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TITLE: Minimally Invasive VAC Therapy with Instillation for Treating Infected Skin-Implant Interfaces in Percutaneous Osseointegrated Devices

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CONTRACTING ORGANIZATION: University of Utah, Salt Lake CIty, UT

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14. ABSTRACT

Percutaneous osseointegrated (OI) prosthetics are a superior alternative to socket-type prosthetics. Sadly, the weak link of this OI technology is high and re-occurring infection rates that originates from the implant post-exit sites. One potential method for treating infected tissue locally is the direct application of negative pressure wound therapy with installation (NPWTi) at the implant exit site. Thus, this proposal's overall goal is to successfully develop a NPWTi treatment plan for infected skin-implant interfaces of percutaneous OI devices. We have designed, developed, and fabricated all necessary implants and tools during this reporting period and obtained required institutional approvals. We also developed bacterial inoculation and animal surgical protocols.

15. SUBJECT TERMS

Percutaneous osseointegrated implants, Infection, Negative pressure treatment with antibiotic instillation, NPWTi

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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

For a significant portion of patients with limb loss, the direct skeletal attachment of artificial limbs via a percutaneous osseointegrated (OI) implant offers a vastly improved alternative to the "socket type suspension" method for prosthetic limb attachment. This system has two components—an intramedullary implant (endoprosthesis) and a percutaneous post (exoprosthesis)—which allows direct transfer of forces to the skeletal system. By abandoning sockets, skeletal OI prosthetic docking obviates many problems inherent to socket suspension systems, such as socket-induced skin breakdown, pressure sores, time-consuming processes for socket attachment, and refitting due to tissue mass fluctuations. This OI attachment platform allows faster docking and donning time, essentially pain-free ambulation, and virtually unlimited prosthesis "wear time" each day. The ability to sit after ambulation, without removing bilateral sockets, and the greater economy of energy and increased endurance, markedly improve the patients' quality of life. Although most percutaneous OI devices used today differ in implant design, device materials, surface methods of osseointegration, and means of skin attachment, they all share a single commonality, which is the "stoma" through which an artificial limb is connected. Clinical reports indicate that most of these percutaneous devices could become infected one or more times during their lifetime and that these infections are usually superficial and can readily be treated with appropriate antibiotics. However, some inadequately treated and frequently recurring infections could lead to chronic bone infection (i.e., osteomyelitis). Clinically, bone necrosis and osteomyelitis have been observed in some deep infection cases and resulted in implant loss.

Antimicrobial resistance is a global health challenge and we have no long-term solutions. A constant and systemic overuse/misuse of antibiotics in these OI populations could increase their antimicrobial resistance and related complications. In order to prevent a chronic state of recurrent infections at the stoma, we proposed a negative pressure wound therapy with instillation (NPWTi), where antibiotics are delivered locally. This technique allows the instillation of antibiotics with pre-selected indwell times directly to the infection site, avoiding systemic toxicity.

Therefore, this study is designed to test the efficacy of NPWTi therapy to successfully treat the infected skin-device interfaces of percutaneous OI devices. The efficiency and effectiveness of the treatment are proposed to be tested in two translational animal models. Study 1 will utilize a Guinea pig model to determine the treatment frequency, while Study 2 will use a pig model to validate the treatment protocol.

There are two aims to this study:

- Specific Aim 1 is used to compare the effectiveness of a commercial NPWTi to systemic IP injection of broad-spectrum antibiotics for treating infected skin-percutaneous device interfaces in a subdermal guinea pig model.
- Specific Aim 2 is used to confirm the efficacy of NPWTi therapy for treating infected skin-percutaneous OI device interfaces in a bone-anchored porcine model.

2. KEYWORDS:

Osseointegrated percutaneous implants, OI prosthetics, Negative pressure wound therapy, Antibiotics instillation, Amputees, Alternative to socket prosthetics.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major goals for Year 1 are:

1. Task 1: Design, manufacture, and sterilize implants – 100% completed (June 2021)
2. Task 2: Acquiring institutional approvals – 100% completed (March 2021)
3. Task 3: To compare the effectiveness of a commercial NPWTi with the systemic IP injection of broad-spectrum antibiotics for treating infected skin-percutaneous device interfaces in a subdermal guinea pig model – 10% completed
4. Task 4: To confirm the efficacy of NPWTi therapy for treating infected skin-percutaneous OI device interfaces in a bone-anchored porcine model – 5% completed

Milestone achieved
There were two deliverables for this reporting period, both are now achieved.

