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A new direction in fighting infection.

MBEC Assay® Kit

PROCEDURAL MANUAL

For High-Throughput Antimicrobial Susceptibility Testing of Biofilms

Version 2.3

For use with the following product codes:

MBEC Assay® Biofilm Inoculator: 96-well base
19113 – 10/case, **19111** – 25/case, **19112** – 100/case

MBEC Assay® Biofilm Inoculator: Trough base
19123 – 10/case, **19121** – 25/case, **19122** – 100/case

MBEC Assay® Biofilm Inoculator: 96-well base & hydroxyapatite coated pegs
19133 – 10/case, **19131** – 25/case, **19132** – 100/case

MBEC Assay® Biofilm Inoculator: Trough base & hydroxyapatite coated pegs
19143 – 10/case, **19141** – 25/case, **19142** – 100/case

MBEC Assay® Biofilm Inoculator: 96-well base & titanium hydroxide coated pegs
19153 – 10/case; **19151** – 25/case; **19152** – 100/case

MBEC Assay® Biofilm Inoculator: Trough base & titanium dioxide coated pegs

19163 – 10/case; **19161** – 25/case; **19162** – 100/case

MBEC Assay® Biofilm Inoculator: 96-well base & cellulose coated pegs

19173 – 10/case; **19171** – 25/case; **19172** – 100/case

MBEC Assay® Biofilm Inoculator: Trough base & cellulose coated pegs

19193 – 10/case; **19191** – 25/case; **19192** – 100/case

Table of Contents

I. PRODUCT DESCRIPTION

The MBEC (Minimum Biofilm Eradication Concentration) Assay® system is a high throughput screening assay used to determine the efficacy of antimicrobials against biofilms of a variety of microorganisms. The MBEC Assay® Biofilm Inoculators are plates consisting of a plastic lid with 96 pegs and one of two corresponding bases: One base contains 96 individual wells (Figure 1), which allow for the growth of a variety of species of microorganisms in one device, while the other base is a corrugated trough base such that all pegs contact the same microorganism(s) (Figure 2). Biofilms are established on the pegs (Figure 3) under batch conditions (no flow of nutrients into or out of an individual well/trough base) with gentle mixing. The established biofilms are transferred to a new 96-well base for antimicrobial efficacy testing. The assay design allows for the simultaneous testing of multiple biocides at multiple concentrations with replicate samples, making it an efficient screening tool. Also, the small well volume is advantageous for testing expensive antimicrobial agents or those with limited availability. The MBEC Assay® system is versatile, and expanded uses of the system are listed in Section II.

Figure 1. The inoculator showing the 96-well base found in products 19111, 19112, 19113, 19131, 19132, 19133, 19151, 19152, 19153, 19171, 19172, and 19173.

Figure 2. The inoculator showing the trough base found in products 19121, 19122, 19123, 19141, 19142, 19143, 19161, 19162, 19163, 19191, 19192, and 19193.

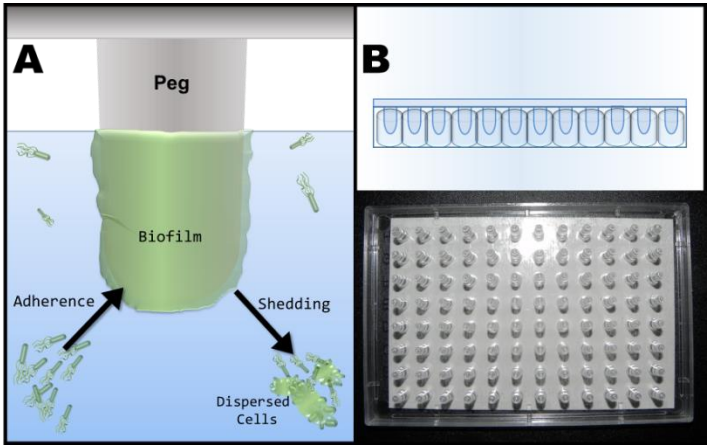

Figure 3. The MBEC Assay® Biofilm Inoculator. A) Biofilms form on the pegs of the MBEC Assay® Biofilm Inoculator when planktonic microbes adhere to the surface. In the presence of shear forces, these microbes become attached and form mature biofilms. Biofilms are encased in 'slime', which is sometimes visible to the naked eye. Dispersed cells are shed from the surface of biofilms, which serve as an inoculum for minimum inhibitory concentration (MIC) determinations. B) The peg lid has 96 identical pegs. The average surface area of each peg on the MBEC Assay® Biofilm Inoculator is 108.9 mm^2. This lid fits into a standard 96-well microtiter base or a trough base with channels that are set up to contain an inoculated growth medium.

II. PRODUCT USES

- Antibiotic, biocide, disinfectant, and heavy metal susceptibility testing of biofilms to determine a minimum inhibitory concentration (MIC), a minimum biocidal concentration (MBC), a minimum biofilm inhibitory concentration (MBIC), and/or a minimum biofilm eradication concentration (MBEC).
- Basic research in the physiology and genetics of biofilms (such as screening to identify mutants impaired in their ability to form biofilms).
- Biofilm growth comparison between different isolates under identical conditions.
- Comparative studies of gene expression in multiple isolates or mutants.
- Selection criteria applied against biofilm microorganisms.
- Checkerboard assays to identify synergisms between anti-biofilm agents.
- Time course studies of biofilm formation/treatment (by removal of pegs from lids).
- Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) to examine biofilm structure.

- Conventional assays for attachment, growth, survival, or metabolic activity to measure the parameters of interest. These can be done while the biofilm is intact on the peg or following sonication to again produce a planktonic population.
- Biofilm generation for downstream "-omics" (transcriptomic, proteomic, genomic, etc.) analysis.

