Designation: E2896 – 12

Standard Test Method for Quantitative Petri Plate Method (QPM) for Determining the Effectiveness of Antimicrobial Towelettes[1]

This standard is issued under the fixed designation E2896; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

This test method provides a standardized approach to quantitatively determine the effectiveness of antimicrobial towelettes (wipes) in treating hard non-porous surfaces contaminated with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. This test method addresses the need for a user-friendly, relevant, and reproducible procedure.[2]

1. Scope

1.1 This test method provides detailed instructions for performing a quantitative evaluation of antimicrobial efficacy of a towelette when challenged against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica*. The method may be used with other microbial strains, though modification may be necessary to accommodate recovery.

1.1.1 Antimicrobial towelettes, designed to decontaminate hard, non-porous surfaces, are diverse in size, matrix composition, and packaging.

1.1.2 Antimicrobial towelettes also vary in label claims and use directions.

1.2 This quantitative method does not differentiate between mechanical removal of inoculum from a surface and chemical inactivation of the test microbe; rather, product efficacy is considered a combination of both attributes of a towelette-based formulation.

1.3 It is the responsibility of the investigator to determine whether Good Laboratory Practices (GLP Standards—40 CFR, Part 160 of FIFRA) are required and to follow them when appropriate.

1.4 This standard may involve the use of hazardous materials, chemicals and infectious microorganisms and should be performed only by persons with formal training in microbiology.

1.5 Strict adherence to the protocol is necessary for the validity of the test results.

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *This standard does not address specific product performance standards established by regulatory authorities; see Section 10, Note 2 for details.*

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *Other Documents:*

AOAC Official Method 961.02, Germicidal Spray Products as Disinfectants. Revised 2012[3]

AOAC Official Method 955.15, Use-Dilution Method for Testing Disinfectants against *Staphylococcus aureus*. Revised 2012[3]

AOAC Official Method 964.02, Use-Dilution Method for Testing Disinfectants against *Pseudomonas aeruginosa*. Revised 2012[3]

AOAC Official Methods 955.14, Use-Dilution Method for Testing Disinfectants against *Salmonella enterica*. Revised 2012[3]

40 CFR, Part 160 Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards[4]

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[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] Samalot-Freire, L., Tomasino, S. F., and Hasan, J. A., The Quantitative Petri Plate Method (QPM): A New Method for Assessing the Efficacy of Antimicrobial Towelettes, Presented at the 125th Annual Meeting of the AOAC International, New Orleans, LA, 2011.

[3] Available from AOAC International, 481 North Frederick Ave., Suite 500, Gaithersburg, Maryland 20877-2417, http://www.aoac.org.

[4] Available from U.S. Government Printing Office Superintendent of Documents, 732 N. Capitol St., NW, Mail Stop: SDE, Washington, DC 20401, http://www.access.gpo.gov.

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3. Terminology

3.1 *Definitions:*

3.1.1 *antimicrobial towelette (wipe), n*—a piece of porous material soaked in an antimicrobial liquid that is meant for decontamination of hard non-porous environmental surfaces by wiping.

3.1.2 *dilution blank, n*—tubes of phosphate buffered saline (PBS) or phosphate buffered dilution water (PBDW) or similar inert phosphate buffer solution.

3.1.3 *carriers, n*—glass petri plates (150 by 20 mm).

3.1.4 *colony forming units (CFU), n*—number of microorganisms that form colonies (clusters of microorganisms visibly growing on the surface of a membrane filter) as a means of enumerating the total number of viable microorganisms in a sample.

3.1.5 *quality control (QC), n*—the procedures, products or services that meet a laboratory's specified standards of quality.

4. Summary of Test Method

4.1 This test method provides detailed instructions for performing a quantitative evaluation of antimicrobial efficacy of a towelette when challenged against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica*. The method may be used with other microbial strains, though modification may be necessary to accommodate recovery.

4.2 Antimicrobial towelettes, for which disinfecting and sanitizing label claims are made, are suitable products to be evaluated with this test method.

4.3 Petri plates, inoculated with a suspension of vegetative bacteria, are used as the test carriers.

4.3.1 Each petri plate is inoculated with five spots (10 µL each) of the test organism and allowed to dry.

4.4 The bacteria are exposed to the test chemical by applying a towelette to the inner bottom surface of a plate using a prescribed pattern of wiping, followed by exposure to the test chemical for a specific contact time. Bacterial population after exposure is determined by neutralizing the residual test substance (liquid), scraping the petri plate surface, and collecting the inoculum-neutralizer suspension. The pooled suspension is serially diluted, filtered and plated onto recovery media (Tryptic Soy Agar).

