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.

**Designation: E2871 − 21**

# Standard Test Method for Determining Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Method[1]

This standard is issued under the fixed designation E2871; 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 perform a quantitative liquid disinfectant efficacy test against bacterial biofilm.

1.2 The test method was optimized and validated for a *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilm grown in the CDC Biofilm Reactor (E3161). The method is suitable for evaluating additional bacteria grown using the procedures outlined in methods with comparable coupon dimensions such as Practice E3161, Test Method E2562, or Test Method E2196.

1.3 Disinfectant preparation and contact time are used in the assessment according to the manufacturer's instructions for use.

1.4 The test method uses a closed system to treat biofilm. A coupon is placed in a single tube for the treatment, neutralization, and harvesting steps to prevent the loss of cells.

1.5 This test method describes a harvesting and analysis procedure which includes vortexing and sonicating treated and untreated control biofilm, and recovery of culturable cells using filtration to lower the limit of detection. Biofilm population density is recorded as $\log_{10}$ colony-forming units per coupon. Efficacy is reported as a $\log_{10}$ reduction of culturable cells.

1.6 Basic microbiology training is required to perform this assay.

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in 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, 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.*

## 2. Referenced Documents

2.1 *ASTM Standards:*[2]

E2196 Test Method for Quantification of ***Pseudomonas aeruginosa*** Biofilm Grown with Medium Shear and Continuous Flow Using Rotating Disk Reactor

E2562 Test Method for Quantification of ***Pseudomonas aeruginosa*** Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

E3161 Practice for Preparing a ***Pseudomonas aeruginosa*** or ***Staphylococcus aureus*** Biofilm using the CDC Biofilm Reactor

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation according to Eaton et al (**1**)[3]

## 3. Terminology

3.1 For definitions of terms used in this test method 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*—Biofilm may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a

[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] 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] 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**

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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 *contact time, n*—predetermined time that the biofilm is exposed to the activity of a disinfectant.

3.2.3 *coupon, n*—biofilm growth surface.

3.2.4 *disinfectant, n*—a physical or chemical agent or process that destroys pathogenic or potentially pathogenic microorganisms in/on surfaces or objects.

3.3 *Acronyms:*

3.3.1 *ATCC*—American Type Culture Collection.

3.3.2 *CDC*—Centers for Disease Control and Prevention.

3.3.3 *CFU*—colony-forming unit.

## 4. Summary of Test Method

4.1 This test method describes the use of the Single Tube Method to evaluate the efficacy of a liquid disinfectant against a *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilm on a hard, non-porous surface grown in the CDC Biofilm Reactor. The test method consists of adding disinfectant (treated) or buffered dilution water (untreated) to individual coupons held in conical tubes. Five coupons are treated with disinfectant and three coupons receive buffered dilution water. Neutralizer is added to the tubes after the appropriate contact time. A combination of vortexing and sonication is used to remove the biofilm from the coupon and disaggregate the clumps. The cell suspension is serially diluted and recovered on an agar medium. The difference in viable plate counts from treated coupons and untreated control coupons is used to calculate the $\log_{10}$ reduction of viable cells.

## 5. Significance and Use

5.1 The Single Tube Method is designed to evaluate the efficacy of disinfectants against biofilm grown in the CDC biofilm reactor following the procedures outlined in Practice E3161. Biofilm grown in the CDC reactor is representative of biofilm that forms under high fluid shear on surfaces conducive to biofilm formation.

5.1.1 Vegetative biofilm bacteria are phenotypically different from suspended planktonic cells of the same genotype. Biofilm growth reactors are engineered to produce biofilm with specific characteristics (**2**). Altering either the engineered system or operating conditions will modify those characteristics as well as the physicochemical environment. The goal in biofilm research and testing is to choose the growth reactor and operating conditions that generate the most relevant biofilm for the particular study.

5.2 The test method was designed to determine the $\log_{10}$ reduction in bacteria after exposure to a disinfectant in a closed system.

5.3 The test method uses 50 mL conical tubes. The conical geometry allows for disinfectant exposure to biofilm on all surfaces of the coupon. For foaming disinfectants or for disinfectants requiring a larger volume of neutralizer, 250 mL conical tubes are used which preserve the required geometry and allow for greater neutralization capacity.

