MBEC™ Assay

For High-Throughput Antimicrobial Susceptibility Testing of Biofilms

PROCEDURAL MANUAL

Version 1.0

For use with the following Product Codes:

MBEC™ Biofilm Inoculator with 96 well base - **19111** - 25/case, **19112** – 100/case
MBEC™ Biofilm Inoculator with 96 well base & Hydroxyapatite coated pegs - **19131** - 25/case, **19132** – 100/case

MBEC™ Biofilm Inoculator with Trough base – **19121** - 25/case, **19122** – 100/case
MBEC™ Biofilm Inoculator with Trough base & Hydroxyapatite coated peg, - **19141** - 25/case, **19142** – 100/case
PRODUCT DESCRIPTION

The MBEC™ (Minimum Biofilm Eradication Concentration) Assay is a high throughput screening assay used to determine the efficacy of antimicrobials against biofilms of a variety of microorganisms. The MBEC™ Biofilm Inoculator consists of a plastic lid with 96 pegs and a corresponding base. There are two types of bases that may be used with the MBEC™ lid. One base contains 96 individual wells (Figure 1). The individual wells allow for the growth of a variety of microorganisms on the same peg lid. The other type of base is a corrugated trough base that can contain only a single microorganism (Figure 2). Biofilms are established on the pegs under batch conditions (no flow of nutrients into or out of an individual well) with gentle mixing. The established biofilm is transferred to a new 96 well plate 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.

Figure 1. A 96 Well Base. Found in products 19111, 19112, 19131, and 19132.

Figure 2. Trough Base. Found in products 19121, 19122, 19141 and 19142.

Figure 3. The MBEC™ Assay. A) Biofilms form on the pegs of the MBEC™ Biofilm Inoculator when planktonic bacteria adsorb to the surface. In the presence of shear, these bacteria become attached and grow to form mature biofilms. Biofilms are encased in ‘slime’, which is sometimes visible to the naked eye. Dispersed cells are also shed from the surface of biofilms, which serve as an inoculum for MIC determinations. B) The peg lid has 96 identical pegs. The average surface area of each peg on the MBEC device is 108.9 mm². This lid fits into a standard 96-well microtiter plate or a trough bottom with channels that are set up to contain an inoculated growth medium. The entire device is placed on a gyrorotary shaker or a rocking platform in an incubator, which provides the shearing force for facilitating the formation of 96 biofilms on the peg lid.
PRODUCT USES

• Antibiotic, biocide, disinfectant and heavy metal susceptibility testing of biofilms to determine a minimum inhibitory concentration (MIC), a minimum biocidal concentration (MBC) and 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)

• Comparative biofilm growth assays of up to 96 different isolates under identical culture conditions

• Comparative studies of gene expression in multiple isolates or mutants

• Selection criteria applied against biofilm microorganisms

• Checkerboard assays to identify synergistic interactions between antimicrobials used to treat biofilms

• Time course studies of biofilm formation or treatment in a single assay, since the pegs are designed to be easily removed from the peg lid

• Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) to examine biofilm structure

• Many conventional assays for attachment, growth, survival, or metabolic activity to measure the parameters of interest to the researcher. These can be done while the biofilm is intact on the peg or following sonication to again produce a planktonic population.

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. Please contact one of our customer service representatives for a methodological design specific to your requirements.

INTRODUCTION TO BIOFILMS

The expertise of Innovotech Inc. is 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 response to the presence of a solid surface as well as other factors such as shear force (flow) as a mechanism to avert 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 adsorb to a surface and become attached, triggering a change in physiology. The bacteria then grow and divide to form layers, clumps or stalk and mushroom shaped microcolonies, all under the control of specific biofilm genes. The production of an extracellular polymeric matrix on the surface further protects the biofilm and can often be seen with the naked eye.

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 one hundred-fold increase in tolerance to antibiotics when compared to the same bacteria in a planktonic state [1, 2, 3]. This is thought to be due to the physiological alteration of the microorganism upon attachment to the surface, as well as to cell specialization that may occur within biofilms.