4.5 Viable CFUs for control and treated carriers are enumerated using membrane filtration.

4.6 The mean $\log_{10}$ density (LD) recovered from treated carriers is compared to the mean $\log_{10}$ density recovered from the control carriers. This calculation is used to determine the efficacy of the product based on the mean $\log_{10}$ reduction (LR) value.

5. Significance and Use

5.1 The glass petri plate provides a closed system for enumeration and easy application of a pre-saturated or impregnated antimicrobial towelette by an analyst.

5.2 Inoculation of carriers (five 10 µL spots of microbial suspension) is conducted using a template and a positive displacement pipette, thereby ensuring a precise inoculum level and uniform distribution of inoculum.

5.3 A single towelette is tested per carrier, thereby ensuring comparable treatment among carriers and eliminating the likelihood of cross-contamination between carriers.

5.4 The circular motion of the product application (wipe outside to inside, lift towelette to invert and wipe inside to outside) is a relevant motion that ensures uniform coverage and contact of disinfectant with the inoculated surface.

5.5 The addition of neutralizer to the treated plates ensures thorough neutralization at the end of the product's contact time. This test method provides a procedure for performing neutralization verification to confirm that the microbicidal and/or microbistatic activity of a test substance has been brought to an undetectable level at the end of the contact time.

5.6 The design of the test method minimizes any loss of viable organisms through carrier wash-off.

5.7 This test method provides for optional use of an organic soil load as dictated by a product's label claim.

5.8 It is optional to adjust (either dilute or concentrate) the inoculum level to achieve desired control carrier counts and to accommodate different product performance standards.

6. Apparatus

6.1 *Biosafety cabinet (BSC, Type B2, Class II)*—Certified, recommended for maintaining an aseptic work environment.

6.2 *Petri plates*—Glass petri plates used as test carriers (150 by 20 mm).

6.3 *Micropipette*—Calibrated.

6.4 *Positive displacement pipette*—Calibrated, used to dispense 10 µL aliquots (5 total spots) of a challenge suspension onto the test carriers.

6.5 *Timer*—Any certified timer that can display time in seconds.

6.6 *Sterile test tubes*—Reusable or disposable 20 by 150 mm for dilution blanks and cultures/subcultures or other appropriate size.

6.7 *Test tube racks*—Any convenient size.

6.8 *Sterile cell scraper*—To scrape carriers for removal of bacteria during harvesting (for example, scraper blade dimensions = 1.8 to 3.0 cm).

6.9 *Sterile plate spreader*—May be used to spread inoculum on plate surface.

6.10 *Cryovial*—To store frozen stock cultures (for example, 1.5 mL capacity).

6.11 *Conical tubes*—Sterile, 50 mL. 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.

6.12 *Vortex mixer.*

6.13 *Serological pipettes*—Sterile single-use pipettes (for example, 25.0, 10.0, 5.0, 2.2, 1.0 mL capacity).

6.14 *Sterile membrane filters*—To filter serial dilutions for cell enumeration (0.22 µm pore size, polyethersulfone). Filtration units (reusable or disposable) may be used.

6.15 *Sterile surgical gloves*—To handle antimicrobial towelette when folding and wiping inoculated petri plate carrier.

6.16 *Autoclave (steam sterilizer)*—To sterilize media and reagents.

7. Media and Reagents

7.1 *Culture Media:*

7.1.1 *Trypticase Soy Broth (TSB)*—For use in rehydrating lyophilized/frozen vegetative culture of test microorganism. Prepare TSB according to manufacturer's instructions.

7.1.2 *AOAC Nutrient Broth (AOAC NB)*—For use in culture maintenance of *Salmonella enterica*. Prepare media in accordance with the instructions from AOAC methods.[3]

7.1.3 *Synthetic Broth (AOAC SB)*—For use in culture and subculture preparation of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella enterica*. Prepare media in accordance with the instructions from AOAC methods.[3]

7.1.4 *Trypticase Soy Agar (TSA)*—For use 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.

7.1.5 *Nutrient Agar (NA)*—For use in propagation. 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).

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

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

7.1.8 *Neutralizer Medium*—To use as chemical neutralizer (for example, letheen broth, letheen broth with 0.1 % sodium thiosulfate).

NOTE 1—Commercially dehydrated media that conform to the recipes provided in AOAC Method 961.02 may be substituted.

7.2 *Reagents:*

7.2.1 *Cryoprotectant solution*—TSB with 15 % v/v glycerol. Sterile solution is used in the preparation of frozen stock cultures.

7.2.2 *Organic soil*—Organic burden added to inoculum, consistent with product label claims (for example, fetal bovine serum, horse serum, heat-inactivated fetal bovine serum).