5.4 Each test includes three untreated control coupons (exposed to buffered dilution water) and five treated coupons (per disinfectant/concentration/contact time combination).

## 6. Apparatus

6.1 *Conical centrifuge tubes,* sterile, any with 50-mL volume capacity and secure leak-proof lids.

NOTE 1—There are slight differences in the dimensions of the conical tubes between manufacturers. The tubes selected for testing must properly accommodate the splashguard inserts (that is, appropriate interior diameter and length) (see Fig. 1).

6.2 *Ultrasonic water bath,* any capable of maintaining a homogeneous sound distribution at 45 kHz ± 5 kHz with a volume large enough to accommodate 50 mL or 250 mL conical tubes in a wet environment.

NOTE 2—Prior to using the sonicating bath for the first time, verify that

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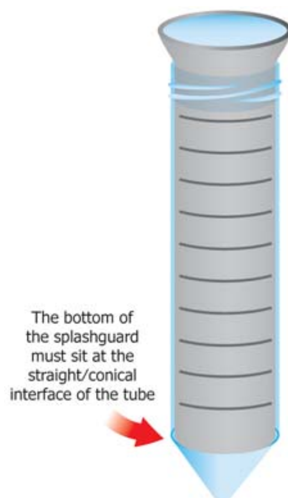


**FIG. 1 Conical tube with splashguard insert**

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the sonicating bath does not kill viable cells by placing the standardized broth culture into sonicator for 60 s, serially dilute, and plate. Compare sonicated counts to a non-sonicated control. The sonicated and non-sonicated counts should be comparable.

6.3 *Test tube rack,* any wire or plastic rack capable of holding 50 mL or 250 mL conical centrifuge tubes.

6.4 *Calibrated micropipettes,* continuously adjustable pipettes with volume capacity of 100 µL and 1000 µL.

6.5 *Sterile pipette tips,* 100 µL and 1000 µL volumes.

6.6 *Bunsen burner,* used to flame-sterilize Allen wrench, forceps, and plate spreader.

6.7 *95 % Ethanol,* used to flame-sterilize Allen wrench and plate spreader.

6.8 *Small Allen wrench,* (1.27 mm, hex) for loosening set screws and pushing coupons out of reactor rods.

6.9 *Timer,* any that can display time in seconds.

6.10 *Vortex mixer,* any vortex that will ensure proper mixing of tubes.

6.11 *Serological pipettes,* sterile single-use pipettes with volume capacity of 1 mL, 5 mL, 10 mL, 25 mL, and 50 mL.

6.12 *Plate spreader,* for spreading aliquots of dilutions on agar plates.

6.13 *Water bath,* any capable of maintaining a constant temperature of 21 °C ± 2 °C.

6.14 *Sterilizer,* any steam sterilizer capable of producing the conditions of sterilization.

6.15 *Colony counter,* any one of several types may be used. A hand tally for recording of the bacterial count is recommended if manual counting is done.

6.16 *Environmental incubator,* any capable of maintaining a temperature of 36 °C ± 2 °C.

6.17 *Appropriate glassware/plasticware,* as required to make media and agar plates.

6.18 *Magnetic stir bars,* sterile, for mixing prepared disinfectant.

6.19 *Magnetic stir plate,* any capable of mixing.

6.20 *Polyethersulfone (PES) filter membranes,* sterile 47 mm diameter membranes with 0.45 µm pore size for microbe recovery from treated coupons. Filtration units (reusable or disposable) may be used.

6.21 *Vacuum source,* in-house line or suitable vacuum pump for filtering.

6.22 *Forceps,* any appropriate for handling membrane filters.

6.23 *Splashguard inserts,* two sizes to fit the 50 mL and 250 mL conical tubes used during coupon deposition.[4] Equivalent splashguards (2.54 cm tapered to 2.39 cm (outer diameter) × 10.48 cm long for the 50 mL conical tubes and 2.50 cm (outer diameter) × 14.80 cm long for the 250 mL conical tubes) from other suppliers may also be used.

