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**Designation: ~~E3161 – 18~~ E3161 – 21**

# Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor[1]

This standard is issued under the fixed designation E3161; 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 practice specifies the parameters for growing a *Pseudomonas aeruginosa* (ATCC 15442) or *Staphylococcus aureus* (ATCC 6538) biofilm that can be used for disinfectant efficacy testing using the Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method (E2871) or in an alternate method capable of accommodating the coupons used in the CDC Biofilm Reactor. The resulting biofilm is representative of generalized situations where biofilm exist on hard, non-porous surfaces under shear rather than being representative of one particular environment. Additional bacteria may be grown using the basic procedure outlined in this document, however, alternative preparation procedures for frozen stock cultures and biofilm generation (for example, medium concentrations, baffle speed, temperature, incubation times, coupon types, etc.) may be necessary.

1.2 This practice uses the CDC Biofilm Reactor created by the Centers for Disease Control and Prevention (**1**).[2] The CDC Biofilm Reactor is a continuously stirred tank reactor (CSTR) with high wall shear. The reactor is versatile and may also be used for growing or characterizing various species of biofilm, or both (**2-4**) provided appropriate adjustments are made to the growth media and operational parameters of the reactor.

1.3 Basic microbiology training is required to perform this practice.

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

1.5 *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.*

1.6 *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:*[3]

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

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[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.

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

[3] 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.

***A Summary of Changes section appears at the end of this standard**

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E2871 Test Method for Determining Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Method

## 3. Terminology

3.1 *Definitions:*

3.1.1 For definition of terms used in this method refer to Terminology E2756.

3.1.2 *batch phase, n*—establishment of the biofilm by operating the reactor without the flow of nutrients (batch phase growth medium), but with mixing.

3.1.3 *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.

3.1.4 *continuously stirred tank reactor (CSTR) phase, n*—establishment of a steady state biofilm population achieved with the continuous flow of nutrients (continuous flow growth medium) in a glass vessel.

3.1.5 *coupon, n*—biofilm sample surface.

## 4. Summary of Practice

4.1 This practice is used for growing a *P. aeruginosa* or *S. aureus* biofilm in the CDC Biofilm Reactor. The biofilm is established by operating the reactor in batch phase (no flow of the nutrients) for 24 h. A steady state population is reached while the reactor operates for an additional 24 h with continuous flow of the nutrients. The residence time of the nutrients in the reactor is set to select for biofilm growth, and is species and reactor parameter specific. During the entire 48 h, the biofilm is exposed to continuous fluid shear from the rotation of a baffled stir bar. Controlling the rate at which the baffle turns determines the intensity of the shear stress to which the coupons are exposed. At the end of the 48 h, the biofilm-laden coupons are used for testing.

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## 5. Significance and Use

5.1 Bacteria that exist in biofilms are phenotypically different from suspended cells of the same genotype. Research has shown that biofilm bacteria are more difficult to kill than suspended bacteria (**4, 5**). Laboratory biofilms are engineered in growth reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. The purpose of this practice is to direct a user in the growth of a *P. aeruginosa* or *S. aureus* biofilm by clearly defining the operational parameters to grow a biofilm that can be assessed for efficacy using the Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method (E2871).

5.2 Operating the CDC Biofilm Reactor at the conditions specified in this method generates biofilm at log densities ($\log_{10}$ CFU per coupon) ranging from 8.0 to 9.5 for *P. aeruginosa* and 7.5 to 9.0 for *S. aureus*. These levels of biofilm are anticipated on surfaces conducive to biofilm formation such as the conditions outlined in this method.

5.2.1 To achieve an *S. aureus* biofilm with a population comparable to that for *P. aeruginosa* using the bacterial liquid growth medium conditions specified here, the *S. aureus* biofilm must be grown at ~~36~~36 °C ±2 °C rather than at room temperature (~~21~~(21 °C ±2 °C).

## 6. Apparatus

6.1 *Culture Tubes and Culture Tube Closures*—any glass or plastic tube with a volume capacity of at least 15 mL.

6.2 *Calibrated Pipetter*—continuously adjustable pipetter with volume capability of 1 mL.

6.3 *Vortex*—any vortex that will ensure proper agitation and mixing of culture tubes.

6.4 *Ultrasonic Water Bath*—any cavitating sonicating bath that operates at 45 ±5 kHz and which has a volume large enough to accommodate 50 mL or 250 mL conical tubes.

6.5 *Analytical Balance*—sensitive to 0.01 g.

6.6 *Sterilizer*—any steam sterilizer that can produce the conditions of sterilization is acceptable.

