Designation: ~~E2799 – 17~~ E2799 – 22

Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay[1]

This standard is issued under the fixed designation E2799; 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~~ Scope*

1.1 This test method specifies the operational parameters required to grow and treat a *Pseudomonas aeruginosa* biofilm in a high throughput screening assay known as the MBEC (trademarked)[2] (Minimum Biofilm Eradication Concentration) Physiology and Genetics Assay. The assay device consists of a plastic lid with ninety-six (96) pegs and a corresponding receiver plate with ninety-six (96) individual wells that have a maximum 200 µL working volume. Biofilm is established on the pegs under batch conditions (that is, no flow of nutrients into or out of an individual well) with gentle mixing. The established biofilm is transferred to a new receiver plate for disinfectant efficacy testing.[3,4] The reactor design allows for the simultaneous testing of multiple disinfectants or one disinfectant with multiple concentrations, and replicate samples, making the assay an efficient screening tool.

1.2 This test method defines the specific operational parameters necessary for growing a *Pseudomonas aeruginosa* biofilm, although the device is versatile and has been used for growing, evaluating and/or studying biofilms of different species as seen in Refs (**1-4**).[5]

1.3 Validation of disinfectant neutralization is included as part of the assay.

1.4 This test method describes how to sample the biofilm and quantify viable cells. Biofilm population density is recorded as $\log_{10}$ colony forming units per surface area. Efficacy is reported as the $\log_{10}$ reduction of viable cells.

1.5 Basic microbiology training is required to perform this assay.

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 *ASTM International takes no position respecting the validity of any patent rights asserted in connection with any item mentioned in this standard. Users of this standard are expressly advised that determination of the validity of any such patent rights, and the risk of infringement of such rights, are entirely their own responsibility.*

[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] The MBEC trademark is held by Innovotech, Inc., Edmonton, Alberta, Canada.

[3] The sole source of supply of the apparatus known to the committee at this time is Innovotech Inc., Edmonton, 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,[1] which you may attend.

[4] The MBEC Assay is covered by a patent. Interested parties are invited to submit information regarding the identification of an alternative(s) to this patented item to the ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[5] The boldface numbers in parentheses refer to a list of references at the end of this standard.

***A Summary of Changes section appears at the end of this 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 and health 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.*

2. Referenced Documents

2.1 *ASTM Standards:*[6]
E1054 Practices for Evaluation of Inactivators of Antimicrobial Agents
E2756 Terminology Relating to Antimicrobial and Antiviral Agents
2.2 *Other Standards:*
Method 9050 C.1.a Buffered Dilution Water Preparation according to Rice et al (**5**)

3. Terminology

3.1 For definitions of terms used in this standard refer to Terminology E2756.

3.2 *Definitions:*

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.

3.2.1.1 *Discussion*—
Biofilms may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a biofilm including, but not limited to, population density, taxonomic diversity, thickness, chemical gradients, chemical composition, consistency, and other materials in the matrix that are not produced by the biofilm microorganisms, are controlled by the physicochemical environment in which it exists.

3.2.2 *disinfectant, n*—chemicals used on inanimate surfaces to rapidly inactivate 99.9 % of the treated microorganisms at a specific concentration and desired exposure time.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *peg, n*—biofilm growth surface (base: 5.0 mm, height: 13.1 mm).

3.3.2 *peg lid, n*—an ~~86~~86 mm × 128 mm plastic surface consisting of ninety-six (96) identical pegs.

3.3.3 *plate, n*—an ~~86~~86 mm × 128 mm standard plate consisting of ninety-six (96) identical wells.

3.3.4 *well, n*—small reservoir with a 50 to 200 µL working volume capacity.

3.4 *Acronyms:*

3.4.1 *ATCC*—American Type Culture Collection

3.4.2 *BGC*—biofilm growth check

3.4.3 *CFU*—colony-forming unit

3.4.4 *MBEC*—minimum biofilm eradication concentration

3.4.5 *rpm*—revolutions per minute

[6] 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.

