A method for growing a biofilm under low shear at the air–liquid interface using the drip flow biofilm reactor

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This protocol describes how to grow a *Pseudomonas aeruginosa* biofilm under low fluid shear close to the air–liquid interface using the drip flow reactor (DFR). The DFR can model environments such as food-processing conveyor belts, catheters, lungs with cystic fibrosis and the oral cavity. The biofilm is established by operating the reactor in batch mode for 6 h. A mature biofilm forms as the reactor operates for an additional 48 h with a continuous flow of nutrients. During continuous flow, the biofilm experiences a low shear as the media drips onto a surface set at a 10° angle. At the end of 54 h, biofilm accumulation is quantified by removing coupons from the reactor channels, rinsing the coupons to remove planktonic cells, scraping the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration. The entire procedure takes 13 h of active time that is distributed over 5 d.

INTRODUCTION

The discovery that bacteria predominantly exist as a biofilm in natural ecosystems[1–3] led to the realization that better-designed systems were needed for growing and testing disinfectant efficacy against bacterial biofilms in the laboratory. A laboratory reactor is a tool used by biofilm researchers to grow biofilms. A biofilm has a unique architecture depending on the conditions under which it forms. For example, a *Pseudomonas fluorescens* biofilm formed in a reactor with high shear is denser and more tightly adhered to the surface as opposed to a *P. fluorescens* biofilm formed in a reactor under low shear, which is fluffy[4]. One biofilm, or one reactor, is not better than another; they are just different from each other and therefore have different applications.

In 'antibiofilm' efficacy testing, the goal is to grow a biofilm that is relevant to the environment where a disinfectant will be applied then test the disinfectant under 'real-use' conditions. Biofilm methods are complex. An efficient approach for standardizing biofilm methods is to partition the method into discrete steps, and then standardize each step separately. This approach results in less methods development, yet yields hundreds of combinations of potential methods. The steps required for testing the efficacy of an 'antibiofilm' treatment are (1) grow a relevant and repeatable biofilm using a biofilm reactor, (2) treat a mature biofilm with a biocide or with antibiotics, (3) remove a representative biofilm sample and (4) analyze the sample for a quantitative and/or qualitative estimate of kill and/or removal as a result of the treatment. Researchers interested in biofilm efficacy testing choose the most relevant combination of grow, treat, sample and analyze methods that best serve their particular needs.

In biofilm research there are four environments of particular interest to be considered when choosing the best biofilm reactor for use: high shear/turbulent flow, moderate shear, low shear/laminar flow and no shear. The CDC Biofilm Reactor[5] simulates a high-shear environment and the rotating disk reactor[6] simulates a moderate-shear environment. A simple procedure, namely the colony biofilm growth method[7], simulates a no-shear environment. This protocol describes how to grow a *Pseudomonas aeruginosa* biofilm under low-shear/laminar flow using the drip flow reactor (DFR).

In engineering terms, DFR is defined as a plug flow reactor, meaning that cell density and nutrient concentration change along the length of the coupon. Plug flow is also found in pipes, tubing or catheters. The flow in a plug flow reactor can be either laminar or turbulent depending on the density and viscosity of the liquid, the flow velocity and the geometry of the reactor. This information is used to calculate a dimensionless number known as the Reynolds number. Defining the type of reactor and the flow that exists within it enables a researcher to choose the most relevant reactor system to be used in a particular study. Additional parameters to be considered when choosing the best reactor system to use are statistical properties, number of individual samples per reactor, amount of biomass produced, and cost and time of operation.

Distinguishing characteristics that make the DFR widely applicable include the following:

- The biofilm is formed close to the air–liquid interface. This reactor system can model environments such as food-processing conveyor belts, catheters[8], lungs with cystic fibrosis and the oral cavity[9].
- Surfaces within the reactor experience mild shear (low fluid velocity over the surface), which yields a biofilm that has a smooth appearance and is loosely attached. Microscopically, the biofilm is sheet-like with few architectural details.
- The gas in the head space may be varied to accommodate the growth of various anaerobic biofilms.
- The residence time in the reactor is short. This characteristic allows for the application of different treatments or molecular labeling schemes.
- Biofilm from the reactor can be easily analyzed using various techniques, including viable plate counts, *in situ* confocal microscopy, microelectrodes or cryosectioning followed by microscopy[10,11].
- The biofilm grown in the DFR can be used for disinfectant and antibiotic efficacy testing[12–14].