III. INTRODUCTION TO BIOFILMS

The expertise of Innovotech Inc. is in microbial biofilms. Biofilms are a cohesive matrix of microorganisms, mucopolysaccharides (slime), and other extracellular constituents that exist in virtually every natural environment. Biofilms form in the presence of a solid surface, combined with a factor such as shear force (flow), as a mechanism to prevent being removed from their surroundings. Biofilm formation is a developmental process that has been likened to differentiation in multicellular organisms, with intercellular signals that regulate growth. A typical biofilm forms when bacteria and/or fungi adsorb to a surface and become attached, triggering a change in physiology. The bacteria and/or fungi then grow and divide to form layers, clumps, or stalk-and-mushroom shaped microcolonies, all under the control of specific biofilm-expressing genes. The production of an extracellular polymeric matrix on the surface further protects the biofilm.

Once formed, biofilms are difficult to remove, as they show an increased tolerance to biocides and antibiotics when compared to planktonic (free-floating) microorganisms. Studies have shown that biofilms may have greater than a thousand-fold increase in tolerance to antibiotics when compared to the same microbe in a planktonic state [1, 2, 3]. This is thought to be due to the physiological alteration of a microorganism upon attachment to a surface, as well as cell specialization that may occur within biofilms.

Microbial biofilms naturally exist on both inanimate and living surfaces. Biofilms may be found just about anywhere, from hard surfaces in food processing facilities to the water lines of dental equipment. The organisms present on these surfaces frequently include a number of normally benign bacteria and fungi; however, these biofilms may also serve as a haven for serious human and animal pathogens. Further, the National Institutes of Health (NIH) estimates that over 80% of microbial infections are due to biofilms, while the Center for Disease Control and Prevention (CDC) estimates that over 60% of clinical infections are caused by biofilms. This includes heart, wound, and middle ear infections; illnesses associated with implanted medical devices such as artificial joints and catheters; and tooth decay and gum diseases (which arise from dental plaque – an oral biofilm). Information on biofilm control properties of existing products, as well as biofilm testing protocols, are not available to most industries as there has been minimal biocide testing for microorganisms in the biofilm state. The MBEC Assay® test allows microorganisms to grow on 96 identical pegs protruding down from a plastic lid. By placing the biofilm-coated pegs into the wells of a microtiter base, an array of antimicrobial compounds with varying concentrations can easily be assessed. This

allows for rapid testing of compounds including antibiotics, disinfectants, biocides, and metals for anti-biofilm activity [2, 4-7]. A broad spectrum of both bacterial and fungal species have been grown using this assay, including *Escherichia coli* [2], *Pseudomonas aeruginosa* [2], *Staphylococcus* spp. [2, 8], *Mycobacterium* spp. [9], *Candida* spp. [10], *Burkholderia* spp. [11, 12], and many more [7]. To date, the MBEC Assay ® system has been featured in more than 100 peer-reviewed publications.

References

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[11] Tomlin, K.L., Malott, R.J., Ramage, G., Storey, D.G., Sokol, P.A., and Ceri, H. (2005) Quorum-sensing mutations affect attachment and stability of *Burholderia cenocepacia* biofilms. Applied and Environmental Microbiology 71, 5208-5218.
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IV. MATERIALS AND EQUIPMENT

- MBEC Assay® Biofilm Inoculator plate (coated or uncoated peg lid with 96-well base or corrugated trough base)
- Sterile 96-well microtiter plates (Recommended manufacturer: Nunc™ Nunclon™ 96-well tissue culture microtiter plates, sterile and individually packaged)
- Sterile 16 × 100 mm glass culture tubes (Recommended manufacturer: FisherBrand borosilicate glass disposable culture tubes)
- Sterile physiological saline solution (e.g., phosphate buffered saline or 0.9% NaCl)
- 0.5 McFarland Barium Sulfate Turbidity Standard or alternative
- Sterile micropipette tips (2-200 µL), in racks of 96
- Sterile 1 mL and 25 mL pipettes
- Sterile 50 mL culture tubes
- Sterile reagent reservoirs
- Agar and broth growth media specific to the microorganism being cultured
- Platform shaker to be set at 110 and 150 revolutions per minute (rpm) for use with 19111, 19112, 19113, 19131, 19132, 19133, 19151, 19152, 19153, 19171, 19172, and 19173 (Recommended manufacturer: New Brunswick Scientific Excella Platform Shaker)
- Rocking table set at 3-5 rotations per minute and 9-16° of inclination for use with 19121, 19122, 19123, 19141, 19142, 19143, 19161, 19162, 19163, 19191, 19192, and 19193 (Recommended manufacturer: Bellco Glass Inc. – Rocker with Tray Platform)
- Single and/or multichannel micropipettes to measure volumes of 10 µL, 20 µL, 100 µL, 150 µL, 180 µL, and 200 µL
  NOTE: *This procedure may be performed using only single channel micropipettes (recommended sizes: 2-20 µL and 20-200 µL); however, 12-channel micropipettes are suggested (recommended sizes: 5-50 µL and 30-300 µL).*
- Ultrasonic cleaner (water bath sonicator) with stainless steel insert tray (Recommended manufacturer: VWR Scientific Aquasonic Model 250)
- Inoculation loop or sterile cotton swab
- Incubator for biofilm growth with temperature settings specific to test microorganisms
- Bunsen burner
- Needle nose pliers
- Pipette bulb or aid
- (Recommended) Biological safety cabinet

V. PRECAUTIONS

- Single use only.
- Follow aseptic techniques and standard laboratory practices throughout all procedures, with special awareness that the plates, once inoculated, could contain potentially pathogenic organisms.
- Decontaminate all biohazardous waste autoclave, incineration, or chemical means prior to disposal in compliance with facility/institutional guidelines.

