US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Quantitative Petri Plate Method (QPM) for
Determining the Effectiveness of Antimicrobial Towelettes
Against Vegetative Bacteria on Inanimate, Hard, Non-porous Surfaces.

SOP Number: MB-33-00

Date Revised: 03-25-14

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Title	Quantitative Petri Plate Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes Against Vegetative Bacteria on Inanimate, Hard, Non-porous Surfaces.
Scope	This test method provides a standardized approach to quantitatively determine the effectiveness of antimicrobial towelettes (wipes) in treating hard non-porous inanimate surfaces contaminated with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. This SOP is based on ASTM Standard E2896-12 (see 15.1) and incorporates modifications recently proposed to ASTM International*. Product efficacy is considered a combination of mechanical removal and chemical inactivation.
Application	The methodology described in this SOP is used to evaluate the performance of towelette products against the prescribed test microbes.

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

Date SOP issued:	
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* 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.

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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-04 (Spectrophotometer), 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, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Any disruption of the *Pseudomonas aeruginosa* pellicle resulting in the dropping or breaking of the pellicle in the culture before or during its removal renders that culture unusable. 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. For example, if the performance standard is a 5 log reduction for *S. aureus* and *P. aeruginosa* and a 4 log reduction for *S. enterica*, the control counts should be between 5.5 to 6.5 logs/carrier for *S. aureus* and *P. aeruginosa* and between 4.5 to 5.5 logs/carrier for *S. enterica*. 2. If sterility and/or viability controls do not yield expected results, the test may be deemed invalid. 3. A high level of contamination, which interferes with reading of the results, may invalidate the test.
9. Data	Data will be archived consistent with SOP ADM-03, Records and Archives.

Management	
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. Verify the volume of dilution blanks and neutralizer tubes in advance and adjust accordingly. 3. Inoculum may be inadvertently aerosolized when lowering the pipette tip onto the glass surface. Do not 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 in this SOP.
11. Special Apparatus and Materials	1. *Test microbes*: a. *Pseudomonas aeruginosa* (ATCC No. 15442). b. *Staphylococcus aureus* (ATCC No. 6538). c. *Salmonella enterica* (ATCC No. 10708). 2. *Culture Media:* a. *Trypticase Soy Broth (TSB).* For rehydrating lyophilized/frozen vegetative culture of *S. aureus* and *P. aeruginosa* and for use in test culture preparation of *S. aureus*, *P. aeruginosa* and *S. enterica*. Prepare TSB according to manufacturer's instructions. b. *AOAC Nutrient Broth (AOAC NB).* For rehydrating lyophilized/frozen vegetative culture of *S. enterica*. Prepare media in accordance with the instructions from the AOAC Method 961.02. c. *Trypticase Soy Agar (TSA).* Used as a recovery medium for bacterial enumeration and purity checks. Prepare TSA according to manufacturer's instructions. Equivalent commercially prepared agar culture medium may be purchased. TSA with 5 % sheep blood may

be substituted.

d. *Nutrient Agar (NA).* Used in propagation of the test microbe. Dissolve 1.5 % Bacto Agar (Difco) in AOAC NB and adjust pH to 7.2 to 7.4 (blue-green with bromothymol blue), autoclave at 121°C for 20 min.

e. *Mannitol Salt Agar (MSA).* Selective solid medium for *S. aureus*. Prepare MSA according to manufacturer's instructions.

f. *Cetrimide Agar (CA).* Selective solid medium for *P. aeruginosa*. Prepare CA according to manufacturer's instructions.

g. *Xylose Lysine* Deoxycholate (*XLD).* Selective solid medium for *S. enterica*. Prepare XLD according to manufacturer's instructions.

h. *Neutralizer Medium.* Used to stop the activity of the disinfectant (for example, letheen broth, letheen broth with 0.1 % sodium thiosulfate).

Note: Commercially dehydrated media that conform to the recipes provided in AOAC Method 961.02 may be substituted.

3. *Reagents*:

a. *Cryoprotectant solution.* Used in the preparation of frozen stock cultures.TSB with 15 % v/v glycerol.

b. *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.

c. *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.

d. *Phosphate-buffered saline stock solution (PBS-SS).*Used in the preparation of PBS. Prepare 10× stock solution of PBS by dissolving 492 g PBS powder in 5 L of deionized water.

e. *Phosphate-buffered saline (PBS) 1× Solution.* Used for dilutions and rinsing. 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.