## 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 *Bacterial Plating Medium*—R2A agar for *P. aeruginosa*, tryptic soy agar (TSA) for *S. aureus*.

7.3 *Buffered Water*—Prepare stock phosphate buffer solution by dissolving 34.0 g $KH_2PO_4$ in 500 mL reagent-grade water, adjust to pH 7.2 ± 0.5 with 1 N NaOH, and dilute to 1 L with reagent-grade water. Prepare stock magnesium chloride solution: 81.1 g $MgCl_2 \cdot 6H_2O$/L reagent-grade water. Filter-sterilize both stock solutions. Prepare buffered dilution water by combining 1.25 mL $KH_2PO_4$ stock solution and 5.0 mL $MgCl_2 \cdot 6H_2O$, dilute to 1 L with reagent-grade water (for final concentrations of 0.0425 g $KH_2PO_4$/L and 0.405 g $MgCl_2 \cdot 6H_2O$/L) and sterilize (**1**). Alternatively, phosphate buffered dilution water (PBDW) or phosphate buffered saline (PBS) may be used for rinse tubes (with 30 mL), control coupon exposure fluid, dilution blanks, and filtration fluid, provided that the same buffer is used for each step.

7.4 *Disinfectant*—antimicrobial test substance to be tested (see 3.2.4).

7.5 *Neutralizer*—One specific to the disinfectant being evaluated as determined for effectiveness and toxicity; see Annex A1 for suggested procedure.

7.6 *Detergent*—laboratory detergent for cleaning coupons and reactor parts.

## 8. Culture/Inoculum Preparation

8.1 Coupons with mature *Pseudomonas aeruginosa* ATCC 15442 or *Staphylococcus aureus* ATCC 6538 biofilm grown according to Practice E3161.

8.1.1 Biofilms are to be grown according to Practice E3161 10.2 through 10.6.

8.1.2 A mature *P. aeruginosa* biofilm will have a mean $\log_{10}$ density (LD) of 8.0 to 9.5 CFU/coupon, with each coupon exhibiting a LD of 8.0 to 9.5.

8.1.3 A mature *S. aureus* biofilm will have a mean LD of 7.5 to 9.0 CFU/coupon, with each coupon exhibiting a LD of 7.5 to 9.0.

## 9. Procedure

9.1 The test is conducted with five treated coupons and three untreated control coupons. An overview of the procedure is shown in Fig. 2.

NOTE 3—Prior to performing the assay for the first time, microscopy or a similar approach may be used to confirm that the equipment used in this method is capable of harvesting and disaggregating the biofilm from the coupon surface (**3**). This should be done using the same conical tubes, neutralized treatment volume, and number of tubes placed in the sonicating bath to replicate the actual test conditions as closely as possible.

9.2 *Reaction tube preparation:*

[4] The sole source of supply of the splashguard known to the committee at this time is BioSurface Technologies, Corp. www.biofilms.biz. 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.

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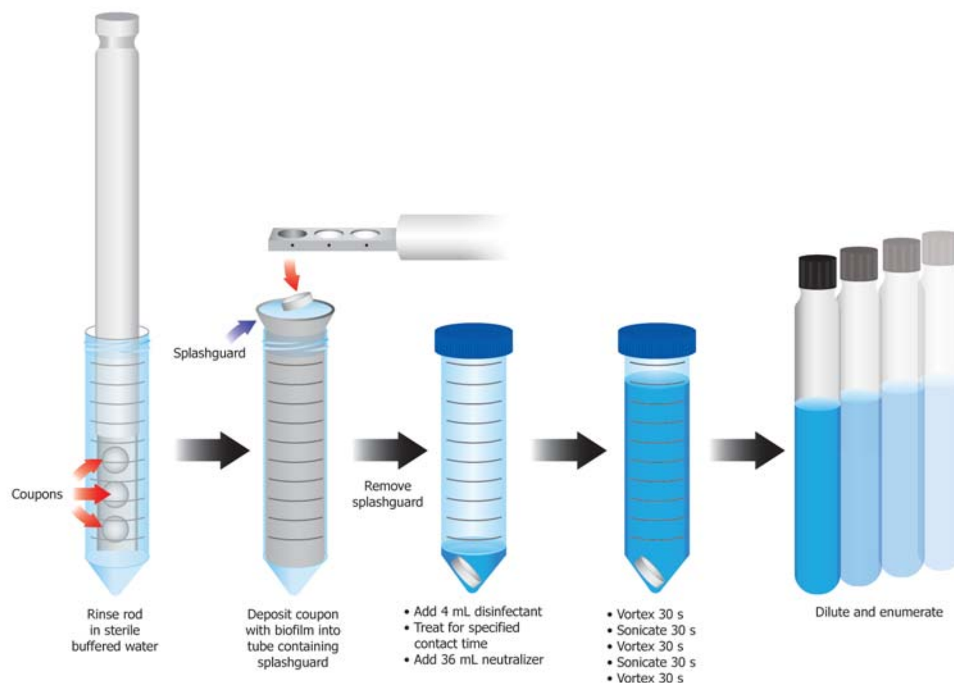