Microbial biofilms naturally exist on 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 in a dentist’s office. 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 Center for Disease Control and Prevention estimates that 60% of clinical infections in the Western world are caused by biofilms. This includes heart and middle ear infections, illnesses associated with implanted medical devices such as artificial joints and catheters, as well as tooth decay and gum disease (which arise from dental plaque – an oral biofilm). Information on biofilm control properties of existing products and protocols are not available to most industries as there has not been biocide testing for biofilm microorganisms.

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The MBEC™ assay, allows microorganisms to grow on 96 identical pegs protruding down from a plastic lid. By placing the biofilms on the pegs into the wells of a microtiter plate, an array of antimicrobial compounds with varying concentrations can easily be assessed. This allows rapid testing of compounds for antibiofilm activity, which includes antibiotics, disinfectants, biocides and metals [2, 4-6]. Many different bacterial and yeast species have been grown using this assay, including Escherichia coli [2], Pseudomonas aeruginosa [2], Staphylococcus spp. [2, 7], Mycobacterium spp. [8], Candida spp. [9], Burkholderia spp. [10, 11] as well as many more. To date, the MBEC™ assay has been featured in more than 100 peer-reviewed publications.

References

PRECAUTIONS
• Single use only.
• Aseptic techniques and good laboratory practice should be observed throughout all procedures, with special awareness that the plates and inoculation lid contain potentially pathogenic organisms.
• All biohazardous waste should be decontaminated by autoclave, incineration, or chemical means prior to disposal in compliance with facility/institutional guidelines.
MATERIALS AND EQUIPMENT

- MBEC™ Biofilm Inoculator (Peg lid with 96-well microtiter plate 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 cotton swabs
- Sterile 16 × 100 mm glass culture tubes  (Recommended manufacturer: FisherBrand, Borosilicate glass disposable culture tubes)
- Sterile physiological saline solution (ex. phosphate buffered saline, 0.9% NaCl)
- 0.5 McFarland Barium Sulfate Turbidity Standard or other 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 for the microorganism to be cultured

General laboratory equipment

- Platform shaker (Set at 110 and 150 revolutions per minute (rpm) for use with 19111, 19112, 19131 and 19132) (Recommended manufacturer: New Brunswick Scientific – Excella Platform Shaker)
- Rocking Table (For use with 19121, 19122, 19141 and 19142. Set at 3-5 rocks per minute and 90°-160° of inclination) (Recommended manufacturer: Bellco Glass Inc. - Rocker with Tray Platform)
- Single channel micropipette
- Multichannel micropipette (2-20 μL and 50-200 μL required, 12 channels recommended)
- Ultrasonic cleaner (water bath sonicator) with stainless steel insert tray (Recommended manufacturer: VWR Scientific, Aquasonic model 250)
- Incubation loop
- Incubator for biofilm growth , with temperature settings specific to test microorganisms
- Bunsen burner
- Needle nose pliers
- Pipette bulb or aid
- Recommended - Biological safety cabinet (Laminar flow hood)

MEDIA AND SOLUTIONS

Stock antimicrobial solutions:
Antibiotic and other antimicrobial stock solutions should be prepared in advance at the highest concentration to be used in the challenge plate. Clinical Laboratory Standards Institute (CLSI) document M100-S22 may be consulted for details of which solvents and diluents to use. Stock solutions of most antibiotics are stable for a minimum of 6 months at -70°C.

Neutralizing and biofilm recovery agents:
For research applications it is appropriate 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. As examples, it is possible to use β-lactamase to neutralize penicillin, or L-cysteine to neutralize Hg²⁺ and some other heavy metal cations. Innovotech Inc. often uses a universal neutralizer in biocide susceptibility assays that is required for regulatory aspects of product development. The neutralizer also contains a surfactant that aids in recovering any remaining biofilm from the device following the challenge. Examples are presented below:
**Universal Neutralizer (for biocide testing)**
1.0 g L-Histidine
1.0 g L-Cysteine
2.0 g Reduced glutathione

Make up to 20 ml in double distilled water. Pass through a syringe with a 0.20 µm filter to sterilize. This solution may be stored at -20°C.

