SOP Number	MB-32-00
Title	Quantitative Petri Plate Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes Against Spores of *Clostridium difficile* (ATCC 43598) on Inanimate, Hard Non-porous Surfaces.
Scope	This quantitative test method is used to evaluate the sporicidal efficacy of antimicrobial towelettes (wipes) in treating hard non-porous surfaces contaminated with spores of *C. difficile* (ATCC 43598). This SOP is based on ASTM Standard E-2896-12 [see 15.1) which was developed for testing vegetative bacteria. This SOP incorporates modifications specific to testing spores of *C. difficile**. Product efficacy is considered a combination of mechanical removal and chemical inactivation.
Application	Data from this method are used to generate the log reduction (LR) values of viable spores of *C. difficile* as the quantitative measure of efficacy for towelette products on an inanimate, hard non-porous surface.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

* Method modifications for vegetative bacteria and to include testing of spores of *C. difficile* were presented to ASTM Subcommittee E35.15, and currently are under consideration by the ASTM Subcommittee at the time of development of this SOP.

TABLE OF CONTENTS

1. Definitions	Abbreviations/definitions are provided in the text.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOP EQ-01(pH meters), EQ-02 (Thermometers/Hygrometers), EQ-03 (Weigh Balances), EQ-05 (Timers), EQ-08 (Oxford Media Dispensor) and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Disinfectant Sample Login, Tracking and Disposal.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. The test organism (*C. difficile* ATCC 43598) must be incubated under strict anaerobic conditions. The presence of oxygen will severely compromise the viability and growth of *C. difficile.* 2. Due to different levels of saturation, different volumes of active ingredient may be deposited on the carriers during wiping, thus adequate neutralization is essential to generate valid results. 3. Inconsistent pressure during wiping process may lead to variable results. In order to ensure consistent pressure, the analysts should practice the wiping procedure prior to product testing.
8. Non-conforming Data	1. The control carrier counts should be between 0.5 and 1.5 logs higher than the performance standard (log reduction of 6.0). 2. For a valid test, the spore suspension used in efficacy testing must meet the criteria specified in SOP MB-28 (Production of *C. difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents).
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Follow time sensitive steps including: the use periods of the inoculated carriers and dilution and filtration of eluates as specified in the method. 2. Place pre-reduced BHIY-HT agar plates (with or without filters) under anaerobic conditions within 50±10 min of opening a pouch. 3. Inoculum may be inadvertently aerosolized when lowering the pipette tip

	onto the glass surface, it is important not to touch the surface of the glass during inoculation. Use the inoculation template for proper placement of inoculum. 4. Use the wiping pattern template to ensure the accuracy of the wiping procedure. 5. Avoid spilling or splattering the neutralizer × test chemical combination outside of the carriers. The method is quantitative and requires the recovery of the entire volume prior to dilution and filtration. 6. Verify the sterility of all filtered reagents and media (for example, PBS, neutralizer) used in the study. 7. Because towelettes are diverse in size, matrix composition, and packaging, the towelette removal and folding process should be practiced in advance of testing. Pre-folded towelettes should be gently unfolded and refolded as described.
11. Special Apparatus and Materials	1. *Test microbe*. Spore suspension of *C. difficile* (ATCC 43598); see SOP-MB-28 for the preparation of *C. difficile* spores. 2. *Recovery Medium.* Brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY–HT). Pre-reduced recovery media (Anaerobe Systems, Morgan Hill, CA) for enumeration of viable *C. difficile* spores. Note: Commercially purchased media (i.e., BHIY-HT) is received in-house with a certificate of analysis which includes sterility and performance verification of the medium conducted by the manufacturer; file certificates in the packing slip binder (as discussed in SOP-ADM-09: Using the EPA Purchase Card). 3. *Reagents* Note: Refer to SOP MB-10, Media and Reagent Preparation and Quality Evaluation, for the sterility verification of reagents used in this SOP. a. *Neutralizer Medium.* Used to stop the activity of the disinfectant (for example, letheen broth, letheen broth with 0.1 % sodium thiosulfate). b. *Phosphate-buffered saline stock solution (PBS-SS).*Prepare 10× stock solution of PBS by dissolving 492 g PBS powder in 5 L of deionized water. c. *Phosphate-buffered saline (PBS) 1× Solution.* Used for rinsing filter units. Dilute 1:10 [1 part (PBS-SS) 10× solution) plus 9 parts deionized water] to obtain 1× solution, distribute into bottles and

autoclave for 20 min at 121°C.

d. *ST-80: Phosphate-buffered saline (PBS) containing 0.1 % Tween 80*. Used for the preparation of dilution blanks. Add 2.0 mL of polysorbate 80 (Tween 80, or equivalent) to approximately 1.5 L of PBS (1X). Mix thoroughly and (using a volumetric flask) bring solution to volume (2 L) with PBS. Distribute into bottles and autoclave for 20 min at 121ºC.

e. *Spore stain*. 5% aqueous malachite green and 0.5% aqueous safranine to differentiate spores from vegetative cells. Spores appear green while vegetative cells appear red.

f. *Soil load*. The recommended soil load to be incorporated in the test suspension is a mixture of the following stock solutions in PBS:

i. *BSA*: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at -20 ± 5°C.

ii. *Yeast Extract*: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at -20 ± 5°C.

iii. *Mucin*: Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS, mix thoroughly until dissolved, and autoclave (15 minutes at 121°C), aliquot and store at -20 ± 5°C.