7.2.3 *De-ionized water*—Purified water with mineral ions removed through pre-treatment, deionization and filters. Alternatively, reagent grade water (ultrapure water) may be used.

7.2.4 *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.

7.2.5 *Phosphate-buffered saline (PBS) 1× Solution*—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.

8. Test Organisms

8.1 *Staphylococcus aureus*—ATCC 6538. The organism is a gram-positive, coccus-shaped bacterium, that when plated onto general growth media (for example, Trypticase Soy Agar) produces small, circular, yellow, glistening colonies within 24 h at 36 ± 1°C.

8.2 *Pseudomonas aeruginosa*—ATCC 15442. The organism is a gram-negative, rod-shaped bacterium, that when plated onto general growth media (for example, Trypticase Soy Agar) produces flat, opaque to off-white, round, spreading colonies within 24 h at 36 ± 1°C.

8.3 *Salmonella enterica (*subsp. *enterica* serovar choleraesuis*)*—ATCC 10708. The organism is a gram-negative, rod-shaped bacterium, that when plated onto general growth media (for example, Trypticase Soy Agar) produces entire, glistening, circular, smooth, translucent, low, convex colonies within 24 h at 36 ± 1°C.

9. Generation of Frozen Stock Cultures

9.1 All cultures are reconstituted per the manufacturer's instructions as presented in sections 9.2 – 9.4.

9.2 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.

9.3 Aseptically transfer the entire rehydrated pellet back into the source tube of TSB or NB. Mix well.

9.4 Incubate for 24 ± 2 h at 36 ± 1°C.

9.5 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.

9.6 To verify purity, conduct streak-isolation for *S. aureus* and *P. aeruginosa* using a general growth medium such as TSA. Use of selective media such as MSA for *S. aureus* and CA for *P. aeruginosa* may be employed as an option for presumptive identification testing.

9.7 Incubate all plates for 24 ± 2 h at 36 ± 1°C.

9.8 Following incubation, add 5 mL cryoprotectant solution to the surface of each agar plate.

9.9 Resuspend the cells in this solution using a sterile plate spreader and aspirate the cell suspension from the surface of the agar.

9.10 Transfer suspension into a sterile vessel.

9.11 Mix the pooled contents of the vessel thoroughly.

9.12 Immediately after mixing, pipette approximately 0.5 to 1.0 mL of the collected suspension into a cryovial.

9.13 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 ATCC culture must be purchased after 18 months to generate new frozen stock cultures.

10. Test Organism Preparation

10.1 Defrost a single cryovial 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 for single use only.

10.2 Add 100 µL of the thawed stock suspension to a tube containing 10 mL of SB (that is, primary) and incubate at 36 ± 1°C for 18 to 24 h.

10.3 For *S. aureus* and *S. enterica* cultures, briefly vortex to mix the 18 to 24 h SB culture prior to transfer. For *P. aeruginosa*, do not vortex the 18 to 24 h culture prior to transfer.

10.4 Inoculate 100 µL per tube of the 18 to 24 h SB culture to an appropriate number (for example, 2 to 3) of tubes containing 10 mL SB for final test culture and incubate at 36 ± 1°C for 18 to 24 h.

10.5 For *S. aureus* and *S. enterica*, using a vortex-style mixer, mix the 18 to 24 h SB test cultures 3 to 4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture tube (that is, upper ¾), leaving behind any debris or clumps, and transfer to a small sterile flask or test tube; pool cultures and swirl to mix. Aliquot culture into sterile test tube(s). Prepare 1 or 2 tubes of culture.

10.6 For *P. aeruginosa*, do not shake the 18 to 24 h SB final test culture. The pellicle from the 18 to 24 h cultures must first be separated from the broth before mixing on a vortex mixer using vacuum suction or by using a pipette to gently aspirate the broth away from the pellicle. Any disruption of the pellicle resulting in its breakage and fragmentation into the suspension renders the suspension unusable for testing. Inspect the suspension visually for signs of pellicle, discarding any containing fragments of pellicle. Decanting the suspension away from the pellicle is an acceptable alternative method for separation.

10.6.1 Using a vortex-style mixer, mix the SB test suspensions 3 to 4 s and let stand 10 min at room temperature before continuing.

10.6.2 Remove the upper portion of each culture tube (that is, upper ¾ or approximately 7 mL), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot suspension into sterile test tube(s).

NOTE 2—Titer adjustment (that is, dilution or concentration of a suspension) can be conducted in order that control counts be within the 0.5 to 1.5 logs greater than the performance standard. Sterile broth used to grow the microorganism shall be used to dilute the suspension. For example, if the titer of the culture is approximately 10^8 CFU/mL, then dilute 10-fold to achieve approximately 10^7 CFU/mL; use the diluted culture to achieve carrier counts in the range of 5.5 to 6.5 logs.