VI. MEDIA AND SOLUTIONS

Organism specific media (OSM):

Prepare agar and broth growth media specific to the microorganism being cultured as per the manufacturer's instructions (common examples are Tryptic Soy Broth/Agar, and Sabouraud Dextrose Broth/Agar).

Stock antimicrobial solutions:

Prepare antibiotic and/or other antimicrobial stock solutions in advance at the highest concentration to be used in the challenge plate. Clinical Laboratory Standards Institute (CLSI) document M100 (current revision) may be consulted for details on which solvents and diluents to use. Most antibiotic stock solutions are stable for a minimum of 6 months at -70°C.

Neutralizing and biofilm recovery agents:

For research applications, it is recommended to employ a neutralizing agent for determination of minimum bactericidal and fungicidal concentrations. These agents reduce toxicity from the carry-over of biologically active compounds from challenge to recovery media. For example, it is possible to use β-lactamase to neutralize penicillin, or L-cysteine to neutralize Hg^{2+} and some other heavy metal cations. Innovotech Inc. often uses a universal neutralizer in biocide susceptibility assays that are required for regulatory aspects of product development. This neutralizer also contains a surfactant that aids in recovering any remaining biofilm from the device following the challenge. Examples of universal neutralizers are presented on the following page.

Universal Neutralizer (for biocide testing)

Ingredients:
1.0 g L-histidine
1.0 g L-cysteine
2.0 g reduced glutathione

Procedure:

1. Make up the universal neutralizer to a total volume of 20 mL in double distilled water. Pass the solution through a syringe with a 0.20 µm filter to sterilize. This solution may be stored at -20°C for up to 1 year.
2. To make surfactant supplemented growth medium, make up 1 liter of the appropriate growth medium (for example, cation adjusted MHB). Supplement this medium with 20.0 g per liter of saponin and 10.0 g per liter of Tween-80. Adjust with dilute NaOH to pH 7.0 ± 0.2 at 20°C.
3. Add 500 µL of the universal neutralizer to each 20 mL of the surfactant supplemented growth medium used for recovery plates (Step 6b below).

D/E Neutralizing Broth – Prepare as per manufacturer's instructions.

NOTE: *There are no standard neutralizer recipes that can effectively work with all antimicrobial treatments. Neutralizer recipes can be adjusted and modified to achieve the optimum recovery results with different antimicrobial agent(s).*

VII. PROCEDURE

The example procedure provided in these instructions describes an assay for testing single or multiple organisms grown as biofilms against a single antimicrobial agent. This protocol may need to be optimized to best suit your needs. Although the majority of microorganisms form biofilms as part of their lifecycle, not all are amenable to growth in the MBEC Assay® Biofilm Inoculator. Please contact Innovotech for help in developing a methodological design specific to your requirements.

The number of days required to complete this protocol is dependent on the growth rate and biofilm formation time of the microorganism being examined.

This protocol has been developed for use with Nunc™ Brand, flat bottomed, 96-well microtiter plates. Figure 4 shows a schematic of the steps to be followed.

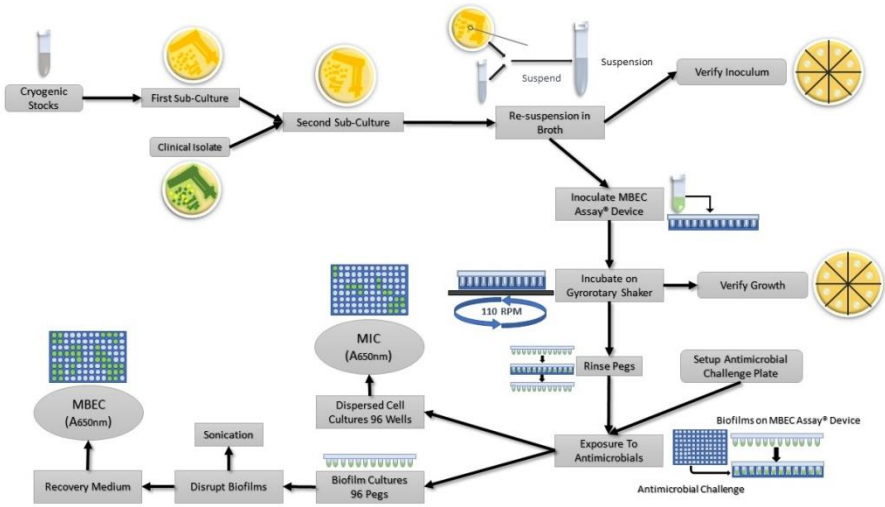

Figure 4. A flow diagram illustrating the experimental process for antimicrobial susceptibility testing using the MBEC Assay® Biofilm Inoculator with a 96-well base.

1. **GROWING SUBCULTURES:**
    a. If using a cryogenic stock (at -70 to -80°C), streak out a first subculture of the desired bacterial or fungal strain on an appropriate agar plate. Incubate at the optimum growth temperature of the microorganism for an appropriate period of time. For most strains, the first subculture may be sealed with Parafilm™ and stored at 4±2°C for up to 14 days after incubation is complete.
    b. Check the first subculture for purity (i.e., only a single colony morphology should be present on the plate).
    c. From the first subculture or from a clinical isolate, streak out a (second) subculture on an appropriate agar plate. Incubate at the optimum growth temperature of the microorganism for an appropriate period of time. It is recommended to use this subculture within 32 hours starting from the time it was first removed from incubation.
    d. Verify the purity of the subculture generated in Step 1c (following the same procedure as Step 1b).
    NOTE: *It is not recommended to grow subcultures on media containing selective agents. Antibiotics and other antimicrobials may trigger an adaptive stress response in microorganisms and/or may increase the accumulation of mutants in the population. This may result in an aberrant susceptibility determination.*

2. INOCULUM PREPARATION:

a. Choose a single, well isolated colony from the subculture created in Step 1c to inoculate a flask containing 100-200 mL of organism specific broth.

b. Incubate the flask on a shaker at ~150 rpm at the appropriate growth conditions. Viable cell density is generally expected to be between 10^7-10^9 CFU/mL (achievable cell density is dependent on the microorganism) and may be checked by serial dilution and spot plating, as follows:

   i. Place 180 µL of sterile saline or buffered water into each well of rows B-H of a fresh 96-well base. Place at least 3 technical replicates of 100 µL (3 x 100 µL) of inoculated broth into row A.

   ii. Prepare a serial dilution (10^0-10^7) by transferring 20 µL from each well in row A to each well in row B and so on down each of the 8 rows, mixing well and discarding tips between each transfer.

   iii. Remove 5-20 µL from each well and spot plate on Organism Specific Agar (OSA) plates.