6.7 *Peristaltic Pump*—pump head that can hold size 16 or equivalent peristaltic pump tubing. Use a separate pump for each reactor.

6.8 *Digital Magnetic Stir Plate*—top plate of at least ~~10.16~~10.16 cm by 10.16 cm that can rotate at a range of ~~60~~60 r/min to 125 r/min ±5 r/min.

6.9 *Silicone Tubing*—multiple sizes: size 16 tubing or equivalent designed for use in a peristaltic pump (used for most connections between CSTR growth medium carboy and the reactor), and size 18 or 25 tubing or equivalent (used for reactor effluent). All sizes must withstand sterilization (for example, platinum cured).

6.10 *Norprene Tubing (or equivalent)*—size 16 or equivalent Norprene tubing. Recommended for use in the peristaltic pump.

6.11 *Glass Flow Break*—any that will connect with size 16 tubing and withstand sterilization, used to prevent microbial contamination of the nutrient reservoir from the biofilm reactor.

6.11.1 *Clamp*—used to hold flow break, extension clamp with 0.5 cm minimum grip size.

6.11.2 *Clamp Stand*—height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

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6.12 *Reactor Components.*[4]

6.12.1 *Berzelius Borosilicate Glass Tall Beaker*—1000 mL without pour spout, ~~9.5~~9.5 cm ±0.5 cm diameter. Barbed outlet spout added at ~~400~~400 mL ±50 mL mark. Spout angled 30° to 45° to ensure drainage. Spout should accommodate size 18 or 25 flexible silicone tubing.

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6.12.2 *Reactor Top*—Fig. 1. Ultra-high molecular weight (UHMW) polyethylene top (10.1 cm diameter tapering to 8.33 cm) equipped with a minimum of 3 holes accommodating 6 to 8 cm long pieces of stainless steel or other rigid autoclavable tubing with outside diameter of ~~5~~5 mm to 8 mm for medium inlet, air exchange and inoculation port. Center hole, 1.27 cm diameter, to accommodate the glass rod used to support the baffle assembly. Eight rod holes, 1.905 cm diameter, notched to accommodate stainless steel rod alignment pin (0.236 cm outside diameter). O-ring, attached to underside of reactor top.

6.12.3 *Polypropylene Rods*—Fig. 2. Eight polypropylene rods, 21.08 cm long, two types: coupon holder machined to hold three coupons (see 6.12.4) at the immersed end, three 316 stainless steel set screws embedded in side to hold coupons in place; and coupon holder blanks, without coupon recesses. Rods fit into holes in reactor top and lock into preformed notches with alignment pin.

6.12.4 *Coupons*—twenty-four cylindrical coupons (for example, borosilicate glass) with a diameter of ~~1.27~~1.27 cm ±0.013 cm, thickness of approximately 3.0 mm.

6.12.5 *Small Allen Wrench (1.27 mm, hex)*—for adjusting set screws.

6.12.6 *Stir Blade Assembly (Baffled Stir Bar)*—Fig. 3. PTFE blade (5.61 cm) fitted into cylindrical PTFE holder (8.13 cm) and held in place with a magnetic stir bar (2.54 cm).

6.12.6.1 PTFE holder fits onto a glass rod (15.8 cm), fitted into the reactor top.

[4] The sole source of supply of the apparatus (CDC Biofilm Reactor) and associated coupons 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,[1] which you may attend. The user may also build the reactor.