- Achieved two milestones
  1. Implants for *in vivo* study (3 months)
  2. Institutional approvals for *in vivo* studies (6-months)

- Developed two surgical protocols
  1. Inoculation method for infecting the percutaneous stoma
  2. Pig forehead surgical technique

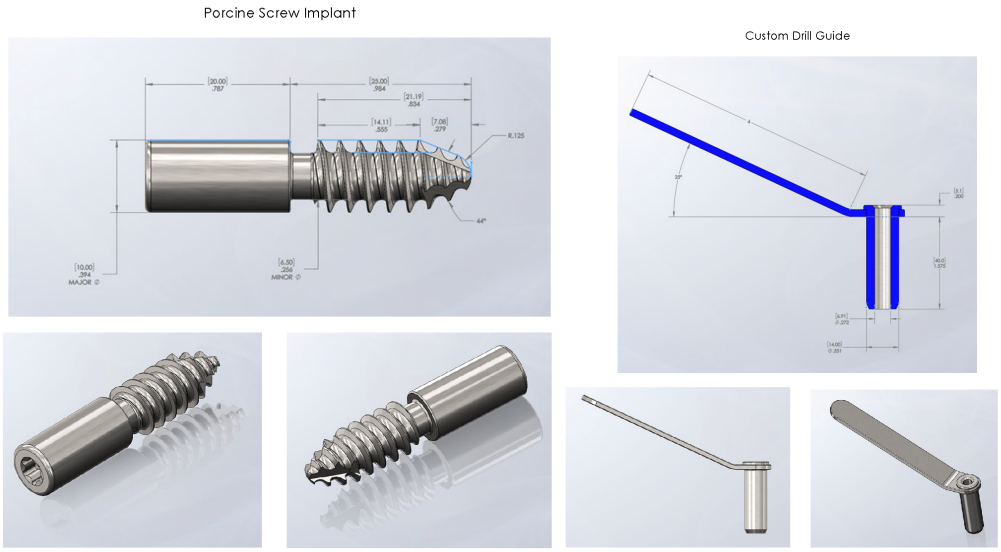
What was accomplished under these goals?

Task 1: Implant Design and Manufacturing (One hundred percent (100%) of this task is now completed)
All of the implants that are needed for the animal studies described in Tasks 3 and 4 are now designed and fabricated.

- Implants were designed in-house. The final engineering drawings and design specifications for guineapig implants were sent to an independent implant manufacturer (Thortex, Inc.) for fabrication (*Figure 1*). All of these implants have now been manufactured; we received ~80 implants to date.

Figure 1: A set of guineapig percutaneous implants: Left image – A porous coated implant with the attachable post. Middle and right images – A smooth subdermal device portions only, coated with oxides.

- For the pig forehead percutaneous implants, a new implant design was made and fabricated with the help of the University of Utah Department of Orthopedic innovation center (*Figure 2*). Additionally, the surgical development step in cadaveric animals revealed a need for a drill guide to prevent over drill and dura penetration. Thus, a drill guide was also custom designed and fabricated.



(a) (b)

Figure 2: (a) - A10 mm forehead implant design for Aim 2. (b) – A custom drill guide design.

Task 2: Institutional Approvals (One hundred percent (100%) of this task is now completed)

The proposed research required two separate animal study protocols. In order to carry out the animal studies, we required three different regulatory approvals from local and federal committees. They were:

1. University of Utah School of Medicine,
2. Salt Lake City Department of Veterans Affairs (Local ACORP), and
3. Department of Army Animal Care and Use Review Office (ACURO).

Our quarterly reports conveyed that all three committees' approvals were obtained for one of the animal study protocols (Guinea pig model). For the second study (pig) protocol, we reported only one local IACUC approval was obtained previously. We now have obtained the second ACURO approval. Thus, this task is now completed.

Briefly, the first protocol approval was obtained through the University of Utah IACUC. Protocol # 20-05002 for the Guinea pig model was approved in its entirety on 25 March 2019. The second approval was obtained through the SLC-DVA Animal Component of Research Protocol (ACORP) committee. The ACORP committee approved this protocol on 13 May 2020. Finally, the approval from the USAMRMC ACURO was obtained on July 2020 (Proposal # OR190083.e001(W81XWH-19-PRORP-ARA, IACUC #A20/05)).

Second approval for the forehead pig (20-05011) model was obtained 04 August 2020 from the University of Utah, and the ACURO application to the DOD's review committee on 02 February 2021, and approval was received on 31 March 2021. As these animals are cared for at the University of Utah animal care unit, the institutional approval from the Salt Lake City Veterans Affairs is not required. This concluded the requirements for one of the animal protocols outlined in Task 2 of the SOW.

Task 3: Guinea pig infection model (Specific Aim 1; on-going)

This task is designed to test Aim 1, which is to compare the effectiveness of a commercial NPWTi to systemic IP injection of broad-spectrum antibiotics for treating infected skin-percutaneous device interfaces in a subdermal guinea pig model. We have made some strides towards this goal. However, this study was delayed for setting up a contract between KCI and the University of Utah to use KCI's NPWTi device in animals, which took longer than anticipated. We are currently in the final stages of negotiation and signing this contract. We expect, next stage to progress without any further delay.

Task 3 is also behind schedule because there were delays in purchasing the Animal Biosafety Level 2 (ABSL-2) housing units for housing. Animal housing units are now ordered (Enviro-Gard™ A & B Systems) and awaiting delivery.