3.4.6 *SC*—sterility control

3.4.7 *TSA*—tryptic soy agar

3.4.8 *TSB*—tryptic soy broth

3.4.9 *UC*—untreated control

4. Summary of Test Method

4.1 This test method describes the use of the MBEC Assay in evaluating the efficacy of a disinfectant against a *Pseudomonas aeruginosa* biofilm. A mature biofilm is established on pegs under batch conditions with very low shear produced by gentle rotation of the device on an orbital shaker. At the end of 24 h of growth, the pegs containing the biofilm are rinsed to remove planktonic cells and the peg lid is placed in a receiver plate. The wells in the receiver plate are filled according to an experimental design that contains the appropriate sterility, growth, and neutralizer controls as well as the disinfectants. After a specified contact time, the peg lid is placed in a receiver plate containing neutralizer, and the entire device is placed in a sonicator to remove the biofilm and disaggregate the clumps. Samples from each well are then diluted, plated and the viable cells enumerated. The $\log_{10}$ reduction in viable cells is calculated by subtracting the mean $\log_{10}$ density for the treated biofilm from the mean $\log_{10}$ density determined for the untreated controls.

5. Significance and Use

5.1 Vegetative biofilm bacteria are phenotypically different from suspended planktonic cells of the same genotype. 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 the particular study.

5.2 The purpose of this test method is to direct a user in how to grow, treat, sample and analyze a *Pseudomonas aeruginosa* biofilm using the MBEC Assay. Microscopically, the biofilm is sheet-like with few architectural details as seen in Harrison et al (**6**). The MBEC Assay was originally designed as a rapid and reproducible assay for evaluating biofilm susceptibility to antibiotics (**2**). The engineering design allows for the simultaneous evaluation of multiple test conditions, making it an efficient method for screening multiple disinfectants or multiple concentrations of the same disinfectant. Additional efficiency is added by including the neutralizer controls within the assay device. The small well volume is advantageous for testing expensive disinfectants, or when only small volumes of the disinfectant are available.

6. Apparatus

6.1 *Inoculating loop*—nichrome wire or disposable plastic.

6.2 *Petri dish*—square ~~100~~100 mm × ~~100~~100 mm × 15 mm, plastic, sterile.

6.3 *Microcentrifuge tubes*—sterile, any with a 1.5 mL volume capacity.

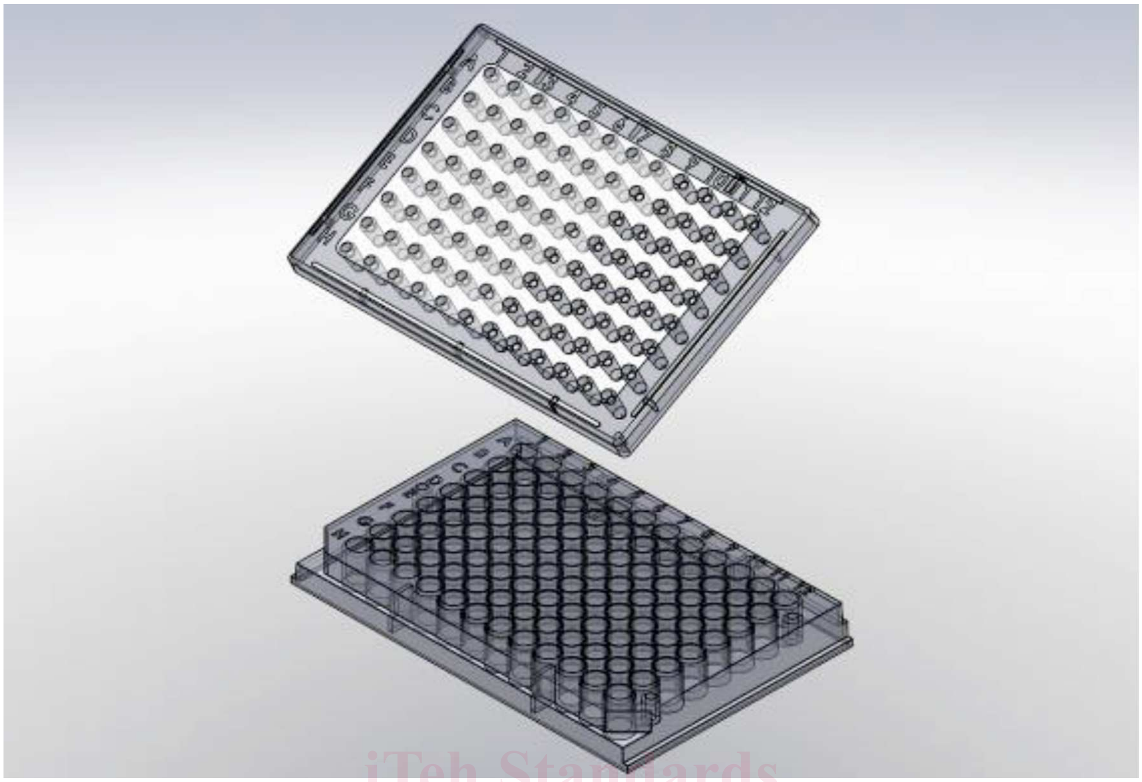
6.4 *96-well microtiter plate*—sterile, ~~86~~86 mm × 128 mm standard plate consisting of ninety-six (96) identical flat bottom wells with a 200 µL working volume.[7]