NOTE: The stock solutions of the soil load are single use only and should not be refrozen once thawed; store up to one year at -20 ± 5°C.

g. *Test substance.* Refer to SOP MB-22, Disinfectant Sample Preparation.

h. *De-ionized water (DI)*. Used in the preparation of media and reagents. Purified water with mineral ions removed through pre-treatment, deionization and filters. Alternatively, reagent grade water (ultrapure water) may be used. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

i. *Tween-80 (polysorbate 80).* To prepare ST-80.

j. *95% Ethyl Alcohol (ETOH).* To clean carriers.

4. *Apparatus*

a. *Biosafety cabinet (BSC, Type B2, Class II).* Recommended for

	maintaining an aseptic work environment. Certified. b. *Carriers*. Sterile glass Petri plates used as test carriers (150 × 20 mm). c. *Calibrated positive displacement pipettes (e.g., 10 µL)*. For carrier inoculation. d. *Micropipettes* (e.g., 200 µL). For serial dilutions and plating. e. *Sterile test tubes.* For dilution blanks and cultures/subcultures or other appropriate size. Reusable or disposable 20 × 150 mm. f. *Test tube racks.* Any convenient size. g. *Forceps.* To handle membrane filters. Straight or curved, non-magnetic or disposable with smooth flat tips. h. *Sterile cell scraper.* To scrape carriers for removal of bacteria during neutralization (for example, scraper blade dimensions = 1.8 to 3.0 cm). i. *Sterile plate spreader.* May be used to spread inoculum on agar surface. j. *Conical tubes.* To collect neutralizer/product/bacterial suspensions from treated carriers and neutralizer/bacterial suspensions from control carriers after inoculum has been dislodged by scraping and neutralized. Sterile, 50 mL. k. *Polyethersulfone membrane filter (PES).* For recovery of test microbe (47 mm diameter and 0.22 µm pore size). Filtration units (reusable or disposable) may be used. l. *Vortex mixer*. To vortex the eluate and rinsing fluid from the carrier to ensure efficient recovery of the test organism(s). m. *Serological pipettes.* Sterile single-use pipettes (for example, 25.0, 10.0, 5.0, 1.0 mL capacity). n. *Sterile surgical gloves.* To handle antimicrobial towelette when folding and wiping carriers. o. *Certified timer*. For managing timed activities, any certified timer that can display time in seconds. p. *Vacuum source*. Used for filtering. In-house line. q. *Autoclave (steam sterilizer).* To sterilize media and reagents. r. *COY Anaerobic chamber*. To provide an anaerobic environment. Supported by a compressed gas mixture consisting of 10%

	Hydrogen, 5% CO_2, and 85% N_2. s. *Anaerobic incubator*. To support the growth of *C. difficile.* Use the incubator at 36 ± 1°C inside the COY anaerobic chamber. t. *Microscope*. To examine spores. With 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option.
12. Procedure and Analysis	1. Perform the neutralization verification assay prior to testing (see Attachment 5). 2. Conduct three independent tests (three test days) or as specified by the study sponsor. The product performance standard for a *C. difficile* claim is a minimum 6 log reduction in viable spores for each test. For each test, evaluate 5 treated and 3 control carriers. 3. Per the request of the study sponsor, soil load may be added to the test suspension.
12.1 Preparation of test organism	For preparation of purified spores and frozen stocks of *C. difficile* (ATCC 43598), refer to SOP MB-28.
12.2 Carrier Preparation	a. Physically screen carriers for any chips, scrapes, or any visible damage. b. Thoroughly clean Petri plates with non-ionic detergent or using a dishwasher cycle. c. After primary cleaning step, rinse once with 95% ethyl alcohol. d. Rinse with DI water three times. Dry plates prior to sterilization. e. Sterilize by autoclaving using a dry cycle for 45 minutes at 121°C. f. Record cleaning and sterilization procedure on the corresponding Media/Reagent Preparation Sheet – Carrier Preparation form (see Section 14).
12.3 Preparation of the final test suspension with soil load	a. Defrost a cryovial of *C. difficile* spores (approximately 10-15 min at room temperature). Each cryovial is single use only. b. Vortex the thawed spore suspension until re-suspended to evenly distribute the spores. c. If soil load is required, to obtain 500 µL of the final test suspension, vortex each component and combine the following: i. 25 µL BSA stock ii. 35 µL yeast extract stock iii. 100 µL mucin stock

