Designation: E3363 – 23

Standard Test Method for Quantitative Performance Evaluation of Antimicrobial Towelettes[1]

This standard is issued under the fixed designation E3363; 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.

1. Scope

1.1 This test method quantitatively determines the effectiveness of various sizes of antimicrobial towelettes in treating hard, non-porous surfaces against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

1.2 This test method may be used to evaluate towelettes for antimicrobial efficacy against additional microorganisms (with necessary modifications).

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

1.3 This test method involves the use of hazardous materials, chemicals, and infectious microorganisms and therefore should be performed only by those trained in microbiological techniques in facilities designed and equipped for work with infectious agents at the appropriate biosafety level, a BSL-2 or higher laboratory; specifications provided in the "Biosafety for Biomedical and Microbiological Laboratories" (BMBL), 6th edition (BMBL).

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

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 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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.8 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E1054 Practices for Evaluation of Inactivators of Antimicrobial Agents

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

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *AOAC Standards:*[3]

Official Method 955.15 Use-Dilution Method for Testing Disinfectants against Staphylococcus aureus. Revised 2013

Official Method 964.02 Use-Dilution Method for Testing Disinfectants against Pseudomonas aeruginosa. Revised 2013

2.3 *Centers for Disease Control:*[4]

Biosafety in Microbiological and Biomedical Laboratories 6th Edition, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health, HHS Publication No. (CDC) 21- 1112, Revised June 2020.

2.4 *U.S. Government Regulations:*[5]

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

[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.

Current edition approved Sept. 1, 2023. Published September 2023. DOI: 10.1520/E3363–23.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] For referenced AOAC standards, visit the AOAC website, www.aoacofficialmethod.org/index.

[4] Available from https://www.cdc.gov/labs/BMBL.html.

[5] Available from U.S. Government Publishing Office (GPO), 732 N. Capitol St., NW, Washington, DC 20401, http://www.gpo.gov.

3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard refer to Terminology E2756.

3.1.2 *antimicrobial towelette (wipe), n*—a piece of porous material pre-saturated or impregnated with an antimicrobial liquid that is meant for decontamination of environmental surfaces by wiping.

3.1.3 *carriers, n*—sterile plastic (for example, polystyrene) Petri plates (150 mm by 15 mm).

3.1.4 *colony forming unit (CFU), n—in microbiology*, a visible mass of cells (algae, bacteria, or fungi) originating from either an individual cell or cluster of cells that have been placed onto or dispersed into a solid or semi-solid nutrient medium and subsequently incubated under prescribed conditions.

3.1.5 *diluted test suspension, n*—microbial suspension with no soil.

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

3.1.7 *final test suspension, n*—microbial suspension with the 3-part soil load (25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock, and 340 µL microbial test suspension).

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

3.1.9 *soiled carrier, n*—Petri plates (see 3.1.3) spotted with sterile 5 % non-heat inactivated fetal bovine serum.

3.1.10 *soil suspension, n*—3-part soil load, comprised of 25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock.

NOTE 1—Fetal bovine serum or other animal serum as desired may be added to the bacterial suspension to achieve the desired level of soil depending on the target regulatory agency and claim desired.

3.1.11 *treated carrier, n*—Petri plates (see 3.1.3) inoculated with final test suspension.

3.1.12 *two carrier (2-carrier) set, n*—one dried soiled carrier and one dried treated carrier.

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 *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The test method may be evaluated with additional microorganisms, though modification might be necessary to accommodate recovery of surviving organism.

4.2 Petri plates, spotted with a suspension of sterile 5 % non-heat-inactivated fetal bovine serum, are used as the soiled carriers. Each soiled carrier is spotted with five spots (10 µL each) of sterile 5 % non-heat-inactivated fetal bovine serum and allowed to dry.

4.3 Petri plates, inoculated with a suspension of vegetative bacteria and 3-part soil, are used as the treated carriers. Each treated carrier is inoculated with five spots (10 µL each) of the final test suspension and allowed to dry.

4.4 One dried soiled carrier and one dried treated carrier are together referred to as a 2-carrier set.

4.5 The dried carriers are exposed to the test substance by applying a pre-saturated towelette to the inner bottom surface of the carriers using a prescribed pattern of wiping, and the treated carrier is allowed to stand for a specific contact time. At the end of the contact time, two aliquots of an appropriate neutralizer are added to the treated carrier, one prior to the initial scraping of the carrier surface and the second aliquot prior to the secondary scraping. Both done with a sterile cell scraper at each instance to dislodge and suspend inoculum remaining on the surface. The inoculum-neutralizer suspension is collected, diluted, and enumerated using membrane filtration and plated onto recovery media (Tryptic Soy Agar).

4.6 Control and treated carrier CFUs are enumerated using membrane filtration (Practice D5465).

4.7 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 plastic Petri plate (carrier) provides a closed system for enumeration and easy application of a pre-saturated or impregnated antimicrobial towelette by an analyst.

5.2 Aliquoting of sterile 5 % non-heat-inactivated fetal bovine serum (five 10 µL spots) onto soiled carriers and inoculation of final test suspension onto treated carriers (five 10 µL spots) is conducted using a template and a positive displacement pipette, thereby ensuring a precise inoculum level and uniform distribution of soil and final test suspension.

5.3 A single towelette is tested per 2-carrier set, eliminating the likelihood of cross contamination between carriers.

5.4 The corkscrew-patterned circular motion of the product application (wipe outside to inside, wipe inside to outside using the wiping template; see Annex A3 – Annex A6) ensures uniform coverage and contact of disinfectant with the inoculated surface.