**FIG. 2 Single Tube Method Overview**

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9.2.1 Prior to sterilization, verify that the splashguards will sit properly in the conical tubes so that the end of the splashguard sits at the straight/conical interface of the tube (see Fig. 1).

9.2.2 Sterilize (autoclave) the splashguards appropriately.

9.2.3 Splashguards are only required for reaction tubes with coupons treated with disinfectants.

9.2.4 For disinfectants requiring larger neutralizer volumes, use 250 mL conical tubes with corresponding splashguards.

9.3 *Prepare Disinfectant:*

9.3.1 When preparing disinfectant, ensure that the disinfectant is adequately mixed. Use within 3 h of preparation or as specified in the manufacturer's instructions.

9.3.2 Evaluate the disinfectant at room temperature (21 °C ±2 °C). If necessary, place disinfectant in water bath prior to use to equilibrate to the appropriate temperature for 10 min -15 min. Record temperature.

9.3.3 Bring the neutralizer to room temperature prior to use.

9.4 *Removal of Coupons from the CDC Biofilm Reactor:*

9.4.1 Prepare sampling materials: reaction tubes with splashguards, rinse tubes, and flame-sterilized Allen wrench.

9.4.2 Turn off growth medium flow and baffled stir bar. After growth medium flow and baffled stir bar have been turned off, use coupons for testing (that is, exposed to disinfectant/control) within 30 min.

NOTE 4—If necessary for experiments in which more than 8 coupons are evaluated or removed from the reactor and evaluated in batches over a period of time, the growth medium flow and baffled stir bar may remain on during coupon removal such that the total amount of time during the CSTR phase does not exceed 24 h ± 2 h. Insert sterile coupon holder blank in place of those rods removed for coupon evaluation to maintain the appropriate flow dynamics within the reactor.

9.4.3 Aseptically remove a randomly selected rod containing coupons with biofilm from the CDC Biofilm Reactor by pulling it straight up and out of the reactor.

9.4.4 Rinse the coupons to remove planktonic cells: orient the rod in a vertical position directly over a 50 mL conical tube containing 30 mL sterile buffered water. With one continuous motion, immerse the rod into the buffered water with minimal to no splashing, then immediately remove. Use a new 50 mL conical tube with 30 mL sterile buffered water for each rod.

9.4.5 Hold the rod with one of the randomly selected coupons centered over an empty, sterile 50 mL or 250 mL conical tube containing a splashguard (for coupons exposed to a disinfectant). During coupon deposition, do not allow the rod to contact the tube or splashguard for treated or control samples. Refer to Fig. 3 for proper rod orientation. If contact occurs, replace the coupon and associated tube and/or splashguard. Loosen the set screw using a flame-sterilized Allen wrench and allow the coupon to drop directly to the bottom of the tube. If the coupon does not freely drop, press in the center of the coupon with the Allen wrench used to loosen the set screw.

9.4.6 Remove an appropriate number of coupons for testing. Obtain a set of five coupons for each treatment and a set of three coupons for the controls (one set of control coupons per reactor run) as described in steps 9.4.3 through 9.4.5.

