US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for

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

SOP Number: MB-19-06

Date Revised: 09-19-22

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Title	Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor
Revisions Made	• Minor editorial changes for clarification purposes. • Removed footnotes in Attachment 1 referencing ASTM E3161-18. • Updated ASTM International Standard reference from 2018 to 2021 and updated version number from E3161-18 to E3161-21.

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Title	Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor
Scope	Describes the methodology used for growing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilm that can be used for disinfectant efficacy testing using the Single Tube Method (SOP MB-20).
Application	For use in the evaluation of antimicrobial products with biofilm claims.

	Approval	Date
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Date SOP issued:	09/19/22
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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. CDC = Centers for Disease Control and Prevention 2. Biofilm = 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. Coupon = biofilm sample surface 4. Residence time = the time required for the entire volume of the reactor to exchange once (during CSTR mode). 5. Batch phase = establishment of the biofilm by operating the reactor without the flow of nutrients (batch phase growth medium), but with mixing. 6. Continuously stirred tank reactor (CSTR) phase = establishment of a steady state biofilm population achieved with the continuous flow of nutrients (continuous flow growth medium) in a glass vessel.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	1. Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-05 (timers), and QC-19 (pipettes) for details on method and frequency of calibration. 2. For new reactors (i.e., new Berzelius beaker with spout), confirm the operating volume of reactor prior to initial use and record results (see section 14). a. Fully assemble the reactor (including rods with coupons and baffle apparatus) and place on a stir plate set to the appropriate speed (e.g., 125±5 r/min for *P.a.* or 60±5 r/min for *S.a.*). Clamp the effluent tubing on the reactor beaker. b. 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. c. Remove the clamp on the effluent tubing and allow the excess fluid to drain out of the reactor. d. Carefully pour the remaining water into a graduated cylinder; this

	remaining water is the operating volume of the reactor. e. 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 equal to 10.8 mL/min 3. Periodic pump calibration: follow manufacturer's instructions for calibrating the pump and document on the appropriate form (see section 14). 4. Periodic residence time verification a. Set up the pump as required to run the biofilm reactor. Using a calibrated timer, pump liquid into an appropriately sized vessel (e.g., at least 500 mL) for 30 min and measure the volume pumped. Using the formula Q = V/RT, ensure the residence time is equal to 30±2 min. Adjust the pump flow rate as necessary. Document the results on the appropriate form (see section 14).
5. Sample Handling and Storage	N/A
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. The rotational speed of the baffled stir bar directly determines the amount of shear stress that the biofilm experiences. Biofilm accumulation on the coupons is sensitive to changes in the baffle's rotational speed. The baffle rotational speed is a critical factor that must be controlled. If baffle speed is not maintained correctly, it may impact the quality of the biofilm. 2. Due to the deterioration of the materials, it may be necessary to change the tubing and filters on the reactor and carboys after 5-6 autoclaving processes. Ensure tubing used in the peristaltic pump is kept free of dirt and grit by wiping with a damp paper towel. 3. Inspect all parts of the reactor system frequently and replace as necessary. 4. Coupons must be screened prior to use. Coupons that are compromised (presence of nicks or scratches) due to repeated use should be replaced. 5. Wide fluctuations in ambient temperature may cause variability in the formation of the biofilm.

<table>
<tr><td></td><td>6. The temperature of the medium (36±2°C) must be maintained prior to and during CSTR phase for S. aureus.</td></tr>
<tr><td>8. Non-conforming Data</td><td>Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.</td></tr>
<tr><td>9. Data Management</td><td>Archive the data consistent with SOP ADM-03, Records and Archives.</td></tr>
<tr><td>10. Cautions</td><td>1. Place waste containers in secondary containment to prevent a spill of biohazardous material.
2. Check all tubing connections prior to initiating the reactor.</td></tr>
<tr><td>11. Special Apparatus and Materials</td><td>1. Test organisms.
a. Pseudomonas aeruginosa (ATCC No. 15442).
b. Staphylococcus aureus (ATCC No. 6538).
2. Cryoprotectant. TSB (30 g/L) with 15% (v/v) glycerol.
3. Growth medium for stock culture generation. Trypticase soy agar (TSA).
4. Bacterial liquid growth media. Tryptic soy broth (TSB).
Table 1. Growth Media Concentrations
<table>
<tr><th></th><th colspan="2">TSB Concentration</th></tr>
<tr><th>Biofilm Phase</th><th>P. aeruginosa</th><th>S. aureus</th></tr>
<tr><td>Inoculum</td><td>300 mg/L</td><td>30 g/L</td></tr>
<tr><td>Batch phase</td><td>300 mg/L</td><td>3 g/L</td></tr>
<tr><td>CSTR</td><td>100 mg/L</td><td>1 g/L</td></tr>
</table>
5. Water. Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.
6. Calibrated micropipettes. Continuously adjustable pipette with volume capability of 1 mL for performing culture transfers.
7. Ultrasonic water bath. For cleaning the CDC Reactor coupons. Any cavitating sonicating bath that operates at 45±5 kHz and has a volume large enough to accommodate 50 mL or 250 mL conical tubes.
8. Peristaltic pump. Pump head that can hold size 16 or equivalent</td></tr>
</table>