While waiting, we carried out some pilot studies for model developments. They included bacterial propagation and quantification protocol for achieving consistencies between batches. We also developed tissue collection and processing protocols. Initially, we tested the ability of the bacteria to form biofilm on the implant surface and the ability to infect the percutaneous exit-site (or stoma). For these additional tasks, we have grown biofilms on titanium coupons for 48 hours and quantified and characterized them using scanning electron microscopes (SEM) and colorimetric assays. We have used three bacterial strains, *Staphylococcus aureus* (Xen 36 – a bioluminescence strain), *Staphylococcus epidermidis* (clinical strain), and *Staphylococcus aureus* (*S. aureus*, ATCC 6538) to grow biofilm on Ti-64 disks using the ASTM method E3161-18 (Appendix 1). While Xen 36 strain failed to produce biofilm on the test coupons, *S. aureus ATCC 6538* did (*Figure 3*). We will proceed with our further studies using *ATCC 6538* strain.

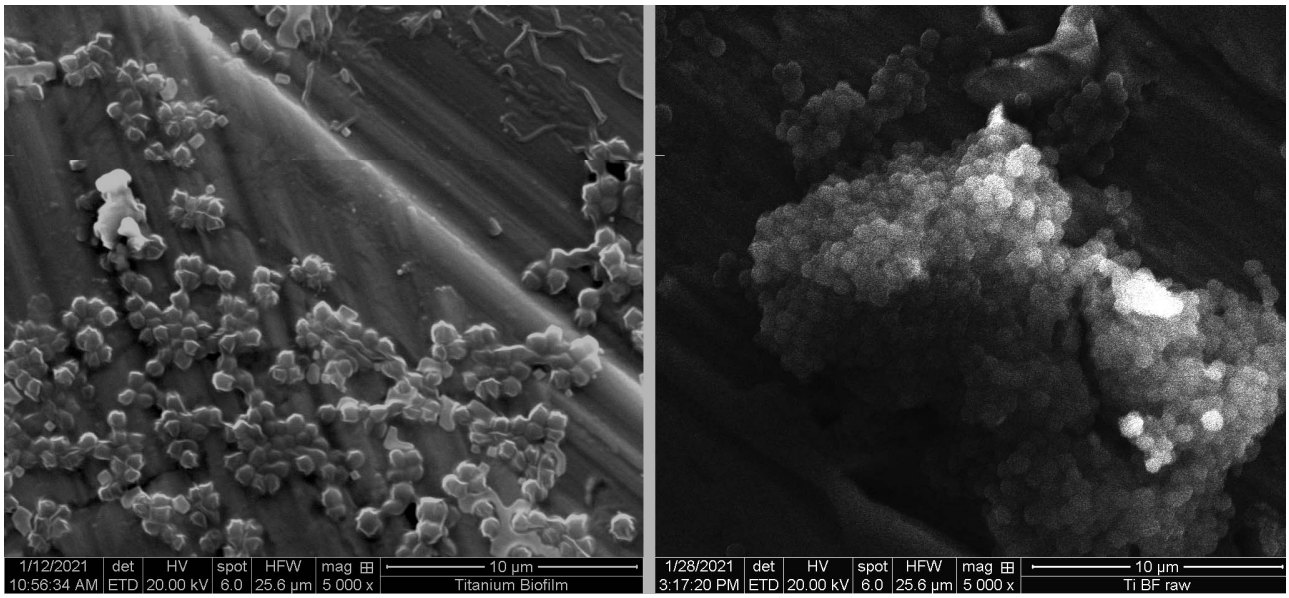
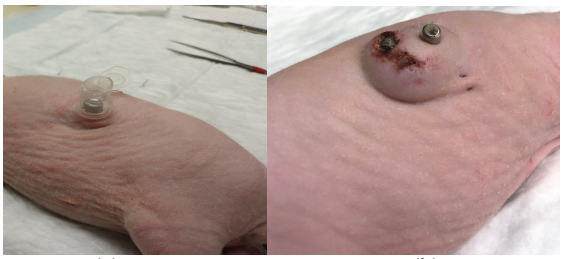


Figure 3: *A set of SEM images showing the ability of the S. aureus (ATCC 6538) to form biofilm on the Ti-64 coupons, which was the same material that was used for fabricating implants given in Figures 1 and 2.*

