1. Introduction

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 irreversibly 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]. 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.

Until recently it has been difficult to study biofilms due to the difficulties in reproducible culture of biofilm organisms in the lab. Many devices have been developed to produce biofilms. However, most have been cumbersome and prone to various technical problems.

In 1996, microbiologists working at the University of Calgary developed a simple batch culture technique to reliably grow 96 equivalent biofilms at a time (the Calgary Biofilm Device) [1, 3]. This method, now commercially available as 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 [1, 3-5]. Many different bacterial and fungal species have been grown using this assay, including *Escherichia coli* [1], *Pseudomonas aeruginosa* [1], *Staphylococcus* spp. [1, 6], *Mycobacterium* spp. [7], *Candida* spp. [8], *Burkholderia* spp. [9, 10] as well as many more. To date, the MBEC™ assay has been featured in more than 100 peer-reviewed publications.

References


2. Product Description

The MBEC™ Physiology and Genetics (P&G) assay consists of two parts. The top half of the device is a polystyrene lid with 96 identical pegs. The average surface area of each peg on the MBEC device is
108.9 mm$^2$. The lid is inserted into the bottom half of the device - a microtiter plate that is set up to contain an inoculated growth medium. The entire device is placed on a gyrorotary shaker in an incubator, which provides the shearing force that facilitates the formation of 96 biofilms on the peg lid. This assay is a highly versatile high-throughput tool for that is amenable to the following applications:

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

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**Figure 1. The MBEC™ Physiology and Genetics (P&G) Assay.**

A) Biofilms form on the polystyrene pegs of the MBEC™ device when planktonic bacteria adsorb to the surface. In the presence of shear, these bacteria become irreversibly attached and grow to form mature biofilms. Biofilms are encased in ‘slime’, which is sometimes visible to the naked eye. Planktonic cells are also shed from the surface of biofilms, which serves as the inoculum for MIC determinations. B) The peg lid has 96 identical plastic pegs. This lid fits into a standard 96-well microtiter plate.
The example procedure provided in these instructions describes an assay for testing single or multiple organisms grown as a 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.

Figure 2. Microbial biofilms formed on the pegs of the MBEC™ P&G Plate. Images were captured using confocal laser-scanning microscopy (CLSM). Biofilms were stained with Syto-9 and propidium iodide (Molecular Probes). The images on the right are 3-dimensional reconstructions of the 2-dimensional averages of z-stacks on the left. A and B) A biofilm of the yeast *Candida tropicalis* 99916, an isolate from the dialysate of a patient at the Foothills Hospital, Calgary, AB, Canada. C and D) A biofilm of the genome-sequenced urinary tract pathogen *Escherichia coli* CFT073. Each image represents an area of 238.1 µm x 238.1 µm from the middle of a single plastic peg from the MBEC™ P&G device.
3. Materials and Equipment

Disclaimer: Mention of trade names or commercial products in this protocol is solely for the purpose of providing specific information and does not imply endorsement by MBEC BioProducts Incorporated.

The following materials are recommended for use in this protocol:

**Materials List**
- Sterile peg lid with 96-well microtiter plate (The MBEC™ P&G Assay)
- Sterile 96-well microtiter plates
  - *Recommended manufacturer*: Nunc™, Nunclon™ Delta Surface 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)
- Sterile micropipette tips (2-200 µl), in racks of 96
  - *Recommended manufacturer*: Diamed Lab Supplies Inc., PRE200MF-GK Premium calibrated tip, 1-200 µl, beveled end, 1000 per pack (fits multichannel pipette recommended below)
- Sterile 1 ml and 25 ml pipettes
- Sterile 50 ml culture tubes
  - *Recommended manufacturer*: BD Falcon®, Bluemax™ polypropylene conical tubes, 30 × 115 mm
- Sterile reagent reservoirs
  - *Recommended manufacturer*: Corning Inc., Costar® 50 ml reagent reservoir
- Agar and broth growth media specific for the microorganism to be cultured