peristaltic pump tubing. Use a separate pump for each reactor.

9. *Digital magnetic stir plate*. Top plate of at least 10.16×10.16 cm that can rotate at a range of 60-125±5 r/min.
10. *Silicon 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 steam sterilization (e.g., platinum cured).
11. *Norprene tubing (or equivalent)*. Size 16 or equivalent Norprene tubing. Recommended for use in the peristaltic pump.
12. *Glass flow break*. Any that will connect with size 16 tubing and withstand steam sterilization, used to prevent microbial contamination of the nutrient reservoir from the biofilm reactor.
13. *Clamp*. Used to hold flow break, extension clamp with 0.5 cm minimum grip size.
14. *Clamp stand*. Height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.
15. *Reactor Components*. For schematic illustrations of CDC reactor components, see ref. 15.1. Reactor components available from BioSurface Technologies, Inc.
    a. *Berzelius borosilicate glass tall beaker*.1000 mL without pour spout, 9.5±0.5 cm diameter. Barbed outlet spout added at 400±50 mL mark. Spout angled 30-45° to ensure drainage. Spout should accommodate size 18 or 25 flexible silicone tubing.
    b. *Reactor top*. UHMW (ultra-high molecular weight) polyethylene top (10.1 cm diameter tapering to 8.33 cm) equipped with a minimum of 3 holes accommodating 6-8 cm long pieces of stainless steel or other rigid autoclavable tubing with OD of 5-8 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 OD). O-ring, attached to underside of reactor top.
    c. *Polypropylene rods*. Eight polypropylene rods, 21.08 cm long, two types: coupon holder machined to hold three coupons (see 11.14d) at the immersed end, three 316 stainless steel set screws embedded in the 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.
    d. *Coupons*. Twenty-four cylindrical coupons (e.g., borosilicate

	glass) with a diameter of 1.27±0.013 cm, thickness of approximately 3.0 mm. e. *Small Allen wrench (1.27 mm hex)*. For adjusting set screws. f. *Stir blade assembly (baffled stir bar)*. Polytetrafluoroethylene (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). PTFE holder fits onto a glass rod (15.8 cm), fitted into the reactor top. The glass rod is held in place with a compression fitting and acts as a support for the moving blade assembly. 16. *Carboys*. Two 20 L autoclavable carboys, one used for waste and one for growth medium. a. *Carboy lids*. Two carboy lids. One carboy lid with at least 2 barbed fittings to accommodate size 16 tubing (one for growth medium and one for bacterial air vent (filter); a third barbed fitting may be used for injecting TSB medium concentrate). 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). i. Carboy lids can be purchased with fittings. 17. *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. 18. *Detergent*. Laboratory detergent for cleaning coupons and reactor parts.
12. Procedure and Analysis	Growing the organism in the reactor is partitioned into 2 phases. The biofilm is established by operating the reactor in batch phase (i.e., no flow) for 24 hours followed by 24 hours with continuous flow of growth medium to form biofilm on coupons (CSTR phase). The coupons are harvested to conduct the efficacy test. 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*. The main components of this procedure are as follows: - Verify reactor operating volume (once per reactor) and periodically calibrate the pump - Clean and screen coupons - Prepare the reactor and steam sterilize the assembled reactor - Steam sterilize the 20 L carboy containing 19 L de-ionized water - Prepare the inoculum - Initiate batch phase