5.5 The addition of neutralizer to the treated carriers at the end of the contact time results in neutralization of the test substance. This standard test method provides a procedure for performing neutralization verification to confirm that the microbicidal, microbistatic, or both types of activity of a test substance has been reduced by 50 % at the end of the contact time (see Annex A1 for neutralization verification procedure).

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

5.7 It is optional to adjust (dilution in PBS) the inoculum to achieve desired control counts of 5.0 $\log_{10}$ CFU/carrier to 6.5 $\log_{10}$ CFU/carrier.

5.8 Include, where applicable, comparisons of the test to other similar procedures such as Practices E1054 and E2362.

6. Apparatus

6.1 *Autoclave (Steam Sterilizer)*—To sterilize media and reagents.

6.2 *Centrifuge (with Rotor Capable of Achieving 5 000 g)*—For test culture preparation.

6.3 *Freezer*—To maintain appropriate temperature of 5 % non-heat-inactivated FBS and 3-part soil reagents.

6.4 *Identification System (Optional)*—For test microbes to appropriately identify bacteria.

6.5 *Micropipette*—100 µL, calibrated.

6.6 *Non-Humidified Incubator*—Capable of maintaining temperature(s) at 36 °C ± 1 °C.

6.7 *Petri Plates*—Pre-sterilized, non-coated plastic (for example, polystyrene) Petri plates used as soiled and treated carriers (150 mm by 15 mm).

6.8 *Positive Displacement Pipette/ Repeater Pipette*—Calibrated, used to dispense 10 µL aliquots of a designated suspension onto a soiled or treated carrier (see Annex A8).

6.9 *Refrigerator*—To maintain appropriate temperature of media, reagents, and test suspension.

6.10 *Sterile Cell Scraper*—To scrape Petri plates for removal of bacteria during neutralizing (for example, scraper blade dimensions = 1.8 cm to 3.0 cm) (see Annex A7).

6.11 *Sterile Forceps*—To handle membrane filters. Straight or curved with smooth flat tips.

6.12 *Sterile Glass Test Tubes*—Reusable or disposable borosilicate glass 20 mm by 150 mm with Morton closures for dilution blanks and cultures/subcultures or other appropriate size.

6.13 *Serological Pipettes*—Sterile single-use pipettes (for example, (25.0, 10.0, 5.0, 2.0, 1.0) mL capacity).

6.14 *Sterile Polyethersulfone Membrane (PES) Filters*—To filter serial dilutions for cell enumeration (0.2 µm pore size). Filtration units (utilizing 0.2 µm pore size PES membrane filters), reusable or disposable, may be used.

6.15 *Test Tube Racks*—Any convenient size.

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

6.17 *Vacuum Source*—For filtering test solutions. In-house line or vacuum pump.

6.18 *Vortex-Style Mixer*—For vortex-mixing of various solutions.

7. Reagents and Materials

7.1 *Conical Tubes*—Sterile, 15 mL and 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.

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

7.3 *Culture Media:*

NOTE 2—All percentages prescribed in this Test Method are volume fraction %.

7.3.1 *10 % Dextrose Solution*—For use in rehydrating lyophilized/frozen vegetative culture of test microorganism. Add 5.0 g dextrose to 50 mL de-ionized water and mix by stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution at 5 °C ± 3 °C for up to 30 days.

7.3.1.1 Prior to inoculation, on the day inoculated, use a calibrated pipette to aseptically add 100 µL of 10 % sterile dextrose (w/v) solution to each 10 mL tube of SB (see 10.2).

7.3.2 *Synthetic Broth (SB)*—For use in rehydrating lyophilized/frozen vegetative culture of test microorganism. Commercial media (HIMEDIA[6], Synthetic Broth, AOAC, #M334-500G). Store prepared SB at 2 °C to 8 °C.

7.3.2.1 Alternatively, SB made in-house per the recipe provided in Annex A14 and AOAC Methods 955.15 and 964.02 may be substituted.

7.3.3 *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's blood (BAP) may be substituted.

7.3.4 *Trypticase Soy Agar with 5 % Sheep's Blood (BAP)*—For performing streak isolation of microbial cultures as a purity check (quality control purposes); may be used as a substitute for TSA.

7.3.5 *Trypticase Soy Broth (TSB), (30 g/L)*—For use in rehydrating lyophilized/frozen vegetative culture of test microorganism. Prepare TSB according to manufacturer's instructions.

7.3.6 *AOAC Nutrient Broth (NB)*—For use in preparation of Nutrient Agar (NA), Commercial media (HIMEDIA, Nutrient Broth, AOAC, #M1680). Store prepared NA at 2 °C to 8 °C.

7.3.7 *Nutrient Agar (NA)*—For use in propagation. Purchase plates from a reputable source or prepare as follows: Dissolve 1.5 % Bacto Agar (Difco[7]) in AOAC Nutrient Broth; final pH should be 7.3 ± 0.1. Steam sterilize at 121 °C for 20 min. See Note 3.

NOTE 3—Commercially dehydrated media that conform to the recipes may be substituted.

7.4 *Reagents:*

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

7.4.2 *Organic Soil*—Organic burden used for both soiled carriers (that is, non-heat inactivated fetal bovine serum) and for creating final test suspension used for treated carriers (that is, 3-part soil (BSA, Yeast Extract, Mucin)).

7.4.2.1 *Bovine Serum Albumin (BSA)*—Required for preparation of final test suspension. Add 0.5 g BSA to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot, and store frozen at –20 °C ± 2 °C for up to one year. Aliquots are single use only; do not refreeze once thawed.

7.4.2.2 *Yeast Extract*—Required for preparation of final test suspension. Add 0.5 g yeast extract to 10 mL of PBS, mix, and

[6] HIMEDIA is a registered trademark of HiMedia Technology Limitied.
[7] DIFCO is a registered trademark of Becton, Dickinson and Company.

pass through a 0.2 µm pore diameter membrane filter, aliquot, and store frozen at –20 °C ± 2 °C for up to one year. Aliquots are single use only; do not refreeze once thawed.