During the last reporting period, fourth-quarter Y1, we performed a pilot animal study to develop a bacterial inoculation protocol. For this, we used four animals. A small incision was made with a scalpel parallel to the spinal column along the dorsum for this infection induction study. After creating a subcutaneous pocket to accommodate the body of the implant (*Figure 1*), the percutaneous implant was inserted, the incision line was closed, and animals were allowed to heal for four weeks. A stoma was then made during the second surgery using a biopsy punch, and a percutaneous post was attached to the subdermal disk. A known concentration of bacteria (*S. aureus*; ATCC 6538; 1 ml of 10^8 CFU) was placed on gauze and held in place on the implant post using Tegaderm wound dressings. However, this single inoculation of bacteria was insufficient to induce any clinical signs of infection (e.g., no inflammation, exudate, redness, etc.) over the 4-week experiment. Second, we carried out the same surgery and used a funnel-like apparatus (centrifuge tubes cut in half) that was "glued" onto the rats back over the stoma with Mastisol liquid adhesive. We then loaded the well with 1 ml of PBS containing the bacteria (10^8 CFU) and let it stand for 30 minutes (*Figure 4*). Following the 30-minute inoculation period, the bacterial solution was wiped off with gauze, and the implant exit site was covered with gauze and Tegaderm dressings. This technique resulted in infection at the stoma. Following this success, we intend to repeat the procedure in 6 guinea pigs. If repeated, we intend to use this inoculation method for all future studies.



(a) (b)

Figure 4: *(a) – A representative photograph showing the bacterial inoculation setup. (b) - A photograph of the infected implant exit site a week after the bacterial inoculation.*

Task 4: Bone anchored pig forehead model – (Specific Aim 2; on-going)

This task is aimed to confirm the efficacy of the NPWTi therapy protocol developed in Task 3 in a bone-anchored porcine model (clinically relevant model). To date, our group has not performed these forehead surgeries. In order to confirm the feasibility and to develop the surgical technique, we have used 2 Yucatan miniature pigs during this quarter. Even though a two-stage surgery is planned for the infection study, we used a single-stage surgical procedure to minimize the pain and distress to the animal. During the sterile surgery, a 10 mm skin punch was used to expose the skull bone at the juncture of the suture lines. Then, using a hand-held drill with the guide, a 5-mm diameter hole was made to penetrate the skull to 15 mm in depth. After which, the fabricated self-tapping bone implant (*Figure 2*) was screwed in place. After inserting the implant, the implant exit site was cleaned and dressed. This surgery was performed in late June. During the short observation time, the implant exit site healed well, and there were no signs of natural infection, to date (*Figure 5*). These animals will be survived for 12 weeks. If successful, these pilot animals could be placed in the negative control group (no infection).

Figure 5: *A representative image showing skin-penetrating implant that was placed on the pig forehead.*

What opportunities for training and professional development has the project provided?

Graduate student training – During the Q2-Q4 Y1, Samantha Style and Andrew Miller have received graduate student training through this study. Also, both of them were trained to perform surgeries by an experienced orthopaedic surgeon, which was specific for this project.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

We are planning to perform surgeries described in Task 3 during the next reporting period, while continue to monitor the pigs.

4. **IMPACT:**

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

8. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Based on the pilot pig study, we may want to change the surgeries for pigs from a two-stage to a single-stage process. If effective, we plan to submit changes to the protocol to both local and ACURO for further approvals.

Actual or anticipated problems or delays and actions or plans to resolve them

- Due to COVID restrictions, acquiring the surgical time and space for the animals are difficult, which may delay our milestones. We are working with the animal care unit to come up with alternative plans.

Changes that had a significant impact on expenditures

- Delays in the large animal study and increasing costs for supplies, animals, and daycare are expected to impact the expenditures. We may re-budget and request approvals.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

N/A

8. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

8. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

1. Name: Jay Agarwal, MD
   Project Role: Principal Investigator
   Research Identifier (ORCID ID): 0000-0002-1209-6703
   Nearest person month worked: 0.6 Months
   Contribution to Project: Dr. Agarwal reviewed all of the animal protocols, helped with surgical technique development and reporting.

2. Name: Sujee Jeyapalina, PhD
   Project Role: Principal Investigator
   Research Identifier (ORCID ID): 0000-0002-2199-7191
   Nearest person month worked: 6.0 Months
   Contribution to Project: Dr. Jeyapalina has prepared all of the institutional approval documents and reports, designed and acquired engineering drawings, and contributed to the methodology developments and project management, surgeries and related tasks.

3. Name: Clark Nielson
   Project Role: Graduate student
   Research Identifier: N/A
   Nearest person month worked: 4.0 - 6.0 Months
   Contribution to Project: Mr. Nielson is responsible for acquiring equipment and supplies for the study and has taken a lead role in optimizing the procedures. He also helps with animal surgeries, daycare, and related tasks.

4. Name: Samantha Steyl
   Project Role: Postgraduate research assistant
   Research Identifier: N/A
   Nearest person month worked: ~3.0 Months
   Contribution to Project: Ms. Steyl developed methodologies for optimizing and delivery of bacteria to the percutaneous implant exit site reproducibly.

5. Name: Andrew Miller
   Project Role: Postgraduate research assistant
   Research Identifier: N/A
   Nearest person month worked: ~2.0 Months
   Contribution to Project: Mr. Miller has been developing methodologies for optimal sample collection and storage and helped to design implants. He undertakes daily animal care and data management.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

What other organizations were involved as partners?