10.7 If soil is required for testing, add the appropriate amount of soil to the pooled test suspension after dilution (if necessary) and prior to carrier inoculation. Swirl to mix, and aliquot into sterile test tube(s).

11. Carrier Inoculation

11.1 Use sterile glass petri plates (150 by 20 mm) as the test carriers.

11.2 Use test suspension prepared in Section 10. Determine titer (optional) of the test suspension and the corresponding OD at 650 nm or other appropriate wavelength.

11.3 Direct plating on TSA may be employed for microbial enumeration of test culture.

11.4 Briefly vortex to mix the test culture immediately prior to titer determination and inoculation of test carriers.

11.5 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); see Appendix X1. A template (an approximate 1 by 1 in. area in the center of the petri plate) is placed beneath the petri plate to standardize the location of the inoculation sites.

11.6 Inoculate a sufficient number of plates to test at least 3 controls (per challenge species per test substance lot) and a minimum of 5 treated carriers per test condition per test substance lot. Prepare extra inoculated petri plates as necessary.

11.7 Dry inocula in an incubator at 36 ± 1°C for 30 to 40 min or until visibly dry. Visually inspect each petri plate to ensure complete drying of the inoculum. Lids must be placed on the petri plates during the drying process.

11.8 Use inoculated petri plates for product testing within 90 min after drying.

12. Carrier Load Enumeration (Control Carrier Counts)

12.1 One petri plate (carrier) is evaluated prior to testing the test wipe to assess inoculated carrier population immediately prior to commencing the test and two petri plates are evaluated immediately following testing to assess inoculated carrier population after the test. Carriers are processed within 90 min after drying to ensure an appropriate microbial challenge at the time of testing.

12.2 For carrier counts, add 20 mL of the neutralizer to each inoculated petri plate, and using a sterile cell scraper (scraper dimensions = 1.8 to 3.0 cm), gently scrape across the middle of the plate with three back and forth motions (6 total motions), and rotating the plate 90 degrees, repeat the scraping procedure using the same cell scraper to dislodge and suspend the inocula (gently swirl to mix). The template used to aid in inoculation is also used during the scraping step to highlight/locate the inoculated areas.

12.3 Use a serological pipette to transfer the suspension into a sterile conical tube (that is, 50 mL). Tilt the petri plate as necessary to collect the suspension.

12.4 Add a second 20-mL aliquot of neutralizer to each petri plate, gently swirl, and remove liquid with a serological pipette, combining it with the first 20 mL aliquot.

12.5 Vortex-mix the 40 mL suspension for 20 to 30 s; this tube is the 10^0 dilution.

12.6 Prepare serial dilutions (for example, final dilutions of 10^{-3}, 10^{-4}, and 10^{-5} for controls) and enumerate inoculum using membrane filtration (add 1 mL of each dilution per filter) on TSA. Pre-wet filter with approximately 10 mL saline solution prior to adding the 1 mL sample. Swirl the entire contents of

the filter unit and apply vacuum. Pass the entire content through the filter, then rinse filter unit with approximately 20 mL saline solution; place each filter on a separate plate of TSA and incubate at 36 ± 1°C for up to 48 h and record CFU/carrier.

12.7 Dilutions resulting in 20 to 200 CFUs per filter are deemed acceptable for counting purposes; all counts up to 200 are used in the calculations. CFUs above 200 are indicated as Too Numerous to Count (TNTC).

12.8 Account for all dilutions and the 40 mL of neutralizer to calculate the viable CFU/carrier.

12.9 A slight decrease in CFU/carrier may be observed over the sampling period. Conduct the testing expeditiously to reduce the potential for loss in the microbe's viability on the carriers.

12.10 Calculate the mean $\log_{10}$ density (LD) of the three carriers. Refer to Section 14 for example calculations.

12.11 The population of a test species on the dried carriers (determined for control counts) must be between 0.5 and 1.5 $\log_{10}$ higher than the defined performance standard to provide statistical confidence in the data. For example, if the performance standard is a 5 $\log_{10}$ reduction, the control counts should be between 5.5 to 6.5 $\log_{10}$.

NOTE 3—On the day of the test, a single uninoculated petri plate should be used as a negative control to verify sterility of the petri plates and the neutralizer. The negative control should be treated the same as the test and control carriers. (For example, add two 20 mL neutralizer (40 mL total), scraping, aspirating, and filtering the entire volume.)

13. Wiping

13.1 Sterility of all filtered reagents and media (for example, PBS, neutralizer) used in the study should be verified prior to use.