	- Initiate CSTR phase - Harvest coupons for efficacy testing (refer to SOP MB-20)
12.1 Borosilicate glass coupon preparation	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, steam sterilize at 121°C for 30 min or using other parameters that ensure sterilization. a. Check each coupon under 20X magnification for scratching, chipping, other damage, or accumulated debris before each use. Discard those with visible damage to surface topography. b. 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. Process coupons individually to minimize the risk of damage to the coupons. c. Rinse coupons with reagent grade water and sonicate for approximately 1 min in reagent grade water. Repeat rinsing and sonication with reagent grade water until no soap is left on the coupons, as demonstrated by a visible lack of suds. d. 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: Coupons may be made out of alternative materials. Adjust the cleaning procedure so that it is appropriate for the coupon material being used.
12.2 Preparation of reactor top	a. Invert the reactor top and place baffled stir bar onto glass rod positioned in the center of the reactor top. b. 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. c. 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 the set screw. If less than 24 coupons are required for testing, substitute one coupon holder blank for each polypropylene rod holding 3 coupons. d. Place rods into reactor top loosely (not yet fitted into notches). e. 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.

	f. Splice the glass flow break into the growth medium tubing line near the reactor top.
12.3 Sterilization of the reactor system	a. 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. b. 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. c. Steam sterilize the empty reactor system at 121°C for 20 min. d. After sterilization, verify that all coupons are in place. If a coupon has fallen out of a rod, aseptically remove the rod with the missing coupon and insert a sterile coupon holder blank into the reactor prior to initiating batch phase; retrieve the fallen coupon with flame sterilized forceps or other sterile instrument.
12.4 Inoculum initiation from frozen stock culture	Refer to SOP MB-02 for the test microbe culture transfer notation. Refer to Attachments 1-2 for stock culture generation and QC. a. For *P. aeruginosa*, defrost a single cryovial and briefly vortex to mix. Add 10 µL of the frozen stock culture to a tube containing 10 mL of sterile TSB (300 mg/L) and vortex to mix. Incubate bacterial suspension at 36±2°C for 24±2 h. b. For *S. aureus*, defrost a single cryovial and briefly vortex to mix. Add 10 µL of the frozen stock culture to a tube containing 10 mL of sterile TSB (30 g/L) and vortex to mix. Incubate bacterial suspension at 36±2°C for 24±2 h. c. Culture tracking: add "-BF-A" after the number of the frozen stock culture vial in the organism transfer number to denote the first transfer (A) of a biofilm culture (BF) (see section 14 for culture tracking form). d. Verify purity of the inoculated tube by streak isolation (e.g., verify appropriate colony morphologies) on TSA with 5% sheep blood (BAP). Incubate plate with the -BF-A culture and examine for purity. Record results of purity check on culture tracking form (see section 14). e. If desired, verify that the culturable bacterial density of the 24±2 h -BF-A culture is at least 10^7 CFU/mL by serial dilution and plating.

12.5 Growth of biofilm in CDC reactor – Batch Phase	a. Clamp the effluent line of the reactor. Aseptically add 500 mL of the cooled batch phase culture medium to the cooled reactor by aseptically removing a rod from the reactor and pouring the batch phase growth medium through the rod opening. Re-insert the rod. i. For *P. aeruginosa*, the batch phase growth medium is 300 mg/L TSB. ii. For *S. aureus*, the batch phase growth medium is 3 g/L TSB. b. Secure the rod alignment pins into the reactor top notches. c. Place reactor onto a stir plate. d. Clamp the flow break in an upright position; leave other tubing clamped and covered with aluminum foil. e. Vortex the 10 mL tube of culture (see sections 12.4a-b) and use 1 mL to inoculate the reactor through one of the available rigid stainless steel tubes in the reactor top. f. Turn on the magnetic stir plate. i. For *P. aeruginosa*, the rotational speed of the baffle is 125±5 r/min. Run the reactor system in batch phase at room temperature (21±2°C) for 24±2 h. Record ambient temperature on the culture tracking form (see section 14). Note: wide fluctuations in ambient temperature may cause variability in the formation of the biofilm. ii. For *S. aureus*, the rotational speed of the baffle is 60±5 r/min. Incubate the reactor system in batch phase at 36±2°C for 24±2 h. g. Culture tracking: add "-BF-B" after the number of the frozen stock culture vial in the organism transfer number to denote the second transfer (B) of a biofilm culture (BF) (see section 14 for culture tracking form).
12.6 *P. aeruginosa* CSTR medium preparation	For *P. aeruginosa* biofilm, run the CSTR phase at room temperature (21±2°C). a. Prepare and sterilize concentrated growth medium (e.g., 40 g/L TSB) separately from 19 L sterile deionized water. b. Add 50 mL of sterile 40 g/L TSB to 19 L sterile water (in carboy), then fill to 20 L with additional sterile water to achieve a final growth medium concentration of 100 mg/L TSB. Other concentration/volume combinations of TSB may be used to achieve a final concentration of 100 mg/L TSB. Shake the carboy