- The reactor can house various surfaces for biofilm growth to further model relevant conditions (for example, the surfaces may be made out of glass, polycarbonate, stainless steel or mild steel).

The DFR was designed as a flexible reactor system that could be easily adapted to model a variety of conditions in the laboratory. Essentially every component of this protocol may be modified, although typically the equipment setup and general approach for establishing a biofilm, through a batch phase of growth followed by growth under a continuous laminar flow of nutrients, will remain the same. Researchers are encouraged to modify the protocol to model the most appropriate biofilm for their research needs. As a cautionary note, changing one operational parameter will often necessitate a change in other operational parameters. For instance, if another type of bacteria is used, the researcher may need to modify the operational temperature, nutrient source and concentration, length of time in batch and continuous flow operation, and the media used for the viable plate counts. If the protocol is modified to fit a specific application, then experiments should be conducted to determine whether the resulting biofilm is repeatable and sufficiently abundant.

The following protocol describes a method for growing a repeatable *P. aeruginosa* biofilm under low shear at room temperature (21 °C ± 2 °C) (ref. 15). In this method, a laboratory biofilm is established on four separate coupons in batch mode for 6 h and is then grown under low shear with a continuous flow of nutrients for 48 h. Biofilm accumulation is quantified by harvesting the biofilm from coupons of a known surface area, disaggregating the cell clumps and performing viable plate counts.

MATERIALS

REAGENTS

- Tryptic Soy Broth (TSB) (Remel, cat. no. 455054)
- KH_2PO_4 (Fisher Scientific, cat. no. P285)
- $MgCl \cdot 6H_2O$ (Fisher Scientific, cat. no. NC9432081)
- 95% Ethanol (Fisher Scientific, cat. no. S73985)
- R2A (Remel, cat. no. 454374)
- Distilled water or water of equal purity

EQUIPMENT

Drip flow biofilm reactor components (BioSurface Technologies Corp., see **Fig. 1**)

- **Base:** 15.24 cm × 12.70 cm × 2.54 cm polysulfone base with four 2.55 cm × 10.16 cm × 1.99 cm channels and four 1.27-cm barbed effluent ports (one at the end of each channel). The underside holds four adjustable legs providing a 10° angle of operation (**Fig. 1**).
- **Cover:** Four 3.44 cm × 12.7 cm × 1.22 cm polycarbonate covers, each with two threaded holes for nylon screws to secure to the reactor base. Each cover has two ports, one for influent media line attachment and another for bacterial air vent attachment (used to allow for the sterile air and gas exchange). O-rings are fitted underneath to seal cover to base during operation (**Fig. 1**).
- **Mininert valves:** Fit into each cover as influent ports to allow for inoculation and media line attachment (**Fig. 1**).
- **Needle:** 1 inch, 21 gauge; fit into mininert valve (**Fig. 1**).
- **Glass coupons:** Four new rectangular glass microscope slides with a surface area of 18.75 cm^2 (25 mm × 75 mm × 1 mm) (**Fig. 1**). ▲ CRITICAL Coupons may be fashioned from other similarly shaped/sized materials; however, the test method must be re-evaluated and standardized accordingly. ▲ CRITICAL Estimated time to assemble the reactor is 1.5 h.

Drip flow reactor system assembly components

- **Glass flow break:** Any that will connect with a tubing of inner diameter (ID) 3.1 mm and can withstand sterilization (**Fig. 2**). Used to prevent back-contamination into the media carboy.
- **Silicone tubing:** Tubings of two different sizes: one with ID 3.1 mm and outer diameter (OD) 3.2 mm, and the other with ID 7.9 mm and OD 9.5 mm. Both types must withstand sterilization (**Fig. 2**).
- **Norprene tubing:** ID of 1.6 mm, capable of withstanding sterilization. This portion of tubing is run through the pump head (**Fig. 2**).