7.4.2.3 *Mucin*—CAS# 84195-52-8; Required for preparation of final test suspension. Add 0.04 g mucin (from bovine submaxillary gland or equivalent) to 10 mL of PBS, mix thoroughly until dissolved (see Note 4), and pass through a 0.2 µm pore diameter membrane filter (do not steam sterilize), aliquot, and store frozen at –20 °C ± 2 °C for up to one year. Aliquots are single use only; do not refreeze once thawed.

NOTE 4—Mucin may require vigorous stirring or vortex-mixing to fully dissolve.

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

7.4.4 *Neutralizer Medium*—To use as chemical neutralizer based on active ingredients (for example, letheen broth, letheen broth with 0.1 % sodium thiosulfate).

7.4.4.1 *Non-Heat-Inactivated Fetal Bovine Serum (FBS)*—(5 % (v/v) FBS diluted in PBS) to be used for soiled carriers (not inoculated with the test microbe).

7.4.5 *Phosphate-Buffered Saline Stock Solution (PBS-SS) (Optional)*—Prepare 10× stock solution of PBS by dissolving 492 g PBS powder in 5 L of deionized water.

7.4.6 *Phosphate-Buffered Saline (PBS) 1× Solution*—Purchase from a reputable supplier or prepare as follows: Dilute 9+1 [1-part (PBS-SS) 10X solution) plus 9 parts deionized water] to obtain 1× solution, distribute into bottles and steam sterilize for 20 min at 121 °C.

7.4.7 *Ethanol*—volume fraction = 70 %. To spray gloves and prepare external surface of towelette container/canister/flatpack for use.

8. Test Organisms

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

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

9. Generation of Frozen Stock Cultures

9.1 Frozen stock cultures are single use only and should be approximately 10^9 CFU/mL.

9.1.1 Prepare new frozen stock cultures from lyophilized cultures of *P. aeruginosa* (ATCC 15442) and *S. aureus* (ATCC 6538) at least every 18 months.

9.1.1.1 New frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source. Begin process at step 9.1.4 below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.

9.1.2 Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube containing 5 mL to 6 mL of TSB (30 g/L), aseptically withdraw 0.5 mL to 1.0 mL and rehydrate the lyophilized culture.

9.1.3 Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly by vortexing. Incubate broth culture at 36 °C ± 2 °C for 24 h ± 2 h.

9.1.4 After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plate for 24 h ± 2 h at 36 °C ± 2 °C.

NOTE 5—Perform a streak isolation of the broth culture onto TSA with 5 % sheep's blood (BAP) as a purity check.

9.1.5 Select three to five isolated colonies (from the TSA plate, 9.1.4) of the test organism and re-suspend into 1 mL of TSB (30 g/L). For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa*—the stock culture should be representative of all phenotypes present on the streak isolation plate (select all phenotypes when resuspending). Spread plate 0.1 mL of the suspension on each of 6 to 10 TSA plates. Incubate the plates for 24 h ± 2 h at 36 °C ± 2 °C.

9.1.5.1 If necessary, to obtain more frozen stock cultures, a larger suspension (for example, 2 mL) may be prepared using the same ratio of TSB (1 mL) to number of colonies (3 colonies to 5 colonies).

9.1.6 Following the incubation of the agar plates from 9.1.5, place approximately 5 mL sterile cryoprotectant solution (TSB with 15 % glycerol) on the surface of each plate.

9.1.7 Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface.

9.1.8 Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold approximately 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

9.1.9 Immediately after mixing, dispense aliquots (0.5 mL to 1.0 mL) of the harvested suspension into cryovials; these represent the frozen stock cultures. Within 60 min after harvesting, store the cryovials at ≤–70 ºC for a maximum of 18 months.

10. Test Organism Preparation

10.1 Defrost a single cryovial at room temperature (for example, 21 °C ± 4 °C) and briefly vortex to mix. Defrosting should be rapid to avoid loss in the viability of the preserved cells (for example, expose to running room temperature water to thaw). Each cryovial is for single use only.

10.2 Prior to inoculation, on the day inoculated, use a calibrated pipette to aseptically add 100 µL of 10 % sterile dextrose (w/v) solution to each 10 mL tube of SB.

10.2.1 Using a calibrated micropipette (6.5), add 100 µL of defrosted stock culture to 10 mL SB with 100 µL 10 % (v/v) dextrose solution (see 7.3.1). Briefly vortex-mix and incubate for 24 h ± 2 h at 36 °C ± 1 °C.

10.3 In addition, inoculate an agar plate (for example, TSA or BAP) from the inoculated tube and streak for isolation. Incubate plate with the test culture.

10.4 Following incubation, use the broth culture to prepare a test suspension for the organism.

10.5 For *P. aeruginosa*, inspect culture prior to harvest; discard if no visible pellicle has formed or if pellicle has been disrupted (fragments in culture). Do not vortex-mix or shake the 24 h ± 2 h test culture. Remove visible pellicle on surface of medium and around associated interior edges of the tube with vacuum suction.

10.5.1 Using a serological pipette, withdraw the remaining broth culture (approximately 7 mL to 8 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL conical centrifuge tube.

10.5.1.1 If necessary, the culture may be harvested from two 10 mL 24 h ± 2 h broth cultures to harvest and centrifuge a maximum of 10 mL of *P. aeruginosa* culture.

10.6 For *S. aureus*, briefly vortex-mix the 24 h ± 2 h culture and transfer the entire contents to a 15 mL conical centrifuge tube.