   NOTE: *For some types of OSA, 20 µL spots may run together, in which case lower volume spots, such as 5 or 10 µL, may be more appropriate.*

c. Dilute the flask culture 10x-1000x in a tube of sterile OSM to adjust the inoculum to an approximate cell density of 10^5 CFU/mL. Vortex the diluted sample for approximately 10 seconds to achieve a homogenous distribution of cells.

d. As an alternate to Steps 2a-c, emulsify 4-5 large, or 5-10 small, colonies from the subculture from Step 1c in sterile saline or distilled, buffered or deionized water in a glass test tube and mix well.

   i. If required, adjust the cell concentration to achieve a turbidity equivalent to a 0.5 McFarland standardized suspension. Alternatively, measure the absorbance using a spectrophotometer at a wavelength of 625 nm, with the diluent used in Step 2d (saline or water) as a blank. Adjust the suspension to achieve an absorbance between 0.08-0.13. Dilute the standardized suspension in OSM to achieve an approximate cell density of 10^5 CFU/mL.

e. Perform an inoculum check by serially diluting and spot plating the diluted culture to confirm the cell density and purity (Steps 2bi-iii). If a mixed culture is present on the agar plate after incubation, re-isolate the test colonies and retest the inoculum (Steps 1 and 2).

3. INOCULATION AND BIOFILM FORMATION:

It is recommended that the following steps be carried out in a biological safety cabinet (if available). However, it is possible to perform these steps using aseptic technique on a bench top for microorganisms that do not require handling in a biological safety cabinet.

3.1. If using an MBEC Assay® Biofilm Inoculator with a **96-Well Base:**

a. Open a sterile MBEC Assay® Biofilm Inoculator with a 96-well base (Product codes: 19111, 19112, 19113, 19131, 19132, 19133, 19151, 19152, 19153, 19171, 19172, or 19173). Pour the inoculum into a reagent reservoir. Using a multichannel pipette, add 150 µL of the inoculum to each well of the 96-well base that is packaged with the MBEC Assay® Biofilm Inoculator peg lid. Place the peg lid onto the 96-well base. Ensure that the orientation of the base matches the orientation of the lid (i.e., peg A1 must be inserted into well A1 of the base, otherwise the device will not fit together correctly).

NOTE: *The volume of inoculum used in this step has been calibrated such that the biofilm covers a surface area that is immersed entirely by the volume of antimicrobials used in the challenge plate set up in Step 4 (below). Using a larger volume of inoculum may lead to biofilm formation higher up on the peg that physically escapes exposure in the challenge step*.

b. Place the device on a platform shaker set at ~110 rpm in a humidified incubator at the appropriate temperature.

c. Incubate for the appropriate time required to achieve a biofilm density of approximately 10^4-10^6 CFU/mL (confirmed as per Step 5 below). Biofilm growth time will vary between strains, however 16±2 hours is sufficient for many microorganisms.

3.2. MBEC Assay® Biofilm Inoculator with a **Trough Base:**

a. Open a sterile MBEC Assay® Biofilm Inoculator with a trough base (Product codes: 19121, 19122, 19123, 19141, 19142, 19143, 19161, 19162, 19163, 19191, 19192, or 19193). Pour the inoculum into a reagent reservoir. Using a sterile pipette, add 22 mL of the inoculum to the trough base. Place the peg lid onto the base.

NOTE: *The volume of inoculum used in this step has been calibrated such that the biofilm covers a surface area that is immersed entirely by the volume of antimicrobials used in the challenge plate set up in Step 4 (below). Using a larger volume of inoculum may lead to biofilm formation higher up on the peg that physically escapes exposure in this challenge step.*

b. Place the device on a rocking table set to between 3 and 5 rocks per minute in a humidified incubator at the appropriate temperature.

NOTE: *It is critical that the angle of the rocking table is set to between 9° and 16° of inclination. The rocking motion must be symmetrical.*

c. Incubate for the appropriate time required to achieve a biofilm density of approximately 10^4-10^6 (determined as per Step 5 below). Biofilm growth time will vary between strains, however 16±2 hours is sufficient for many microorganisms.

4. PREPARATION OF THE ANTIMICROBIAL CHALLENGE PLATE:

Using a sterile 96-well microtiter plate, the following steps describe how to aseptically prepare the challenge plate. Figure 5 provides a sample plate layout and the steps below describe how to set up a challenge plate with this layout. The steps must be modified appropriately if a different layout is used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	100	100	100	100	100	100	100	100	100		
B	50	50	50	50	50	50	50	50	50	50		
C	25	25	25	25	25	25	25	25	25	25	SC	SC
D	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	SC	SC
E	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	SC	SC
F	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	3.13	GC	BGC
G	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	1.56	GC	BGC
H	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	0.78	GC	BGC

