Designation: E3180 – 18

Standard Test Method for Quantification of a *Bacillus subtilis* Biofilm Comprised of Vegetative Cells and Spores Grown Using the Colony Biofilm Model[1]

This standard is issued under the fixed designation E3180; 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 specifies the operational parameters required to grow and quantify a *Bacillus subtilis* biofilm comprised of vegetative cells and endospores (spores) using the colony biofilm method (CBM).[2,3] The resulting biofilm is representative of static environments that can develop a sporulating biofilm rather than being representative of one particular environment.

1.2 This test method utilizes a modified CBM to grow the biofilm. The CBM uses a semipermeable membrane on an agar plate as the biofilm growth surface and nutrient source.[2,3] In this test method, membranes are inoculated and incubated for a total of 8 days to promote sporulation within the biofilm.

1.3 This test method describes how to sample and analyze the biofilm for vegetative cells and spores. Biofilm population is expressed as total colony forming units (CFU) and spores per membrane.

1.4 Basic microbiology training is required to perform this test method.

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

1.6 *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.*

1.7 *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:*[4]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

3. Terminology

3.1 *Definitions*—For definitions of terms used in this Test Method see Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *biofilm, n*—microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

3.2.2 *spore, n*—a dormant, robust, and non-metabolically active structure produced by certain bacteria enabling prolonged survival and greater resistance to deleterious environmental factors.

4. Summary of Test Method

4.1 This test method utilizes a modified CBM to grow the biofilm. The CBM uses an inoculated semipermeable membrane on an agar plate as the biofilm growth surface and nutrient source.[2] In the published method, the inoculated membranes are transferred to fresh agar plates regularly (usually every 8-24 h) to provide new nutrients to the membrane-grown cells and are incubated for a total of approximately 48 h before sampling or subjecting the biofilm to further tests.[3] In this test method, membranes are inoculated and incubated for 24 h before transferring the membrane to a new media plate. This initial growth step is similar to the published CBM and allows the colony biofilm to grow and spread across

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[2] Anderl, J. N., Franklin, M. J., & Stewart, P. S. (2000). Role of Antibiotic Penetration Limitation in Klebsiella pneumoniae Biofilm Resistance to Ampicillin and Ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, 44(7) 1818–1824.

[3] Merritt, J.H., Kadouri, D.E., and O'Toole, G.A. (2011). Growing and analyzing static biofilms. *Current Protocols in Microbiology*. 22:B:1B.1:1B.1.1–1B.1.18

[4] 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.

the membrane. After the transfer, the colony biofilms are incubated for an additional seven (7) days on the same media plate without additional transfers to fresh medium as is typically performed in the published CBM. These growth conditions induce sporulation to occur within the biofilm.

4.2 After the growth phase, the biofilms grown on membranes are sampled by removing the biofilm from the membrane surface and disaggregating the biofilm into a homogeneous cell suspension. The disaggregated cell suspension is split into two fractions. One fraction is directly diluted and plated for viable cell enumeration. The other fraction is heat shocked prior to diluting and plating to kill the vegetative cells and enumerate the spores in the sample.

5. Significance and Use

5.1 Biofilm characteristics such as thickness, matrix architecture, and population are dependent on factors such as shear and nutrient availability and composition. Additionally, sporulation can occur within biofilms, including those formed by *Bacillus subtilis*.[5] The purpose of this test method is to define the parameters to grow and enumerate a *B. subtilis* biofilm comprised of vegetative cells and spores embedded in extracellular polymeric substance (EPS). This type of biofilm could provide a greater challenge to antimicrobials than vegetative biofilm or spores alone. The biofilm generated using this method is suitable for efficacy testing of antimicrobials.

6. Apparatus

6.1 *Biological safety cabinet,*

6.2 *Environmental chamber*—capable of maintaining temperature at 32.5 ± 2.5 °C,

6.3 *Shaker,* capable of maintaining 100 RPMs,

6.4 *Variable volume pipetters,*

6.5 *UV lamp,*

6.6 *Vortex mixer*—any vortex mixer that will ensure proper agitation and mixing of test tubes,

6.7 *Ultrasonic bath*—capable of maintaining 40 ± 5 kHz.

6.8 *Timer,*

6.9 *Recirculating water bath,*

6.10 *Thermometer or thermocouple,* and

6.11 *Incinerator or Bunsen burner.*

7. Reagents and Materials

7.1 *Reagents:* [6]

7.1.1 *Tryptic soy broth (TSB)*—purchased pre-made or prepared according to manufacturer's instructions for a concentration of 30 g/L.

7.1.2 *Tryptic soy agar (TSA)*—purchased pre-made plates or prepared according to manufacturer's instructions for a concentration of 40 g/L and poured into sterile 100 × 15 mm petri plates.

7.1.3 *Sterile water*—purchased or sterilized distilled or reverse osmosis (RO) water.

7.1.4 *Tween 80*—prepared to a concentration of 1% v/v in distilled or RO water and filter sterilized.

7.2 *Materials:*

7.2.1 *Inoculating loops*—sterile,

7.2.2 *Test tubes*—sterile, 50 mL volume capacity,

7.2.3 *Test tubes*—sterile, any with a volume capacity of 10 mL and a minimum diameter of 16 mm and a cap that can withstand 100 °C. Recommended size is 25 × 150 mm borosilicate glass with a threaded opening.

7.2.4 *Membrane filters*—0.22 µm nitrocellulose, 13 mm diameter,

7.2.5 *Beads*—sterile, 6 mm borosilicate glass, and

7.2.6 *Forceps*—sterile.

7.3 *Test organism:*

7.3.1 *Bacillus subtilis,* ATCC 35021.[7]

8. Culture Preparation

8.1 *Bacillus subtilis* is the organism used in this test.

8.1.1 The source culture may originate from a frozen culture or spore suspension.

8.1.2 Streak a TSA plate for isolation with the source culture and incubate at 32.5 ± 2.5 °C for 18-24 h.

NOTE 1—Alternative strains can be substituted with the appropriate modifications to the method (that is, heat shocking temperature, etc). Repeatability and reproducibility coefficients based on testing performed using *B. subtilis* ATCC 35021 will not apply to testing performed using other strains.

9. Procedure

9.1 *Inoculum Preparation:*

9.1.1 Aseptically remove an isolated colony from a TSA plate and place into 20 mL of TSB in a 50 mL sterile tube.

9.1.2 Incubate the culture on a shaker in an environmental chamber at 32.5 ± 2.5 °C for 18 h -24 h at 100 RPMs.

9.1.2.1 Culturable cell density should equal $\geq 10^7$ CFU/mL and can be checked by serial dilution and plating.

9.2 *Inoculation of Membranes:*

9.2.1 Prior to inoculation, decontaminate the membrane filters in a biological safety cabinet under an UV lamp for 30 min per side.

9.2.2 Using sterilized forceps, aseptically place the membranes on TSA plates.

NOTE 2—Multiple membranes can be placed on one plate provided they do not touch and there is sufficient room between them for manipulations (five (5) per plate works well).

[5] Branda, S.S., Gonzalez-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R.(2001). Fruiting body formation by Bacillus subtilis. *Proc. Natl .Acad. Sci.* U.S.A. 98: 11621-11626.

[6] *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 *Annual 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.

[7] The sole source of supply of ATCC 50321 is ATCC (https://www.atcc.org/). 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.