▲ CRITICAL Other tubing materials and sizes may be used, provided the test method is carefully re-evaluated. Ensure that ID changes are taken into consideration for flow rate calculations if norprene tubing substitutions are made.

- **Bacterial air vents:** Autoclavable, 0.2 μm pore size, spliced into the tubing on waste carboy and nutrient carboy. A small air vent is attached to each reactor channel cover (**Fig. 2**).
- **Teflon thread seal tape:** Wrapped around effluent ports, if needed, to prevent leakage from effluent port connectors.
- **Carboys:** Two 20-liter autoclavable carboys, used for waste and nutrients (**Fig. 2**).
- **Carboy lids:** One carboy lid with at least two barbed fittings to accommodate a tubing of ID 3.1 mm (one for the nutrient line and one for the bacterial air vent). One carboy lid with at least two 1-cm holes bored in the same manner (one for effluent waste and one for the bacterial air vent). Carboy lids can be purchased pre-drilled and/or fitted, or can be purchased for drilling/fitting in-house by the user.
- **Peristaltic pump and four pump heads:** Capable of holding a tubing with ID 3.1 mm and OD 3.2 mm and calibrated to operate at a flow rate of 200 ml per h (**Fig. 2**).
- **Clamp stand and clamp:** Clamp with minimum 0.5 cm grip size used to hold the glass flow break and media inlet tubing in a vertical position.

Inoculum preparation

- **Environmental shaker:** Capable of maintaining a temperature of 35 °C ± 2 °C.

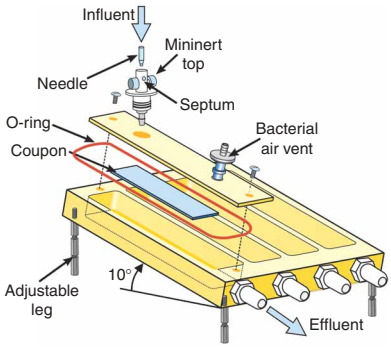

Figure 1 | Schematic diagram of a drip flow reactor showing its various components. Reprinted with permission from ref. 15.

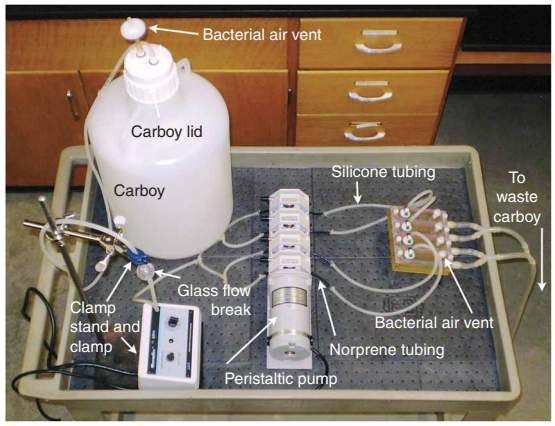

Figure 2 | Assembled DFR apparatus. Reprinted with permission from ref. 15.

- **250-ml Erlenmyer flask:** Capped with foil and sterilized.