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 the correct pH (7.0 ± 0.2 at 20°C).

Add 500 µl of the universal neutralizer to each 20 ml of the surfactant supplemented growth medium used for recovery plates (Section 4.5 below).

**D/E Neutralizing Broth** – prepare as per manufacturer’s instructions

**Organism specific media:**
Prepare agar and broth growth media specific for the microorganism to be cultured as per manufacturer’s instructions (i.e. Tryptic Soy Broth/Agar, Sabouraud Dextrose Broth/Agar).

**PROCEDURES**
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 bottom, 96-well microtiter plates.

![Figure 4](image-url)

**Figure 4.** A flow diagram representing the steps in the experimental process for 96 well base antimicrobial susceptibility testing using the MBEC™ assay.
1: GROWING SUBCULTURES
   a. If using a cryogenic stock (at -70°C), streak out a first sub-culture 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 bacterial strains, the sub-culture may be wrapped with Parafilm™ and stored at 4±2°C for up to 14 days.
   b. Check the first sub-culture for purity (i.e. only a single colony morphology should be present on the plate).
   c. From the first sub-culture or from a clinical isolate, streak out a second sub-culture on an appropriate agar plate. Incubate at the optimum growth temperature of the microorganism for an appropriate period of time. The second sub-culture should be used within 24 h starting from the time it was first removed from incubation.
   d. Verify the purity of the second sub-culture.

   NOTE: It is not recommended to grow subcultures on media containing selective agents. Antibiotics and other antimicrobials may trigger an adaptive stress response in bacteria and/or may increase the accumulation of mutants in the population. This may result in an aberrant susceptibility determination.

2: INOCULUM PREPARATION:
   a. Choose approximately 4-5 large or 5-10 small, well isolated colonies from an 18-24 hour culture.
   b. Inoculate a flask containing 100-200 mL of organism specific broth and incubate on a shaker at 150 rpm at the appropriate growth conditions. Viable cell density should be between 10^7-10^9 CFU/mL (cell density is dependent on the microorganism) and may be checked by serial dilution and spot plating.
   c. Dilute the flask culture 10x-1000x in a tube of sterile organism specific media 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. Alternatively, emulsify the colonies from the second sub-culture in sterile distilled deionised water in a glass test tube and mix well. If required, adjust to achieve a turbidity equivalent to a 0.5 McFarland standardized suspension. Dilute the standardized suspension in organism specific media to achieve an approximate cell density of 10^5.
   e. Perform an inoculum check by serially diluting and spot plating the diluted culture to confirm the cell density.

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 use aseptic technique on a bench top.

A. MBEC™ Biofilm Inoculator with 96 well Base:
   a. Open a sterile MBEC™ Biofilm Inoculator with a 96 well base (Product code: 19111 and 19112). 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 packaged with the MBEC™ Biofilm Inoculator. Place the peg lid onto the microtiter base. Ensure that the orientation of the plate matches the orientation of the lid (i.e. peg A1 must be inserted into well A1 of the microtiter plate, 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 high on the peg that physically escapes exposure in this challenge step.**

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

c. Incubate for the appropriate time required to achieve an inoculum density of approximately $10^4$ - $10^6$ CFU/mL (determined as per step 4 below). Biofilm growth time will vary between strains, however 16±2 hours is sufficient for many bacterial organisms.

**B. MBEC™ Biofilm Inoculator with Trough Base:**

a. Open a sterile MBEC™ Biofilm Inoculator with a trough base (Product code 19121 and 19122). 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 high 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 90° and 160° of inclination. This motion must be symmetrical.

c. Incubate for the appropriate time required to achieve an inoculum density of approximately $10^4$ - $10^6$ (determined as per step 4 below). Biofilm growth time will vary between strains, however 16±2 hours is sufficient for many bacterial organisms.