13.2 Wiping of inoculated carriers:

13.2.1 If possible, perform assay inside the BSC.

13.2.2 Record ambient temperature and humidity.

13.2.3 Clean/disinfect the cap/lid area of the wipe container with 70% (v/v) ethanol.

13.2.4 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. 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.

13.2.5 Use a new pair of sterile gloves when preparing to handle each test towelette. 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 final dimensions of approximately 2 by 2 in.

NOTE 4—For larger towelettes, additional folding may be necessary in order to produce dimensions of approximately 2 by 2 in. For small towelettes (1 by 1 in.), fold in half.

NOTE 5—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.

13.2.6 Application is a timed exercise; use a calibrated timer to track contact time.

13.2.7 Avoid contact of the towelette with the inside rim of the petri plate as much as possible.

13.2.8 Remove lid and secure the petri plate in one hand (non-wiping hand).

13.2.9 Wipe 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. Lift towelette from plate and invert the last fold to expose an unused area (fold) of the wipe. Position the inverted towelette at the center of plate and perform three circular revolutions from center to outer margin of the plate. The wipe pattern should be consistent from plate to plate with consistent pressure typical of towelette use. Wipe with the folded edge of the towelette. Refer to Appendix X2 for illustrations of wiping procedure.

13.2.10 The entire wiping process should not take longer than 10 s per petri plate.

13.2.11 The contact time begins immediately upon completion of the entire wiping procedure.

13.2.12 Use one antimicrobial towelette per petri plate; discard the towelette after use.

13.2.13 Treated petri plates, with lids on, must be kept undisturbed and horizontal during the contact time.

13.3 After contact time has elapsed, add 20 mL of the neutralizer to the treated petri plate, and using a sterile cell scraper (scraper dimensions = 1.8 to 3.0 cm), 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). The template used to aid in inoculation is also used during the scraping step to highlight/locate the inoculated areas.

13.4 Remove the suspension using a serological pipette and transfer into a sterile conical tube (that is, 50 mL). Tilt the petri plate as necessary to collect as much of the suspension as possible.

13.5 Add a second 20-mL aliquot of neutralizer to the petri plate, gently swirl, and remove liquid with a serological pipette, combining it with the previous 20 mL aliquot.

13.6 Vortex-mix the 40-mL suspension for 20 to 30 s. (This tube is considered to be the 10^0 dilution.)

13.7 Within 30 min of neutralization, prepare serial dilutions and enumerate the suspension using membrane filtration. 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.

13.8 Pre-wet filter with approximately 10 mL of saline solution prior to use.

13.9 Rinse the 10° tube (40 mL suspension) with approximately 20 mL of saline solution after initial filtration and filter through the same membrane. Transfer the filter to the surface of TSA.

13.10 Pass the entire volume of each dilution tube through a pre-wetted filter, rinse filter unit with approximately 20 mL of saline solution. Transfer the filter to the surface of TSA.

13.11 Incubate at 36 ± 1°C for up to 48 h, and record CFU/filter. Plates can be inspected at 24 h. If filters have sparse colonies or no colonies, then plates are incubated for up to 48 h. Final counts are recorded at 48 h. A range of 20 to 200 colonies per filter is suitable for counting.

13.12 Account for all dilutions and the 40 mL of neutralizer to calculate the viable CFU/filter.

13.13 Calculate mean $\log_{10}$ densities (LD). Refer to Section 14 for calculations.

14. Calculations

14.1 To calculate CFU/mL associated with each carrier when three (3) serial dilutions are plated, use the following example formula (weighted mean approach) for calculating CFU/mL where 10^{-x}, 10^{-y}, and 10^{-z} are the final dilutions plated:

$$\frac{(CFU \text{ for } 10^{-1}) + (CFU \text{ for } 10^{-2}) + (CFU \text{ for } 10^{-3})}{10^{-1} + 10^{-2} + 10^{-3}} \quad (1)$$

Example:

$$\frac{200\ CFU + 20\ CFU + 2\ CFU = 222 \text{ total CFU}}{0.1 + 0.01 + 0.001 = 0.111} = 222/0.111$$

$$= 2000 \quad \text{CFU/mL}$$

NOTE 6—All counts up to 200 should be used in the calculations. In addition, adjust calculations to account for final volume of suspension in conical tube (39 or 40 mL) and/or the serially diluted sample (9 or 10 mL) when they are used to calculate CFU/mL.

14.2 To calculate *CFU/carrier*, multiply the CFU/mL recovered from a carrier by the volume of suspension (that is, 40 mL).

14.3 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.

14.4 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.

14.5 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.

