuge tube for each culture tube, and vortex the microcentrifuge tubes for 20 s.

12.3 Transfer 0.2-mL aliquots from the microcentrifuge tubes to separate sterile 15-mL centrifuge tubes containing 5.0 mL of the modified neutralizing solution (7.5).

12.4 Vortex the centrifuge tubes for 20 s and then maintain them at 4 ± 2 °C until after completion of step 13.2.

12.5 Perform steps 12.5.1 – 12.5.3 either immediately before or after step 13.3.

12.5.1 To ensure the disaggregation of any biofilm clumps present within the neutralized inocula, it is subjected to a combination of vortexing and sonication similar to the process performed on the test specimen to remove adherent bacteria in step 13.3.

12.5.2 Vortex the centrifuge tubes containing the neutralized inocula for 1 min each, followed by sonication for 5 min, and vortexing for an additional 1 min each.

12.5.3 Determine the titer of planktonic bacteria that survived exposure to the test specimens using techniques outlined in Section 14.

13. Recovery of Adherent Bacteria from Test Specimens

13.1 At the conclusion of step 12.4, slowly invert the 50-mL centrifuge tubes containing the test specimens (12.1.2) five times to remove any non- or loosely adherent bacterial cells from the surfaces of the test specimens.