**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.

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**Figure 5.** The above plate layout represents a serial two-fold dilution gradient of a single antimicrobial in the challenge plate. This is only one example. The antimicrobial challenge plate 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 plate is 200 μL. This is to ensure complete submersion of the biofilm in the antimicrobial. SC=Sterility Control, GC=Growth Control, BGC=Biofilm Growth Check

a. Prepare sufficient biocide stock solution of disinfectant for the experiment.
b. Add 200 µL of sterile organism specific media to each well in columns 11 and 12 of the challenge plate. These wells will serve as the sterility controls, growth controls and biofilm growth checks.

c. Add 100 µL of the appropriate diluent for the biocide to each well in columns 1-10, rows B-H.

d. Add 200 µL of the biocide working solution (the highest concentration of biocide required for testing) to each well in columns 1 to 10, row A of the microtitre plate.

e. Add 100 µL of the biocide working solution to each well in column 1-10, rows B and C.

f. Using a multichannel micropipette, mix the contents of columns 1 to 10, 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.

g. Mix the contents of columns 1-10, row D and transfer 100 µL from each well to the corresponding wells in row E. Serially repeat this mix and transfer process down the length of the microtitre plate until reaching row H. **NOTE: Perform this for challenge columns (columns 1 to 10) only.**

h. Using a multichannel pipette, remove 100 µL from each well in columns 1-10, row H and discarded.

i. Add 100 µL of diluent to each well in columns 1-10, rows C-H so that each well of the challenge plate contains 200 µL.

j. Aseptically cover the freshly prepared challenge plate and let stand at room temperature for 30 minutes to equilibrate prior to use. **NOTE: The challenge plate should be prepared fresh the day of the challenge.**

5: BIOFILM GROWTH CHECK

a. Prepare rinse plates by filling each well of a new, sterile 96 well plate with 200 µL of sterile saline.

b. After removing the MBEC™ Biofilm Inoculator from the incubator following biofilm formation, rinse dispersed cells from the biofilm(s) that have formed on the peg of the MBEC™ device by placing the lid into the rinse plate 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 with sterilized (flamed) pliers.

d. Place each peg into 200 µL of neutralizer recovery media in row A of a new, sterile 96 well microtiter plate.

e. Transfer the plate to the sonicator and sonicate on high for 30 minutes to dislodge the biofilm. The plate(s) are placed in a dry stainless steel insert tray which sits on the water of the sonicator. The vibrations created in the water by the sonicator transfer through the insert tray to actively sonicate the contents of the 96 well recovery plate(s).

f. Following sonication, place 180 µL of sterile saline or buffered water into each well of rows B-H.

g. Prepare a serial dilution (10⁰-10⁷) by moving 20 µL down each of the 8 rows.

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

i. Incubate the plates for 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 (Step 5.b.), transfer the MBEC™ lid to the challenge plate prepared in step 4 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 of a new, sterile 96 well microtiter plate.

c. After the challenge, transfer the MBEC™ lid to the neutralizer recovery plate and let stand for 30 minutes to equilibrate prior to the next step(s).

d. After the equilibration time, transfer the plate to the sonicator and sonicate on high for 30 minutes to dislodge the biofilm. The plate(s) are placed in a dry stainless steel insert tray which sits in the water of the sonicator. The vibrations created in the water by the sonicator transfer through the insert tray to actively sonicate the contents of the 96 well recovery plate(s).

7: DETERMINATION OF MBC

a. After the MBEC™ peg lid has been removed from the challenge plate, remove 20 µL from each well of the challenge plate and add into the corresponding wells of a fresh, sterile 96 well microtiter plate containing 180 µL of organism specific broth (OSB) in each well.

b. Cover the plate and incubate at appropriate growth conditions.

c. Following sufficient growth time (growth control wells should have visible growth), determine the MBC (Minimum Biocidal Concentration) for the antimicrobial for each of the organisms tested.

d. To determine the MBC values, use a plate reader to visually check for turbidity in the wells of the challenge plate. Alternatively, use an automated plate reader to obtain optical density measurements at 650 nm (OD₆₅₀). 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 a suitable period of incubation.