**Required Equipment List**
- Biological safety cabinet (Laminar flow hood)
- Ultrasonic cleaner (a water bath sonicator with stainless steel insert tray)
  - *Recommended manufacturer*: VWR Scientific, Aquasonic model 250HT.
- Single and multichannel micropipettes (for the latter, 2-20 and 50-300 µl with 12 channels recommended)
  - *Recommended manufacturer*: Thermo Electron Corporation, Finnpipette®. It is not recommended to use a digital micropipette for this protocol.
- Inoculation loop
- Bunsen burner
- Gyrorotary shaker
  - *Recommended manufacturer*: New Brunswick Scientific, gyrorotary shaker model C2
- Needle nose pliers
- Ethanol lamp and lighter
- Pipette aid or pipette bulb
- McFarland Standards

### 3.1 Stock antimicrobial solutions

Antibiotic and other antimicrobial stock solutions should be prepared in advance at 5 × the highest concentration to be used in the challenge plate. For example, de-ionized water or an appropriate solvent is used to prepare stock solutions of antibiotics at 5120 µg ml⁻¹ of active agent. Consult Clinical Laboratory Standards Institute (CLSI) document M100-S8 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.

### 3.2 Neutralizing 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 Hg^{2+} and some other heavy metal cations. Innovotech Inc. uses a universal neutralizer in biocide susceptibility assays that is required for regulatory aspects of product development. This example is 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 20º 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).

### 4. Standard Experimental Protocol

An overview of this experimental protocol is provided in Figure 3. The number of days required to complete this protocol is dependent on the growth rate of the microorganism being examined. The protocol has been divided into 6 sequential steps, each of which is detailed in the sections below.

This protocol has been developed for use with Nunc Brand, flat bottom, 96-well microtiter plates. These microplates have a maximum volume of 300 µl per well. The medium and buffer volumes listed here may need to be adjusted for different brands of microtiter plates.

![Figure 3. A flow diagram representing the experimental process for high-throughput antimicrobial susceptibility testing using the MBEC™ P&G assay. This standard protocol may be broken into a series of small steps, each of which is detailed in the sections below.](image)

4.1 Step 1: Grow sub-cultures of the desired microorganism

**Figure 4. Grow fresh sub-cultures.** These are fresh streak plates grown from either a cryogenic stock or from a clinical isolate. Second sub-cultures will be used for biofilm cultivation.

1. 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 first sub-culture may be wrapped with Parafilm™ and stored at 4°C for up to 14 days.

2. Check the first sub-culture for purity (ie. only a single colony morphology should be present on the plate).

3. 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.

4. Verify the purity of the second sub-culture.

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.

4.2 Step 2: Inoculate the MBEC™ P&G assay

**Figure 5. Inoculate the MBEC™ P&G assay.** A fresh second sub-culture is used to create an inoculum that matches a 1.0 McFarland Standard. This solution is diluted 1 in 30 with growth medium. 150 µl of the 1 in 30 dilution is added to each well of the microtiter plate. The device is placed on a shaker.
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:

1. Open a sterile 96-well microtiter plate. For each MBEC™ P&G assay, fill 4 ‘columns’ of the microtiter plate from ‘rows’ A to F with 180 µl of a physiological saline solution.

2. Put 1.5 ml (plus 1.0 ml for each additional MBEC™ device being inoculated at the same time) of the desired broth growth medium into a sterile glass test tube.

3. Using a sterile cotton swab, collect the bacterial colonies on the surface of the second agar sub-culture. Cover the tip of the cotton swab with a thin layer of bacteria.

4. Dip the cotton swab into the broth to suspend the bacteria. The goal is to create a suspension that matches a 1.0 McFarland standard (ie. $3 \times 10^8$ cfu ml$^{-1}$). Be careful not to get clumps of bacteria in the solution.