8. SPECIAL REPORTING REQUIREMENTS

Minimally Invasive V.A.C. Therapy with Instillation for Treating Infected Skin-Implant Interfaces in Percutaneous Osseointegrated Devices
OR190083/W81XWH2010606

PI: Jay Agarwal, MD **Org:** University of Utah /Isabella Johnsen **Award Amount:** $748,965

Study/Product Aim(s)

We hypothesize that superficially infected skin-device interfaces of percutaneous OI devices can be efficiently and effectively treated with negative pressure wound therapy with antibiotic instillation (NPWTi).

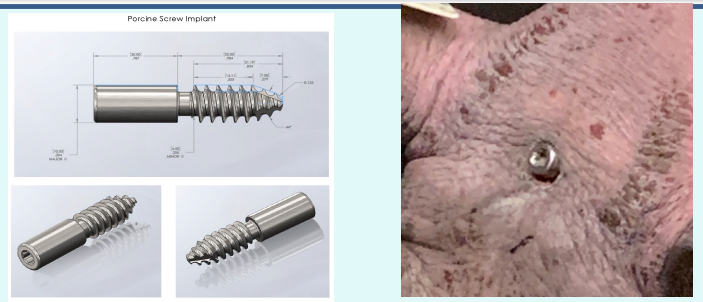
This will be tested using two aims:

Specific Aim 1 will compare the effectiveness of NPWTi with the systemic IP injection of broad-spectrum antibiotics for treating infected skin-percutaneous device interfaces in a subdermal guinea pig model.

Specific Aim 2 will confirm the efficacy of NPWTi therapy for treating infected skin-percutaneous OI device interfaces in a bone-anchored porcine model.

Approach

Our approach includes a two-stage surgery to implant percutaneous devices in a guinea pig/pig model, followed by an inoculation of 10^8 CFU of Seattle 1945 *Staphylococcus aureus* directly into the periprosthetic tissues to induce infection. Post-infection, each animal will be treated with a combination of NPWT therapy with or without antibiotic instillation. An effective treatment protocol will then be considered for human applications.



(a) (b)

Figure : (a) An engineering drawing of pig forehead implant design (b) and a pilot animal implanted with this implant to the forehead (4-weelk post surgery).

Accomplishment: (1) Institutional approvals for animal studies (100% completed); (2) – Completed implant designs (100% completed); (3) – Completed surgical planning steps (100%); (4) – Implant manufacturing (100 % completed). (5) – Guinea pig infection model development (6% completed). (6) – Surgeries were carried out in n=5 pigs.

Timeline and Cost

Activities CY	20	21	22
Completion of guinea pig surgeries			
Completion of data analysis			
Completion of pig surgeries and analysis			
Manuscript preparation and submission/clinical translation			
Estimated Budget ($K)	**$290**	**$239**	**$220**

Updated: 16 February 2021

Goals/Milestones (Example)

CY20 Goal – IACUC approvals
✓ Local and DOD approvals
CY21 Goals – Completion of guinea pig study
☐Completion of guinea pig surgeries
☐Collect data/analyses
CY21 Goal – Define the treatment protocol
☐ Submission of manuscript
CY22 Goal – Confirm the treatment protocol in pigs
☐ Completion of pig surgeries and analysis

Comments/Challenges/Issues/Concerns

- N/A

Budget Expenditure to Date

Projected Expenditure: $748,965
Actual Expenditure: ~$244,000

Appendix 1

Designation: E3161 – 18

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

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

E2871 Test Method for Evaluating Disinfectant Efficacy Against ***Pseudomonas aeruginosa*** Biofilm Grown in CDC Biofilm Reactor Using 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

[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.

Current edition approved April 1, 2018. Published June 2018. DOI: 10.1520/E3161–18.

[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.

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.

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 ±2 °C rather than at room temperature (21 ±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 by 10.16 cm that can rotate at a range of 60 to 125 ±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.

6.12 *Reactor Components.*[4]

6.12.1 *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° to 45° to ensure drainage. Spout should accommodate size 18 or 25 flexible silicone tubing.

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 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 ±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.