	or use an appropriately sized sterile stir bar on a magnetic stir plate to thoroughly mix the contents. c. Aseptically connect the tubing from the reactor to the carboy containing the CSTR growth medium (100 mg/L TSB). Record ambient temperature on the culture tracking form (see section 14).
12.7 *S. aureus* CSTR medium preparation	For *S. aureus* biofilm, run the CSTR phase at 36±2°C. a. Prepare and sterilize concentrated growth medium (e.g., 40 g/L TSB) separately from 19 L sterile deionized water. b. Add 0.5 L of sterile 40 g/L TSB to 19 L sterile water (in carboy), then fill to 20 L with additional sterile water to achieve a final growth medium concentration of 1 g/L TSB. Other concentration/volume combinations of TSB may be used to achieve a final concentration of 1 g/L TSB. Shake the carboy or use an appropriately sized sterile stir bar on a magnetic stir plate to thoroughly mix the contents. c. For *S. aureus*, the continuous flow growth medium entering the reactor must be at 36±2°C; therefore, preheating of the continuous flow growth medium is required. i. The following are examples of methods that may be used to preheat the continuous flow growth medium if an incubator large enough to contain the entire assembled reactor system is not available: (1) place the continuous flow growth medium carboy into an incubator for 2 to 3 days prior to use to bring the medium to 36±2°C, or (2) coil approximately 12 to 15 feet of size 16 tubing inside the incubator and prime the tubing with the continuous flow growth medium (1 g/L TSB) at least 1 day prior to use; refer to Attachment 3. ii. The temperature of the medium for *S. aureus* (36±2°C) must be maintained prior to and during CSTR.
12.8 Growth of biofilm in CDC reactor – CSTR phase	a. Aseptically connect the growth medium tubing to the carboy containing the continuous flow growth medium. b. Pump a continuous flow of growth medium into the reactor to achieve a 30±2 min residence time based on the reactor's operating volume (see 4.2). Attach tubing from the effluent spout to a waste carboy and remove the clamp. i. The effluent spout on the beaker allows overflow to occur, maintaining a constant growth medium concentration in the reactor during CSTR phase.

	c. For *P. aeruginosa*, operate the reactor in CSTR mode for 24±2 h at room temperature (21±2°C) with a baffle speed of 125±5 r/min. d. For *S. aureus*, operate the reactor in CSTR mode for 24±2 h at 36±2°C with a baffle speed of 60±5 r/min. e. Culture tracking: add "-BF-C" after the number of the frozen stock culture vial in the organism transfer number to denote the third step (C) in production of a biofilm culture (BF) (see section 14 for culture tracking form). f. Use the procedure in MB-20 to sample the biofilm and evaluate for efficacy. Use coupons for testing in MB-20 within 30 min after growth medium flow and baffled stir bar have been turned off.
13. Data Analysis/ Calculations	N/A
14. Forms and Data Sheets	1. Attachment 1: Procedures for Maintenance of Vegetative Bacterial Cultures – Preparation of Frozen Stock Cultures 2. Attachment 2: Typical Growth Characteristics of strains of *P. aeruginosa* and *S. aureus* 3. Attachment 3: Reactor/Tubing Setup for Generation of *S. aureus* Biofilm 4. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Biofilm Organism Culture Tracking Form — MB-19-06_F1.docx Test Microbe Confirmation Sheet (Quality Control) — MB-19-06_F2.docx Biofilm Reactor Operating Volume Verification Form — MB-19-06_F3.docx Pump Calibration Form — MB-19-06_F4.docx Biofilm Reactor Residence Time Verification Form — MB-19-06_F5.docx
15. References	1. ASTM International, 2021. E3161-21: Standard Practice for Preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor. 2. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's

	Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015. 4. Standard Methods for the Examination of Water and Wastewater. 23rd Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC.