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

15. Precision and Bias

15.1 Precision and bias statement cannot be determined at this time.

16. Keywords

16.1 antimicrobial towelettes; cell scraper; membrane filtration; product application; *Pseudomonas aeruginosa*; Quantitative Petri Plate Method (QPM); *Salmonella enterica*; *Staphylococcus aureus*; test culture; wiping contaminated surfaces

APPENDIXES

(Nonmandatory Information)

X1. INOCULATION AND SCRAPING TEMPLATE

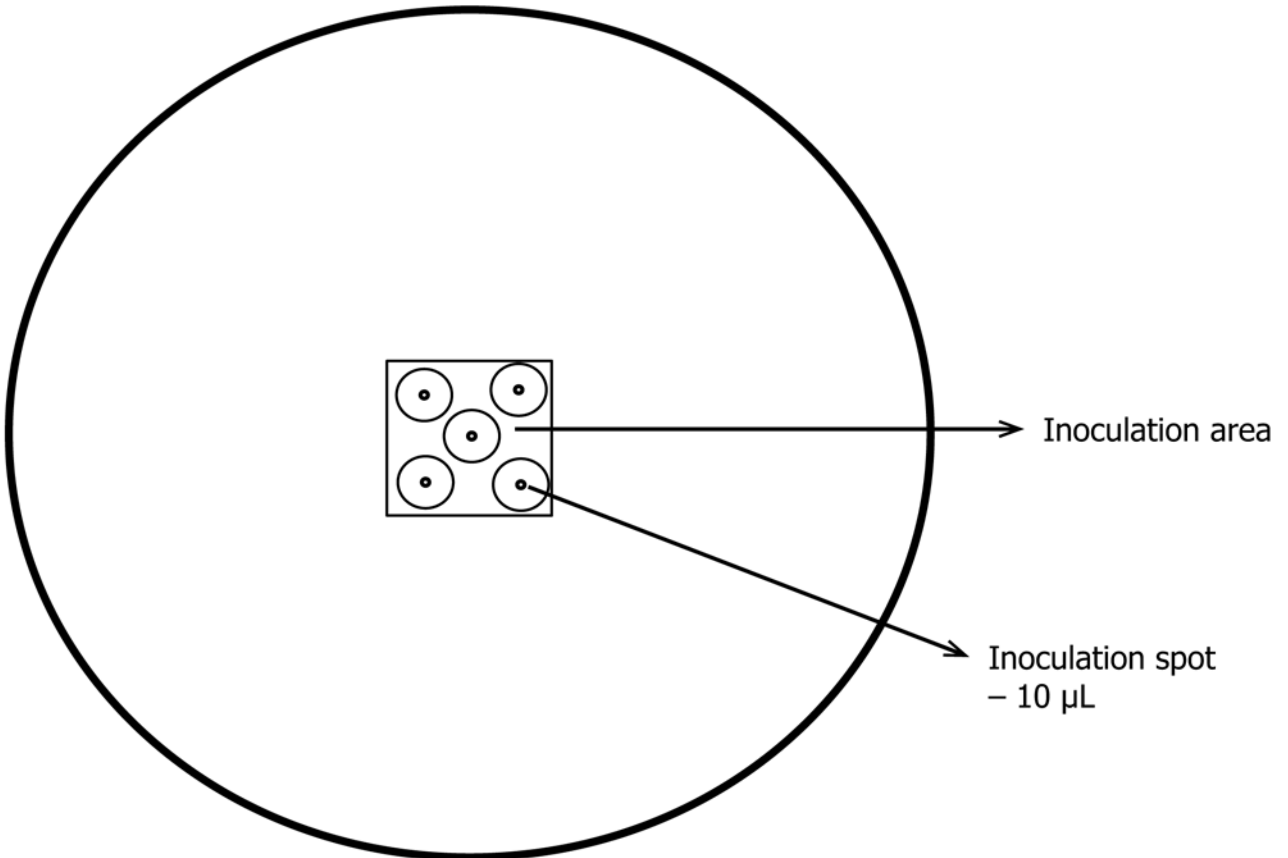

FIG. X1.1 Inoculation and Scraping Template

X2. WIPING PROCEDURE SCHEMATICS

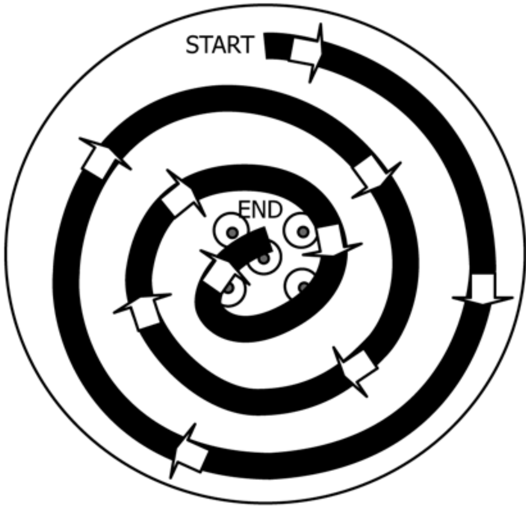

FIG. X2.1 Wiping from Outer Region to Center (Inoculated) Region

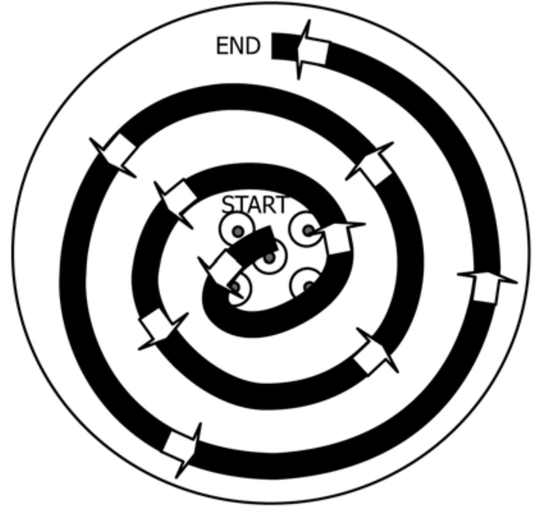

FIG. X2.2 Wiping from Center (Inoculated) Region to Outer Region

X3. PICTURE OF STERILE SCRAPER

FIG. X3.1 Sterile Scraper

X4. PICTORIAL PRESENTATION OF QPM

FIG. X4.1 Dried Inoculum on Petri Plate

FIG. X4.2 Folding of Antimicrobial Towelette-1

FIG. X4.3 Folding of Antimicrobial Towelette-2

FIG. X4.4 Wiping of Plate

FIG. X4.5 Adding Neutralizer

FIG. X4.6 Scraping

FIG. X4.7 Collecting Mixture

X5. NEUTRALIZATION VERIFICATION PROCEDURE

X5.1 Materials

X5.1.1 Sterile petri plates.

X5.1.2 Conical tubes.

X5.1.3 Sterile membrane filters.

X5.1.4 Sterile surgical gloves.

X5.1.5 Neutralizer medium.

X5.1.6 TSA plates.

X5.1.7 Test culture—prepared in accordance with Section 10 of this test method.

X5.1.8 Antimicrobial towelette.

X5.1.9 Phosphate-buffered saline (PBS) 1X solution.

X5.2 Method

X5.2.1 Prepare test organism in accordance with Section 10 of the QPM.

X5.2.2 Add organic soil load to inoculum if specified.

