



Designation: E3435 – 24

Standard Practice for Testing Antimicrobial or Disinfectant Efficacy Against Biofilms Grown on a Medical Device or Surface by the Biofilm Surface Test Protocol (BSTP)¹

This standard is issued under the fixed designation E3435; 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 The Biofilm Surface Test Protocol (BSTP) is a high-throughput screening approach used to determine the antimicrobial activity of either liquid agents or agents impregnated, coated, or otherwise incorporated into a medical device or surface. This practice may also be used to evaluate surface-modified devices that contain no antimicrobial agent. A key feature of this practice is the growth and challenge of biofilms on a relevant surface of interest, as opposed to a predetermined material such as polystyrene (see, for instance, Test Method E2799).

1.2 The BSTP incorporates key salient features to provide a simple, robust challenge of the device/surface while simulating the real-world environment to which the device will be exposed. This practice mimics the host environment (for example, by pre-conditioning the device/surface with a relevant medium, such as serum, artificial urine, or artificial mucous), the scope of organisms to which the device will be exposed (for example, clinical isolates), and the conformation of the device as it will be used and presented to the challenge organisms in a clinical or environmental setting (for example, if desired, testing can evaluate both the intraluminal and extraluminal surface of a catheter or be limited to the extraluminal surface). Variations of this assay have been correlated to *in vivo* models and human clinical trials (1-3).² Corrosion, encrustation, or biofouling of devices or surfaces can also be evaluated using this practice.

1.3 This practice is versatile and can be used for growing and evaluating biofilms of many different organisms, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Staphylococcus*

aureus, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Clavibacter michiganensis*, *Fusarium* sp. *Verticillium dahlia*, and *Botrytis cinerea* (see Refs 4-8). Other examples from unpublished studies include *Salmonella enterica*, *Proteus mirabilis*, and *Listeria monocytogenes*. Appropriate modifications to the practice may be required when testing organisms not specified herein.

1.4 Validation of disinfectant neutralization can be included as part of the assay.

1.5 This practice describes how to sample a biofilm and quantify viable cells. Biofilm population density is recorded as log₁₀ colony forming units per device. To test for any antimicrobial activity due to leaching, suspended bacterial population density is reported as log₁₀ colony forming units per volume. Efficacy is reported as the log₁₀ reduction of viable cells. Alternatively, or additionally, qualitative or semi-quantitative assessments can be utilized, such as colorimetric staining, microscopy, or turbidimetric assessment of the minimal biofilm eradication concentration (MBEC; see Test Method E2799).

1.6 This practice should be performed only by those trained in microbiological techniques.

1.7 It is the responsibility of the investigator to determine whether Good Laboratory Practices (GLPs) are required and to follow them when appropriate.

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

1.9 *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.*

1.10 *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 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.

Current edition approved Oct. 1, 2024. Published October 2024. DOI: 10.1520/E3435-24.

² The boldface numbers in parentheses refers to the list of references at the end of this standard.

2. Referenced Documents

2.1 ASTM Standards:³

E1054 Practices for Evaluation of Inactivators of Antimicrobial Agents

E2721 Practice for Evaluation of Effectiveness of Decontamination Procedures for Surfaces When Challenged with Droplets Containing Human Pathogenic Viruses (Withdrawn 2024)⁴

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E2799 Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay

E3321 Test Method for Intraluminal Catheter Model used to Evaluate Antimicrobial Urinary Catheters for Prevention of *Escherichia coli* Biofilm Growth

3. Terminology

3.1 Definitions:

3.1.1 For definitions of terms used in this practice, refer to Terminology **E2756**.

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *plate, n*—an 86 mm by 128 mm standard plate consisting of twelve (12) identical wells.

3.2.2 *test article, n*—biofilm sample surface with or without antimicrobial properties.

3.2.2.1 *Discussion*—Test article dimensions may vary as described in **Annex A1**.

3.2.3 *test article lid, n*—an 86 mm by 128 mm plastic surface capable of mounting up to twelve (12) separate test articles.

3.2.4 *well, n*—small reservoir with a 5 mL maximum working volume capacity.

3.3 Acronyms:

3.3.1 *AUM*: artificial urine medium

3.3.2 *BGM*: biofilm growth medium

3.3.3 *BSA*: bovine serum albumin

3.3.4 *BSTM*: biofilm surface test materials device

3.3.5 *BSTP*: biofilm surface test protocol

3.3.6 *CFU*: colony forming unit

3.3.7 *D/E*: Dey/Engley neutralizing broth

3.3.8 *df*: dilution factor

3.3.9 *ET tube*: endotracheal tube

3.3.10 *OSA*: organism specific agar

3.3.11 *OSB*: organism specific broth

3.3.12 *RPM*: revolutions per minute

3.3.13 *SC*: sterility control

3.3.14 *TSA*: tryptic soy agar

3.3.15 *TSB*: tryptic soy broth

3.3.16 *UC*: untreated control

³ For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at www.astm.org/contact. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

⁴ The last approved version of this historical standard is referenced on www.astm.org.