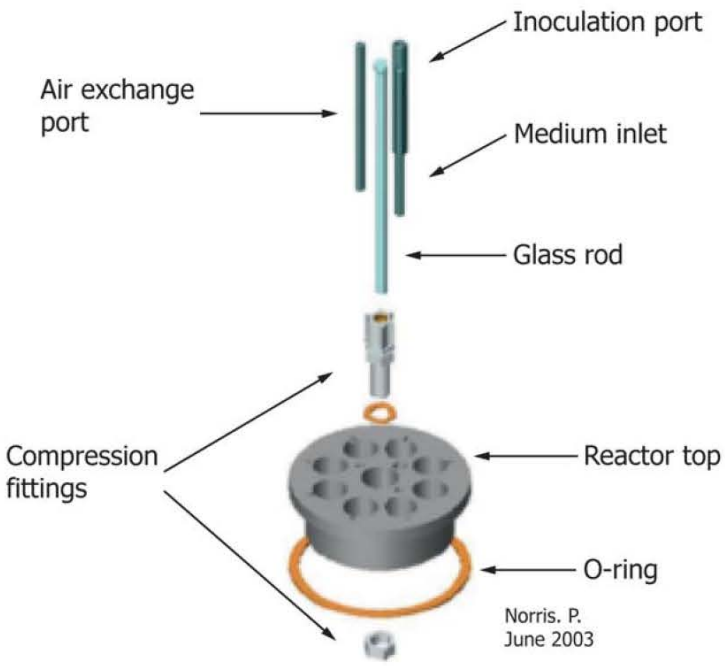


FIG. 1 Expanded Schematic of Reactor Top

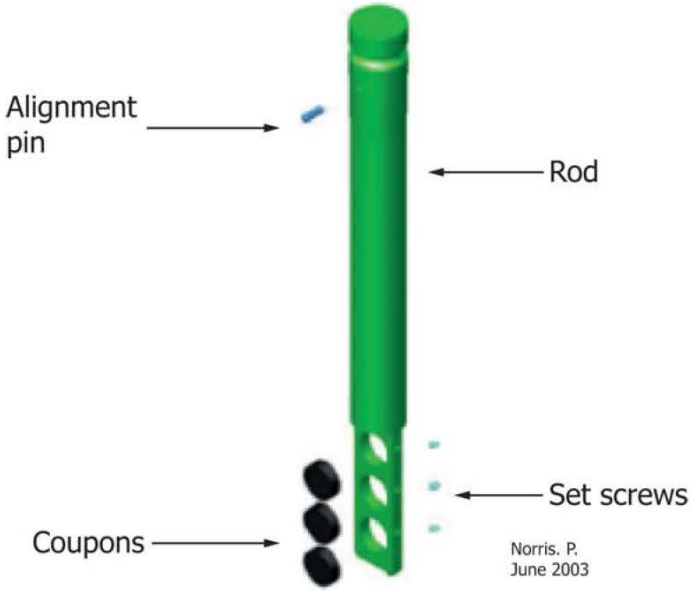


FIG. 2 Expanded Schematic of Rod and Coupons

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.

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).

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.

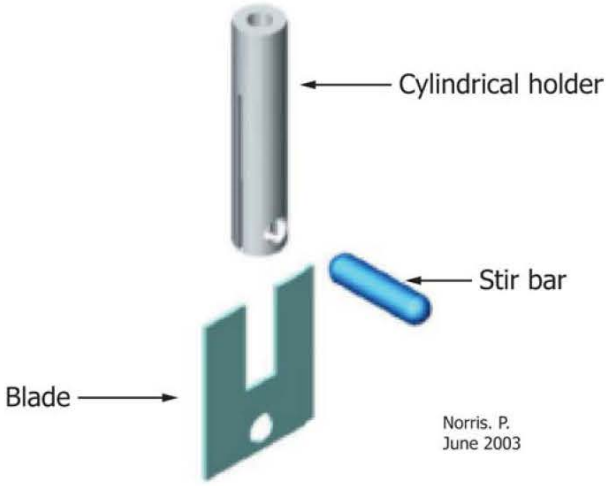


FIG. 3 Expanded Schematic of Baffled Stir Bar

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

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.