	iv. 340 µL spore suspension d. If different total volumes of the final test suspension are required, scale each component listed in 12.3c, maintaining the correct ratio of each component. Following the addition of the soil load, vortex the final test suspension. Note: Processing of spores can be conducted in an aerobic environment (for example, inside a BSC); all incubation for growth, however, **must** be performed anaerobically.
12.4 Carrier Inoculation	Use sterile glass Petri plates (150 × 20 mm) as the test carriers. Inoculate a sufficient number of carriers for testing, carrier load enumeration, sterility controls and extras. a. Use test suspension prepared in Section 12.3. b. Determine titer (optional) of the test suspension. c. Briefly vortex to mix the test suspension immediately prior to titer determination and inoculation of test carriers. d. Place the template (an approximate 2.5 cm × 2.5 cm area in the center of the Petri plate) beneath the Petri plate to standardize the location of the inoculation site; see Attachment 1. Inoculate the inside bottom surface of each plate with five 10 µL spots using a positive displacement pipette in an hourglass pattern (2 spots – 1 spot – 2 spots pattern). e. Do not use carriers where inoculum spots have coalesced. Inspect inoculated carriers prior to the drying procedure. f. Inoculate 3 control carriers and 5 treated carriers per test condition per test substance lot. Prepare extra inoculated Petri plates as necessary. g. Set lids ajar and dry plates in an incubator at 36 ± 1°C for 30 ± 2 minutes. Visually inspect each Petri plate to ensure complete drying of the inoculum. h. Use inoculated carriers for testing within 90 minutes after drying.

12.5 Carrier Load Enumeration (control carrier counts)	a. One Petri plate (carrier) is evaluated prior to the product test; two Petri plates (carriers) are evaluated immediately following the test. b. Place inoculation template under inoculated plate for guidance during the neutralization process. c. Add 20 mL of the neutralizer to each inoculated Petri plate. Using a sterile cell scraper, gently scrape across the middle of the plate with three back and forth motions (6 total motions). Rotate the plate 90 degrees and repeat the scraping procedure using the same cell scraper to dislodge and suspend the inoculum. Gently swirl to mix. d. Transfer the suspension using a pipette into a sterile conical tube. Tilt the Petri plate as necessary to collect the suspension. e. Add a second 20 mL aliquot of neutralizer to each Petri plate, gently swirl. Using a pipette remove liquid combining it with the first 20 mL aliquot. f. Vortex mix the 40 mL suspension for 10 ± 5 s; this tube is the 10^0 dilution. g. Prepare serial dilutions using ST-80 (for example, final dilutions of 10^{-3}, 10^{-4}, and 10^{-5} for controls). h. Pre-wet filter with approximately 10 mL of PBS. Add the entire volume of the dilution tube to the filter unit. Swirl the entire contents of the filter unit and apply vacuum. Rinse filter unit with approximately 20 mL of PBS with vacuum. i. Open pouch of pre-reduced plates. Place pre-reduced BHIY-HT plates (with or without filters) under anaerobic conditions within 50±10 min of opening a pouch. j. Using sterile forceps place each membrane filter onto the agar plate. Inspect each filter; no air bubbles should be present. Ensure proper adherence to the surface of the agar plate. k. Incubate BHIY-HT plates with membrane filters under anaerobic conditions at 36±1°C for 48± 4 h. l. After 48± 4 h, count and record number of colonies on each plate on the Control Carrier Counts Dilution Scheme and Results test sheet. Colony counts in excess of 200 should be recorded as Too Numerous to Count (TNTC).
12.6 Towelette Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Clean/disinfect the cap/lid area or pouch area of the wipe container

	with 70% (v/v) ethanol. c. For canisters, gently roll and/or invert 3 to 4 times to distribute liquid in advance of removing the towelettes. For products on single pouch, gently squeeze the pouch to redistribute the liquid, use sterile gloves to remove towelettes from pouch. For multi-count containers only, use sterile gloves to remove 2 to 3 wipes from the container and discard. d. Change gloves as necessary to maintain sterility.
12.7 Test Procedure (Wiping)	a. Perform assay inside the BSC. b. Record ambient temperature and humidity on the Test Information and Carrier Inoculation Sheet (see section 14). c. Use a new pair of sterile gloves to handle each test towelette. d. Remove a towelette from the container and gently fold it in half. Rotate the folded towelette 90 degrees and fold in half again to target a final dimension of approximately 5 cm × 5 cm for wiping. Note: For larger towelettes, multiple folds may be necessary to produce a final dimension of approximately 5 cm × 5 cm. For small towelettes (2.5 cm × 2.5 cm), fold in half. e. Use only smooth folded edge for wiping. f. Place wiping template under each Petri plate and use throughout the entire wiping procedure. g. Avoid contact of the towelette with the inside wall of the Petri plate. h. Remove lid and secure the Petri plate in one hand (non-wiping hand). i. Use a calibrated timer to track contact time. Start the timer and initiate the wiping procedure. j. Using the folded edge of the towelette, wipe the inoculated surface with consistent pressure, using a "corkscrew" pattern by starting with three (3) revolutions from the outer margin of plate inward toward inoculated area in the center of plate. Without lifting the towelette, continue three circular revolutions from center to the outer margin of the plate. Note: The wipe pattern should be consistent from plate to plate with consistent pressure typical of towelette use. Refer to Attachment 2 for illustrations of wiping procedure.