Figure 5. A sample challenge plate layout. The above layout represents a serial two-fold dilution gradient of a single antimicrobial (n=10). The numbers in columns 1 to 10 refer to the percentage of undiluted sample with 100 representing 100% concentration of the stock solution, 50 representing a 50% concentration of the stock solution and so on. SC = Sterility Control, GC = Growth Control, BGC = Biofilm Growth Check. This is only one example - the antimicrobial challenge layout may be set up in any manner desired with any combination of antimicrobials at any desired concentration(s). It is important that the final volume in each well of the challenge base is 200 µL. This is to ensure complete submersion of the biofilm in the antimicrobial.

a. Prepare a sufficient volume of stock solution of the sample to be tested (e.g., an antibiotic, biocide, disinfectant, etc.).
b. Add 200 µL of sterile OSM to the GC, BGC, and three of the SC marked wells in columns 11 and 12 (see Figure 5). Add 200 µL of the test sample working solution (the highest concentration required for testing) to the remaining three SC wells. These wells serve as the sterility controls, growth controls, and biofilm growth checks.
c. Add 100 µL of the appropriate diluent for the test sample to each well in columns 1-10 of rows B-H.

d. Add 200 µL of the test sample working solution (the highest concentration of sample required for testing) to each well in columns 1-10 of row A.
e. Add 100 µL of the test sample working solution to each well in columns 1-10 of rows B and C.
f. Using a multichannel micropipette, if available (otherwise use of single channel micropipettes is acceptable), mix the contents of columns 1 to 10 of row C by pipetting up and down. After mixing, transfer 100 µL from the wells in row C to the corresponding wells in row D. Discard pipette tips after the transfer.
g. Mix the contents of columns 1-10 of row D and transfer 100 µL from each well to the corresponding wells in row E, then discard the pipette tips. Repeat this mix-and-transfer process down the length of the microtitre base until reaching row H.
NOTE: *Perform this for challenge columns (e.g., columns 1 to 10) only.*
h. Using a multichannel pipette, remove 100 µL from each well in columns 1-10 of row H and discard the liquid appropriately.
NOTE: *After this step, the wells in columns 1-10 of rows C-H will contain 100 µL.*
i. Add 100 µL of diluent to each well in columns 1-10 of rows C-H so that each well of the challenge base contains 200 µL.
j. Aseptically cover the freshly prepared challenge base and let it stand at room temperature for 30 minutes to equilibrate prior to use.
NOTE: *Prepare the challenge plate fresh on the day of the challenge.*

5. BIOFILM GROWTH CHECK

a. Prepare one rinse plate by filling each well of a new sterile 96-well base with 200 µL of sterile saline, and aseptically covering the base until used.
b. After removing the MBEC Assay® Biofilm Inoculator from the incubator following biofilm formation, rinse dispersed cells from the biofilms that have formed on the pegs of the MBEC Assay® device by placing the lid into the rinse base for approximately 10 seconds.
c. Following the rinse step, and prior to the antimicrobial challenge, break off the biofilm growth check (BGC) pegs (F12, G12, and H12 in Figure 5) with sterilized (flamed) needle nose pliers.
    i. Grasp the base of the peg with the pliers, and gently rock back and forth until the peg breaks away from the lid.
d. Place each peg broken off in Step 5c into 200 µL of neutralizer recovery media in row A of a new sterile 96-well microtiter base, aseptically cover the recovery base, and place the MBEC Assay® lid with biofilm growth onto the challenge base (see Step 6a).

e. Transfer the recovery plate from Step 5d to the sonicator and sonicate on high for 30 ± 5 minutes to dislodge the biofilm.
NOTE: *The plate is placed on a DRY stainless steel insert tray that floats on the water in the sonicator, OR in a sealed container that sits in the water of the sonicator. The vibrations created in the water by the sonicator transfer through the insert tray/container to actively sonicate the contents of the 96-well recovery plate.*
f. Following sonication, place 180 µL of sterile saline or buffered water into each well of rows B-H, directly below the BGC pegs in row A of the recovery base.
g. Prepare a serial dilution and spot plate per Step 2bi-iii.
h. Incubate the plates for the appropriate time and temperature, and count colonies to determine the biofilm density on the pegs (Biofilm Growth Check).

6. ANTIMICROBIAL CHALLENGE AND RECOVERY OF THE BIOFILM

a. Following the rinse step and after breaking off the BGC pegs (in Step 5c), transfer the MBEC Assay® lid with biofilm growth to the challenge base prepared in Step 4 (per Step 5d) and incubate at the appropriate temperature for the desired contact time.
b. Prepare the neutralizer/recovery plate by placing 200 µL of the appropriate neutralizer recovery solution in each well (including GC and SC wells) of a new sterile 96-well microtiter base, and aseptically covering the base until used.
c. After the challenge, transfer the MBEC Assay® lid to the neutralizer/ recovery base and retain the challenge base, covering it with a non-pegged lid or plate sticker until needed, as it will be used in Sections 7 and 8.
d. Transfer the neutralizer/recovery plate to the sonicator and sonicate on high for 30 ± 5 minutes to dislodge the biofilm. For this step, the plate is placed in a dry stainless steel insert tray which sits in the water of the sonicator (see note in Step 5e). After sonication, this plate will be used for Sections 9 and 10.