10.7 Centrifuge the 24 h ± 2 h harvested broth cultures at 5,000 g for 20 min.

10.8 Remove the supernatant without disrupting the pellet. Resuspend the pellet in 5 mL to 10 mL PBS to target control counts that are 5.0 CFU/carrier to 6.5 $\log_{10}$ CFU/carrier. The resuspended pellet is the diluted test suspension.

10.8.1 If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in 5 mL to 10 mL PBS. If necessary, add a small portion of the total volume (5 mL to 10 mL) of PBS to the pellet to aid in the disaggregation.

10.8.2 Use this diluted test suspension within 30 min to prepare the final test suspension.

10.8.3 If desired, determine titer of the diluted test suspension and the corresponding Optical Density at 650 nm or another appropriate wavelength.

10.9 Vortex-mix the diluted test suspension for 10 s to 30 s.

10.10 To obtain the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette: 25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock. This is the soil suspension.

10.10.1 Vortex-mix the soil suspension for 10 s prior to adding the diluted test suspension.

10.10.1.1 Vortex-mix the diluted test suspension for 10 s. Combine 340 µL diluted test suspension to the soil suspension. This is the final test suspension.

10.10.1.2 Use this final test suspension to inoculate carriers within 30 min of preparation.

10.10.2 If necessary, the final test suspension can be refrigerated for up to 2 h (at 2 °C to 8 °C) after initial preparation.

10.10.3 Streak inoculate an agar plate of non-selective medium (for example, TSA or BAP) with a loopful of the final test suspension. Incubate plate with the treated and control carrier plates and examine for purity after incubation at 36 °C ± 1 °C for 72 h ± 4 h.

11. Carrier Inoculation

11.1 Use sterile plastic Petri plates (150 mm by 20 mm) for the soiled and treated carriers. See Annex A13 for pictorial representation of test assay.

11.2 *Soiled Carriers:*

11.2.1 Use sterile 5 % non-heat-inactivated FBS as the organic soil.

11.2.2 Prepare soiled carriers at room temperature (21 °C ± 4 °C).

11.2.3 Using the inoculation template placed beneath the Petri plates to standardize the location of the soil placement sites (see Annex A2), apply five 10 µL spots of 5 % FBS to the inside bottom surface of each Petri plate using a positive displacement/ repeater pipette (6.8).

11.2.4 Inoculate one soiled carrier for each treated carrier (2-carrier set). Prepare extra soiled carriers, as necessary.

11.2.5 Streak inoculate an agar plate of non-selective medium (for example, TSA or BAP) with a loopful of remaining 5 % FBS. Incubate plate with the treated and control carrier plates and examine for purity after incubation at 36 °C ± 1 °C for 72 h ± 4 h.

11.3 *Treated Carriers:*

11.3.1 Use final test suspension as prepared in Section 10 within 30 min of preparation at room temperature (21 °C ± 4 °C) or within 2 h of initial preparation if refrigerated at 5 °C ± 3 °C (10.10.1.2 and 10.10.2).

11.3.2 Inoculate treated carriers at room temperature (21 °C ± 4 °C).

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

11.3.4 Using the inoculation template placed beneath the Petri plates to standardize the location of the inoculation sites (see Annex A2), using the final test suspension, inoculate the inside bottom surface of each Petri plate with five 10 µL spots using a positive displacement pipette (6.8).

11.3.5 Inoculate enough plates with the final test suspension for at least 3 controls (per challenge organism) and a minimum of 5 treated carriers per test condition. Prepare extra inoculated treated carriers, as necessary.

11.4 Dry soiled and inoculated treated carriers in a non-humidified incubator with lids ajar, as shown in Annex A9, at 36 °C ± 1 °C for 30 min to 45 min until soil and inoculation spots appear visibly dry. Visually inspect each carrier to ensure complete drying.

11.4.1 Do not use carriers in which soil or inoculation spots have coalesced.

11.4.2 Assay (wipe) and neutralize soiled and inoculated treated carriers for product testing within 60 min after drying.

11.4.2.1 If holding time (60 min) for carriers is exceeded, additional carrier inoculations may be conducted within the same day using the same sterile 5 % non-heat inactivated FBS and final test suspension (if previously refrigerated at 2 °C to 8 °C and used within 2 h of initial preparation of final test suspension (10.10.2)).

12. Carrier Load Enumeration (Control Carrier Counts)

12.1 One control carrier is evaluated immediately prior to commencing the test and two control carriers are evaluated

immediately following the test to assess inoculated treated carrier population (carrier counts). All carriers must be processed (assayed and neutralized) within 60 min after drying to ensure an appropriate microbial challenge at the time of testing (see 11.4.2.1).

12.1.1 The two control carriers evaluated immediately following the test should be processed after neutralization of all treated carriers is completed.

12.1.2 Prior to commencing the test, the first control carrier is evaluated, appropriately diluted (see 13.8 and 13.9), filtered and plated (see 13.10 – 13.17).

12.1.2.1 If serial dilutions are not filtered and plated immediately, keep the PBS dilution tubes at 2 °C to 8 °C until this step can be completed.

12.2 For control carrier counts, add 20 mL of the neutralizer to each control carrier. Gently swirl to spread neutralizer across the entire inner bottom surface of the carrier. Use a sterile cell scraper to gently scrape across the middle of the carrier (where inocula were added) with 6 to 8 passes (a pass is defined as moving over the carrier from one side to the other (for example, left to right, top to bottom) and gently swirl to mix. For example, if scraping started on the left side of the carrier and moved to the right side (one pass), then back to left (2 passes), then back to right (3 passes) and then back to left, a total of 4 passes would have occurred.