4. Summary of Practice

4.1 The assay device⁵ consists of a plastic lid that accommodates the attachment of a test article and a corresponding receiver plate with an appropriate number of individual wells (typically 12, though other multi-well configurations can be used depending upon the size of device to be tested). The wells are filled with growth medium, and biofilm is established on the surface of the test article. The surface-associated biofilms cultured on the lid of the Biofilm Surface Test Materials (BSTM) Device⁶ can then be conveniently transferred to subsequent receiver plates for treatment, rinse, neutralization, and recovery. This practice can assess the population of the challenge species that survives planktonic exposure to the test article as well as the population that survives in an adherent/biofilm state.

4.2 This practice describes how to evaluate and quantitatively measure the efficacy of antimicrobial liquids or coatings on medical device surfaces using the BSTP. The test article (that is, catheter, ET tube, stent tubing, or material coupon) is attached to the lid of the device. Tubing is attached either in the shape of a "U" (to test only the extraluminal surface) or at one end only (to test both the intraluminal and extraluminal surfaces). Optionally, the test articles can be pre-treated at this point (for example, to test the effects of encrustation or to monitor corrosion) by exposing them to a relevant medium (for example, serum or AUM) in the wells of a receiver plate.

4.3 A known inoculum containing the species to be tested is placed into the wells of a receiver plate, leaving certain wells empty (or adding sterile medium) to serve as sterility controls. A mature biofilm is then established on the test articles under batch conditions (that is, no flow of nutrients into or out of an individual well), with low shear produced by the gentle rotation of the device on an orbital shaker. The density of the inoculum in each well is determined at this point, which allows the confirmation of growth and – if applicable – quantitation of any antimicrobial effects, due to leaching chemistry, on the planktonic cells.

4.4 After a desired growth time (typically, 1 day to 3 days), the test surfaces (attached to the device lid) containing the biofilm are rinsed to remove planktonic cells. If testing a liquid antimicrobial or disinfectant, the lid is placed into a receiver plate containing test and control solutions at this point and incubated for the desired contact time, before rinsing again and placing the lid in a receiver plate filled with a neutralizing recovery medium. If testing an antimicrobial surface/coating, the biofilm growth time was also the contact time, in which case the lid is rinsed and placed into a receiver plate filled with

⁵ The sole source of supply for detailed instructions for the custom manufacturing of the apparatus, or purchasing pre-made apparatus (that is, pegged or slotted lids to accommodate test article attachment) known to the committee at this time is BSTMLab (Alberta, Canada). 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.

⁶ Biofilm Surface Test Materials (BSTM) Device is a registered trademark of BSTMLab.

a neutralizing recovery medium. The entire device is then placed in a sonicator to remove the biofilm and disaggregate the clumps.

4.5 Finally, samples from each well are diluted, plated, and the viable cells enumerated. The log reduction in viable cells is calculated by subtracting the mean $\log_{10}$ density for the coated (or treated) devices from the $\log_{10}$ density of the uncoated (or control) devices. Alternatively, biofilms can be assessed by semi-quantitative or qualitative approaches, such as turbidity of the recovery medium or a visual or microscopic examination for staining or biofouling.

5. Significance and Use

5.1 Vegetative biofilm bacteria are phenotypically different from suspended planktonic cells of the same genotype (9). Biofilm is the etiological agent of many implant and device-related infections and, once established, microorganisms in biofilm can be up to 1000 times more tolerant to antibiotic therapy.

5.2 Biofilm growth reactors are engineered to produce biofilms with specific characteristics. Altering either the engineered system or operating conditions will modify those characteristics. The goal in biofilm research and efficacy testing is to choose the growth reactor that generates the most relevant biofilm for a particular study (10). Common systems used to grow biofilms in laboratories include flow cells, drip flow reactors, spinning-disk reactors, and tube biofilm reactors. These specialized models have several advantages, including growth of biofilms to high population densities and controlled fluid dynamics. The purpose of this practice is to direct a user in how to grow, treat, sample, and analyze a biofilm on the surface of virtually any medical device or material of interest, using the more simplistic BSTP.

5.3 The BSTP was originally designed as a rapid and reproducible assay for evaluating biofilm growth on surfaces (9 and 11). The design allows for the simultaneous evaluation of multiple parameters—such as testing multiple disinfectants or multiple concentrations of the same disinfectant, testing multiple surfaces (for example, coated versus uncoated), multiple challenge organisms, various growth media, growth/challenge times, and so on—making it an efficient screening tool. In addition to the throughput afforded by the use of a multi-well plate, the BSTP allows for extremely facile rinses, media changes, challenges, and recovery steps by virtue of immobilizing the test devices/surfaces to the lid of the multi-well plate. Moreover, the BSTP requires no specialized reactors or equipment and, as it does not depend upon continuous flow, uses relatively small volumes of media and other reagents.