7. DETERMINATION OF MINIMUM BIOCIDAL CONCENTRATION (MBC)

a. Fill every well of a new sterile 96-well microtiter base with 180 µL of OSM.
b. After the MBEC Assay® peg lid has been removed from the challenge plate (Step 6c), remove 20 µL from each well of the challenge base and add it to the corresponding well of the base prepared in Step 7a.
c. Cover the base with a sterile non-pegged lid and incubate it under appropriate growth conditions. Retain the challenge base, as it will be used in Step 8, aseptically covering the base until used.

d. Following sufficient growth time (i.e., growth control wells should have visible growth), determine the MBC for the antimicrobial against the organism(s) tested.
   i. Sterility wells should be clear - if SC wells contain growth, all results are inconclusive and it is recommended that testing be repeated, ensuring aseptic technique is carefully followed.
   ii. To determine the MBC values, visually check for turbidity in the wells of the MBC plate. Alternatively, use an automated plate reader to obtain optical density measurements at 650 nm (OD_{650}).
   NOTE: *The MBC value represents the lowest concentration of antimicrobial which kills 99.9% of the population of the dispersed cells that have been shed from the biofilm. Clear wells are evidence of effective biocidal concentration following the chosen contact time.*

8. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

a. Following Step 6c, place a fresh sterile non-pegged 96-well microtiter lid or a plate sticker on the challenge plate and incubate it under appropriate growth conditions, until the growth control wells become turbid.
b. Following sufficient growth time (i.e., growth control wells should have visible growth) determine the MIC for the antimicrobial against the organism(s) tested.
   NOTE: *Due to the optical density/opacity of some antimicrobial agents, determination of MIC may not be possible.*
   i. Sterility wells should be clear - if SC wells contain growth, results are inconclusive and it is recommended that testing be repeated, ensuring aseptic technique is carefully followed.
   ii. To determine the MIC values, visually check for turbidity in the wells of the challenge plate. Alternatively, use an automated plate reader to obtain optical density measurements at OD_{650}.
   NOTE: *The MIC is defined as the minimum concentration of antimicrobial that inhibits growth of the dispersed cells from the biofilm. Clear wells are evidence of inhibition following the chosen contact time.*

9. DETERMINATION OF LOG_{10} REDUCTION

a. Following sonication of the neutralizer/recovery plate (Step 6d), place 100 µL from each well of row A into the 12 empty wells of row A in a fresh 96-well microtiter base, which will be used for serial dilution. Place 100 µL from each well of row B (of the recovery base) into the 12 empty wells of row A in a second fresh 96-well microtiter base. Continue doing this until samples from all 8 rows of the challenge base are transferred into row A of eight fresh 96-well microtiter bases. Place 180 µL of sterile saline or buffered water in the remaining rows (B-H) for all eight 96-well serial dilution bases.

b. Perform serial dilutions and spot plating of all eight bases per Step 2bii-iii.

c. Incubate the plates for an appropriate time and at an appropriate temperature, and then count colonies to determine the quantity of viable microorganisms remaining on the pegs following the challenge.

   i. Count the number of colonies on the spot plates according to the plating method used. Count colonies from a spot where the individual colonies are visibly distinct from each other within the plated spot and where preferably more than one colony is present. The section in which the countable spot is located gives the order of magnitude for the cell enumeration, i.e., which dilution, from 10^0-10^7, to use in subsequent calculations.

d. Calculate the log density for one peg as follows:

$$\text{Log}_{10}\left(\frac{\text{CFU}}{\text{peg}}\right) = \text{Log}_{10}\left[\left(\frac{X}{B}\right)(D)(V)+1\right]$$

Where:
X = CFU counted on spot plate
B = volume plated (e.g., 0.02, 0.01 or 0.005 mL)
V=well volume (0.20 mL)
D = Dilution (Ex. 10^4)

e. The log density per unit area (mm^2) may also be calculated as follows:

$$\text{Log}_{10}\left(\frac{\text{CFU}}{\text{mm}^2}\right) = \text{Log}_{10}\left[\left(\frac{X}{B}\right)\left(\frac{V}{A}\right)(D)+1\right]$$

Where:
X = CFU counted on spot plate
B = volume plated (e.g., 0.02, 0.01, or 0.005 mL)
V = well volume (0.20 mL)
A = biofilm surface area (46.63 mm^2)
D = Dilution (Ex. 10^4)

f. Calculate the overall biofilm accumulation by calculating the mean of the log densities for the chosen number of replicates.

g. Calculate the Log_{10} Reduction for each dilution as follows:

$$Log_{10}\text{ Reduction} = \text{Mean } Log_{10} \text{ Untreated Control Pegs} - \text{Mean } Log_{10} \text{ Treated Pegs}$$

10. DETERMINATION OF MINIMUM BIOFILM ERADICATION CONCENTRATION (MBEC)

a. Discard the MBEC Assay® lid appropriately.
NOTE: *Treat the lid as a biohazard*.

b. Following removal of 100 µL for the determination of Log_{10} Reduction (Step 9a), add 100 µL of sterile OSB into each well of the neutralizer/recovery base. Cover the base with a fresh non-pegged lid or a sterile plate sticker. Incubate the refilled plate under appropriate conditions.

c. Following sufficient growth time (i.e., growth control wells should have visible growth) determine the MBEC for the antimicrobial against the organism(s) tested.

d. To determine the MBEC values, visually check for turbidity in the wells of the neutralizer/recovery plate. Alternatively, use an automated plate reader to obtain optical density measurements at 650 nm (OD_{650}).
NOTE I: *The MBEC is defined as the minimum concentration of antimicrobial that eradicates the biofilm. Clear wells are evidence of eradication following the chosen contact time.*
Note II: *If the neutralizer is turbid, it may not be possible to determine the MBEC by measuring the optical density. In this case, following Step 10c, spot 10 µL from each well on OSA plates and incubate the plates under appropriate growth conditions. Following incubation, score each spot +/- for growth.*

VIII. MICROSCOPY

The following protocols may be used to prepare biofilms for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). You may include pegs for microscopy in the MBEC Assay® testing by appropriately modifying the plate layout. If using a challenge plate with the same or a similar layout to that in Figure 5, pegs in columns 12 of rows A and B may be used for microscopy.
NOTE: *It is easiest to remove pegs for microscopy from the outer pegs on the lid; i.e., rows A or H and columns 1 or 12.*

The following procedures may be performed immediately following biofilm formation and rinsing (prior to Step 5c above) or may be performed following the antimicrobial challenge, but prior to recovery (i.e., prior to neutralization and sonication - immediately following Step 6a above).