	k. The entire wiping process should be conducted within 6 ± 2 seconds. l. After wiping replace the lid on the plate and discard towelette. Allow plates to remain undisturbed in a horizontal position for the duration of the contact time. m. Add 20 mL of the neutralizer, sequentially to the treated plates, within ± 5 seconds (for products with contact time of 1-10 minutes) and within ± 2 seconds (for products with contact time of ≤ 1 minute) after contact time has elapsed. n. Using a sterile cell scraper, gently scrape across the middle of the plate with three back and forth motions (6 total motions). Rotate the plate 90 degrees, and repeat scraping. Gently swirl to mix. o. Using a pipette, remove suspension and transfer into a sterile conical tube. Tilt the Petri plate as necessary to collect as much of the suspension as possible. p. Add a second 20 mL aliquot of neutralizer to the Petri plate, gently swirl, and using a pipette remove liquid, combining it with the previous 20 mL aliquot. q. Vortex mix the 40 mL suspension for 10 ± 5s. (This tube is considered 10^0 dilution).
12.8 Dilution and Recovery	a. Within 30 min of collecting the neutralized suspension, prepare serial dilutions in ST-80 (for example, 10^{-1}, 10^{-2}, and 10^{-3}.) b. Filter dilutions as in 12.5 h. Repeat filtration for all dilution tubes. c. Incubate BHIY-HT plates with membrane filters (from treated carriers) under anaerobic conditions at 36 ±1°C for 2-5 days. If zero colonies or only a few colonies are observed, continue with incubation through 5 days. d. After incubation, count and record number of colonies on each plate on the Treated Carriers Results Sheet. Colony counts in excess of 200 should be recorded as Too Numerous to Count (TNTC). If no colonies present, record as zero.
12.9 Carrier sterility control	a. On the day of the test, a single uninoculated Petri plate (carrier) should be used as a negative control to verify sterility of the Petri plates and the neutralizer. b. Add 20 mL of the neutralizer to a sterile Petri plate; gently scrape the surface as described in 12.7n. Collect liquid using a pipette into a sterile conical tube. Repeat with the addition of 20 mL of

<table>
<tr><td></td><td>neutralizer. Collect suspension with a pipette and combine it with the previous 20 mL.
c. Pre-wet filter with approximately 10 mL of PBS prior to use. Vortex mixture for approximately10 s.
d. Pass the entire volume of pooled mixture (in conical tubes) through the pre-wetted filter, rinse filter unit with approximately 20 mL of PBS.
e. Transfer the filter to the surface of BHIY-HT.
f. Incubate sterility control plate along with treated and control plates at 36 ±1°C for 5 days. The acceptance criterion is no growth.</td></tr>
<tr><td>12.10 Confirmatory Steps for Test Microbes</td><td>a. Inspect the growth from one of the membrane filters for purity and typical characteristics of the test microbe (see Table 1). Observe growth from a typical colony on a membrane filter using spore staining or under phase contrast microscopy.
Table 1. Characteristics of C. difficile (ATCC 43598)
<table>
<tr><th colspan="2">Typical Diagnostic Characteristics</th></tr>
<tr><td>Filter plated onto BHIY-HT</td><td>Growth circular, entire edge, convex, smooth and grey colonies.*</td></tr>
<tr><td>Phase-contrast microscopy</td><td>Spores appear bright and ovular while vegetative cells appear dark and rod-shaped.</td></tr>
<tr><td>Spore staining</td><td>Spores appear green while vegetative cells appear red.</td></tr>
</table>
*At 48±4 hr.
b. Record results on the Test Microbe Confirmation Sheet (see section 14).</td></tr>
<tr><td>12.11 Re-use of Petri plates (carriers)</td><td>a. Pre-clean (with a towelette) inoculated Petri plates prior to autoclaving.
b. Sterilize by autoclaving using a dry cycle for 45 minutes at 121° C.
c. Refer to section 12.2, for carrier preparation.</td></tr>
<tr><td>13. Data Analysis/ Calculations</td><td>Calculations will be computed using a Microsoft Excel spreadsheet (see Form 9, Section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts up to 200 and their associated dilutions will be included in the calculations.
a. To calculate CFU/carrier when three (3) serial dilutions are plated, use the following example formula:</td></tr>
</table>

$$\frac{CFU\ for\ 10^{-x} + CFU\ for\ 10^{-y} + CFU\ for\ 10^{-z}}{(a \times 10^{-x}) + (b \times 10^{-y}) + (c \times 10^{-z})} \times D$$

where 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions filtered, "a" "b" and "c" are the volumes filtered at each dilution (typically 9 or 10 mL), and "D" is the volume of medium originally in the vial with the carrier (39 or 40 mL).