12.2.1 The inoculation template may be used under the carriers to highlight/locate inoculation spots for recovery.

12.3 Use a serological pipette to transfer the suspension into a sterile 50 mL conical tube. Carefully tilt the carrier as necessary to collect the suspension.

12.4 Add a second 20 mL aliquot of neutralizer to the control carrier. Gently swirl to spread neutralizer across the entire inner bottom surface of the carrier. Use a second sterile cell scraper to gently scrape across the middle of the carrier (where inocula were added) with 6 to 8 total passes (see 12.2) and gently swirl to mix. Use a serological pipette to transfer suspension to the same 50 mL conical tube to combine it with the first 20 mL aliquot.

12.5 Vortex-mix the 40 mL suspension for 10 s ± 5 s; this tube is the 10^0 dilution.

12.6 Prepare serial dilutions and enumerate inoculum using membrane filtration and plating on TSA.

12.7 Prior to filtration, pre-wet each 0.2 µm PES membrane filter with approximately 10 mL sterile PBS; apply vacuum to filter contents.

12.7.1 Add the sample to pre-wetted PES membrane filter in the filter unit.

12.7.1.1 For dilution tubes, rinse tube once with approximately 10 mL sterile PBS, briefly vortex-mix, and pour into filter unit.

12.7.2 Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

12.7.3 Rinse the inside surface of the funnel unit with at least 20 mL sterile PBS and filter the contents.

12.8 Use sterile forceps to gently place each filter (13.17) on a separate TSA plate and incubate at 36 °C ± 1 °C for 48 h ± 4 h and record CFU/carrier.

12.9 Dilutions resulting in up to 200 CFUs per filter are deemed acceptable for counting purposes; all counts up to 200 are used in the calculations (refer to Practice D5465). CFUs greater than 200 are indicated as Too Numerous to Count (TNTC).

12.10 Calculate the viable CFU/carrier. Account for all dilutions that result in up to 200 CFU and the 40 mL neutralizer volume in the calculations.

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

13. Procedure

13.1 Verify the sterility of all filtered reagents and media (for example, PBS, neutralizer) used in the study prior to or concurrent with testing.

13.2 It is recommended to perform assay inside the Biological Safety Cabinet (BSC).

13.3 *Towelette Sample Preparation (for formulated products):*

13.3.1 Record ambient temperature and humidity.

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

13.3.3 Ensure an even distribution (for example, invert to mix 3 to 5 times) of liquid and wipes in advance of removing towelettes and prior to opening the container.

NOTE 6—If possible, remove 3 to 5 wipes from towelette container/packaging and discard prior to commencing the test.

13.3.4 Change gloves as necessary to maintain sterility.

13.3.5 Use a new pair of sterile gloves (or non-sterile gloves sprayed with 70 % (v/v) ethanol) when preparing to handle each test towelette.

13.3.5.1 Gloves sprayed with 70 % (v/v) ethanol must be completely dry before proceeding with step 13.3.6.

13.3.6 Remove one towelette from the container and prior to wiping, unfold towelette completely if folded or wrinkled. Then, gently fold the towelette symmetrically in half along the short edge of the towelette (if rectangular in shape). Rotate the folded towelette 90° and again, symmetrically fold in half along the short edge of the towelette. Repeat to achieve a target of approximately 2.5 cm by 2.5 cm to 6.4 cm by 6.4 cm. See Annex A10 for folding schematic.

NOTE 7—Wipes smaller than 5.1 cm by 5.1 cm may be folded once to fit the dimensions.

13.3.7 Hold towelette between the thumb and the index and middle fingers, minimizing contact with the towelette (Annex A11). Do not squeeze the towelette. Do not position a finger in between folds. Use only smooth folded edge for wiping.

NOTE 8—Towelettes of rectangular shape should have the first fold made symmetrically along the short edge of the towelette.

NOTE 9—Excessive folding can result in over expression of liquid.

NOTE 10—Because towelettes are diverse in size, matrix composition, and packaging, the towelette removal and folding process should be practiced in advance of testing and documented accordingly.

13.4 *Wiping:*

13.4.1 Application is a timed exercise; use a calibrated timer to track the approximate wiping application time and the

contact time. Initiate wiping beginning with the soiled carrier of the first 2-carrier set. The contact time begins after completion of wiping of the inoculated treated carrier.

13.4.1.1 Discard the soiled carrier after wiping; soiled carriers are not evaluated for recovery of the test microbe.

13.4.2 Avoid contact of the towelette with the inside rim of the carrier.

13.4.3 Remove lid and secure the carrier in one hand (non-wiping hand) with wiping template securely positioned under the carrier.

13.4.4 While maintaining contact of the folded edge of the towelette (without lifting from surface) wipe inoculated surface with consistent pressure following the "corkscrew" pattern on the wiping template by starting with three (3) revolutions from the outer margin of the plate inward towards inoculated area. Then without lifting the wipe, continue reverse wiping and perform three circular revolutions from center to outer margin of the plate (ending at the initial starting point of wiping) to complete the wiping process. Annex A3 and Annex A4 (clockwise wiping) or Annex A5 and Annex A6 (counterclockwise wiping) illustrate the wiping template (to scale).

13.4.5 To assist in maintaining a consistent pattern of wiping and coverage, avoid holding the towelette perpendicularly to the surface of the carrier; per Annex A12, hold the towelette at a visually estimated 30° to 45° angle from the carrier surface.

13.4.6 The wipe pattern and pressure used while wiping should be consistent from plate to plate.

13.4.7 The towelette and hand holding towelette should remain pointing in the same direction throughout the wiping process and does not rotate.

13.4.8 The entire wiping process should take approximately 6 s to 8 s per carrier (3 s to 4 s inwards, 3 s to 4 s outwards). The conclusion of the wiping process begins the contact time.