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 (for example, 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. *Cryovial.* To store frozen stock cultures (for example, 1.5 mL capacity). s. *Incubator.* To maintain appropriate temperature for recovery. t. *Vitek 2 Compact*. For microbe confirmation.
12. Procedure and Analysis	1. Generate frozen stock cultures as described in section 12.1. 2. Prior to testing, perform the neutralization verification assay (see 12.11) to determine a suitable neutralizer for testing. Neutralization verification is conducted for each test chemical to determine the proper neutralizer for the active ingredient. 3. If results from the neutralization assay are inconclusive, the assay may be repeated with a different neutralizer. 4. The neutralization assay is conducted once per product, it does not need to be conducted for every efficacy evaluation. 5. Conduct three independent tests (three test days) or as specified by the study sponsor. For each test, evaluate 5 treated and 3 control carriers.
12.1 Frozen Stock Culture Preparation	All cultures are reconstituted per the manufacturer's instructions: a. Using tubes containing 5 to 6 mL of TSB for *S. aureus* and *P. aeruginosa* and NB for *S. enterica*, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. b. Aseptically transfer the entire rehydrated pellet back into the source tube of TSB or NB. Mix well. c. Incubate for 24 ± 2 h at 36 ± 1°C. d. Using a sterile plate spreader, inoculate a sufficient number of TSA plates for *S. aureus* and *P. aeruginosa* or NA for *S. enterica* (for example, 5 to 10 plates per organism) with 100 µL each of the culture.

	e. Verify purity, by conducting a streak-isolation for *S. aureus* and *P. aeruginosa* using a general growth medium such as TSA. Use of selective media such as mannitol salt agar (MSA) for *S. aureus*, cetrimide agar (CA) for *P. aeruginosa* and xylose lysine deoxycholate (XLD) for *S. enterica* may be used as an option for presumptive identification testing. f. Incubate all plates for 24 ± 2 h at 36 ± 1°C. g. Following incubation, add 5 mL cryoprotectant solution to the surface of each TSA plate. h. Resuspend the cells in this solution by using a sterile plate spreader and aspirate the cell suspension from the surface of the agar. Transfer suspension into a sterile vessel. i. Mix the pooled contents of the vessel thoroughly. j. Immediately after mixing, pipette approximately 0.5 to 1.0 mL of the collected suspension into a cryovial. k. Place cryovials in a –70°C (or lower) freezer for long term storage; these are the frozen stock suspensions that may be used for testing for up to 18 months after preparation. A new stock suspension must be initiated from a new ATCC vial after 18 months to generate new frozen stock cultures. l. Record information on the Test Microbe Transfer Tracking Form (see section 14).
12.2 Carrier Preparation	a. Physically screen carriers (Petri plates) for any chips, scrapes, or any visible damage. b. Thoroughly clean Petri plates with non-ionic detergent by hand or use a dishwasher cycle. c. After cleaning, 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 Test Culture Preparation	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Defrost a single cryovial of a frozen stock culture at room temperature and briefly vortex to mix. Defrosting should be rapid to avoid loss in the viability of the preserved cells (for example, expose to running

water to thaw). Each cryovial is single use only.

b. Add 100 µL of the thawed stock suspension to a tube containing 10 mL of TSB. Incubate at 36 ±1°C for 18 to 24 h.

c. After incubation, (for *P. aeruginosa only*), remove the pellicle from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipette, or by vacuum removal. Avoid harvesting pellicle from the bottom of the tube. Transfer test culture after pellicle removal into sterile test tubes and visually inspect for pellicle fragments. Presence of pellicle in the final culture makes it unusable for testing. Using a vortex-style mixer, mix the TSB test culture tubes for 3 to 4 s and let stand 10±1 min at room temperature before continuing. Proceed as in 12.3e.

d. After incubation, (for *S. aureus* and *S. enterica*), using a vortex-style mixer, mix the TSB test culture tubes for 3 to 4 s and let stand 10±1 min at room temperature. Proceed as in 12.3e.

e. Remove the upper portion of each culture tube (that is, upper $^3/_4$), leaving behind any debris or clumps, and transfer to a test tube; pool cultures and swirl to mix. Aliquot test organism into sterile test tube(s).