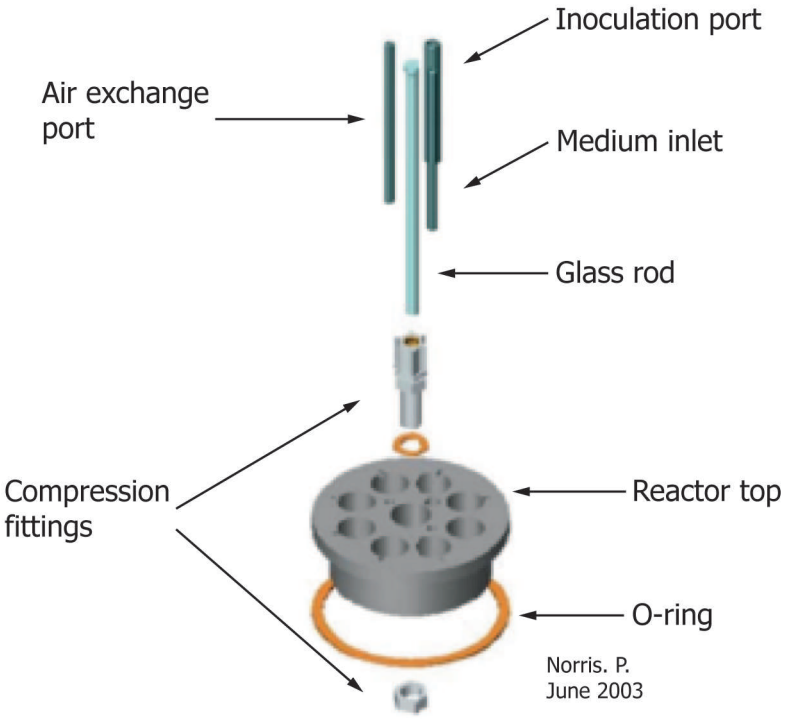


**FIG. 1 Expanded Schematic of Reactor Top**

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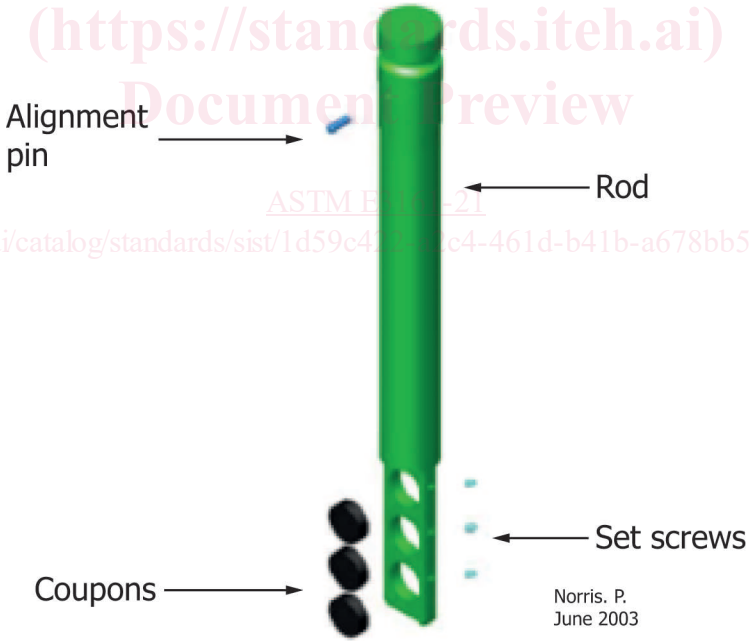


**FIG. 2 Expanded Schematic of Rod and Coupons**

6.12.6.2 The glass rod is held in place with a compression fitting and acts as a support for the moving blade assembly.

6.13 *Carboys*—two 20 L autoclavable carboys, one used for waste and one used for growth medium.

6.13.1 *Carboy Lids*—two.

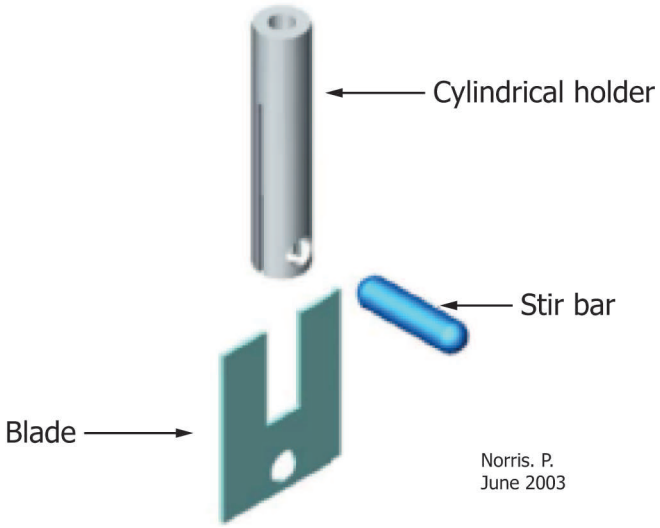


**FIG. 3 Expanded Schematic of Baffled Stir Bar**

6.13.1.1 One carboy lid with at least 2 barbed fittings to accommodate size 16 tubing (one for growth medium and one for bacterial air vent).

6.13.1.2 One carboy lid with at least two 1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).

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NOTE 1—Carboy lids can be purchased with fittings.

6.13.2 *Bacterial Air Vent (Filter)*—autoclavable 0.2 µm pore size, to be spliced into tubing on waste carboy, growth medium carboy and reactor top, recommended diameter 37 mm.

6.14 Fig. 4 illustrates a schematic of the assembled system.

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

## 7. Reagents and Materials

7.1 *Purity of Water*—All references to water as diluent or reagent shall mean de-ionized water or water of equal purity.