5. Repeat step 2, parts 3 and 4 as many times as required to match the optical standard.

6. Put 29 ml of the appropriate broth growth medium (e.g. TSB) into a sterile 50 ml polypropylene or glass tube. To this, add 1.0 ml of the 1.0 McFarland standard bacterial suspension. This 30 fold dilution of the 1.0 McFarland standard (ie. $1 \times 10^7$ cfu ml$^{-1}$) serves as the inoculum for the MBEC™ device.

7. Open the sterile package of the MBEC™ P&G assay. Pour the inoculum into a reagent reservoir. Using the multichannel pipette, add 150 µl of the inoculum to each well of the 96-well tissue culture plate packaged with the MBEC™ P&G assay. Place the peg lid into the microtiter plate. 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). Label the device appropriately.

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 3 (below). Using a larger volume of inoculum may lead to biofilm formation high on the peg that physically escapes exposure in this challenge step.

8. Place the device on the gyrorotary shaker in a humidified incubator at the appropriate temperature. The shaker should be set to between 100 and 150 revolutions per minute (rpm).

9. Serially dilute (ten-fold) a sample of the inoculum (do 3 or 4 replicates). These are controls used to verify the starting cell number in the inoculum.

10. Spot plate the serial 10 fold dilutions of the inoculum from $10^{-6}$ to $10^{-1}$ on an appropriately labeled series of agar plates. Incubate the spot plates for an appropriate period of time and score for growth.
4.3 Step 3: Set up the antimicrobial challenge plate

The following section describes how to set up 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. 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.

1. Get a brand new, sterile 96-well microtitre plate and open it in the laminar flow hood.

2. Setup a working solution of the desired antimicrobial in the appropriate growth medium. Do not dilute the antimicrobial by more than 20% (ie. no more than 1 part stock antimicrobial solution per 4 parts of growth medium). The working solution of the antimicrobial should be made at a concentration equal to the highest concentration to be tested in the challenge plate.

3. Add 200 µl of growth medium to ‘column’ 1 and ‘column’ 12 of the challenge plate. These will serve as sterility and growth controls, respectively.

4. Add 100 µl of growth medium to ‘columns’ 3 to 11 of the microtitre plate.

5. Add 200 µl of the working solution to ‘column’ 2 of the microtitre plate.

6. Add 100 µl of the working solution to ‘column’ 3 and ‘column’ 4 of the microtitre plate.

7. Using the multichannel micropipette, mix the contents of ‘column’ 4 by pipetting up and down. After mixing, transfer 100 µl from the wells in ‘column’ 4 to the corresponding wells in ‘column’ 5.

8. Mix and transfer 100 µl from ‘column’ 5 to ‘column’ 6. Serially repeat this mix and transfer process down the length of the microtitre plate until reaching ‘column’ 11.

9. Mix the contents of column 11 up and down. Extract 100 µl from each well in ‘column’ 11 and discard.

10. Add 100 µl of growth media to the wells in ‘columns’ 4 through 11.

11. Replace the lid on the challenge plate. Gently tap the plate to facilitate mixing of biocide/antibiotic and media.
4.4 Step 4: Expose the biofilms

**Figure 8. Expose the biofilms to the antimicrobials.** The MBEC™ P&G assay is removed from the gyrorotary shaker and the biofilms are rinsed in a physiological saline solution. The rinsed biofilms are then immersed in the antimicrobials of the challenge plate and incubated for the desired exposure time.

1. Setup a sterile microtiter plate with 200 µl of physiological saline solution in every well. This plate will be used to rinse the pegs to remove loosely adherent planktonic cells from the biofilm (this is termed a ‘rinse plate’).

2. This step will be used to determine biofilm growth on four sample pegs and from four wells of the planktonic cultures. Setup a sterile microtiter plate with 200 µl of physiological saline solution in 4 ‘columns’ of row A for each MBEC™ device inoculated (ie. 1 microtitre plate is required for every 3 MBEC™ devices). Fill rows B to F with 180 µl of physiological saline solution. In a second microtitre plate, fill 4 ‘columns’ from rows A to H with 180 µl of physiological saline solution for each MBEC™ device inoculated. The first microtitre plate will be used to do serial dilutions of biofilm cultures, the second will be used to check the growth of planktonic cells in the wells of the microtiter plate that contained the inoculum.