Note: All counts up to 200 should be used in the calculations.

b. Calculate the *log_{10} density (LD)* recovered from each individual carrier (control carriers and treated carriers) by taking the $\log_{10}$ of the CFU/carrier. This equals the LD/carrier.

c. Calculate the mean log density across *control carriers* for each test by determining the average of the LD/carrier recovered from all control carriers. This equals the mean LD for control carriers.

d. Calculate the mean log density across *treated carriers* for each test by determining the average of the LD/carrier recovered from all treated carriers. This equals the mean LD for treated carriers.

e. Calculate the *log_{10} reduction (LR)* by subtracting the mean LD of the treated carriers from the mean LD of the control carriers.

14. Forms and Data Sheets

1. Test Sheets. Test sheets are stored separately from the SOP under the following file names:

Organism Culture Tracking Form	MB-32-00_F1.docx
Test Microbe Confirmation Sheet (Quality Control)	MB-32-00_F2.docx
Test Information and Carrier Inoculation Sheet	MB-32-00_F3.docx
Treated Carriers Results Sheet	MB-32-00_F4.docx
Time Recording Sheet for Carrier Transfers	MB-32-00_F5.docx
Control Carrier Counts Dilution Scheme and Results	MB-32-00_F6.docx
Test Microbe Culture Titer (Optional)	MB-32-00_F7.docx
Test Microbe Confirmation Sheet	MB-32-00_F8.docx
Treated Carriers and Control Carrier Count Spreadsheet (MS Excel spreadsheet)	MB-32-00_F9.xlsx
Media/Reagent Preparation Sheet – Carrier Preparation	MB-32-00_F10.xlsx