X5.2.3 Prepare up to three serial dilutions (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the microbial test suspension. Inoculate the sterile petri plates with 0.1 mL of test suspension after addition of neutralizer or PBS to yield a final count of 20 to 200 CFU/plate. Follow steps in X5.3, X5.4, X5.5, X5.6, X5.7 and X5.8.

X5.3 Test Organism Viability Control (TVC)

X5.3.1 Add 20 mL of PBS to a sterile petri plate.

X5.3.2 Immediately (within 10 to 15 s) inoculate with 0.1 mL of diluted suspension of the test organism dilution (for example, 10^{-4}, 10^{-5} and 10^{-6}). Gently swirl to mix.

X5.3.3 Up to three petri plates are used, one per dilution (for example, 10^{-4}, 10^{-5} and 10^{-6}) of the test organism.

X5.3.4 With a pipette, transfer PBS and test suspension mixture into a sterile conical tube.

X5.3.5 Add an additional 20 mL of PBS to the petri plate, swirl to mix and pool with mixture in conical tube from step X5.3.4.

X5.3.6 Follow steps 13.8 and 13.9.

X5.3.7 Incubate the plates at 36 ± 1°C for up to 48 h; count CFU on the filters and record results.

X5.4 Neutralizer Effectiveness Control (NEC)

X5.4.1 Wipe surface of sterile petri plate according to the QPM.

X5.4.2 Immediately after the contact time has been reached (10 ± 5 s), add 20 mL of the desired neutralizer, and swirl to mix.

X5.4.3 Within 10 to 15 s, inoculate mixture in petri plate with 0.1 mL of test suspension dilution. Gently swirl to mix. Up to three petri plates are used, one per test organism dilution (for example, 10^{-4}, 10^{-5}, and 10^{-6}).

X5.4.4 With a pipette, transfer test substance-neutralizer-test suspension mixture into a sterile conical tube.

X5.4.5 Add an additional 20 mL of the neutralizer to the petri plate, swirl to mix and pool with mixture in conical tube from step X5.4.4.

X5.4.6 Hold the mixture for a minimum of 5 min at 20 to 25°C prior to filtration. Proceed as in X5.7.