13.4.9 Use one antimicrobial towelette per 2-carrier set; discard the towelette in biohazard bin after use.

13.4.10 Treated carriers, with lids ajar, must be kept undisturbed and horizontal during the contact time.

13.4.11 For products with a contact time of >1 min (10 min, maximum), neutralize within ±5 s of the contact time.

13.4.12 For products with a contact time of ≤1 min, neutralize within ±3 s of the contact time.

13.4.13 At the completion of the contact time, add 20 mL of the neutralizer to the treated carrier. Gently swirl to spread neutralizer across the entire inner bottom surface of the carrier. Use a sterile cell scraper to gently scrape across the middle of the carrier to dislodge and suspend the inoculum with 6 to 8 total passes (see 12.2) and gently swirl to mix.

13.4.13.1 The inoculation template may be used below the carriers to highlight/locate inoculation spots for recovery.

13.5 Use a serological pipette to transfer the suspension into a sterile 50 mL conical tube. Carefully tilt the carrier as necessary to collect the suspension.

13.6 Add a second 20 mL aliquot of neutralizer to the treated carrier. Gently swirl to spread neutralizer across the entire inner bottom surface of the carrier. Use a second sterile cell scraper to gently scrape across the middle of the carrier (where inocula were added) with 6 to 8 total passes (see 12.2) and gently swirl to mix. Use a serological pipette to transfer suspension to the same 50 mL conical tube to combine it with the first 20 mL aliquot.

13.7 Vortex-mix the 40 mL suspension for 10 s ± 5 s (this tube is considered the 10^0 dilution).

13.8 Within 30 min of neutralization and vortex-mixing, prepare serial dilutions as necessary to achieve countable colonies in the target range of up to 200 colonies per filter for both treated and control carriers.

13.9 Serial dilutions are then enumerated using membrane filtration. For treated carriers, filter the entire contents of the 40 mL suspension (the 100 dilution); the entire contents of other dilutions may be filtered as necessary.

13.10 Initiate filtration of samples within 30 min of preparing serial dilutions.

13.11 Turn on vacuum and leave on for the duration of the filtration process.

13.12 Use separate membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.

13.13 Prior to filtration, pre-wet each 0.2 µm pore size PES membrane filter with approximately 10 mL of PBS immediately prior to use.

13.14 Rinse each dilution tube once with approximately 10 mL PBS, briefly vortex mix and pour the rinse into the same filter unit.

13.15 Rinse 40 mL (10^0) suspension tubes with approximately 20 mL PBS, briefly vortex-mix and pour the rinse into the same filter unit.

13.16 Rinse the inside of each funnel unit with approximately 20 mL PBS and filter the rinsing liquid through the same filter membrane. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

13.17 Aseptically remove the membrane filter with sterile forceps and place it carefully over the surface of the recovery medium. Avoid trapping any air bubbles between the filter and the agar surface.

13.18 For control carriers, incubate at 36 °C ± 1 °C for 48 h ± 4 h and record CFU/filter.

13.19 For treated carriers, incubate at 36 °C ± 1 °C for 72 h ± 4 h and record CFU/filter.

13.20 If desired, plates can be scored initially after 24 h of incubation.

13.21 *Sterility Controls:*

13.21.1 On the day of the test, filter ~20 mL of neutralizer and ~20 mL of the PBS used in the test using two separate membrane filters and place on TSA.

13.21.2 Incubate sterility filters along with a single, empty plate of recovery medium for 72 h ± 4 h at 36 °C ± 1 °C, record sterility results.

13.22 Calculate the viable CFU/carrier. Dilutions resulting in up to 200 CFUs per filter are deemed acceptable for counting purposes; all counts up to 200 are used in the calculations (Practice D5465). CFUs greater than 200 are indicated as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

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

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

14. Calculations

14.1 Use values with at least three significant figures when performing calculations (for example, log density, mean log density). Report log reduction values with at least two significant figures (for example, round up to nearest tenth).

14.2 The $\log_{10}$ density (LD) for each treated and control carrier is calculated as follows:

$$\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i \times D_i)}\right] \times V\right\}, \quad (1)$$

where:

Y = CFU per filter,
C = volume filtered,
V = total volume of neutralizer,
D = 10^{-k},
k = dilution,
n = number of dilutions, and
i = lower limit of summation (the fewest number of dilutions).

14.3 Calculate the mean LD for three control carriers as follows (where CC = control carrier):

$$\text{Mean LD} = [\mathrm{Log}_{10}(\text{CC 1}) + \mathrm{Log}_{10}(\text{CC 2}) + \mathrm{Log}_{10}(\text{CC 3})] / 3 \quad (2)$$

14.4 Calculate the mean LD for each set of treated carriers as follows (where TC = treated carrier):

$$\text{Mean LD} = [\mathrm{Log}_{10}(\text{TC1}) + \mathrm{Log}_{10}(\text{TC2}) + \mathrm{Log}_{10}(\text{TC3}) + \mathrm{Log}_{10}(\text{TC4}) + \mathrm{Log}_{10}(\text{TC5})] / 5 \quad (3)$$

NOTE 11—For the purpose of calculation, if no organism is recovered from a test carrier, the log density for that carrier is 0 provided that the entire contents of the 10^0 dilution were filtered.

14.5 Calculate the LR for each test chemical as follows (where CC = control carrier; TC = treated carrier):

$$\text{LR} = \text{Mean LD(CCs)} - \text{Mean LD(TCs)} \quad (4)$$

NOTE 12—If no organism is recovered from each of the five test carriers, the log reduction is greater than or equal to the mean control carrier log density.

15. Precision and Bias[8]

15.1 *Precision*[9]—The repeatability standard deviation within a single laboratory was determined.