Note —Titer adjustment (that is, dilution of the suspension) is conducted to obtain control counts within 0.5 to 1.5 logs/carrier greater than the performance standard. If dilution is necessary, use TSB to dilute the suspension.

f. Add the appropriate amount of soil to the pooled test suspension. Swirl to mix, and aliquot into sterile test tube(s).

g. 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 test culture

h. If different total volumes of the final test suspension are required, scale each component listed in 12.3g, maintaining the correct ratio of each component. Following the addition of the soil load, vortex the final test suspension.

i. Determine titer and OD at 650 nm wavelength of the test suspension, if necessary (these steps are optional). Use sterile TSB to calibrate the

	spectrophotometer.
12.4 Test culture titer (optional)	a. Determine titer (optional) of the test organism. Use test culture prepared in Section 12.3. b. Briefly vortex mix the test culture immediately prior to titer determination and inoculation of test carriers. c. Prepare serial dilutions, by taking a sample of the test culture (section 12.2g) and dilute out to 10^{-6}. d. Plate 0.1 mL of dilutions 10^{-4}, 10^{-5} and 10^{-6} in duplicate onto TSA; these dilutions should achieve 20-200 CFU/mL. e. Incubate titer plates alongside treated and control carrier plates.
12.5 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. Place the inoculation 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). c. Do not use carriers where inoculum spots have coalesced. Inspect inoculated carriers prior to the drying procedure. d. Inoculate 3 control carriers and 5 treated carriers per test condition per test substance lot. Prepare extra inoculated Petri plates as necessary. e. Carefully transfer carrier into the incubator without disturbing the inoculation spots. 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. f. Use inoculated carriers for testing within 90 minutes after drying.
12.6 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 the scraping template (see Attachment 1) 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 mixture 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 the 40 mL suspension for 10 ± 5 s; this tube is considered the 10^0 dilution. g. Prepare serial dilutions using PBS (for example, final dilutions of 10^{-3} and 10^{-4} for controls) and enumerate inoculum using membrane filtration (entire sample from dilution tube is filtered). 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. Using sterile forceps place each membrane filter onto the agar plate. Inspect each filter; no air bubbles should be present to ensure proper adherence to the surface of the agar plate. j. Incubate TSA plates with membrane filters (from treated carriers) at 36 ±1°C for up to 48 h. Inspect plates at 24 h, if zero colonies or only a few colonies are observed, continue incubation for up to 48 h. k. After incubation, 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).
12.7 Towelette Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Clean/disinfect the cap/lid area of the wipe container with 70% (v/v) ethanol. c. For multi-count containers only, use sterile gloves to remove 2 to 3 wipes from the container and discard. For canisters, gently roll and/or invert 3 to 4 times to distribute liquid in advance of removing towelettes. d. If possible, have another person hold the towelette container to avoid contamination of the gloves when removing the wipes. Change gloves as necessary to maintain sterility of the product.

12.8 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 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 the wiping template (see Attachment 1) 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 is completed, replace the lid on the plate and discard towelettes in biohazard bin. Allow plates to remain undisturbed in a horizontal position for the duration of the contact time. m. Following the contact time, 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 Petri 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 the 10^0 dilution).
12.9 Dilution and Recovery	a. Within 30 min of collecting the neutralized suspension, prepare serial dilutions in PBS. Serial dilutions (out to 10^{-3}) of the 40-mL suspension may be necessary to achieve countable CFUs/filter. Suitable CFU range equals up to 200 colonies per filter. Plating dilutions of 10^{-1}, 10^{-2}, and 10^{-3} for treated petri plates is recommended. b. Filter dilutions as in 12.6 h. Entire volume in dilution tubes is filtered. Continue filtration for all dilution tubes. c. Incubate TSA plates with membrane filters (from treated carriers) at 36 ±1°C for up to 48 h. Inspect plates at 24 h, if zero colonies or only a few colonies are observed, continue incubation for up to 48 h. 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.
12.10 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 12.8n. Collect liquid using a pipette into a sterile conical tube. Repeat with the addition of 20 mL of 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 suspension 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 a TSA plate. f. Incubate sterility control plate along with treated and control plates at 36 ±1°C for up to 48 h. The acceptance criterion is no growth.
12.11 Neutralization Confirmation	a. Prepare test suspension in accordance with Section12.3. b. Prepare serial dilutions using PBS as the dilution blank (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the test suspension. c. Treatment plates are inoculated with 0.1 mL of test suspension after addition of neutralizer or PBS to yield a final count of 20 to 200 CFU/plate. d. See Table 1 for summary of neutralization assay. e. **Organism Titer Control (OTC)**. 1. Add 20 mL of PBS to a sterile Petri plate. 2. Inoculate with 0.1 mL of appropriate dilutions (for example, 10^{-4}, 10^{-5} and 10^{-6}). Gently swirl to mix. 3. Up to three Petri plates are used, one per dilution (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the test suspension. 4. With a pipette, transfer PBS × test suspension mixture into a sterile conical tube. 5. Add an additional 20 mL of PBS to the Petri plate, swirl to mix and pool with mixture in conical tube from step e.4. 6. 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 h. f. **Neutralizer Effectiveness Control (NEC)** 1. Wipe surface of sterile Petri plate according to steps 12.8 d through. 2. Immediately (within 10 ± 5 s) after the contact time has elapsed, add 20 mL of the desired neutralizer, and swirl to mix. 3. 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}). 4. With a pipette, transfer test substance × neutralizer × test