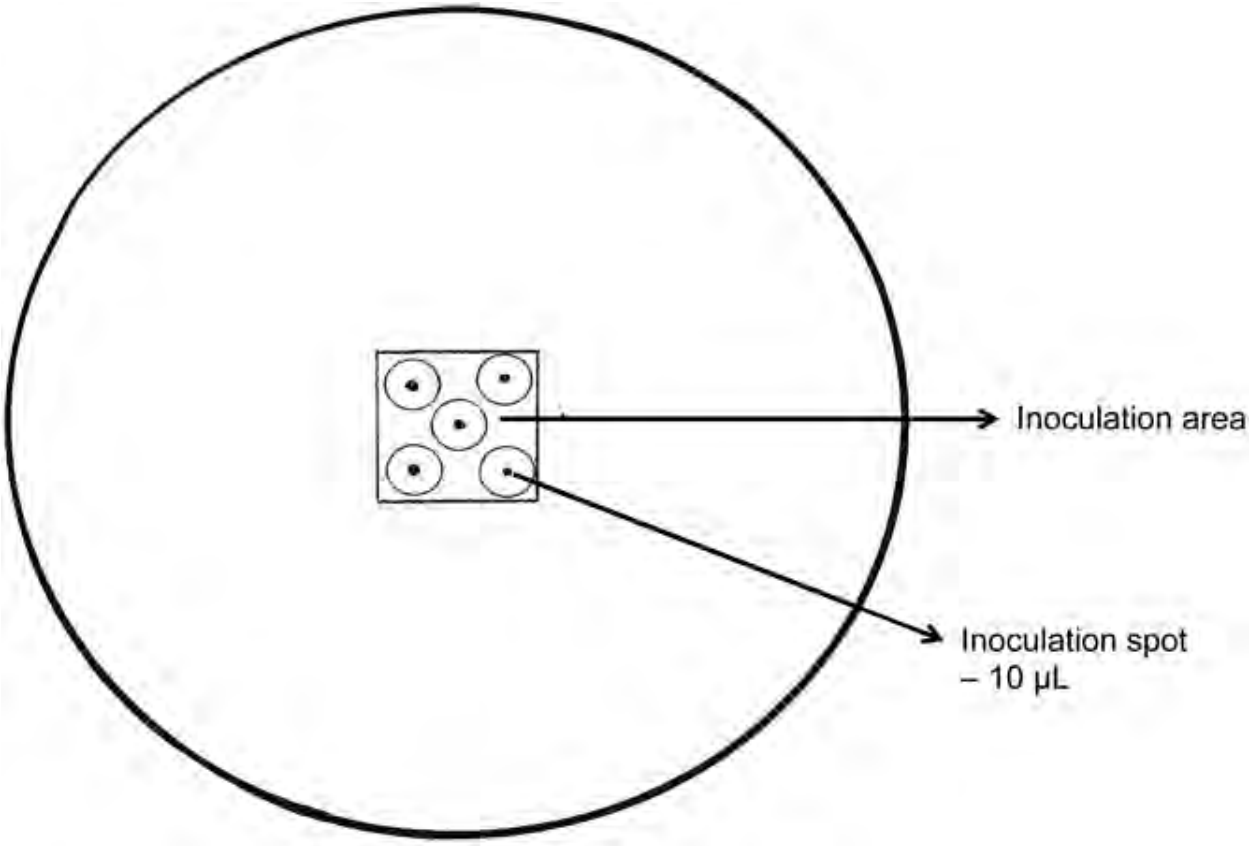
15. References

1. ASTM E2896-12, Standard Test Method for Quantitative Petri Plate

	Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes. ASTM International, West Conshohocken, PA. 2012. 2. ASTM E2839-11, Standard Test Method for Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents. ASTM International, West Conshohocken, PA. 2011. 3. EPA Guidance for the Efficacy Evaluation of Products with Sporicidal Claims against *Clostridium difficile*, http://www.epa.gov/oppad001/ cdif-guidance.html, 2012. 4. Hasan, J. A., Japal, K. M., Christensen, E. R., & Samalot-Freire, L. C., "Development of methodology to generate *Clostridium difficile* spores for use in the efficacy evaluation of disinfectants, a precollaborative investigation," J. AOAC Int, Vol 94, 2011, pp. 259–272. 5. Standard Methods for the Examination of Water and Wastewater, American Public Health Association, Washington, D.C. 2012. 6. Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Ed., Centers for Disease Control and Prevention, and National Institute of Health, Washington DC. 2009.