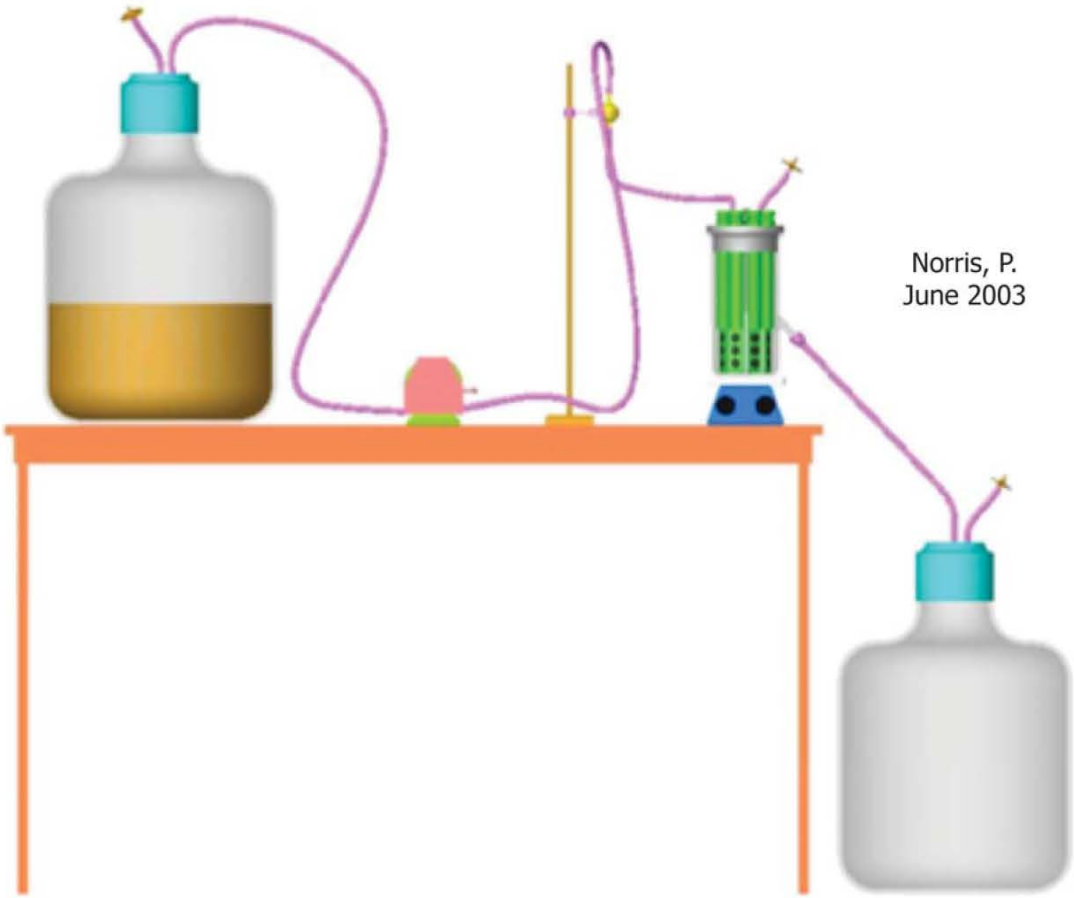


FIG. 4 Schematic of the Completely Assembled Reactor System

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:*

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 ±5 r/min for *P. aeruginosa* or 60±5 r/min for *S. aureus*). Clamp the effluent tubing on the reactor beaker.

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.

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 ±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.

9.4.3 Steam sterilize the empty reactor system at 121 °C for 20 min.

NOTE 4—Due to the deterioration of the materials, it may be necessary to change the tubing and filters on the reactor and carboys after 5 to 6 autoclaving processes. Ensure tubing used in the peristaltic pump is kept free of dirt and grit by wiping with a damp paper towel. Inspect all parts of the reactor system frequently and replace as necessary.

9.5 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.

10. Procedure

10.1 *Frozen Stock Culture Preparation*—Frozen stock cultures are single use only and should be approximately 10^9 CFU/mL.

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

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

10.1.3 Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly by vortexing. Incubate broth culture at 36 ±2 °C for 24 ±2 h.

NOTE 5—Growth in TSB is necessary to generate culture of sufficient titer for streak isolation.

10.1.4 After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plates for 18 to 24 h at 36 ±2 °C.

10.1.5 Select 3 to 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 18 to 24 h at 36 ±2 °C.

10.1.6 Following the incubation of the agar plates from 10.1.5, place approximately 5 mL sterile cryoprotectant solution (TSB with 15 % glycerol) equilibrated to 20 ±5 °C on the surface of each plate.

10.1.7 Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface.

10.1.8 Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. 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.

10.1.9 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 ≤ -70 °C for a maximum of 18 months, then reinitiate with a new lyophilized culture.

NOTE 6—New frozen stock cultures may be initiated one time using an existing, unexpired frozen stock culture as the source.

10.2 *Inoculum Initiation from Frozen Stock Culture:*

10.2.1 *P. aeruginosa* (ATCC 15442).

10.2.1.1 Defrost a single cryovial and briefly vortex to mix. Add 10 µL *P. aeruginosa* 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. Culturable bacterial density should be at least 10^7 CFU/mL and should be checked by serial dilution and plating. Verify purity of the inoculated tube by streak isolation (for example, verify appropriate colony morphologies).

10.2.2 *S. aureus* (ATCC 6538).

10.2.2.1 Defrost a single cryovial and briefly vortex to mix. Add 10 µL *S. aureus* frozen stock culture to a tube containing 10 mL sterile TSB (30 g/L) and vortex to mix. Incubate bacterial suspension at 36 ±2 °C for 24 ±2 h. Culturable bacterial density should be at least 10^7 CFU/mL and should be checked by serial dilution and plating. Verify purity of the inoculated tube by streak isolation (for example, verify appropriate colony morphologies).

10.3 *Growth of Biofilm in the CDC Reactor – Batch Phase:*

10.3.1 Clamp the effluent line of the reactor. Aseptically add 500 mL of the cooled batch phase growth 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.

10.3.1.1 For *P. aeruginosa*, the batch phase growth medium is 300 mg/L TSB.

10.3.1.2 For *S. aureus*, the batch phase growth medium is 3 g/L TSB.

10.3.2 Secure the rod alignment pins into the reactor top notches.

10.3.3 Place reactor onto a stir plate. Clamp flow break in upright position; leave other tubing clamped and covered with aluminum foil.