6. Apparatus

6.1 *Appropriate Glassware*—As required to generate media and agar plates.

6.2 *Bath Sonicator*—Any capable of an average sonic power of 180 W in a dry environment (12).

6.3 *Biosafety Cabinet*—Class I, II, or III rated.

6.4 *Colony Counter*—Any one of several types may be used. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.5 *Conical Tubes*—Sterile, any with a volume capacity of 50 mL.

6.6 *Device Components*—The BSTM Device is shown in Fig. 1; options for mounting and material configurations are summarized in Annex A1.

6.7 *Disposable Filter Units*—Bottle-top or syringe-fitted, sterile, with 0.2 μm pore size.

6.8 *Incubator*—Any capable of maintaining the desired temperature (typically, 26 °C to 37 °C depending upon the tested organism). Use of a humidified incubator is recommended, particularly in arid environments or when growing fastidious organisms.

6.9 *Inoculating Loop*.

6.10 *Microtiter Plate, 96-well*—Sterile, 86 mm by 128 mm standard plate consisting of ninety-six (96) identical flat bottom wells with a 50 μL to 300 μL working volume.

6.11 *Micro Pipettor*—Continuously adjustable pipette with working volume of 20 μL to 200 μL .

6.12 *Orbital Shaker*—Capable of maintaining an orbit of 110 rpm to 200 rpm and the desired temperature (typically, 26 °C to 37 °C depending upon the tested organism).

6.13 *Petri Dish*—100 mm by 15 mm, plastic, sterile.

6.14 *Pipettes, Serological*—Sterile single-use pipettes with volume capacity of 25 mL.

6.15 *Sterile Reagent Reservoir*—50 mL, polystyrene.

6.16 *Sterilizer*—Any steam sterilizer capable of producing the conditions of sterilization.

6.17 *Vortex*—Any vortex that will ensure proper agitation and mixing of microfuge tubes.

7. Media, Reagents, and Test Organisms

7.1 *Organism Specific Agar (OSA)*—Tryptic Soy Agar (TSA) works well for all organisms listed below, but can be substituted with any other rich medium deemed suitable for growing the tested organism(s). See Annex A2 for recipe.

7.2 *Organism Specific Broth (OSB)*—Tryptic Soy Broth (TSB) works well for all organisms listed below, but can be substituted with any other rich medium deemed suitable for growing the tested organism(s). See Annex A2 for recipe.

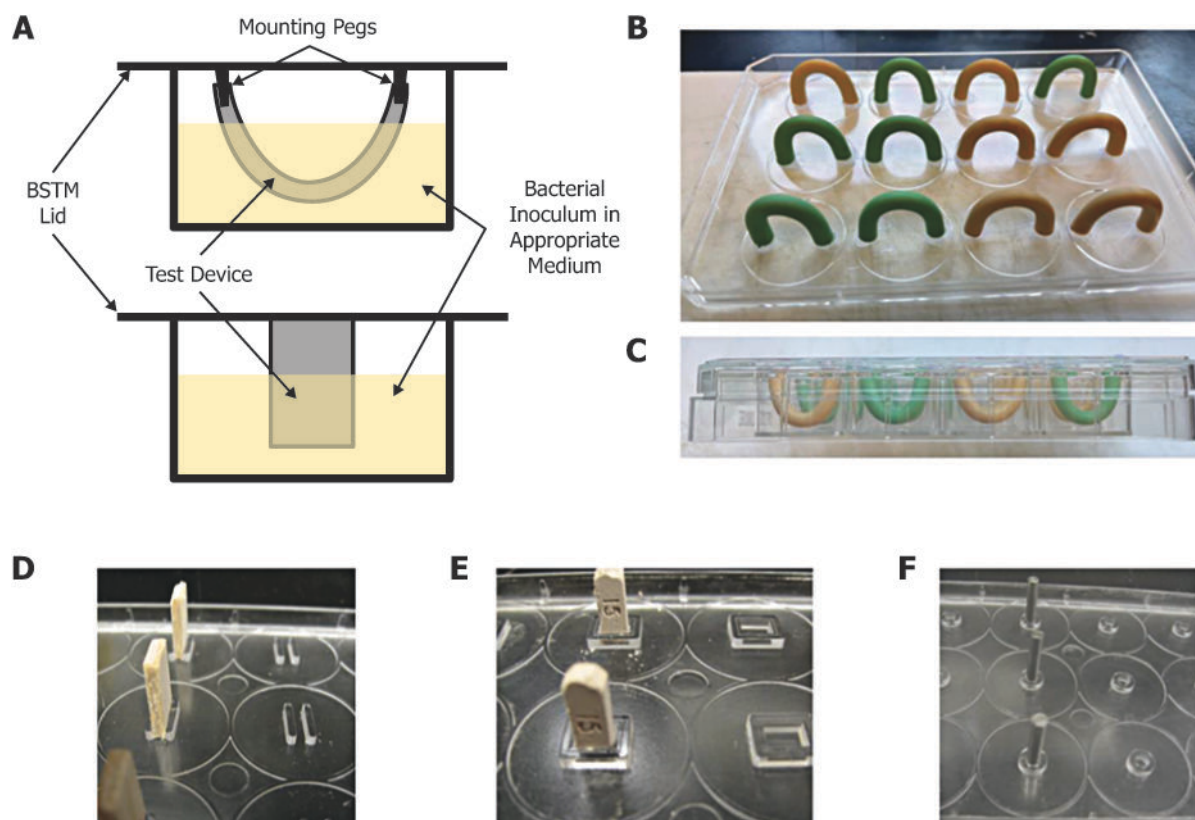
NOTE 1—Other media may be used where appropriate.