3. Following the desired period of incubation, remove the MBEC™ P&G assay from the gyrorotary shaker and into the laminar flow hood. Remove the peg lid from the microtiter plate and submerse the pegs in the wells of the rinse plate. Let the rinse plate sit for 1 to 2 minutes while performing step 4 below.

4. Use a micropipette to transfer 20 µl of the planktonic culture (in the microtiter plate of the MBEC™ P&G assay) into the 180 µl of saline in row A’ of the latter plate set up in step 2 (immediately above). Repeat this three more times for a total of 4 × 20 µl aliquots.

5. Discard the planktonic culture into the appropriate biohazard waste.

6. In the laminar flow hood, dip a pair of pliers into 95% ethanol. Flame the pliers using the ethanol lamp in the flow hood. Be cautious when using the ethanol lamp. Do not light the ethanol lamp and do not flame the pliers before your hands have dried following disinfection using 70% ethanol.

7. Using the flamed pliers, break off pegs A1, C1, E1 and G1 from the lid of the MBEC™ device and immerse them in the 200 µl of saline in row A (and each in a different ‘column’) of the first plate setup in step 2.

8. Using the flamed pliers, break off pegs B1, D1, F1 and H1 and discard.

9. Insert the peg lid of the MBEC™ P&G assay into the challenge plate. Place the challenge plate in the appropriate incubator for the desired exposure time. Incubations may be carried out at alternative temperatures, taking into consideration extended times for MIC determinations.
10. Place the microtitre plate containing the sample pegs in the tray of the ultrasonic cleaner (the sonicator). Sonicate on the setting ‘high’ for 5 to 30 minutes (the time required depends on the microorganism being assayed). The vibrations created in the water by the sonicator transfer first through the water, then through the steel insert tray and finally to the MBEC™ device to vibrationally disrupt biofilms from the surface of the 96 pegs into the saline.

11. Serially dilute 20 µl aliquots of the planktonic cultures (from step 4) in the wells of the corresponding microtitre plate. Once sonication is complete, repeat this serial dilution process with the biofilm cultures.

12. Spot plate the serial 10 fold dilutions of the planktonic and biofilm cultures from 10⁻⁸ to 10⁻³ and 10⁻⁵ to 10⁰ on an appropriately labeled series of agar plates. Incubate the spot plates for an appropriate period of time and score for growth.

4.5 Step 5: Neutralize and recover

Figure 9. Neutralize the antimicrobials and recover surviving biofilm bacteria. After exposure, biofilms are rinsed twice in physiological saline. The biofilms are then transferred to a microtiter plate containing a neutralizing agent and recovery medium. The biofilms are disrupted into this by sonication on a water table sonicator.

1. Add 200 µl of the appropriate recovery medium (containing a neutralizing agent, see example in section 3.2) to each well of a brand new 96-well microtiter plate. This plate is termed the ‘recovery plate’.

2. Prepare 2 rinse plates for every MBEC™ P&G assay used.

3. Remove the challenge plate from the incubator and place in the laminar flow hood (or use careful aseptic technique). Remove the peg lid and immerse the pegs in the physiological saline of a rinse plate. Cover the challenge plate with the sterile lid of the rinse plate. After approximately 1 min, transfer the peg lid from the first rinse plate into the second rinse plate. Cover the challenge plate and retain for an MIC determination if appropriate.

4. Transfer the peg lid from the second rinse plate into the recovery plate setup above. Transfer the recovery plate (containing the pegs of the MBEC™ device) onto the tray of the sonicator. Sonicate on high for 5 to 30 min. (depending on the thickness of the biofilm). The vibrations will disrupt biofilms from the surface of the 96 pegs into the recovery plate.