10.3.4 Vortex the 10 mL tube of culture (see sections 10.2.1.1 to 10.2.2.1) and use 1 mL to inoculate the reactor through one of the available rigid stainless steel ports in the reactor top.

10.3.5 Turn on the magnetic stir plate.

10.3.5.1 For *P. aeruginosa*, the rotational speed of the baffled stir bar is 125 ±5 r/min. Run the reactor system in batch phase at room temperature (21±2 °C) for 24 ±2 h.

NOTE 7—Wide fluctuations in ambient temperature may cause variability in the formation of the biofilm.

10.3.5.2 For *S. aureus*, the rotational speed of the baffled stir bar is 60 ±5 r/min. Incubate the reactor system in batch phase at 36 ±2 °C for 24 ±2 h.

NOTE 8—The rotational speed of the baffled stir bar directly determines the amount of shear stress that the biofilm experiences. Ruggedness testing using the CDC Biofilm Reactor with hard, non-porous coupons (3) showed that 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; refer to sections 10.3.5.1 and 10.3.5.2.

10.4 *P. aeruginosa CSTR Medium Preparation:*

10.4.1 For *P. aeruginosa*, run CSTR phase at room temperature (21 ±2 °C).

10.4.2 Prepare and sterilize concentrated growth medium (for example, 40 g/L TSB) separately from 19 L sterile deionized water.

10.4.3 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.

NOTE 9—Other concentration/volume combinations of TSB may be

used to achieve a final concentration of 100 mg/L TSB.

10.4.4 Shake the carboy or use an appropriately-sized sterile stir bar on a magnetic stir plate to thoroughly mix the contents.

10.4.5 Aseptically connect the tubing from the reactor to the carboy containing the CSTR growth medium (100 mg/L TSB).

10.5 *S. aureus CSTR Medium Preparation:*

10.5.1 For *S. aureus* biofilm, run the CSTR phase at 36 ±2 °C.

10.5.2 Prepare and sterilize concentrated growth medium (for example, 40 g/L TSB) separately from 19 L sterile deionized water.

10.5.3 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.

NOTE 10—Other concentration/volume combinations of TSB may be used to achieve a final concentration of 1 g/L TSB.

10.5.4 Shake the carboy or use an appropriately-sized sterile stir bar on a magnetic stir plate to thoroughly mix the contents.

10.5.5 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.

10.5.5.1 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 ft 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 Fig. 5 and Fig. 6, respectively.

10.5.5.2 The temperature of the medium for *S. aureus* (36 ±2 °C) must be maintained prior to and during CSTR phase for *S. aureus*.

10.6 *Growth of Biofilm in CDC Reactor – CSTR Phase:*

10.6.1 Aseptically connect the growth medium tubing to the carboy containing the continuous flow growth medium.

10.6.2 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 section 9.1).

10.6.3 Attach tubing from the effluent spout to a waste carboy and remove clamp.

10.6.3.1 The effluent spout on the beaker allows overflow to occur, maintaining a constant growth medium concentration in the reactor during CSTR mode.

10.6.4 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.

10.6.5 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.

10.6.6 Use the procedure in Test Method E2871 to sample the biofilm and evaluate for efficacy. Use coupons for testing in Test Method E2871 within 30 min after growth medium flow and baffled stir bar have been turned off. Biofilm-laden coupons generated in this practice may also be used in alternate methods capable of accommodating the coupons used in the CDC Biofilm Reactor.

FIG. 5 Equilibrate Continuous Flow Growth Medium by Placing Carboy into an Incubator 2 to 3 Days Prior to Use

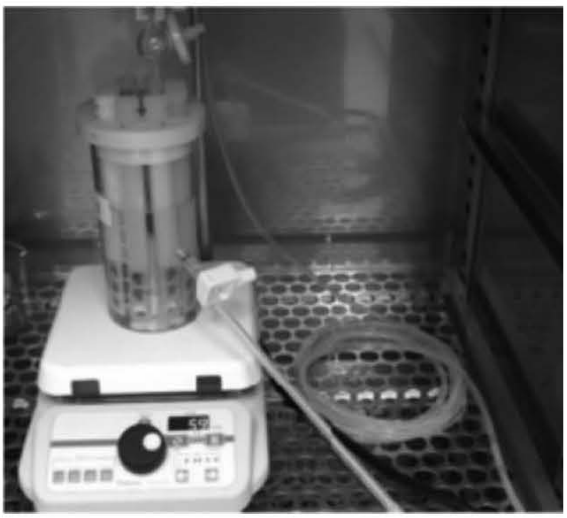


FIG. 6 Equilibrate Continuous Flow Growth Medium by Placing Approximately 12 to 15 Feet of Coiled Tubing Primed with Continuous Flow Growth Medium in an Incubator 1 Day Prior to Use

11. Precision and Bias

11.1 Use randomization whenever possible to reduce the potential for systematic bias.

12. Keywords

12.1 biofilm; coupon; growth reactor; *Pseudomonas aeruginosa*; reactor; *Staphylococcus aureus*

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