8: DETERMINATION OF MIC

a. Place a sterile regular 96 well microtiter plate lid or a plate sticker on the challenge plate and incubate at appropriate growth conditions.

b. Following sufficient growth time (growth control wells should have visible growth) determine the MIC (Minimum Inhibitory Concentration) values for the antimicrobial for the organisms shed from the biofilm during the challenge incubation. **NOTE:** Due to the optical density/opacity potential of some antimicrobial agents, determination of MIC may not be possible.

c. To determine the MIC values, use a plate reader to visually check for turbidity in the wells of the challenge plate. Alternatively, use an automated plate reader to obtain optical density measurements at 650 nm (OD₆₅₀). 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 a suitable period of incubation.

9: DETERMINATION OF LOG₁₀ REDUCTION

a. Following sonication, place 100 µL from each well of the MBEC™ Biofilm Inoculator plate into the first 12 empty wells of Row A of a 96 well microtiter plate. Place 180 µL of sterile saline or buffered water in the remaining rows.

b. Prepare a serial dilution (10⁰-10⁷) by moving 20 µL down each of the 8 rows.

c. Remove 10-20 µL from each well and spot plate on Organism Specific Agar (OSA) plates.

d. Incubate the plates for appropriate time and temperature and count colonies to determine the amount of biofilm remaining on the pegs following the challenge.
i. Count the appropriate number of colonies on the spot plates according to the plating method used. An appropriate number of colonies that would be viable for counting would be a 20 µL spot where the individual colonies are visibly distinct from each other within the plated spot. The section in which this spot is located would give the order of magnitude for the cell enumeration; 10^6-10^7.

ii. Calculate the arithmetic mean of the colonies counted on the duplicate plates.

e. Calculate the log density for one peg as follows:
   \[
   \log_{10}(\text{CFU/peg}) = \log_{10}\left(\frac{X}{B}(D)\right)
   \]
   Where:
   - X = mean CFU counted on spot plates
   - B = volume plated (Ex. 0.02 mL)
   - D = Dilution

f. The log density per mm² may also be calculated as follows:
   \[
   \log_{10}(\text{CFU/mm}^2) = \log_{10}\left(\frac{X}{B}(V/A)(D)+1\right)
   \]
   Where:
   - X = mean CFU counted on spot plates
   - B = volume plated (Ex. 0.02 mL)
   - V = well volume (0.20 mL)
   - A = peg surface area (46.63 mm²)
   - D = Dilution

g. Calculate the overall biofilm accumulation by calculating the mean of the log densities for any number of replicates.

h. Calculate the Log_{10} Reduction for each dilution as follows:
   \[
   \text{Log}_{10} \text{ Reduction} = \text{Mean Log}_{10} \text{ Untreated Controls} - \text{Mean Log}_{10} \text{ Treated Pegs}
   \]

10: DETERMINATION OF MBEC

a. Discard the MBEC™ lid appropriately. NOTE: The lid should be treated as a biohazard at this point.

b. Add 100 µL of sterile OSB into each well of the neutralizer recovery plate (to replace the volume removed for spot plating in the previous step). Cover the plate with a fresh non-pegged lid or a sterile plate sticker. Incubate the refilled plate at the appropriate conditions.

c. Following sufficient growth time (growth control wells should have visible growth) determine the MBEC (Minimum Biofilm Eradication Concentration) values for the antimicrobial agents.

d. To determine the MBEC values, use a plate reader to visually check for turbidity in the wells of the challenge plate. Alternatively, use an automated plate reader to obtain optical density measurements at 650 nm (OD_{650}). The MBEC is defined as the minimum concentration of antimicrobial that eradicates the biofilm. Clear wells are evidence of eradication following a suitable period of incubation.
MICROSCOPY
The following protocols may be used to prepare biofilms for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Pegs for microscopy may be included in the MBEC™ assay according to your desired plate layout. If using a challenge plate with the same or similar layout as in the example experimental procedure above, pegs for microscopy may be added to columns 11 or 12 of the challenge plate in Figure 5. **NOTE:** It is easiest to remove pegs for microscopy from the outer pegs on the lid; e.g. Rows A or H and columns 1 or 12.

The following procedures may be performed immediately following biofilm formation and rinsing (prior to step 4.a. above) or may be performed following the antimicrobial challenge, but prior to recovery (i.e. sonication) (immediately following step 4.c. above).