1. **SCANNING ELECTRON MICROSCOPY (SEM)**
   a. Break the appropriate pegs from the MBEC Assay® Biofilm Inoculator lid with sterilized (flamed) needle nose pliers per Section VII.5.c.i.
   NOTE: *With the pliers, grasp the base of the peg to prevent disruption of the biofilm.*
   b. Place each peg into an empty receiver vial. In a fume hood, add primary fixative (5% glutaraldehyde in 0.1M Na cacodylate buffer, pH=7.5) to each vial to completely cover the peg. Cap each vial and incubate at 4±2°C for 16-24 hours.
   c. Decant the fixative with a disposable pipette and discard all liquid waste in an appropriate organic waste container.
   d. Loosely cap the samples and place them in a fume hood to air dry for 72-96 hours.
   e. Mount the samples on aluminum stubs as follows: Apply epoxy resin to the flat, upper surface of each stub. Then, using forceps, carefully affix the pegs to the stubs, ensuring that they are appropriately labelled.
   f. Follow an appropriate procedure for use of the SEM.
   NOTE: *Example SEM images are shown in Figure 6.*

2. **CONFOCAL LASER SCANNING MICROSCOPY (CLSM)**
   a. Break the appropriate pegs from the MBEC Assay® Biofilm Inoculator lid with sterilized (flamed) needle nose pliers per Section VII.5.c.i.
   NOTE: *With the pliers, grasp the base of the peg to prevent disruption of the biofilm.*
   b. Place each peg into an empty receiver vial and stain with a live/dead viability stain containing 3.34 mM SYTO-9 and 20 mM propidium iodide (Recommended kit: FilmTracer™ LIVE/DEAD® Biofilm Viability Kit, Invitrogen – Catalog # L10316).
   c. Follow the staining instructions for the stain system selected.
   d. Wash the fluorescently stained pegs twice with 0.9% saline.
   e. Immediately examine the viability of biofilm cells using a CLSM. To minimize artefacts associated with simultaneous dual wavelength excitation, scan all samples sequentially, frame-by-frame, first at 488 nm and then at 561 nm. A 63X oil objective lens is recommended.
   f. If using an inverted CLSM, the pegs may be placed in a glass bottomed Petri dish to obtain images (Recommended manufacturer: Mat Tek Corp. – 50 mm glass bottom dish, part # P50G-1.5 14F).

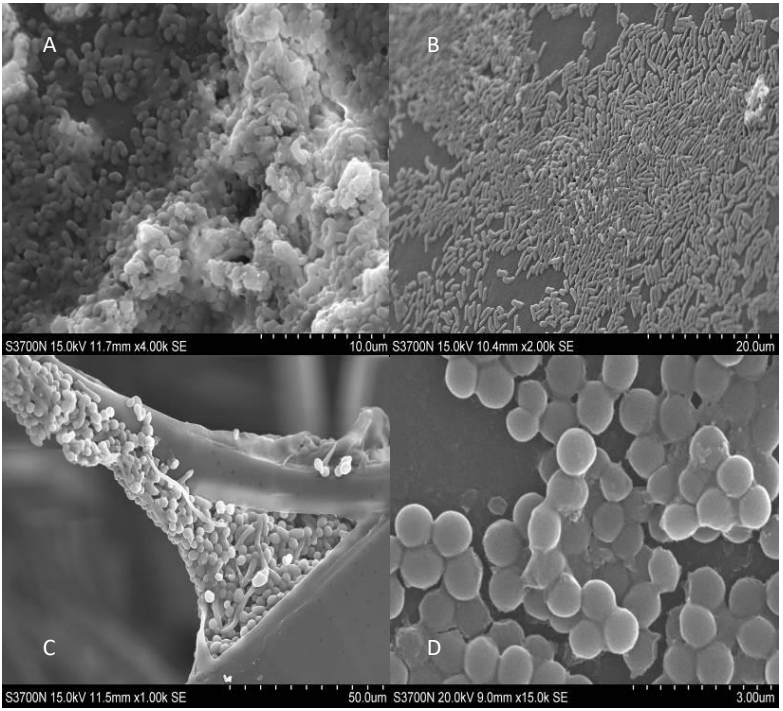

Figure 6. Biofilms formed on the pegs of the MBEC Assay® Biofilm Inoculator. Biofilms were grown in rich medium for 24 to 48h, then fixed and dehydrated. Images were captured by scanning electron microscopy (SEM). Organisms imaged are A) *Aggregatibacter actinomycetemcomitans*, B) *Candida albicans*, C) *Klebsiella pneumoniae*, and D) *Staphylococcus aureus*.

IX. SURFACE COATING THE MBEC ASSAY® BIOFILM INOCULATOR

The surface of the MBEC Assay® Biofilm Inoculator lid may be coated with a number of materials to facilitate the growth of fastidious microorganisms. Hydroxyapatite-coated lids facilitate biofilm growth by microorganisms such as *Candida* spp., and have also proven beneficial when studying bone or dental applications (Product codes: 19133, 19131, or 19132 for cases of 10, 25, or 100, respectively, with 96-well bases; 19143, 19141, or 19142 for cases of 10, 25, or 100, respectively, with trough bases). Titanium dioxide-coated lids provide a great platform to test titanium dioxide's effect on biofilm formation, as titanium dioxide has been reported as an antimicrobial substance due to its photocatalytic effect (Product codes: 19153, 19151, or 19152 for cases of 10, 25, or 100, respectively, with 96-well bases; 19163, 19161, or 19162 for cases of 10, 25, or 100, respectively, with trough bases). Cellulose-coated lids promote biofilm formation by plant pathogens that may not

grow readily on uncoated plastic surfaces (Product codes: 19173, 19171, or 19172 for cases of 10, 25, or 100, respectively, with 96-well bases; 19193, 19191, or 19192 for cases of 10, 25, or 100, respectively, with trough bases). All these coated products are available for purchase through Innovotech's website and may be used following the same procedures as above. The MBEC Assay® lid may also be coated with poly-L lysine, collagen, etc. For additional protocols, to purchase specialized plates, or to request custom coatings, please contact Innovotech.