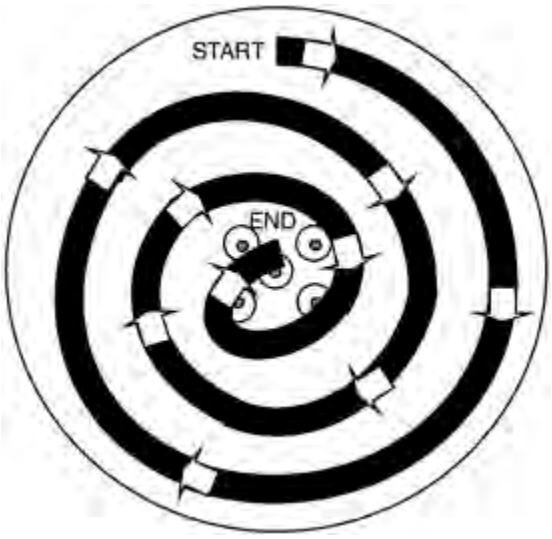
Attachment 1

INOCULATION, WIPING AND SCRAPING TEMPLATE

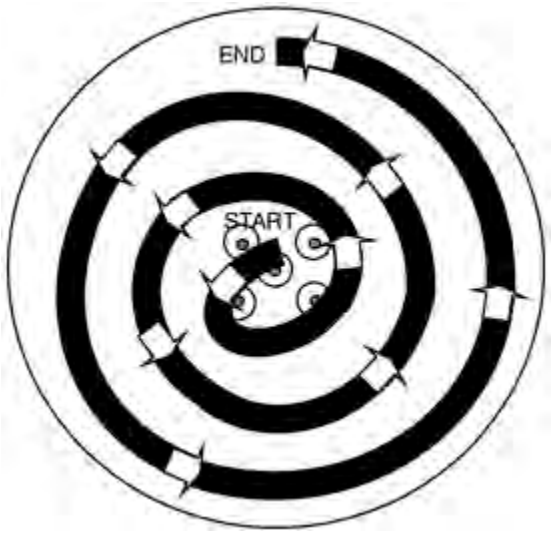


Attachment 2

WIPING PROCEDURE SCHEMATICS



Wiping from Outer Region to Center (Inoculated) Region

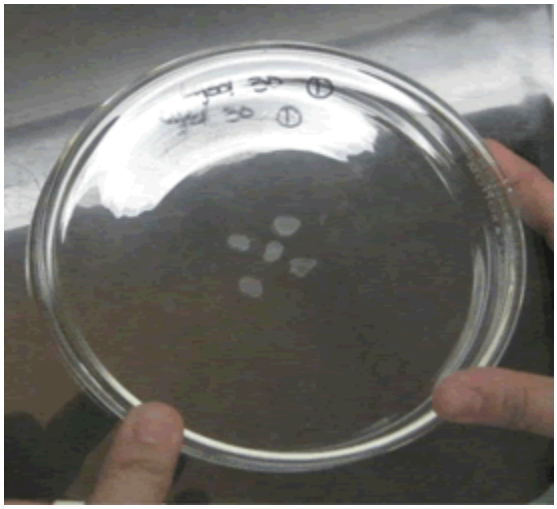


Wiping from Center (Inoculated) Region to Outer Region

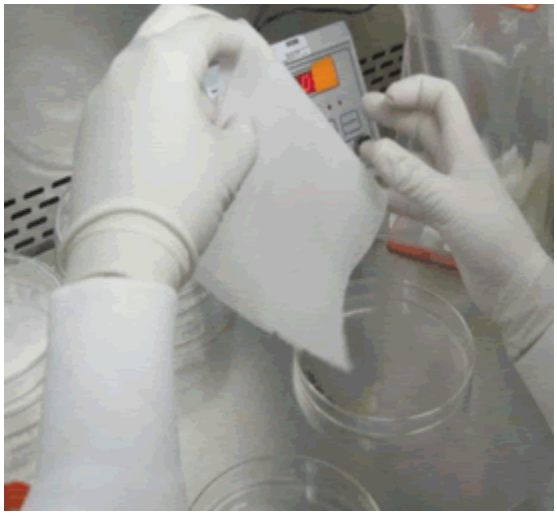
Attachment 3

PICTURE OF STERILE SCRAPER

Attachment 4
PICTORIAL PRESENTATION OF QPM

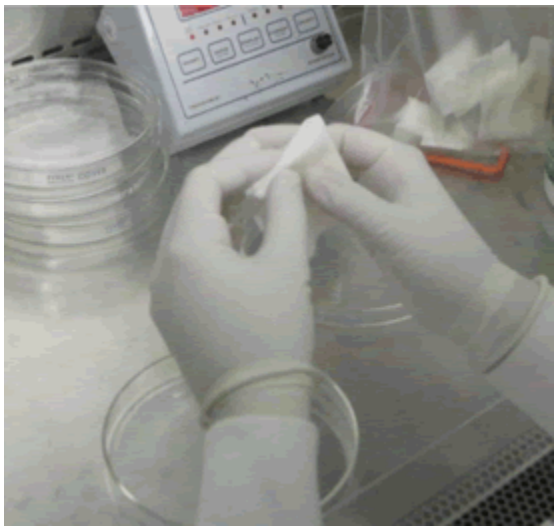


Dried Inoculum on Petri Plate

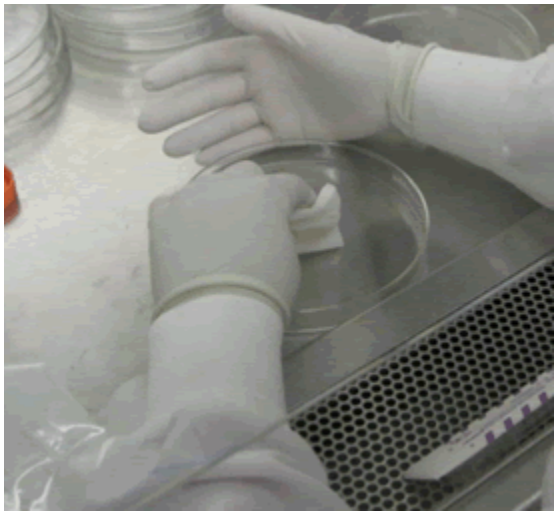


Folding of Antimicrobial Towelette-1

Attachment 4 (continued)

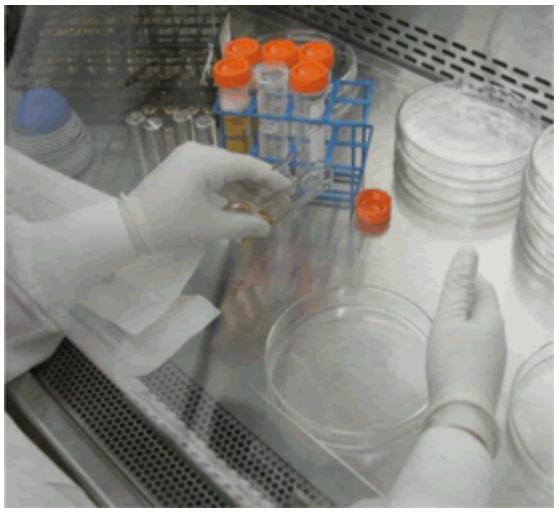


Folding of Antimicrobial Towelette-2



Wiping of Plate

Attachment 4 (continued)



Adding Neutralizer

Scraping

Attachment 4 (continued)

Collecting Mixture

Attachment 5.

NEUTRALIZATION VERIFICATION ASSAY:

1. Prepare test suspension in accordance with Sections 12.1 and 12.2.
2. Prepare serial dilutions using ST-80 as the dilution blank (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the test suspension.
3. Treatment plates are inoculated with 0.1 mL of test suspension after addition of neutralizer or ST-80 to yield a final count of 20 to 200 CFU/plate.
4. See Table 2 for summary of neutralization assay.
5. **Organism Titer Control (OTC)**.
    a. Add 20 mL of ST-80 to a sterile Petri plate.
    b. Inoculate with 0.1 mL of appropriate dilutions (for example, 10^{-4}, 10^{-5} and 10^{-6}). Gently swirl to mix.
    c. Up to three Petri plates are used, one per dilution (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the test suspension.
    d. With a pipette, transfer ST-80 × test suspension mixture into a sterile conical tube.
    e. Add an additional 20 mL of ST-80 to the Petri plate, swirl to mix and pool with mixture in conical tube from step 5.d.
    f. Hold the pooled mixture for 5-10 minutes at 20 to 25°C prior to filtration. No further dilutions are necessary. Proceed as in step 8.
6. **Neutralizer Effectiveness Control (NEC)**
    a. Wipe surface of sterile Petri plate according to step 12.7d-i.
    b. Immediately (within 10 ± 5 s) after the contact time has been reached, add 20 mL of the desired neutralizer, and swirl to mix.
    c. Within 10 to 15 s after addition of neutralizer, inoculate mixture in Petri plate with 0.1 mL of appropriate test suspension dilution. Gently swirl to mix. Up to three Petri plates are used, one per dilution (for example, 10^{-4}, 10^{-5}, and 10^{-6}).
    d. With a pipette, transfer test substance × neutralizer × test suspension mixture into a sterile conical tube.
    e. Add an additional 20 mL of the neutralizer to the Petri plate, swirl to mix and pool with mixture in conical tube from step 6.d.
    f. Hold the pooled mixture for 5-10 minutes at 20 to 25°C prior to filtration. No further dilutions are necessary. Proceed as in step 8.