	suspension mixture into a sterile conical tube. 5. Add an additional 20 mL of the neutralizer to the Petri plate, swirl to mix and pool with mixture in conical tube from step f.4. 6. 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 h. g. **Neutralizer Toxicity Control (NTC)** 1. Add 20 mL of the neutralizer to a sterile Petri plate. 2. 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}). 3. With a pipette, transfer the neutralizer × test suspension mixture into a sterile conical tube. 4. Add an additional 20 mL of the neutralizer to the Petri plate, swirl to mix and pool with neutralizer in conical tube from step g.4. 5. 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 h. h. **Filtration and Recovery** 1. Pre-wet filter with approximately 10 mL of PBS prior to use. 2. Vortex mixtures (in conical tubes) for approximately10 s. 3. 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. 4. Transfer the filter to the surface of a TSA plate. 5. Incubate plates at 36 ± 1ºC for up to 48 hours. 6. 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. i. **Carrier Sterility Control (CSC)** 1. To ensure sterility of the test materials, add 20 mL of the

neutralizer to sterile Petri plate and swirl.

2. Collect in a sterile conical tube.
3. Add an additional 20 mL of the neutralizer and swirl. Pool with conical tube from step i.2.
4. Follow step h.

j. **Anticipated Outcomes**

1. The number of CFU in the *Organism Titer Control* should be in the range of 20 to 200 CFU/mL.
2. Calculate Log Density (LD) values for each set of controls.
3. 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.
4. 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.
5. 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.
6. If all the above criteria are met, the neutralizer is appropriate. If the criteria are not met, then another neutralizer or a mixture of neutralizers should be identified and verified prior to product evaluation.

TABLE 1 Summary of Neutralization Verification Assay

Treatments/Controls	Addition to Petri plates[A]				Treatment Description	Anticipated Outcomes
	Test Chemical	Neutralizer	Test Organism	PBS		
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).

12.12 Confirmatory Steps for Test Microbes

a. Inspect the growth on the filters for purity and typical characteristics of the test microbe (see Table 2). Gram stain growth from one plate with a representative dilution per treated carrier type (see Table 2). Record results on the Test Microbe Confirmation Sheet (see section 14).

b. Isolation streaks may be performed for additional verification of the test organism, as deemed necessary. See Table 2 for General diagnostic characteristics for *P. aeruginosa*, *S. aureus* and *S. enterica.*

Table 2. General diagnostic characteristics for *P. aeruginosa*, *S. aureus* and *S. enterica*. (see ref. 15)

Aspect	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	Negative	Positive	Negative
Mannitol Salt Agar	No Growth	Circular, small, yellow colonies, agar turning fluorescent yellow	No Growth
Cetrimide Agar	Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	No Growth
Xylose lysine deoxycholate (XLD) agar	No Growth	No Growth	Round, clear red colonies with black centers
flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	Small, circular, yellow or white, glistening, beta hemolytic	Entire, glistening, circular, smooth, translucent, low convex, non-hemolytic	Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic
Typical Microscopic Characteristics (Gram stain results)			
Cell appearance	Straight or slightly curved rods, single polar flagella, rods formed in chains	Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	Rod shaped, flagellated.