9.4.7 After removing the coupons for testing, gently remove the splashguard from each tube using sterile forceps. Cap the reaction tube to mitigate dehydration. Splashguards are not required for control coupons.

9.5 *Conduct Efficacy Evaluation:*

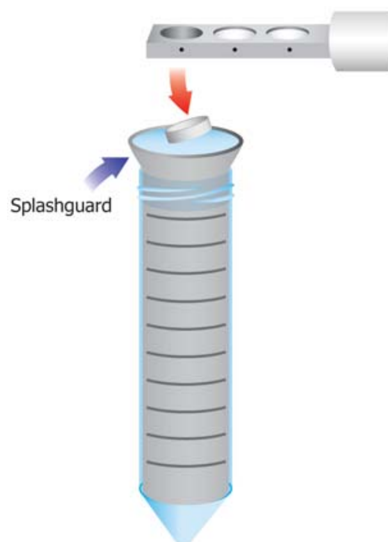


FIG. 3 Proper rod placement during coupon deposition.

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9.5.1 Slowly pipette 4 mL of previously prepared disinfectant (treatment) or buffered dilution water (untreated control) down the side of each of the conical tubes containing the coupons, avoiding direct contact with the coupon during application and being careful to completely cover the coupon. Record the time of coupon exposure and the room temperature (21 °C ± 2 °C). Refer to Fig. 4 for proper treatment application positioning. Process coupons treated with disinfectant first followed by controls.

NOTE 5—If necessary for experiments in which more than 8 coupons are evaluated, evaluate one control coupon prior to the start of the disinfectant treatment(s) and the remaining two control coupons after the disinfectant treatment(s).

NOTE 6—For a 10-min contact time, a 1-min interval between coupons is recommended.

9.5.2 Immediately after deposition of disinfectant or control substance, gently swirl the tube 1-2 times to fully expose the biofilm on the coupon to the liquid, ensuring the coupon is fully covered by the test substance and that there are no air bubbles trapped beneath the coupon. The coupon is invalid if it is not fully exposed to the test substance due to trapped air bubbles; replace with new coupon and tube if this occurs. For those test substances that cause effervescence, the presence of the effervescence does not invalidate the coupon

FIG. 4 Proper treatment application positioning.

9.5.3 Allow tubes to remain at room temperature (21°C ± 2 °C) for the duration of the contact time.

9.5.4 At the end of the contact time, add the appropriate volume of neutralizer (for example, 36 mL or 196 mL) to each tube. Replace the cap and briefly vortex the tube.

9.6 *Remove and Disaggregate Biofilm:*

9.6.1 Vortex each tube on the highest setting, ensuring a complete vortex for 30 s ± 5 s.

9.6.2 Place all tubes into a wire or plastic conical tube rack and suspend the rack in the ultrasonic water bath so that the liquid level in the tubes is even with the water level in the tank of the bath. Do not allow the tubes or the rack to touch the bottom or sides of the ultrasonic water bath. Within the conical tube rack, allow space between the tubes.

9.6.3 Sonicate the tubes at 45 kHz ± 5 kHz for 30 s ± 5 s at room temperature (21 °C ± 2 °C) (use normal mode if sonicator has variable settings).

9.6.4 Vortex the tubes as described in 9.6.1.

9.6.5 Sonicate the tubes as described in 9.6.2 and 9.6.3.

9.6.6 Vortex the tubes as described in 9.6.1. These tubes are the $10^0$ dilution.

NOTE 7—The results from an interlaboratory study of this test method demonstrated the importance of following the disaggregation and removal protocol exactly as written including using the recommended ultrasonic water bath and vortex mixer.

9.7 *Dilute and Recover Disaggregated Biofilm Samples:*

9.7.1 Dilute and recover treated samples followed by control samples. Initiate dilutions within 30 min of neutralization.

9.7.2 Serially dilute the disaggregated biofilm from control samples in buffered water. Test coupons may be serially diluted, if necessary, to achieve countable filters in the target range of 20 CFU-200 CFU.

9.7.3 For treated coupons, filter at least 25 % of the total volume of neutralizer + disinfectant from the $10^0$ reaction tube

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