Sampling supplies

- **Homogenizer:** Any. Capable of mixing at 20,500 ± 5,000 r.p.m.
- **Homogenizer probe:** Any. Capable of mixing at 20,500 ± 5,000 r.p.m. in 50-ml volume and able to withstand autoclaving or other means of sterilization.
- **Vortex:** Any vortex mixer that will ensure proper agitation and mixing of culture tubes.
- **Teflon, metal or rubber spatulas:** Sterile, required for scraping biofilm from the coupon surface.
- **Bunsen or alcohol burner:** Used to flame-sterilize the inoculating loop and other instruments. 95% ethanol is used to flame-sterilize hemostats or forceps.
- **Culture tubes and culture tube closures:** Any with a volume capability of 10 ml and a minimum diameter of 16 mm.
- **Petri dish:** 100 mm × 15 mm, plastic, sterile and empty, used to transport coupons from the reactor to the work station.
- **Stainless steel hemostat clamp or forceps:** Used for aseptic handling of coupons.
- **Glass beakers:** Sterile, any with a volume capacity of 100 ml containing 45 ml sterile dilution water.
- **Conical-bottom sterile disposable plastic centrifuge tubes:** With a volume capacity of 50 ml (containing 45 ml sterile dilution water).
- **Incubator:** Used to incubate plate cultures. Any incubator that can maintain a temperature of 35 °C ± 2 °C.

REAGENT SETUP

Inoculum culture media 100 ml of 3,000 mg TSB liter^{-1}. Sterilize for 20 min on liquid cycle. Prepare and use on the same day.

Batch culture media 100 ml of 3,000 mg TSB liter^{-1}. Sterilize for 20 min on liquid cycle. Prepare and use on the same day.

Continuous flow media Prepare 20 liters of continuous flow nutrient broth. To prevent caramelization that can occur with longer autoclave times required for larger volumes, prepare and sterilize a small, concentrated volume of TSB separate from sterilization of the 20 liters reagent grade water. Aseptically pour the concentrated broth into the carboy of sterile water to make a total of 20 liters of growth media at a concentration equal to 270 mg TSB liter^{-1}. Water can be sterilized 2 d before use. Add the sterile, concentrated broth to the 20 liters of sterile water right before starting continuous flow in the reactor.

Buffered dilution water 0.0425 g liter^{-1} KH_2PO_4 distilled water, filter-sterilized and 0.405 g liter^{-1} $MgCl \cdot 6H_2O$ distilled water, filter sterilized. Aseptically fill sterile, capped conical vials and beakers with 45 ml of sterile dilution water[16]. Can be prepared and stored at room temperature (21 °C ± 2 °C) for up to 2 weeks.

Bacterial plating medium R2A agar prepared according to the manufacturer's recommendations. Prepare 3 d before use and store at 4 °C.

70% ethanol Add 368 ml of 95% ethanol to 132 ml of sterile water. Can be prepared and stored at room temperature for up to 2 weeks.

Bacterial culture and inoculum preparation:

(1) A frozen stock culture of *P. aeruginosa* (ATCC 700888) is streaked for isolation on R2A agar.
(2) Incubate the plate at 35 °C for 17–24 h.
(3) Using a flame-sterilized loop, pick an isolated colony from the streak plate and stir into inoculum culture media (preparation described in REGENT SETUP). Incubate at 35 °C while shaking for 18–24 h.
(4) The viable bacterial density of the inoculum should be equal to 10^8 cfu ml^{-1}. Dilute and plate a sample from the inoculum flask to confirm the bacterial density[17].

! CAUTION All microorganisms should be handled according to the biosafety recommendations specific for each individual species. Decontamination of all media and equipment used during experimentation is required before disposal of media and reuse of equipment. **▲ CRITICAL** Estimated time for reagent setup is 5 h (plus 24 h of incubation time).

EQUIPMENT SETUP

Set the pump flow rates (Refer to **Fig. 2** for tubing configuration and pump placement; estimated time is 30 min)

(1) Set pump flow rates before performing the experiment. Use the same ID tubing to set the flow rate as will be used in the experiment.
(2) To set the flow rate, turn on the pump and allow it to warm up for 1–2 h.
(3) After warm-up, turn off the pump and carefully assemble the tubing through the pump heads. Seat firmly to prevent creeping.