X. FREQUENTLY ASKED QUESTIONS

1. *Which product would you recommend I use*?

We recommend the MBEC Assay® Biofilm Inoculator with 96 well bases to most customers. It is useful for many commonly used bacterial species such *Acinetobacter* spp., *Escherichia* spp., *Klebsiella* spp., *Staphylococcus* spp., some *Streptococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Salmonella* spp., and *Enterobacter* spp.

The MBEC Assay® Biofilm Inoculator with 96 well bases & hydroxyapatite-coated pegs is used to simulate bone/teeth, so we recommend this product for dental pathogens (e.g., *Streptococcus mutans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*). Hydroxyapatite coated pegs typically result in greater biofilm growth compared to uncoated pegs, so we also recommend the MBEC Assay® Biofilm Inoculator with 96 well bases & hydroxyapatite-coated pegs for strains that form weaker biofilms (*Candida* spp*., Citrobacter* spp*., Enterococcus faecalis, Listeria* spp*., Nocardia* spp., fungus, yeast, and anaerobic strains).

The trough base is recommended for strains that prefer side-to-side flow against the MBEC Assay® pegs rather than rotary motion; this typically works well with plant pathogens.

If you are uncertain about which product to order, contact info@innovotech.ca.

2. *What is the difference between the 96 well base and trough base products*?

The 96 well base has separate wells which allow you to test different strains in different wells. The trough base doesn't have separate wells; it's a tray with open longitudinal rows where inoculated media flows from side to side on a rocker shaker, which only allows for one strain per plate.

We sell the MBEC Assay® Kits with 96 well bases to 95% of our customers. The trough base is mainly used for plant pathogen strains, where a larger fluid volume flowing against the pegs is needed.

3. *What type of ultrasonic cleaners do you recommend?*

Any ultrasonic cleaner should work. This is an example of the ones available from VWR:

https://us.vwr.com/store/product/12626090/vwr-ultrasonic-cleaners-220v;jsessionid=CypugE60bBCOtLmyua3WwYnn.estore4a. The size of the sonicator needed is based on the size of experiments you typically run in your lab.

4. *How do I perform the recovery/sonication step?*

You need to use a solid tray such as the Solid Insert Tray for 3510, VWR Catalogue #100491-720 (https://us.vwr.com/store/product?keyword=100491-720). Make sure the water bath of the ultrasonic cleaner is full of water. Place the stainless steel tray in the water bath. Place the MBEC Assay® Kits on the stainless steel tray (don't stack more than two plates high), and then turn on the sonicator for 30 ± 5 min.

5. *How do I break the MBEC Assay® pegs off the lid for biofilm growth check or SEM analysis?*

Using a flame-sterile pair of needle nose pliers, grasp the base of the peg and snap the peg off by twisting the pliers. The biofilm only grows on the top third portion of the peg (the portion immersed in the inoculated media), thus there is no risk of disrupting the attached biomass.

6. *I'm having trouble growing a biofilm. What can I do?*

Some troubleshooting tips for growing biofilms:

– Ensure that the humidity in your incubator is 75%-90% to avoid drying out the wells over the incubation period.
– Place your MBEC Assay® plate on the proper shaker at the proper rpm speed (110 rpm is recommended for 96 well bases).
– Start with the right inoculum concentration (10^5-10^6 CFU/mL).
– Use the proper media and growth condition for the strain you are growing (some strains require the use of a 5% CO_2 incubator, or specific growth factors, such as glucose)
– Try using the MBEC Assay® Biofilm Inoculator with 96 well bases & hydroxyapatite-coated pegs. Using hydroxyapatite-coated pegs typically results in greater biofilm growth than the standard uncoated pegs.

If you would like more specific information or additional help, please email info@innovotech.ca with detailed information about the strain(s) and conditions you are using.

7. *What safety information is there about your products?*

Our products are not toxic and do not fall under the Toxic Substances Control Act (TSCA) regulation. Our products are not dangerous goods.

8. *What are the storage conditions for your products and what are their expiry dates?*

Our products are stored at room temperature. They expire 3 years after their manufacture date.

9. *Is MBEC Assay® testing available as an independent third-party validation service?*

Absolutely! Through our contract research division, we perform antimicrobial validation testing using the MBEC Assay® kit in-house for our clients for competitive prices and with a quick turnaround time.

XI. COMPANY INFORMATION

Innovotech Inc. was first formed in 2001 as a research and product development company, and has world recognized expertise in microbial biofilms.

The company also has developed assays for the analysis of surface coatings using the Biofilm Eradication Surface Test, or BEST Assay™ system. This assay has been used extensively in the development of surface coatings for catheters and other implanted devices. For contract testing services, please contact Innovotech.

XII. WARRANTY

Products purchased from Innovotech Inc. are warrantied to meet stated product specifications and to conform to label descriptions when used and stored properly. Unless otherwise stated, this warranty is limited to one year from the date of sale for products used, handled, and stored according to Innovotech Inc. instructions. Liability of Innovotech Inc. is limited to replacement of the product or refund of the purchase price. If you are not satisfied with the performance of a product purchased from Innovotech Inc., please contact us.

XIII. CONTACT INFORMATION

To tell us how Innovotech Inc. can better meet your needs, contact us about your specific biofilm problem, or ask us about our products and services:

Website: http://www.innovotech.ca
Contact: info@innovotech.ca or sales@innovotech.ca

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