5. After sonication, remove the peg lid from the recovery plate and replace the original lid of the microtire plate. The lid of the MBEC™ device may now be discarded into autoclave garbage.

6. Place the recovery plate in the incubator and incubate a minimum of 24 to 72 h, depending on the organism being examined.
4.6 Step 6: Determine MIC and MBEC

Figure 10. Determine MIC and MBEC values. MIC determinations are made by evaluating the growth in challenge plates after a suitable period of incubation. MBEC determinations may be accomplished by spot plating serial dilutions of the recovery medium onto agar (to get viable cell counts) or by qualitatively evaluating growth in the wells of the microtitre plate containing the recovery plate after a suitable period of incubation.

1. To determine the minimum inhibitory concentration (MIC) values, check for turbidity (visually) in the wells of the challenge plate. Alternatively, use a microtiter plate reader to obtain optical density measurements at 650 nm (OD\textsubscript{650}). The MIC is defined as the minimum concentration of antibiotic that inhibits growth of the organism. Clear wells (OD\textsubscript{650} < 0.1) are evidence of inhibition following a suitable period of incubation.

2. To determine the minimum biofilm eradication concentration (MBEC) values, check for turbidity (visually) in the wells of the recovery plate. Alternatively, use a microtiter plate reader to obtain optical density measurements at 650 nm (OD\textsubscript{650}). Clear wells (OD\textsubscript{650} < 0.1) are evidence of biofilm eradication.

5. Viable cell counting

5.1 Protocol Amendments

For viable cell counts of biofilms after treatment with an antimicrobial, transfer 100 \( \mu l \) of the recovery media (containing the sonicated biofilms) from the recovery plate to row A of a serial dilution plate. This plate additionally set up to contain 180 \( \mu l \) of physiological saline solution in each well of rows B to F. Serially dilute 20 \( \mu l \) from row A using the multichannel pipette. Ensure that the tips on the multichannel pipette are changed between transfers to each row in the microtiter plate. Spot plate biofilm cultures (which have been serially diluted ten-fold) on appropriately labeled agar plates. Incubate for a minimum of 48 hours to ensure maximum recovery of the surviving microorganisms.

Following incubation, enumerate bacteria recovered on plates. Use the formulas in the following section to determine killing of the biofilm population.
5.2 Calculating Death and Survival

To calculate log-kill, use the following formula:

\[
\text{log-kill} = \log_{10}(\text{initial cfu/ml}) - \log_{10}(\text{remaining cfu/ml after exposure})
\]

Alternatively,

\[
\text{log-kill} = \log_{10}\left[\frac{1}{1 - \text{kill \ (as \ a \ decimal)}}\right]
\]

To calculate percent kill, use the following formula:

\[
\text{% kill} = \left[1 - \frac{\text{remaining cfu/ml}}{\text{initial cfu/ml}}\right] \times 100
\]

To calculate percent survival, use the following formula:

\[
\text{% survival} = \left[\frac{\text{remaining cfu/ml after exposure}}{\text{initial cfu/ml}}\right] \times 100
\]

To calculate log percent survival, use the following formula:

\[
\text{log \ % \ survival} = \log_{10}(\text{% survival})
\]

6. Microscopy

For many microscopy techniques, it may be desirable to fix the biofilms to the surface of the pegs of the MBEC™ Assay. The following protocols may be used to prepare biofilms for scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). In the standard experimental procedure above, each challenge plate has eight growth controls (before exposure). Four of these are used for growth controls. The remaining four may be used for microscopy instead of being discarded.

6.1 Fixing Biofilms for Scanning Electron Microscopy (SEM)

Preparing Working Solutions

Wear protective gloves in the following steps and handle these highly toxic chemicals in a fume hood.

Cacodylate buffer 0.1 M

- Dissolve 16 g of cacodylic acid in 1 liter of double distilled H₂O.
- Adjust to pH 7.2.