X5.5 Optional: Test Chemical Control (TCC)

X5.5.1 Wipe surface of sterile petri plate according to the QPM.

X5.5.2 Inoculate immediately the center of petri plate with 0.1 mL of test organism dilution. The contact time (as specified for the candidate towelette product) begins following inoculation. Up to three petri plates are used, one per test suspension dilution (for example, 10^{-4}, $10\text{-}5^{-5}$, and 10^{-6}).

X5.5.3 Immediately after the contact time has been reached, add 20 mL of PBS, and swirl to mix.

X5.5.4 With a pipette, transfer test chemical-test suspension-PBS mixture into a sterile conical tube.

X5.5.5 Add an additional 20 mL of PBS to the petri plate, swirl to mix and pool with mixture in conical tube from step X5.5.4.

X5.5.6 Hold the mixture for a minimum of 5 min at 20 to 25°C prior to filtration. Proceed as in X5.7.

X5.6 Neutralizer Toxicity Control (NTC)

X5.6.1 Add 20 mL of neutralizer to a sterile petri plate.

X5.6.2 Immediately (within 10 to 15 s) inoculate the neutralizer with 0.1 mL of test suspension dilution. Gently swirl to mix. Up to three petri plates are used, one per test organism dilution (for example, 10^{-4}, 10^{-5}, and 10^{-6}).

X5.6.3 With a pipette, transfer the neutralizer-test suspension mixture into a sterile conical tube.

X5.6.4 Add an additional 20 mL of the neutralizer to the petri plate, swirl to mix and pool with neutralizer in conical tube from step X5.6.3.

X5.6.5 Hold the mixture for a minimum of 5 min at 20 to 25°C prior to filtration. Proceed as in X5.7.

X5.7 Filtration and Recovery

X5.7.1 Vortex mixtures for 10 s and pass them separately through membrane filters. Wash each filter with approximately 40 mL PBS. Place each filter onto general growth recovery medium such as TSA. Incubate the plates at 36 ± 1°C for up to 48 h; count CFU on the filters and record results.

X5.8 Carrier Sterility Control (CSC)

X5.8.1 To ensure sterility of the test materials, add 20 mL of the neutralizer to sterile petri plate and swirl.

X5.8.2 Collect in a sterile conical tube.

X5.8.3 Add an additional 20 mL of the neutralizer and swirl.

X5.8.4 Pool with conical tube from step X5.8.2.

X5.8.5 Follow steps 13.8 and 13.9.

X5.8.6 Incubate plate at 36 ± 1°C for up to 48 h. Record filter to verify sterility of plate and neutralizer; no CFUs should be noted on the plate.

X5.9 Anticipated Outcomes

X5.9.1 The number of CFU in the *Test Organism Viability Control* should be in the range of 20 to 200 CFU/mL.

X5.9.2 Calculate Log Density (LD) values for each set of controls.

X5.9.3 The LD count in the *Neutralizer Effectiveness Control* should be within 1 log as compared to the CFU count in the *Test Organism Viability Control*. More than 1 log difference would indicate that the neutralizer is not appropriately inactivating the test substance.

X5.9.4 The LD count in the *Neutralizer Toxicity Control* should be within 1 log as compared to the CFU count in the *Test Organism Viability Control*. More than 1 log difference would indicate that the neutralizer itself is harmful to the viability of the test organism.

X5.9.5 No CFU or significantly less CFU than the CFU of the *Test Organism Viability Control* should be present on the *Test Chemical Control*.

X5.9.6 No CFU 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.

X5.9.7 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.

TABLE X5.1 Summary of Neutralization Verification Assay

Treatments/Controls	Addition to petri plates[A]				Treatment Description	Anticipated Outcomes
	Test Chemical	Neutralizer	Test Organism	PBS		
Test Organism Viability Control (TVC)	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 1 log difference
Neutralizer Toxicity Control (NTC)	N/A	√	√	N/A	To measure if neutralizer has any microbicidal activity **(C)**	**A-C** = within 0 to 1 log difference
Optional: Test Chemical Control (TCC)	√	N/A	√	√	To demonstrate that the residual AI is effective **(D)**	No growth or significantly less than **A**
Carrier Sterility Control (CSC)	N/A	√	N/A	N/A	Sterility assessment of carrier and neutralizer	No growth

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

RELATED MATERIAL

Eaton, A. D., Clesceri, L. S., Rice, E. W., Greenberg, A. E. and Franson, M. A. H. eds., *Standard Methods for the Examination of Water and Wastewater, 21st Edition*, American Public Health Association, Washington, DC, 2005.

ASTM Test Methods E1054 for Evaluation of Inactivators of Antimicrobial Agents

ASTM Practice E2362 for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection

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