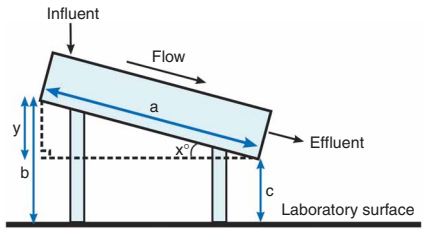

Figure 3 | Side view of the DFR used for calculating the reactor angle. In this schematic, *a* is the measured length (cm) of the reactor base, *b* is the measured length (cm) from the bottom corner of the influent end of the reactor base to the laboratory surface, *c* is the measured length (cm) from the bottom corner of the effluent end of the reactor base to the laboratory surface, *y* is the difference between *b* and *c*, and *x* is the required angle. Reprinted, with permission, from ref. 15.

(4) Place one end of the tubing into a beaker of water and each of the four other ends into separate graduated cylinders.
(5) Set the pump to the approximate desired flow (or start with 1 on the dial) and turn it on.
(6) Let the water flow through the tubing and start the timer when drops begin to fall into the graduated cylinders. Time for 10 min.
(7) Divide the volume in each cylinder by 10 min to determine the flow rate in milliliter per minute for the corresponding channel. Adjust the dial accordingly until the desired flow rate of 0.8 ml min^{-1} per channel is achieved.

Adjust the reactor angle (estimated time is 10 min)

(1) Measure *a*, the length of the reactor base, in cm (**Fig. 3**).
(2) Angle (x) equals 10°.
(3) Calculate y using equation (1):

$$y = a(\sin(x)) \quad (1)$$

where: *a* is the measured length of reactor base in cm, x the required angle and y the difference between length *b* and length *c* in cm.
(4) For a 10° angle, $y = a * 0.17365$.
(5) Tighten the legs near the effluent port until hand-tight, measure length *c*.
(6) Determine length *b* using equation (2):

$$b = y + c \quad (2)$$

where *b* is the measured length (cm) from the bottom corner of the influent end of the reactor base to the laboratory surface; *c* the measured length (cm) from the bottom corner of effluent end of the reactor base to the laboratory surface; y = the difference between length *b* and length *c* in cm.
(7) Adjust the legs near the influent port accordingly. Mark to note the required adjustment.

Prepare the reactor for autoclaving (estimated time is 20 min)

(1) Use new coupons for every experiment.
(2) Insert a coupon into each reactor channel, positioning one end of the slide directly under the influent media port and allowing the opposite end of the slide to rest on the pegs near the effluent port of the channel.
(3) Place the channel covers onto the base and loosely screw in the nylon screws.
(4) Attach the bacterial air vents.
(5) Splice the glass flow break into the area of the media tubing line that will be near the carboy top when attached.
(6) Configure the media tubing so that four individual lines result. Each of the four lines will be fed through a pump head and then attached to a needle, which is inserted through the mininert valves feeding the channels as shown in **Figures 1** and **2**.
(7) To sterilize the reactor, remove the adjustable legs from the reactor base. Wrap all exposed tubing ends and openings with aluminum foil, clamp all effluent tubing and place the assembled reactor into an autoclave tray. Cover the entire tray with aluminum foil.

Sterilize the reactor (estimated time is 40 min)

- Sterilize the reactor system for 20 min on the liquid cycle. Remove immediately after the cycle is over to prevent cracking of the reactor base.

PROCEDURE

Batch phase ● TIMING 30 min plus 6 h of incubation

1| Place the cooled reactor in a flat, level position on the bench top.

2| Clamp the flow break in upright position; leave the other tubing clamped and foiled.

3| Attach the effluent tubing to the waste carboy.

4| Aseptically add 15 ml of 3,000 mg TSB liter^{-1} to each channel using a sterile serological pipette. Pipette 1 ml of the *P. aeruginosa* inoculum (see REAGENT SETUP) into each channel and tighten the cover securely (hand-tight) with nylon screws.

5| Incubate for 6 h on the bench top.

Continuous flow phase ● TIMING 30 min plus 48 h of incubation

6| After 6 h of batch conditions, unclamp the effluent tubing and attach the legs to the DFR.

7| Adjust the legs on the reactor base until the required lengths (*b* and *c*; see EQUIPMENT SETUP) are achieved so that the reactor slopes 10° downward.