NOTE 1—Alignment corner must be in the H12 position of the plate for proper alignment with the MBEC lid (see Fig. 1).

6.5 *Vortex*—any vortex that will ensure proper agitation and mixing of microfuge tubes.

6.6 *Bath sonicator*—any capable of an average sonic power of 180 W in a dry environment (**7**).

[7] The sole source of microtiter plates (Nunclon (trademarked) Catalogue No. 167008) that provide reproducible results is Thermo Fisher Scientific, Waltham, MA, USA, www.thermofisher.com. 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,[1] which you may attend.



96-well tissue culture plate (bottom) and
corresponding 96-peg lid (top).

FIG. 1 MBEC Assay Device

6.7 *Stainless steel insert tray*—for bath sonicator.

6.8 *Bunsen burner*—used to flame-sterilize inoculating loop (if metal) and other instruments.

6.9 *95 % Ethanol*—used to flame-sterilize pliers.

6.10 *4 in. bent needle nose pliers*—for aseptic removal and handling of pegs.

6.11 *Pipette(s)*—continuously adjustable pipette(s) with volume capacity of 1 mL.

6.12 *Micropipette(s)*—continuously adjustable pipette(s) with working volume of 10 µL to 200 µL.

6.13 *Sterile pipette tips*—200 µL and 1000 µL volumes.

6.14 *Sterile reagent reservoir*—50 mL polystyrene.

6.15 *Analytical balance*—sensitive to 0.01 g.

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

6.17 *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.

6.18 *Environmental incubator*—capable of maintaining a temperature of ~~35~~35 °C ± ~~2°C~~2 °C and relative humidity between ~~35~~35 % and 85 %.

6.19 *Orbital shaker*—capable of maintaining an orbit of ~~110~~110 rpm to 150 rpm.

6.20 *Reactor components*—the MBEC Assay device is shown in Fig. 1. Fig. 2 is a diagram of the challenge plate.

6.21 *Appropriate glassware*—as required to make media and agar plates.

6.22 *Erlenmeyer flask*—used for growing broth inoculum.

7. Reagents and Materials

7.1 *Purity of Water*—all references to water as diluent or reagent shall mean distilled water or water of equal purity.

7.2 *Culture Media:*

7.2.1 *Bacterial Growth Broth*—Tryptic soy broth (TSB) prepared according to manufacturer's directions.

7.2.2 *Bacterial Plating Medium*—Tryptic soy agar (TSA) prepared according to manufacturer's directions.

7.3 *Buffered Water*—0.0425 g KH_2PO_4/L distilled water, filter-sterilized and 0.4055 g $MgCl{\cdot}6H_2O$/L distilled water; filter-sterilized (Method 9050 C.1.a).

7.4 *Neutralizer*—appropriate to the disinfectant being evaluated (see Test Method E1054).

7.5 *Disinfectant*—stock concentration.

8. Culture/Inoculum Preparation

8.1 *Pseudomonas aeruginosa* ATCC 15442 is the organism used in this test.