15.2 Two statements concerning the precision of this method can be made for 150 mm pre-sterilized plastic non-coated Petri plate carriers, one for the mean log density (LD) of the control carriers per test and one for the log reduction (LR) of one treatment.

15.3 A study was completed to determine the repeatability of an untreated control towelette wetted with PBS and a treated towelette wetted with PBS + 0.1 % Tween 80. Both towelettes were tested against *P. aeruginosa* and *S. aureus*. The test parameters included three untreated controls and three treated samples enumerated per organism, per test day.

15.4 The control carriers consistently met the acceptance criteria (mean of carriers on each test day between 5.0 to 6.5 $\log_{10}$ (CFU/carrier)).

15.5 The overall mean control carrier counts (control log densities) for *P. aeruginosa* and *S. aureus* were 6.12 and 6.23 $\log_{10}$ (CFU/carrier), respectively. The repeatability SD for the control carriers was 0.2665 for *P. aeruginosa* and 0.0852 for *S. aureus*.

15.6 Data from the treatment provided a log reduction (LR) suitable for determining the method's performance. Mean LR values for *P. aeruginosa* and *S. aureus* were 1.56 and 1.72, respectively. The repeatability SD for the treated carriers was 0.2227 for *P. aeruginosa* and 0.1580 for *S. aureus*.

15.7 *Bias*—No information can be presented on the bias of this procedure for measuring either mean.

16. Reproducibility

16.1 *Reproducibility*—An interlaboratory study of this test method will be conducted and a complete precision statement is expected to be available within five years after this method receives ASTM approval.

17. Keywords

17.1 antimicrobial towelettes; cell scraper; efficacy methods; membrane filtration; Pseudomonas aeruginosa; Quantitative Towelette Method (QTM); Staphylococcus aureus; test culture; wiping contaminated surfaces

[8] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:E35-2005. Contact ASTM Customer Service at service@astm.org.

[9] An interlaboratory study of this test method will be conducted and a complete precision statement is expected to be available on or before December 31st, 2027.

ANNEXES

(Mandatory Information)

A1. NEUTRALIZATION VERIFICATION PROCEDURE

A1.1 Materials

A1.1.1 Antimicrobial towelette.

A1.1.2 Conical tubes, 15 mL, 50 mL.

A1.1.3 Neutralizer medium.

A1.1.4 Phosphate-buffered saline (PBS) 1× solution.

A1.1.5 Pre-sterilized plastic (for example, polystyrene) Petri plates (as used in the test).

A1.1.6 Sterile polyethersulfone membrane (PES) filters—0.2 µm pore size. Filtration units (utilizing 0.2 µm pore size PES membrane filters), reusable or disposable, may be used.

A1.1.7 TSA plates.

A1.1.8 Test culture—prepared in accordance with Section 10.

A1.1.9 Soil load—3-part soil load (comprised of 25 µL BSA stock, 35 µL yeast extract stock, 100 µL mucin stock).

A1.2 Method

A1.2.1 Prepare test organism in accordance with 10.1 – 10.9. Conduct neutralization verification procedure prior to or concurrent with test.

A1.2.2 Prepare *Test Suspension A (without soil load)*. Prepare up to three serial dilutions (for example, 10^{-4}, 10^{-5}, and 10^{-6}) of the microbial test suspension using PBS; conduct preliminary tests as necessary to determine the appropriate dilution(s). Serially dilute the microbial test suspension with PBS. Select appropriate dilution(s) of *Test Suspension A* so that after the addition of the soil load, the *Test Suspension B* will achieve an average challenge of 20 CFU to 200 CFU per 50 µL. Use *Test Suspension A* within 30 min of preparation.

A1.2.2.1 When directed, inoculate carriers and reaction vessels with 50 µL of test suspension to target a final count of 20 CFU to 200 CFU/plate. Follow steps in A1.3.1 – A1.3.4.

A1.2.2.2 Two separate serial dilutions of *Test Suspension A* may be used to prepare three different concentrations of *Test Suspension B* to ensure at least one dilution with an average challenge of 20 CFU to 200 CFU per 50 µL.

A1.2.2.3 A calibration curve (Optical Density at 650 nm) may be used to estimate the number of viable organismsin *Test Suspension A*.

A1.2.3 Prepare *Test Suspension B (with soil load)*. Prepare the 3-part soil load: using a vortex, mix each component and combine in the following order: 25 µL bovine serum albumin (BSA), 35 µL yeast extract stock, and 100 µL of mucin stock; then vortex-mix the solution. Add 340 µL of diluted *Test Suspension A* to the prepared soil load (7.4.2 – 7.4.2.3) and vortex-mix for 10 s. Ensure *Test Suspension B* provides an average challenge of 20 CFU to 200 CFU per 50 µL.

A1.2.3.1 The volumes of each component of the 3-part soil load and the volume of *Test Suspension A* may be scaled up, as necessary, to accommodate the test.

A1.2.4 Prepare 5 % FBS in accordance with 7.4.4.1 and apply to carriers as described in 11.2.3.

A1.2.5 Conduct steps (for example, addition of organism and neutralizer) at timed intervals (for example, 1 min intervals) to ensure consistent time of contact.

A1.3 Carrier Preparation

A1.3.1 For each dilution prepared, use the inoculation template to inoculate 3 carriers with five 10 µL spots of 5 % FBS. Prepare extra carriers, as necessary.

A1.3.2 For each dilution tested, reserve 3 blank sterile carriers (no 5 % FBS, no test organism, no 3-part soil load). Reserve extra carriers, as necessary.

A1.3.3 Carriers inoculated with 5 % FBS are dried as described in 11.4.

A1.3.4 See Table A1.1 for summary of Neutralization Verification Assay.