*After 24±2 hours
(1) Test organism may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.

c. If confirmatory testing determines that the identity of the unknown was not the test organism, indicate on the results sheet that a contaminant was present. The test must be repeated.

12.13 Re-use of Petri plates (carriers)

a. Pre-clean (with a towelette) used and unused inoculated Petri plates prior to autoclaving.

b. Sterilize by autoclaving using a kill cycle (liquid cycle) for 3 hours at 121° C.

c. Refer to section 12.2 for carrier preparation.

13. Data Analysis/ Calculations	Calculations are computed using a Microsoft Excel *spreadsheet* (see Form 9, Section 14). Both electronic and hard copies of the spreadsheet are retained. Counts up to 200 and their associated dilutions are included in the calculations. a. To calculate CFU/carrier when three (3) serial dilutions are plated, use the following example formula: $$\frac{CFU\ for\ 10^{-x} + CFU\ for\ 10^{-y} + CFU\ for\ 10^{-z}}{(a \times 10^{-x}) + (b \times 10^{-y}) + (c \times 10^{-z})} \times D$$ 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:

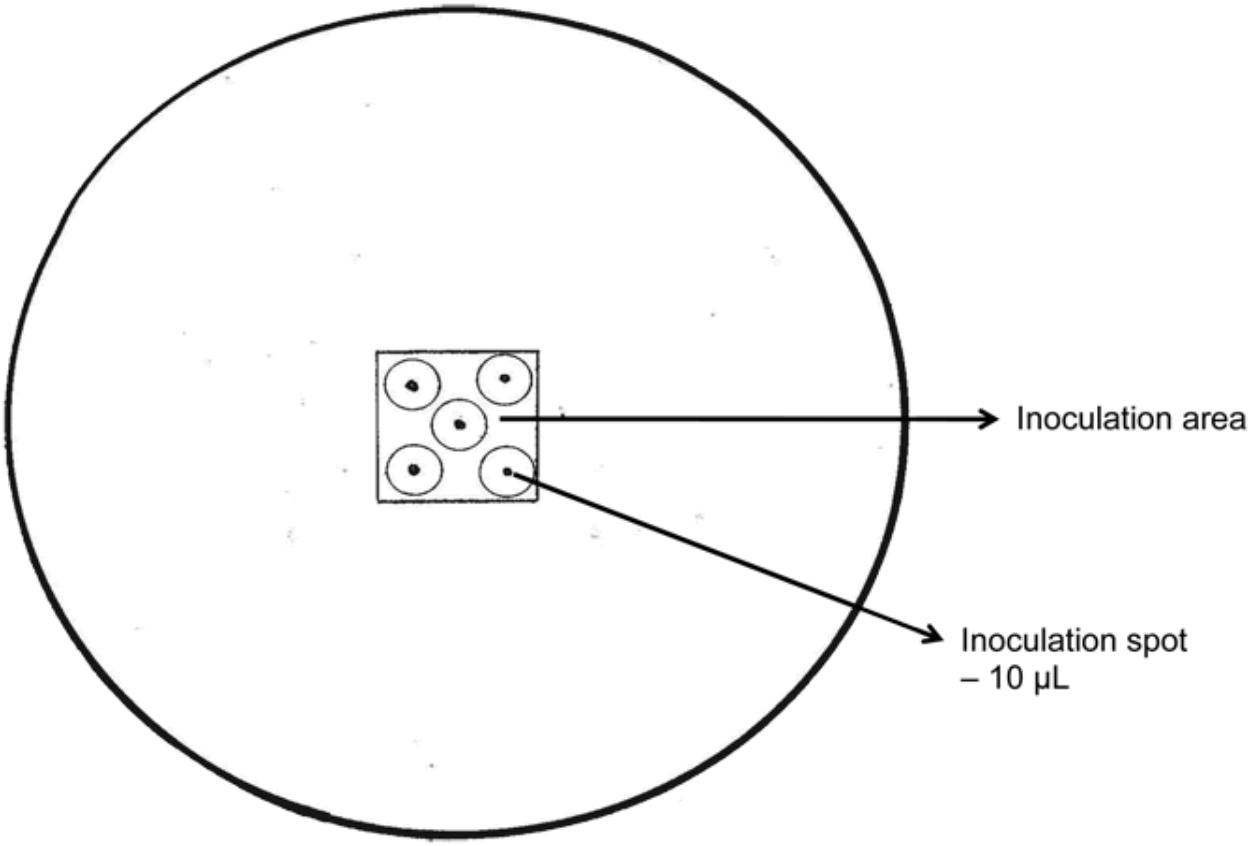
Organism Culture Tracking Form	MB-33-00_F1.docx
Test Microbe Confirmation Sheet (Quality Control)	MB-33-00_F2.docx
Test Information and Carrier Inoculation Sheet	MB-33-00_F3.docx
Treated Carriers Results Sheet	MB-33-00_F4.docx
Time Recording Sheet for Treated Carriers	MB-33-00_F5.docx
Control Carrier Counts Dilution Scheme and Results	MB-33-00_F6.docx
Test Microbe Culture Titer (Optional)	MB-33-00_F7.docx
Test Microbe Confirmation Sheet	MB-33-00_F8.docx
Treated Carriers and Control Carrier Count Spreadsheet (MS Excel spreadsheet)	MB-33-00_F9.xlsx
Media/Reagent Preparation Sheet – Carrier Preparation	MB-33-00_F10.xlsx