Glutaraldehyde 2.5% in cacodylate buffer

- Dissolve 2 ml of 70% glutaraldehyde in 52 ml of cacodylate buffer.
- This yields a 2.5% solution.
- It is also possible to use a 5% solution (2 ml of glutaraldehyde into 26 ml of cacodylate buffer).

Standard protocol

This fixing technique is destructive to biofilms. However, this allows for an examination of the cell structure of the underlying bacteria.

1. Break pegs from the MBEC™-P&G device using a pair of flamed pliers.
2. Rinse pegs in 0.9% saline for 1 min. This disrupts loosely-adherent planktonic bacteria.

3. Fix the pegs in 2.5% glutaraldehyde in 0.1 M cacodylic acid (pH 7.2). Pegs are placed in this solution at 4°C for 16 h.

4. Following this fixing step, wash the pegs once in 0.1 M cacodylic acid for approximately 10 min.

5. Wash the pegs once in double distilled water for approximately 10 min.

6. Dehydrate the pegs in 70% ethanol for 15 to 20 minutes.

7. Air dry for a minimum of 24 h.

8. Mount specimens and examine by SEM.

Alternative protocol

This fixing technique is less destructive. It is possible to observe the extracellular polymeric matrix and some (albeit dehydrated) biofilm structure.

1. Break pegs from the MBEC™-P&G device using a pair of flamed pliers.

2. Rinse pegs in 0.9% saline for 2 min. This disrupts loosely-adherent planktonic bacteria.

3. Fix the pegs in 2.5% glutaraldehyde in 0.1 M cacodylic acid (pH 7.2). Pegs are placed in this solution at 20°C for 2 to 3 h.

4. Air dry for at least 120 h.

5. Mount specimens and examine by SEM.

6.2 Fixing Biofilms for Confocal Scanning Laser Microscopy (CLSM)

Glutaraldehyde 5% in phosphate buffered saline

- Dissolve 2 ml of 70% glutaraldehyde in 26 ml of phosphate buffered saline.
- This yields a 5% solution.

Standard protocol

1. Break pegs from the MBEC™-P&G device using a pair of flamed pliers.

2. Rinse pegs in 0.9% saline for 1 min. This disrupts loosely-adherent planktonic bacteria.

3. Fix the pegs in 5% glutaraldehyde in phosphate buffered saline (pH 7.2). Pegs are placed in this solution at 30°C for 0.5 to 1 h.

4. Rinse pegs in 0.9% saline for 1 min.

5. Stain pegs with the appropriate fluorophores and examine using the confocal laser scanning microscope.
7.0 Surface Coating the MBEC™ P&G Assay

The surface of the MBEC™ assay 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 a solution of 1.0% L-lysine. The peg lid may also be coated with hydroxyapatite, collagen, or platinum. For additional protocols or to purchase specialized plates please contact one of our customer service representatives.

8.0 Quality Controls for Research and Development Applications

An important quality control using this research and development tool is to test microorganisms grown in the MBEC™ P&G assay for equivalent biofilm formation across the different rows of pegs on the lid of the device. This is accomplished growing a biofilm in the MBEC™ P&G assay as described in sections 4.1 and 4.2 above. The biofilms are then disrupted by sonication into neutralization medium (or a physiological saline solution) then plated for viable cell counting as described in section 5. The biofilm cell density on each peg is determined by enumerating spot plates after a suitable period of incubation. These values are arranged in columns in a spreadsheet corresponding to rows in the MBEC™ P&G assay. The viable cell counts are then analyzed using one-way analysis of variance (ANOVA). Highly reproducible results from this assay are attained when growth conditions are optimized to produce statistically equivalent biofilms (i.e. \( p \geq 0.05 \) that the mean growths in each row of pegs are equivalent).

9.0 Company Information

MBEC BioProducts Incorporated was first formed in 1996 as a research and product Development Company with world recognized expertise in bacterial biofilms. MBEC BioProducts 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

10. 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.
11. 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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12. Acknowledgements

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