8.2 Using a cryogenic stock (at 70°C), streak out a subculture of *P. aeruginosa* on TSA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100	100	100	50:N	N	UC				SC
B	50	50	50	50	50	50:N	N	UC				SC
C	25	25	25	25	25	50:N	N	UC				SC
D	12.5	12.5	12.5	12.5	12.5	50:N	N	UC				BGC
E	6.25	6.25	6.25	6.25	6.25	50:N	N	UC				BGC
F	3.13	3.13	3.13	3.13	3.13	50:N	N	UC				BGC
G	1.56	1.56	1.56	1.56	1.56	50:N	N	UC				BGC
H	0.78	0.78	0.78	0.78	0.78	50:N	N	UC				BGC

Columns 1 through 5 are test disinfectant (n=5). Column 6 serves as the neutralizer effectiveness control. Column 7 serves as the neutralizer toxicity control (N). Column 8 is the untreated control for each row (UC). Column 12, rows A to C are sterility controls for each experiment (SC), rows D to H are the biofilm growth check controls (BGC). Lined out cells are spare (columns 9, 10 and 11). The numbers in columns 1 to 5 refer to the percentage of undiluted sample with 100 representing 100 % concentration of the stock solution, 50 representing a 50 % concentration of the stock solution and so on.

FIG. 2 Challenge Plate Preparation

8.3 Incubate at 35 ± 2°C for 16 to 18 h.

8.4 Aseptically remove isolated colony from streak plate and inoculate 200 mL of sterile bacterial growth broth (TSB).

8.5 Incubate flask at 35 ± 2°C and 150 ± 10 rpm for 16 to 18 h. Viable bacterial density should be $\geq 10^8$ CFU/mL and may be checked by serial dilution and plating.

8.6 Pipette 10 µL from the incubation flask into 100 mL of TSB to adjust the inoculum to an approximate cell density of 10^5 CFU/mL. Vortex the diluted sample for approximately 10 s to achieve a homogeneous distribution of cells.

8.7 Perform 10-fold serial dilutions of the inoculum from 8.6 in triplicate.

8.8 Spot plate 20 µL of the serial dilutions from 10^0 to 10^{-7} on an appropriately labelled series of TSA plates. Incubate the plates at 35 ± 2°C for 16 to 18 h and enumerate (**8**).

9. Procedure

9.1 An overview of the procedure is shown in Fig. 3.

9.2 *Growth of Biofilm:*

9.2.1 Open the sterile package containing the MBEC device.

9.2.2 Transfer 25 mL of the inoculum prepared in 8.6 into a sterile reagent reservoir.

9.2.3 Using a micropipette, add 150 µL of the inoculum to each well (exclude columns 9 to 11 and A12, B12, and C12) of the 96-well tissue culture plate packaged with the MBEC device.

