Designation: E2362 – 15

Standard Practice for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection[1]

This standard is issued under the fixed designation E2362; 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 practice is designed to evaluate the antimicrobial activity of pre-saturated or impregnated towelettes when used as a hard surface disinfectant.

1.2 It is the responsibility of the investigator to determine whether Good Laboratory Practices (GLP's) are required and to follow them when appropriate.

1.3 This practice should be performed only by those trained in microbiological techniques.

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

1.5 Appropriate modifications to the practice may be required when testing organisms not specified herein.

1.6 *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 to determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

2.2 *Federal Standard*

40 CFR, Part 160 Good Laboratory Practice Standards[3]

3. Terminology

3.1 *carrier, n*—a transportable surface onto which a test organism will be inoculated and dried. The carrier will be treated with the test substance and subcultured for survivors.

3.2 *CFU, n*—colony forming units

3.3 *disinfectant, n*—a physical or chemical agent or process that destroys pathogenic or potentially pathogenic microorganisms in/on surfaces or objects.

3.4 *impregnated, adj*—saturated with test substance.

3.5 *neutralizer, n*—a component used to render an active agent incapable of destroying organisms by chemical or physical means.

3.6 *pre-saturated, adj*—to be filled or impregnated with test substance prior to the time of its intended use.

3.7 *towelette, n*—A paper, cloth or non-woven blend material used as a transporter for a cleaning and/or disinfection agent.

4. Summary of Practice[4]

4.1 A towelette impregnated or pre-saturated with a test substance is used to treat a carrier which has been inoculated with a test organism after an aliquot of a test organism has been inoculated, evenly distributed to an inoculation area of approximately one square inch (approximately 625 mm), and dried onto the carrier. The carrier is wiped using the pre-saturated or impregnated towelette simulating the application of the test substance and then held for a pre-determined contact time. After the specified contact time, the test substance remaining on the carrier is neutralized and the carrier is subcultured to recover surviving test organism.

5. Significance and Use

5.1 This practice may be used to determine if a pre-saturated or impregnated towelette demonstrates antimicrobial effectiveness as a disinfectant on hard surfaces. This practice provides survivor results in the form of a qualitative endpoint (growth

[1] This practice 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 Oct. 1, 2015 Published October 2015. Originally approved in 2004. Last previous edition approved in 2009 as E2362 – 09. DOI: 10.1520/E2362-15.

[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] Available from the Superintendent of Documents, U.S. Government Printing Office, Washington D.C. 20402

[4] United States Environmental Protection Agency, Standard Operating Procedure for Disinfectant Towelette Test Against *Staphylococcus aureus, Pseudomonas aeruginosa, and Salmonella enterica*, EPA/OPP Microbiology Laboratory, Ft. Meade, MD. SOP# MB09-05, Revised 1/30/13.

Copyright © ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959. United States

positive versus growth negative). The results generated by following this practice do not provide for specific quantitative reductions.

6. Apparatus

6.1 *Incubator*—any calibrated incubator that maintains a temperature specific for propagation of organisms. (for example, bacteria and mycobacteria at 36 ± 1 °C and fungi at 27.5 ± 2.5 °C).

6.2 *Sterilizer*—any suitable, calibrated steam sterilizer that produces the conditions of sterilization is acceptable.

6.3 *Test Towelettes*—with instructions for use.

6.4 *Timer (Stop-clock)*—a calibrated timer that displays min and s.

6.5 *Spectrophotometer*—calibrated to 650 nm.

6.6 *Mixer*—a vortex mixer is recommended.

6.7 *pH meter*—a calibrated pH meter to determine the pH of media.

6.8 *Nonporous Test Carriers*—borosilicate glass slides, 25 mm × 75 mm slides, pre-cleaned (or other hard surfaces and sizes as appropriate).

6.9 *Glass Culture Tubes*—20 mm × 150 mm, 25 mm × 150 mm, and 38 mm × 100 mm or 38 mm × 200 mm without lip, or equivalent, sterile.

6.10 *Culture Tube Closures*—appropriate size nontoxic closures.

6.11 *Petri Dishes*—100 mm × 15 mm, glass and plastic, sterile.

6.12 *Balance*—a calibrated balance sensitive to 0.1 g.

6.13 *Micropipettor*—calibrated for dispensing 10 µL.

6.14 *Forceps*—sterilizable or pre-sterilized.

6.15 *Sterilizer Apparatus*—a bunsen burner or other appropriate heat sterilizer.

6.16 *Bacteriological Culture Loop*— 4 mm inside diameter loop of platinum or platinum alloy wire or sterile disposable plastic loops of appropriate size.

6.17 *Colony Counter*—any one of several types may be used, for example Quebec.

6.18 *Gloves*—sterile gloves not possessing antimicrobial properties.

6.19 *Pipette*—sterile volumetric pipettes.

6.20 *Glass Jars*—100 mL or other appropriate vessel.

6.21 *Filter Paper*—9 cm (Whatman No. 2, or equivalent) sterilized prior to use.

6.22 *Thermometer*—calibrated thermometer.

6.23 *Glass Beads*—3 –5 mm sterile beads.

6.24 *Gauze*—sterile cotton gauze.

6.25 *Hemacytometer*—calibrated hemacytometer.