7.2 *Culture Media:*

7.2.1 *Cryoprotectant*—Tryptic Soy Broth (30 g/L) with 15 % (v/v) glycerol.

7.2.2 *Bacterial Liquid Growth Medium*—Tryptic Soy Broth (TSB).

7.2.2.1 For *P. aeruginosa*, use 300 mg/L TSB for the inoculum and batch phase reactor operations, and 100 mg/L TSB for the continuous flow reactor operation.

7.2.2.2 For *S. aureus*, use 30 g/L TSB for the inoculum, 3 g/L TSB for the batch phase reactor operation, and 1 g/L TSB for the continuous flow reactor operation.

7.2.3 *Growth Medium for Stock Culture Generation*—Trypticase Soy Agar (TSA).

## 8. Preparation of Apparatus

8.1 *Preparation of Borosilicate Glass Coupons:*

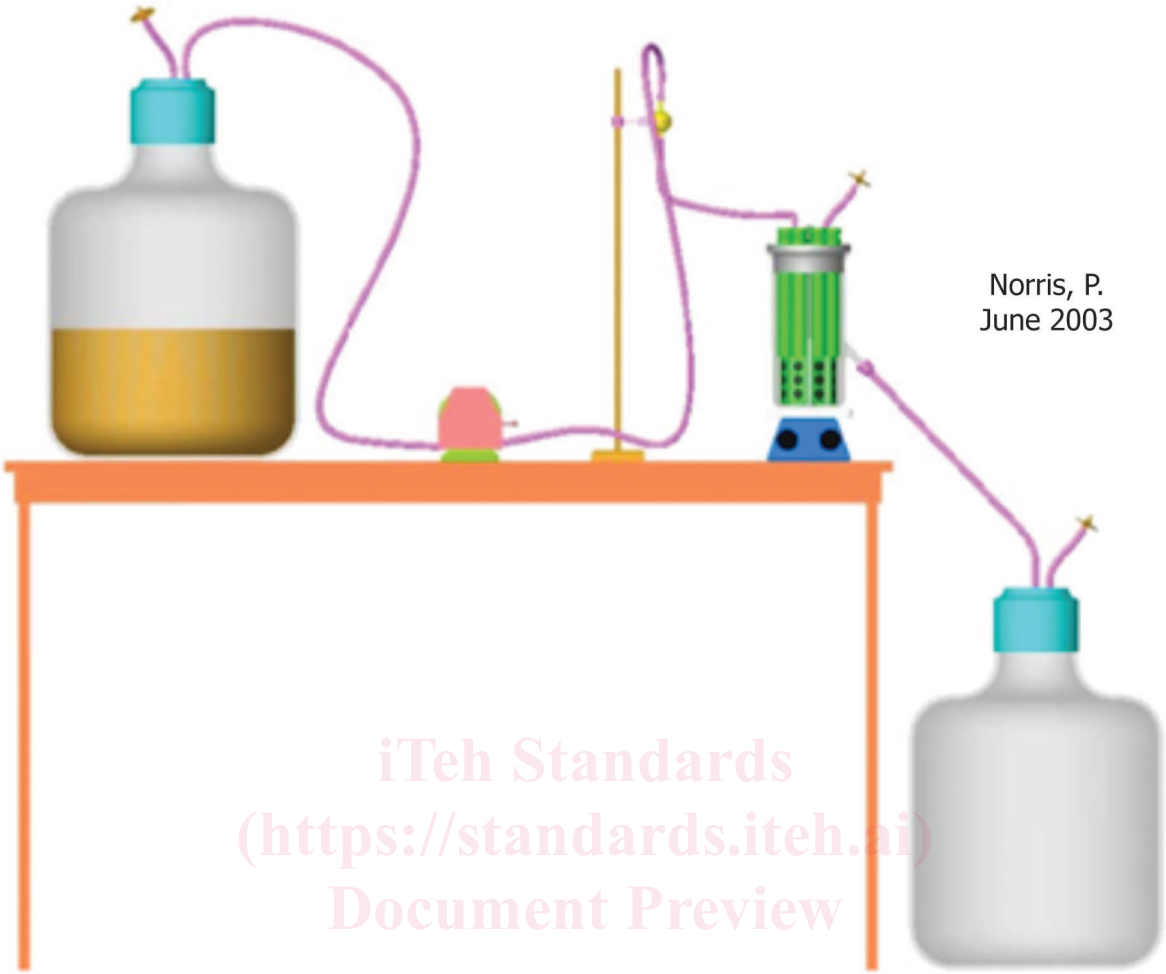


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**FIG. 4 Schematic of the Completely Assembled Reactor System**

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8.1.1 Coupons may be used repeatedly with proper cleaning and screening between each use. After use in the reactor, place contaminated coupons in an appropriate vessel, cover with liquid (e.g., water), and, along with the other parts of the contaminated reactor system, autoclave at 121°C for 30 min or using other parameters that ensure sterilization.