1: SCANNING ELECTRON MICROSCOPY (SEM)
   a. Break the appropriate pegs from the MBEC™ Biofilm Inoculator lid with sterilized (flamed) needle nose pliers. **NOTE:** grasp with the pliers at 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 the vials and incubate at 4±2°C for 16-24 hours.
   c. Decant the fixative with a disposable pipette and discard all liquid waste in appropriate organic waste containers.
   d. Loosely cap the samples and place in a fume hood to air dry for 72-96 hours.
   e. Mount the samples on aluminum stubs by applying epoxy resin to the flat, upper surface of each stub. Using forceps, carefully affix the pegs to appropriately labelled stubs.
   f. Use an appropriate procedure for use of the SEM.

2: CONFOCAL LASER SCANNING MICROSCOPY (CLSM)
   a. Break the appropriate pegs from the MBEC™ Biofilm Inoculator lid with sterilized (flamed) needle nose pliers. **NOTE:** grasp with the pliers at 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 Kit containing 3.35 µM SYTO-9 and 20 µM propidium iodide (Recommended kit: Invitrogen, Cat # L10316).
   c. Follow the staining instructions for the stain of your choice.
   d. Wash the fluorescently stained pegs twice with 0.9% saline.
   e. The viability of biofilm cells can be examined immediately using a confocal laser-scanning microscope. To minimize artefacts associated with simultaneous dual wavelength excitation, all samples should be sequentially scanned, frame-by-frame, first at 488 nm and then at 561 nm. A ×63 oil objective is recommended.
   f. If using an inverted CLSM, the pegs may be placed in a glass bottom petri dish to obtain images (Recommended manufacturer: Mat Tek Corp, 50mm glass bottom dish, part # P50G-1.5 14F)
Figure 6. Bacterial biofilms formed on the pegs of the MBEC™ Biofilm Inoculator Plate. Biofilms were grown in rich medium for 24 to 48 h, then fixed and dehydrated. Images were captured by scanning electron microscopy (SEM). A) *Aggregatibacter actinomycetemcomitans*, B) *Candida albicans*, C) *Klebsiella pneumoniae*, D) *Staphylococcus aureus*.

**SURFACE COATING THE MBEC™ ASSAY**

The surface of the MBEC™ Biofilm Inoculator lid may be coated with a number of materials to facilitate the growth of fastidious microorganisms. For example, biofilm formation by certain Candida spp. is enhanced by coating the pegs with hydroxyapatite. Hydroxyapatite coated plates are available for purchase through Innovotech (Product code: 19131 for case of 25 with 96 well base, 19132 for case of 100 with 96 well base, 19141 for case of 25 with trough base, and 19142 for case of 100 with trough base) and may be used following the same procedures as above. The MBEC™ lid may also be coated with poly-L lysine, cellulose, collagen, or platinum. For additional protocols or to purchase specialized plates please contact one of our customer service representatives.

**COMPANY INFORMATION**

Innovotech Incorporated was first formed in 2001 as a research and product Development Company with world recognized expertise in bacterial biofilms. Innovotech Inc. holds several patents. The assay methods and protocols presented in this document are protected by the following patents:

- US Patent # 6,599,714 – Method of Growing and Analyzing a Biofilm
- US Patent # 6,410,256 – Method of making biofilms
- US Patent # 6,326,190 – Biofilm Assay
• US Patent # 6,051,423 – Biofilm Assay

The company also holds patents on the analysis of surface coatings using the MBEC™ Biofilm Eradication Surface Test, or B.E.S.T. Test. This assay has been used extensively in the development of surface coatings for catheters and other implanted devices. This technology is covered by the following patents:

• US Patent # 6,599,696 – Effects of Materials and Surface Coatings on Encrustation and Biofilm Formation
• US Patent # 6,596,505 – Apparatus and Methods for Testing Effects of Materials and Surface Coatings on the Formation of Biofilms

WARRANTY

Products purchased from Innovotech Inc. are warranted 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 our head office.

CONTACT INFORMATION

Tell us how Innovotech Inc. can better meet your needs. Contact us about your specific biofilm problem. Ask us about our products and services.

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