A1.4 Reaction Vessel Preparation

A1.4.1 For each dilution prepared, prepare three reaction vessels (50 mL conical tubes) filled with 40 mL neutralizer medium, each.

A1.4.2 For each dilution prepared, prepare three reaction vessels (50 mL conical tubes) filled with 40 mL PBS, each.

A1.5 Neutralizer Effectiveness Assay

A1.5.1 For each dilution of *Test Suspension B* prepared, wipe three two-carrier sets (comprised of 1 carrier inoculated with 5 % FBS and 1 blank sterile carrier) with one antimicrobial towelette per two-carrier set. Wipe carriers according to Section 13.

A1.5.2 Carriers are wiped sequentially. Carrier #1 of the two-carrier set, inoculated with five 10 µL spots of 5 % FBS, is wiped first, then, carrier #2, a blank, sterile carrier, is wiped. Once carrier #2 is wiped, the plate is left undisturbed for the duration of the contact time. For multiple contact times, assess at the shortest contact time.

A1.5.3 Immediately after the contact time (±3 s for contact times <1 min; ±5 s for contact times 1 min to 10 min), add 20 mL of the neutralizer to carrier #2 and swirl to mix.

A1.5.4 Immediately (approximately 10 s to 15 s), add 50 µL of the diluted test organism *Test Suspension B* to the carrier. Gently swirl to mix.

A1.5.5 With a serological pipette, transfer test substance-neutralizer-diluted test organism mixture into a sterile 50 mL conical tube.

TABLE A1.1 Summary of Neutralization Verification Assay

	Neutralizer Verification Components	Treatment Description	Towelette Use	Soiled Carrier (5% diluted non-heat-inactivated FBS)	Treated Carrier (sterile, blank; no 5% FBS, no test organism, no 3-part soil load)	Reaction Vessel (50 mL Conical Tube)	Anticipated Outcomes
1.	Neutralizer Effectiveness Assay (NEA)	Inoculum titer for comparative purposes				 Empty, used to collect NEA suspension	The recovered number of CFU is atleast 50% of the **Organism Titer Control.**
2.	Neutralizer Toxicity Control (NTC)	To measure if neutralizer has any microbicidal activity	N/A	N/A	N/A	 Filled with 40 mL neutralizer medium	The recovered number of CFU is atleast 50% of the **Organism Titer Control.**
3.	Organism Titer Control (OTC)	To measure if active ingredient is effectively neutralized	N/A	N/A	N/A	 Filled with 40 mL PBS	The recovered number of CFU is atleast 20 to 200 CFU/filter.

A1.5.6 Add an additional 20 mL of the neutralizer to the carrier, swirl to mix. With a serological pipette, transfer and pool with mixture in conical tube from step A1.5.5.

A1.5.7 Hold the mixture for a minimum of 5 min (approximate time it takes to process all samples after neutralization in sections 13.4.11 – 13.7 at room temperature (21 °C ± 4 °C) prior to filtration. No further dilutions are necessary. Proceed as in A1.8.

A1.6 Neutralizer Toxicity Control (NTC)

A1.6.1 For each dilution tested, use three reaction vessels filled with neutralizer medium as prepared in A1.4.1. In timed intervals, inoculate each reaction vessel with 50 µL of *Test Suspension B*. Vortex-mix for 10 s.

A1.6.2 Hold the mixture for a minimum of 5 min (approximate time it takes to process all samples after neutralization in sections 13.4.11 – 13.7) at room temperature (21 °C ± 4 °C) prior to filtration. No further dilutions are necessary. Proceed as in A1.8.

A1.7 Test Organism Titer Control

A1.7.1 For each dilution tested, use three reaction vessels filled with PBS as prepared in A1.4.2. In timed intervals, inoculate each reaction vessel with 50 µL of *Test Suspension B*. Vortex-mix for 10 s.

A1.7.2 Hold the mixture for a minimum of 5 min (approximate time it takes to process all samples after neutralization in sections 13.4.11 – 13.7) at room temperature (21 °C ± 4 °C) prior to filtration. No further dilutions are necessary. Proceed as detailed in A1.8 for filtration.

A1.8 Filtration and Recovery

A1.8.1 At the conclusion of the holding period, vortex-mix each reaction vessel (50 mL conical tube) for 10 s. Filter each mixture through a separate, pre-wetted 0.2 µm pore size PES membrane filter. Pre-wet each PES membrane filter with approximately 10 mL of PBS immediately prior to use.

A1.8.2 Rinse each reaction vessel with ~20 mL PBS and vortex-mix; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of each funnel unit with ~20 mL PBS and filter the rinsing liquid through the same filter membrane.

A1.8.3 Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium. Incubate the plates at 36 °C ± 1 °C for 72 h ± 4 h; count CFU on filters and record results. If desired, plates can be scored initially after 24 h of incubation (refer to 13.20).

A1.8.4 After incubation, record results as CFU per filter. Colony counts in excess of 200 should be recorded as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

A1.8.5 Anticipated Outcomes

A1.8.5.1 The number of CFU in the Organism Titer Control should be in the range of 20 CFU to 200 CFU/filter.

A1.8.6 Compare the average CFU of the Titer Control with the average CFU of the Neutralizer Toxicity Control and Neutralizer Effectiveness treatment. Determine the percent recovery in CFU.

A1.8.7 For determining the suitability of the neutralizer, ensure that the average CFU in the Neutralizer Toxicity Control is at least 50 % of the Organism Titer Control. A count lower than 50 % indicates that the neutralizer is harmful to the test organism.

A1.8.8 Average CFU for the Neutralizer Toxicity Control that are greater than the Titer Control (for example, 120 % of the Titer Control) are also deemed valid.