8.1.2 Check each coupon under 20× magnification for scratching, chipping, other damage, or accumulated debris before each use. Discard those with visible damage to surface topography.

8.1.3 For initial use and re-use, sonicate coupons in individual tubes or welled plates for approximately 5 min in detergent diluted per the manufacturer's instructions. The soapy water must completely cover the coupons.

NOTE 2—Process coupons individually to minimize the risk of damage to the coupons.

8.1.4 Rinse coupons with reagent grade water and sonicate for approximately 1 min in reagent grade water.

8.1.5 Repeat rinsing and sonication with reagent grade water until no soap is left on the coupons, as demonstrated by a lack of visible suds. Once the coupons are clean, wear gloves to prevent oils and other residue from contaminating the surface. Store screened and cleaned coupons in a Petri dish.

NOTE 3—Coupons may be made out of alternative materials. Adjust the cleaning procedure so that it is appropriate for the coupon material being used.

8.2 *Preparation of Reactor Top:*

8.2.1 Invert the reactor top and place baffled stir bar onto glass rod positioned in the center of the reactor top.

8.2.2 Invert the reactor beaker and place onto the assembled top. Turn the reactor over so that the reactor top is upright. The baffled stir bar is designed to allow it to rotate freely.

8.2.3 Place a cleaned and screened coupon into each hole in the reactor rods, leaving the top of the coupon flush with the inside rod surface. Tighten set screw. If less than 24 coupons are required for testing, substitute one coupon holder blank for each polyproplyene rod holding three (3) coupons.

8.2.4 Place rods into reactor top loosely (not yet fitted into notches).

8.2.5 Connect the bacterial air vent by fitting the vent to a small section of appropriately sized tubing and attach to one of the rigid tubes on the reactor top.

8.2.6 Splice the glass flow break into the growth medium tubing line near the reactor top.

## 9. Calibration and Standardization

9.1 Confirm the operating volume of each reactor (that is, new Berzelius beaker with spout) prior to initial use.

9.1.1 Fully assemble the reactor (including rods with coupons and baffle apparatus) and place on a stir plate set to the appropriate speed (for example, 125 r/min ±5 r/min for *P. aeruginosa* or ~~60±5 r/min~~ 60 r/min ± 5 r/min for *S. aureus*). Clamp the effluent tubing on the reactor beaker.

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9.1.2 Remove one of the rods and fill the reactor with water, higher than the level of the glass spout and reinsert the rod. Turn on the stir plate to the appropriate baffle speed.

9.1.3 Remove the clamp on the effluent tubing and allow the excess fluid to drain out of the reactor.

9.1.4 Carefully pour the remaining water into a graduated cylinder; this remaining water is the operating volume of the reactor.

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9.1.5 Use the operating volume of the reactor to determine the appropriate pump flow rate using the formula $Q=V/RT$, where $Q$ = flow rate (volume of fluid which passes through the tubing into the reactor per unit time), $V$ = operating volume of reactor, and $RT$ = residence time. For example: if the operating volume equals 325 mL and the residence time equals 30 min, then the pump flow rate should be set to equal 10.8 mL/min.

9.2 Periodic pump calibration: Follow manufacturer's instructions for calibrating pumps.

9.3 Periodic residence time verification.

9.3.1 Set up the pump as required to run the biofilm reactor.

9.3.1.1 Using a calibrated timer, pump liquid into an appropriate sized vessel (for example, at least 500 mL) for 30 min and measure the volume pumped.

9.3.1.2 Using the formula $Q=V/RT$, ensure the residence time is equal to ~~30~~30 min ±2 min. Adjust the pump flow rate as necessary.

9.4 *Sterilization of the Reactor System:*

9.4.1 Ensure that the reactor top is securely on the beaker before sterilization. To allow for pressure to escape, do not set rod alignment pins in notches during sterilization.

9.4.2 Cover the ends of the injection ports, the growth medium tubing connected to the growth medium carboy, the entire reactor top, and the effluent tubing with aluminum foil. Cover any extra openings on the reactor top with aluminum foil or plastic caps to maintain sterility after autoclaving.