Attachment 1

Procedures for Maintenance of Vegetative Bacterial Cultures – Preparation of Frozen Stock Cultures

(Refer to SOP MB-02 for establishment of the organism control number.)

A. Frozen stock cultures are single use only and should be approximately 10^9 CFU/mL.

B. Prepare new stock cultures from lyophilized cultures of *P. aeruginosa* (ATCC 15442) and *S. aureus* (ATCC 6538) at least every 18 months.

   i. New frozen stock culture may be initiated <u>one</u> time using an existing, unexpired frozen stock culture as the source. Begin process at step E by streaking a loopful of the frozen stock culture onto a TSA plate.

C. Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB (30 g/L), aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture.

D. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly by vortexing. Incubate broth culture at 36±2ºC for 24±2 h. Growth in TSB is necessary to generate culture of sufficient titer for streak isolation.

E. After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plates for 24±2 h at 36±2°C.

   i. Perform a streak isolation of the broth culture onto BAP as a purity check and streak the broth culture onto the appropriate selective media. Refer to appropriate selective media in Attachment 2. Incubate all plates for 24±2 h at 36±2°C.

   ii. Record results at the end of the incubation timeframe. Refer to Attachment 2 for results on selective media and diagnostic characteristics of the test microbes.

F. Select 3-5 isolated colonies of the test organism and re-suspend into 1 mL of TSB (30 g/L). For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa* – the stock culture should be representative of all phenotypes present on the streak isolation plate. Spread plate 0.1 mL of the suspension on each of 6 to 10 TSA plates. Incubate the plates for 24±2 h at 36±2°C.

   i. If necessary to obtain more frozen stock cultures, a larger suspension (e.g., 2 mL) may be prepared using the same ratio of TSB (1 mL) to number of colonies (3-5 colonies).

   ii. Using the TSB suspension, perform a streak isolation of the suspension onto a BAP as a purity check and streak on the appropriate selective media (refer to Attachment 2).

   iii. Incubate all plates for 24±2 h at 36±2°C. Record results. Refer to Attachment 2 for results on selective media and diagnostic characteristics of the test microbes.

G. Following the incubation of the agar plates from step F, place approximately 5 mL sterile

cryoprotectant solution (TSB with 15% glycerol) equilibrated to 20±5°C on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL.

H. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

I. Immediately after mixing, dispense aliquots (0.5-1 mL) of the harvested suspension into cryovials; these represent the frozen stock cultures. Within 60 min after harvesting, store the cryovials at ≤-70ºC for a maximum 18 months then reinitiate with a new lyophilized culture.

   i. For QC purposes, perform a streak isolation of the pooled culture onto a BAP as a purity check and streak on appropriate selective media (refer to Table 1).

   ii. Incubate all plates for 24±2 h at 36±2°C.

   iii. Record results. Refer to Attachment 2 for results on selective media and diagnostic characteristics of the test microbes.

   iv. After incubation, perform a Gram stain on growth from the BAP; observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

   v. Conduct Vitek confirmation from growth taken from the BAP. Conduct VITEK according to the manufacturer's instructions.

   vi. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

J. If the characteristics of the organism are not consistent with the information in Attachment 2 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