8| Aseptically connect the influent nutrient tubing line to the carboy containing the continuous flow nutrient broth. Feed each of the four lines through a pump head and connect a sterile needle to the end of each tube.

9| Aseptically insert the sterile needles through the mininert valves in the channel covers.
! CAUTION Use standard precautions when using needles.

10| Turn on the pump and allow the media to slowly drip onto the bacterial cells attached to the coupon.

11| The media should flow downward from the influent port to the effluent port. Periodically check the reactor for proper drainage and also check the effluent tubing for leaks.
! CAUTION Given the large volumes of liquids that are used in this protocol, it is strongly recommended that the entire system be placed in a tub or tray to contain any leaks that may occur.

12| Operate the reactor in continuous flow mode for 48 h.

Sample the biofilm ● TIMING sample four coupons is 2.5 h plus 24 h of incubation

13| Prepare the sampling materials: vortex, homogenizer, sterile beakers, sterile centrifuge tubes, culture tubes, pipettes, empty sterile Petri dish, sterile spatulas, and flame-sterilized stainless steel hemostat or forceps.
! CAUTION Be sure to wear proper personal protective equipment, including a lab coat, safety glasses and gloves. Researchers may choose to move the reactor into a laminar flow hood before harvesting and analyzing the biofilm samples.

14| Carefully loosen the channel cover screws and remove the channel cover. Aseptically remove one of the coupons by gently lifting the coupon out of the channel with sterile forceps. Hold the coupon over a sterile Petri dish while moving it to the sampling area.

15| Be careful not to disturb the attached biofilm while holding the coupon. Flame-sterilize the hemostat between each coupon sampling.

16| Rinse the slide in 45 ml of sterile buffered water to remove planktonic cells. Hold the slide and centrifuge tube at 45° angle. Uncap the tube and gently immerse the slide with a fluid motion until the slide is completely covered. Immediately reverse motion to remove the slide.

17| Scrape the biofilm-covered coupon surface in downward direction for approximately 15 s, using the flat end of a sterile spatula or scraper, into the beaker containing 45 ml of sterile dilution buffer. Rinse the spatula or scraper by stirring it in the beaker. Repeat the scraping and rinsing process 3–4 times, ensuring full coverage of the coupon surface.

18| Rinse the coupon by holding it at 60° angle over the sterile beaker and pipetting 1 ml of sterile dilution water over the surface of the coupon. Repeat for a total of five rinses. The final volume in the beaker is 50 ml.

19| Disaggregate the scraped biofilm sample. Homogenize the sample in the beaker at 20,500 ± 5,000 r.p.m. for 30 s.

20| After every sampling, clean the homogenizing probe as follows: homogenize a dilution blank for 30 s at the same r.p.m., homogenize a tube containing 70% ethanol for 15 s, remove the probe and let it sit in the ethanol tube for 1 min, shake any

remaining ethanol off the probe, reattach it, homogenize a dilution blank for 30 s and finally homogenize a second dilution blank for 30 s. Now the probe is ready to homogenize the next sample tube. Discard 70% ethanol at the end of sampling.
▲ CRITICAL STEP Homogenizing the sample disaggregates the biofilm clumps to form a homogenous cell suspension. Improper disaggregation will result in an underestimation of the viable cells present in the sample.

21| Serially dilute the sample 1:10 in buffered dilution water using sterile culture tubes.

22| Plate each dilution in duplicate using any accepted plating technique such as spread, spiral or drop plating for colony growth[17,18].

23| Incubate the plates for 17–24 h at 35 °C ± 2 °C.

24| Count the appropriate number of colonies according to the plating method used (estimated time for enumerating plates is 30 min).