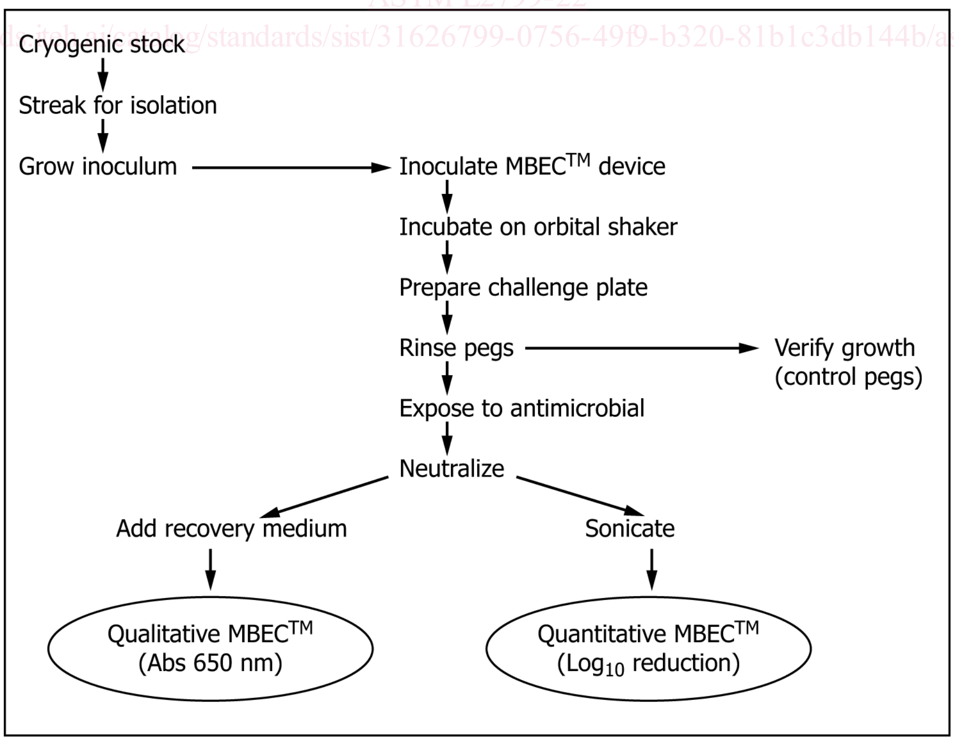


FIG. 3 A Flow Diagram Representing the MBEC Assay for Disinfectant Testing

NOTE 2—Wells A12, B12, and C12 serve as sterility controls and must NOT be filled with inoculum. Columns 9 to 11 are spare, empty wells.

9.2.4 Place the peg lid onto the microtiter plate. Ensure that the orientation of the plate matches the orientation of the lid (that is, peg A1 must be inserted into well A1 of the microtiter plate, otherwise the device will not fit together correctly, see Fig. 1). Label the device appropriately.

NOTE 3—Volume of inoculum used in this step has been calibrated such that the biofilm covers a surface area that is entirely immersed by the volume of antimicrobial used in the challenge plate setup (Section 9.4). Using a larger volume of inoculum might lead to biofilm formation high on the peg that physically escapes exposure during the challenge step.

9.2.5 Place the device on the orbital shaker in a humidified incubator (to prevent evaporation). Set shaker to 110 ± 10 rpm to prevent spillover. Incubate at 35 ± 2°C for 16 to 18 h.

9.3 *Biofilm Growth Check:*

9.3.1 Using flame-sterilized pliers held flush against lid to minimize contact with attached biofilm, break off five (5) pegs D12, E12, F12, G12, and H12.

9.3.2 Place each peg into a separate sterile microfuge tube that contains 1.0 mL of buffered water.

9.3.3 Float a stainless steel insert tray in the center of a sonicator. Place the peg-containing tubes in the tray and sonicate on high for 30 ± 5 min (**7**).

9.3.4 Serially dilute by transferring 0.1 mL to sterile microfuge tubes containing 0.9 mL buffered water and spot plate on TSA (**7**). This serves as a biofilm growth check.

9.4 *Preparation of Challenge Plate:*

9.4.1 Using a sterile 96-well microtiter plate, the next steps will describe how to aseptically prepare the challenge plate (Fig. 2).

9.4.2 Prepare 100 mL stock solution of disinfectant.

NOTE 4—(*Optional*) Measure disinfectant concentration to ensure accuracy (**9**).

9.4.3 Add 200 µL of sterile TSB to well A12 of the challenge plate. This will serve as the device sterility control (SC).

9.4.4 Add 200 µL of sterile neutralizer to column 7 and well B12. These serve as the neutralizer toxicity control (N) and sterility control.

9.4.5 Add 100 µL of sterile neutralizer to column 6, followed by 100 µL of stock disinfectant. This serves as the neutralizer effectiveness control.

9.4.6 Add 200 µL of buffered water to column 8 and well C12. These serve as the untreated control (UC) and buffered water sterility control.

9.4.7 Add 100 µL of buffered water to columns 1 through 5 (rows B through H) of the microtiter plate.

9.4.8 Add 200 µL of the disinfectant stock solution to columns 1 through 5 (row A) of the microtiter plate.

9.4.9 Add 100 µL of the disinfectant stock solution to columns 1 through 5 (row B and row C) of the microtiter plate.

9.4.10 Using a multichannel micropipette, mix the contents of columns 1 through 5 (row C) by pipetting up and down at least twice.

9.4.11 After mixing, transfer 100 µL from the wells in row C to the corresponding wells in row D. Discard the pipette tips.

9.4.12 Using fresh tips, mix the contents in row D, columns 1 through 5 by pipetting up and down at least twice.