Attachment 2

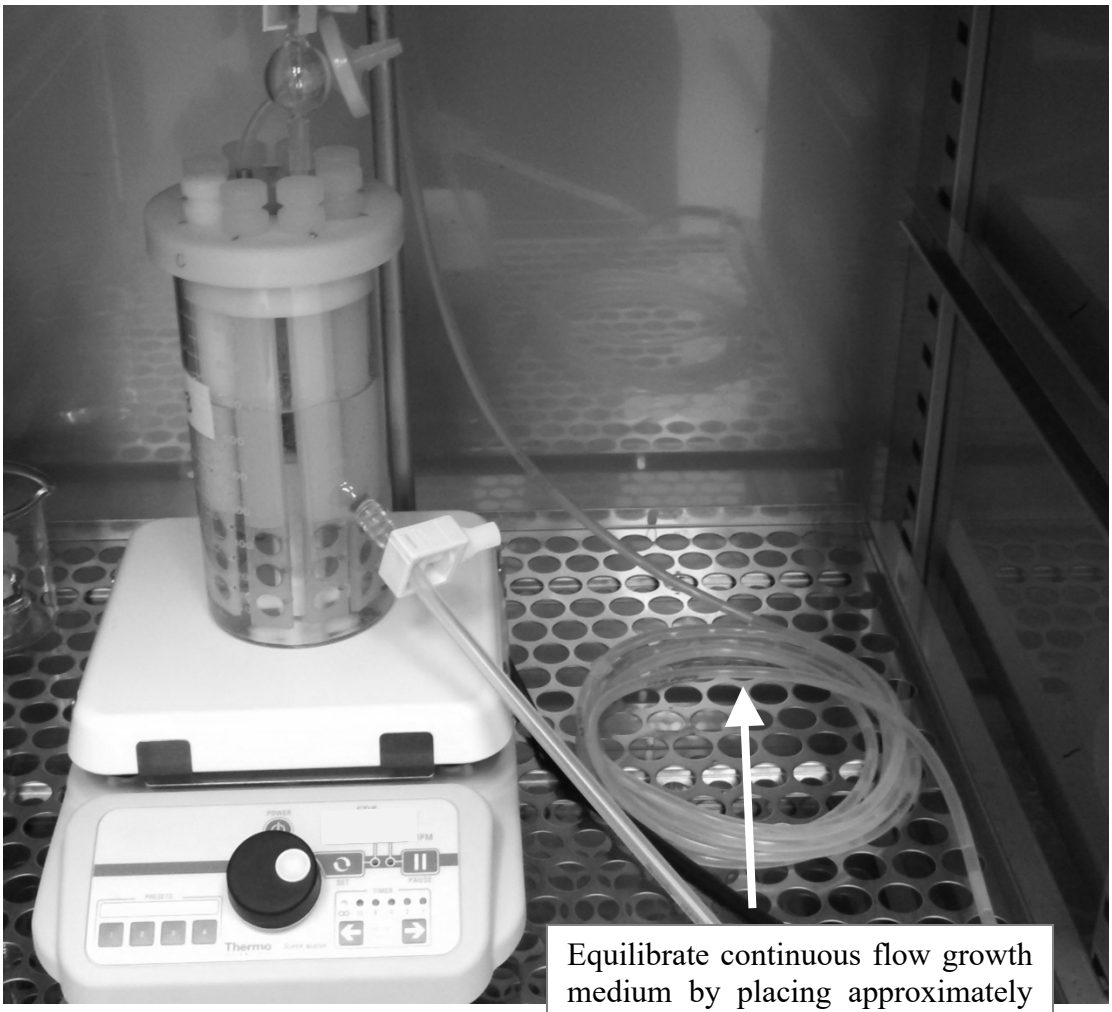
Typical Growth Characteristics of strains of *P. aeruginosa* (see ref. 15.3) and *S. aureus* (see ref. 15.4).

		*P. aeruginosa**	*S. aureus**
Gram stain reaction		Negative	Positive
Typical Growth Characteristics on Solid Media			
Selective Media	Mannitol Salt	No Growth	Circular, small, yellow colonies, agar turning fluorescent yellow
	Cetrimide	Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth
Blood agar (BAP)		Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	Small, circular, yellow or white, glistening, beta hemolytic
Typical Microscopic Characteristics			
Cell appearance		Straight or slightly curved rods, single polar flagella, rods formed in chains; 0.5-1.0 µm in diameter by 1.5-5.0 µm in length	Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters; 0.5-1.0 µm in diameter

*After 24±2 hours (1) *P. aeruginosa* may display two phenotypes.

Attachment 3

Reactor/Tubing Setup for Generation of *S. aureus* Biofilm (inside 36±2°C incubator)



Equilibrate continuous flow growth medium by placing approximately 12-15 ft of coiled tubing primed with continuous flow growth medium in an incubator 1 day prior to use.