7. **Neutralizer Toxicity Control (NTC)**

   a. Add 20 mL of the neutralizer to a sterile Petri plate.

   b. Immediately (within 10 to 15 s) inoculate the neutralizer in the Petri plate with 0.1 mL of the appropriate test suspension dilution. Gently swirl to mix. Up to three Petri plates are used, one per test suspension dilution (for example, 10^{-4}, 10^{-5}, and 10^{-6}).

   c. With a pipette, transfer the neutralizer × test suspension mixture into a sterile conical tube.

   d. Add an additional 20 mL of the neutralizer to the Petri plate, swirl to mix and pool with neutralizer in conical tube from step 7.d.

   e. Hold the pooled mixture for 5-10 minutes at 20 to 25°C prior to filtration. No further dilutions are necessary. Proceed as in step 8.

8. **Filtration and Recovery**

   a. Pre-wet filter with approximately 10 mL of PBS prior to use.

   b. Vortex mixtures (in conical tubes) for approximately10 s.

   c. Pass the entire volume of each pooled mixture (in conical tubes) through a pre-wetted filter, rinse filter unit with approximately 20 mL of PBS.

   d. Prior to placing the filter onto BHIY-HT, open pouch of pre-reduced plates. Place pre-reduced BHIY-HT plates (with or without filters) under anaerobic conditions within 50±10 min of opening a pouch.

   e. Transfer the filter to the surface of BHIY-HT.

   f. Incubate plates under anaerobic conditions at 36 ± 1ºC for 48 ± 4 hours.

   g. Record results as CFU per filter after incubation. Colony counts in excess of 200 should be recorded as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

9. **Carrier Sterility Control (CSC)**

   a. To ensure sterility of the test materials, add 20 mL of the neutralizer to sterile Petri plate and swirl.

   b. Collect in a sterile conical tube.

   c. Add an additional 20 mL of the neutralizer and swirl. Pool with conical tube from step 9.b.

   d. Follow step 8.

10. **Anticipated Outcomes**

   a. The number of CFU in the *Organism Titer Control* should be in the range of 20 to 200 CFU/mL.

   b. Calculate Log Density (LD) values for each set of controls.

c. The LD count in the *Neutralizer Effectiveness Control* should be within 0.5 log as compared to the LD in the *Organism Titer Control*. More than 0.5 log difference would indicate that the neutralizer is not appropriately inactivating the test substance.

d. The LD count in the *Neutralizer Toxicity Control* should be within 0.5 log as compared to the LD in the *Organism Titer Control*. More than 0.5 log difference would indicate that the neutralizer itself is harmful to the viability of the test organism.

e. No growth should be present on the *Carrier Sterility Control*. If colonies are present on filter membrane the test is invalidated since it indicates the presence of contamination associated with the Petri plate or the neutralizer, or both.

f. If all the above criteria are met, the neutralization process is verified. If the criteria are not met, then another neutralizer or a mixture of neutralizers should be identified and verified prior to product evaluation.

TABLE 2 Summary of Neutralization Verification Assay

Treatments/Controls	Addition to Petri plates[A]				Treatment Description	Anticipated Outcomes
	Test Chemical	Neutralizer	Test Organism	ST80		
Organism Titer Control (OTC)	N/A	N/A	√	√	Inoculum titer for comparative purposes **(A)**	**A** = 20 to 200 CFU/mL
Neutralizer Effectiveness Control (NEC)	√	√	√	N/A	To measure if antimicrobial is effectively neutralized **(B)**	**A-B** = within 0 to 0.5 log difference
Neutralizer Toxicity Control (NTC)	N/A	√	√	N/A	To measure if neutralizer has any microbicidal activity **(C)**	**A-C** = within 0 to 0.5 log difference
Carrier Sterility Control (CSC)	N/A	√	N/A	N/A	Sterility assessment of carrier and neutralizer	No growth

[A] A total of up to ten sterile Petri plates are used in one single study (one organism per test substance per test condition).