15. References

1. ASTM Method, E2896-12 – Standard test Method for Quantitative Petri Plate Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes. ASTM International, West Conshohocken, PA.
2. Official Methods of Analysis. Method 955.14 – *Salmonella enterica*. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD.
3. Official Methods of Analysis. Methods 955.15 – *Staphylococcus aureus*. Posted September 2013. AOAC INTERNATIONAL, Gaithersburg, MD.
4. Official Methods of Analysis. Method 964.02 – *Pseudomonas aeruginosa*. Posted September 2013. AOAC INTERNATIONAL, Gaithersburg, MD.
5. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. P. aeruginosa p. 164, S. enterica p. 447.
6. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. S. aureus p. 1015.
7. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011.

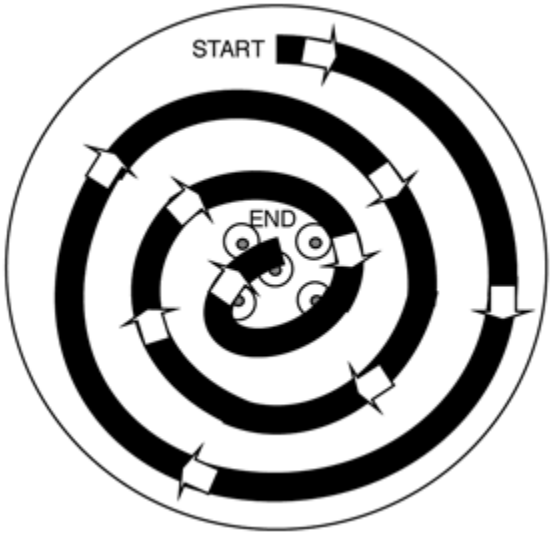
Attachment 1

INOCULATION, WIPING AND SCRAPING TEMPLATE

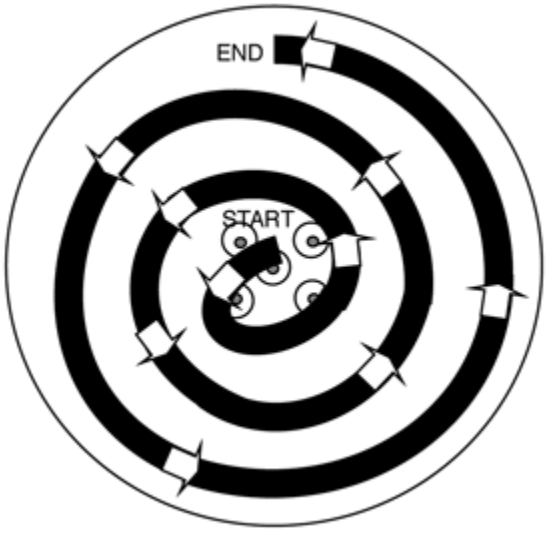


Attachment 2

WIPING PROCEDURE SCHEMATICS



Wiping from Outer Region to Center (Inoculated) Region

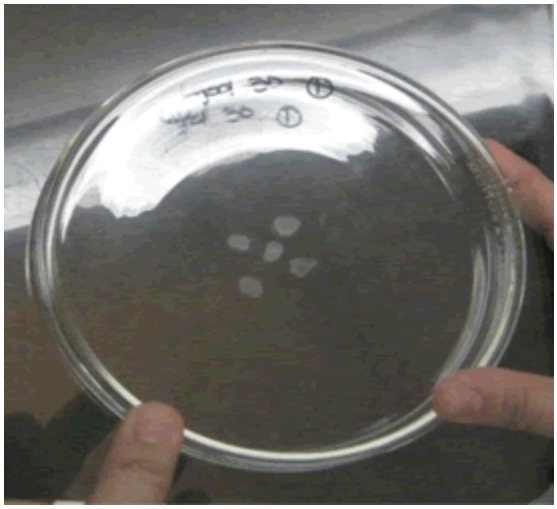


Wiping from Center (Inoculated) Region to Outer Region

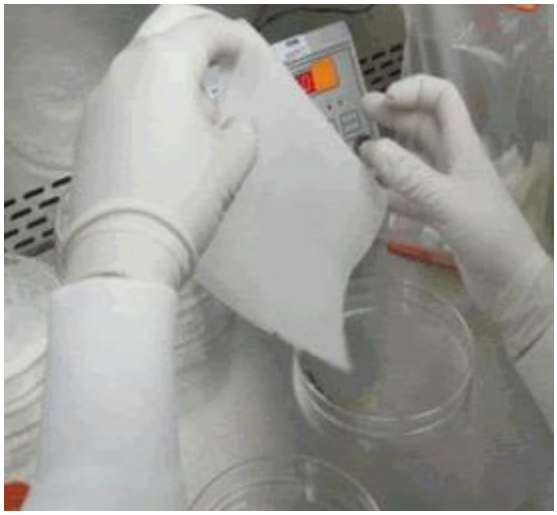
Attachment 3

PICTURE OF STERILE SCRAPER

Attachment 4
PICTORIAL PRESENTATION OF QPM

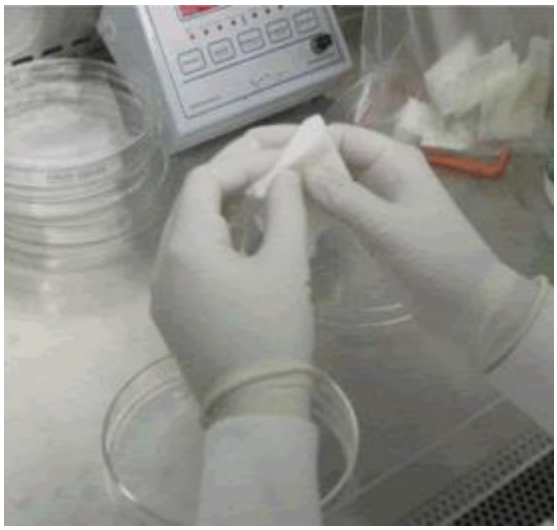


Dried Inoculum on Petri Plate

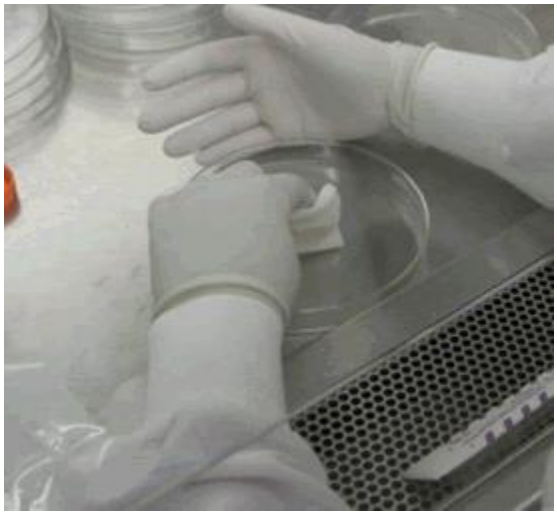


Folding of Antimicrobial Towelette-1

Attachment 4 (continued)



Folding of Antimicrobial Towelette-2



Wiping of Plate

Attachment 4 (continued)

Adding Neutralizer

Scraping

Attachment 4 (continued)

Collecting Mixture