7.3 *Biofilm Growth Medium*—See Table 1.

7.4 *Organic Soil Matrix*—See Table 1.

7.5 *Neutralizing Recovery Medium*—Dey/Engley Neutralizing Broth, or one specific to the antimicrobial under evaluation as determined according to Test Method E1054. See Annex A3 for recipe.

7.6 *Dilution and Rinse Fluid*—Saline (sterile solution of 0.9 % w/v sodium chloride in water).



A device typically has 12 wells, although alternative configurations (for example, 6-well plates) can be used. In panel (A), a cross-section of an individual well is represented schematically for two common configurations: in the upper well, a test article (for example, catheter) is attached to two mounting pegs, forming a “U” shape such that the inoculum only contacts the extraluminal surface; in the lower well, a test article (for example, ET tube or material coupon, 15 mm in length) is mounted to the lid, allowing the inoculum to contact the extraluminal surface and, if applicable, the luminal surface. (B) Example of a device lid with control (green) versus antimicrobial-coated (beige) Foley catheters (size 12-French, 35 mm in length) attached in the “U” configuration. (C) Side-on view of the device lid from (B) inserted into the 12-well receiver plate. (D) Example of a material coupon (in this case, wood) mounted to a “slot” version of a BSTM lid. (E) Example of a formed material (in this case, concrete) mounted to a “square slot” version of a BSTM lid. (F) Example of a surgical stainless-steel pin mounted to a “round slot” version of a BSTM lid. Alternatively, materials can be mounted to a conventional lid with appropriate glue (for example, cyanoacrylate). See Annex A1 for typical dimensions of tested materials.

FIG. 1 Biofilm Surface Test Materials (BSTM) Device

organisms listed below; may be substituted for other diluents, such as buffered water, where appropriate.

7.7 *PBS*—Sterile solution of Phosphate Buffered Saline, pH 7.4.

8. Culture Preparation

NOTE 2—The following approach works well for the organisms listed in Table 1. Other organisms may require alternative culture conditions.

8.1 Using a cryogenic stock (at -70°C), streak out a primary culture of the organism on OSA.

8.2 Incubate at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 16 h to 24 h.

8.3 From the first sub-culture, streak out a secondary sub-culture on OSA.

8.4 Incubate at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 16 h to 24 h.

8.5 Aseptically remove 1 to 5 isolated colonies from the second sub-culture plate and inoculate 5 mL of sterile OSB in a 50 mL conical tube (or similar).

8.6 Incubate the tube at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and 200 rpm for 16 h to 24 h. Viable bacterial density should reach $10^{9 \pm 1}$ CFU/mL, while yeast should reach $10^{7 \pm 1}$ CFU/mL.

8.7 *Viable Density Should be Checked by Serial Dilution and Plating:*

8.7.1 In triplicate, transfer 100 μL of the culture to the first row (row A) of an empty, sterile 96 well microtiter plate.

8.7.2 Add 180 μL of sterile saline to the remaining rows.

8.7.3 Serially dilute with a multichannel pipettor (10^0 to 10^{-7} dilution factors – df) by successively transferring 20 μL from one row to the next.

NOTE 3—Fresh tips must be used between each transfer during the dilution and dispensing steps to ensure accurate dilution and to prevent cross contamination.

8.7.3.1 Mix the contents of each well by pipetting up and down at least three times between dilutions.

8.7.4 After mixing each row, discard the pipette tips.

8.7.5 Using fresh tips, continue the dilution procedure.

8.8 Using a single channel pipettor, remove 20 μL from each well and plate on OSA.

NOTE 4—One OSA plate can be used for each dilution series by dividing individual plates into eight pie-shaped sections and labelling the sections 10^0 to 10^{-7} df (13).