Calculations (estimated time for data entry and calculations is 1 h)

25| Calculate the log density for one coupon using equation (3):

$$\mathrm{LOG}_{10}\left(\frac{\mathrm{cfu}}{\mathrm{cm}^2}\right) = \mathrm{LOG}_{10}\left[\left(\frac{(\text{mean cfu/plate})}{\text{vol. of sample plated}}\right) \times \left(\frac{\text{volume scraped into}}{\text{surface area scraped}}\right) \times (\text{dilution})\right] \quad (3)$$

26| Calculate the overall biofilm accumulation by taking the mean of the log densities.
? TROUBLESHOOTING

● **TIMING**
Reagent preparation: 5 h (plus 24 h of incubation time)
Reactor assembly: 1.5 h
Setting the flow rates: 30 min
Adjusting the angle: 10 min
Preparing the reactor for sterilization: 20 min
Autoclaving the reactor: 40 min
Steps 1–5, batch phase preparation: 30 min (plus 6 h incubation time)
Steps 6–12, continuous flow preparation: 30 min (plus 48 h incubation time)
Steps 13–23, sampling of four biofilm coupons: 2.5 h (plus 24 h incubation time)
Step 24, enumerating plates: 30 min
Steps 25 and 26, data entry and calculations: 1 h

? TROUBLESHOOTING

Tubing
Most problems that arise with this reactor system are because of issues with compromised tubing; therefore, it is necessary to replace all the tubing periodically. Ensure the tubing and plastic connectors are autoclavable before use. To prevent leakage, ensure there is a tight connection between the tubing and the luer lock connectors (where the needles will be attached)—tighten if necessary using plastic ties. Check to ensure that there are no obstructions of flow in any of the tubing lines. Be sure to gently seat the tubing in the pump heads to avoid clamping and puncture of the tubing lines. Blockage of the effluent tubing may occur if the tubing is too narrow when fungal or very thick biofilms are being grown. This will result in backflow into the channels and leakage.

Reactor base
It is important to follow proper autoclaving procedures to prevent premature deterioration of the reactor system. The reactor should be autoclaved for 20 min on the liquid cycle, with the cover screws loosened and the legs removed. Check the entire base for cracks, paying close attention to the effluent connectors. Verify that channels are clean and are cleared of anything that could plug the effluent flow.

Miscellaneous
(1) During batch mode, ensure that the reactor is sitting as level as possible and that all slides are completely covered with inoculum.
(2) Be sure that effluent lines are attached to the waste carboy and effluent clamps are removed before beginning the continuous flow.
(3) Change air vents periodically or if they become wet (as this will prevent gas transfer).
(4) Replace membrane septa in the mininert valves as necessary.

ANTICIPATED RESULTS

When a *P. aeruginosa* biofilm was grown in the DFR according to the above procedure, the mean viable biofilm log density was 9.36, the estimated within-experiment variance was 0.05065 (estimated with 53 degrees of freedom) and the estimated between-experiment variance was 0.02711 (estimated with 15 degrees of freedom). The estimated repeatability standard deviation was 0.28, of which 65% was attributable to within-experiment variation and 35% was attributable to between-experiment variation. These numbers were calculated using unpublished data collected at the Center for Biofilm Engineering (**Supplementary Data 1** online). This repeatability standard deviation pertains to a protocol that samples only one coupon per experiment. The repeatability standard deviation for a protocol that requires sampling n coupons per experiment is $[(0.05065/n) + 0.02711]^{1/2}$. For example, if the protocol specifies $n = 4$ coupons and the reported log density is the mean of the four individual coupon log densities, then the repeatability standard deviation is 0.20, of which 32% is attributable to within-experiment variation and 68% is attributable to between-experiment variation.

Experiments performed to optimize the protocol presented in this paper showed the following: (1) the DFR and protocol were robust in the sense that the mean log density of biofilm bacteria on a coupon was little affected if small perturbations were made to the operational temperature, continuous flow nutrient concentration, pump flow rate and the angle at which the reactor sat during continuous flow, (2) among the four factors listed above, temperature and flow rate had the greatest effect on the mean log density, (3) nutrient concentrations above 270 mg TSB liter^{-1} caused a decrease in the mean log density, potentially because the denser biofilm that grows at the higher nutrient concentrations begins to slough more quickly and (4) temperature had the greatest effect on the visual appearance of the biofilm.

Note: Supplementary information is available via the HTML version of this article.

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COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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