6.26 *Glass Wool*—sterile grease free glass wool.

6.27 *Hot air oven*—ability to maintain ≥180°C.

6.28 *Refrigerator*—calibrated to maintain 5 ± 3°C.

6.29 *Ultra-Cold Freezer,* Calibrated to maintain ≤ -70°C

6.30 *Glass Tissue Grinder or Macerator,* sterile.

6.31 *Sterile cryovials,* (for example, 1.5 mL with screw cap)

6.32 *Centrifuge,* calibrated.

7. Reagents

7.1 *Culture Media—Bacteria*

7.1.1 Nutrient Broth or Synthetic Broth—*Pseudomonas aeruginosa*,

7.1.2 Cystine Trypticase Agar—*Pseudomonas aeruginosa*,

7.1.3 Synthetic Broth—*Salmonella enterica and Staphylococcus aureus*.

7.1.4 Fluid Thioglycollate Broth.

7.1.5 Tryptic Soy Broth (TSB)

7.1.6 Tryptic Soy Broth with 15% v/v glycerol (Cyroprotectant solution)

7.2 *Culture Media—Mycobacteria*

7.2.1 Middlebrook 7H11 or 7H9 Agar Slants.

7.2.2 Modified Proskauer-Beck Broth.

7.3 *Culture Media—Fungi*

7.3.1 Sabouraud Dextrose Agar plates/Glucose Agar plates.

7.3.2 Sabouraud Dextrose Agar slants/Glucose Agar slants.

7.4 *Neutralizing Subculture Media*—A neutralizing growth medium capable of supporting the growth of the test organism following exposure to the test material in accordance with E1054. For Mycobacterium, horse serum (which may be supplemented with additional neutralizers) is recommended.

7.5 *Subculture Agar*

7.5.1 Tryptic Soy Agar with or without sheep blood—Bacteria.

7.5.2 Middlebrook 7H11 Agar—Mycobacteria.

7.5.3 Sabouraud Dextrose Agar or Glucose Agar—Fungi.

7.6 *Subculture Media*—Mycobacteria

7.6.1 Modified Proskauer-Beck Broth[5]

7.6.2 Kirchner's Medium[5]

7.6.3 Middlebrook 7H9 Broth or TB broth

7.7 Other subculture agars, broths and neutralizers may be used where appropriate.

7.8 *Soil*—Blood Serum, such as heat inactivated fetal bovine serum or other appropriate alternative soil.

7.9 *Dilution Fluid*—sterile phosphate buffered water (PBDW), sterile saline or Butterfield's Buffer. (See Specification D1193.)

7.10 Sterile saline + 0.05% v/v Triton X-100

7.11 Sterile 0.1% v/v Polysorbate (Tween) 80

7.12 *Carrier Preparation Solutions*—70 to 95 % isopropyl alcohol, deionized or distilled water.

[5] AOAC Official Method 965.12 Tubcerculocidal Activity of Disinfectants. AOAC International, Chapter 6.

8. Test Organisms

8.1 *Bacterial Test Organisms:*

8.1.1 *Staphylococcus aureus* (ATCC 6538), *Salmonella enterica* (ATCC 10708), and *Pseudomonas aeruginosa* (ATCC 15442)-received lyophilized.

8.1.2 Other bacterial organisms may be tested using appropriate culture and subculture procedures.

8.2 *Mycobacterial Test Organism:*

8.2.1 *Mycobacterium bovis*—(BCG) (Organon teknika or ATCC 35743)

8.2.2 Other mycobacterial strains may be tested using appropriate culture and subculture procedures.

8.3 *Fungal Test Organisms:*

8.3.1 *Trichophyton mentagrophytes* (ATCC 9533)

8.3.2 Other fungi may be tested using appropriate culture and subculture procedures.

9. Preparation of Organism

9.1 *Bacteria*[6]–Preparation of frozen stock cultures for S. enterica, S. aureus, and P. aeruginosa.—Using a tube containing 5–6 mL TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix well. Incubate for 24 ± 2 h at 36 ± 1°C. Using a sterile spreader, inoculate a sufficient number of TSA plates (for example, 5 to 10 plates per organism) with 100 µL each of the culture. Incubate plates at 36 ± 1ºC for 24 ± 2 h. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol) to the surface of each agar plate. Resuspend the cells in this solution using a sterile spreader or a sterile swab and aspirate the cell suspension from the surface of the agar. Transfer suspension into a sterile vessel. Repeat by adding another 5 mL cryoprotectant to the agar plates, resuspend the cells, aspirate suspension and pool with the initial cell suspension. Alternately, 10 mL cryoprotectant solution may be added per plate for resuspending with subsequent aspiration. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, pipet approximately 1.0 mL quantities of the diluted suspension into cryovials. Place and store cryovials in –70°C or below freezer; these are the frozen stock cultures. Each cryovial is considered as single use only. Store stock cultures up to 18 months. Reinitiate stocks using a new lyophilized culture.

9.1.1 *Bacteria Inoculum Preparation*—For *S. aureus* and *S. enterica*, defrost a single cryovial at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock to a tube containing 10 mL synthetic broth and then vortex to mix. Incubate at 36 ± 1°C for 24 ± 2 h. Briefly vortex the 24 h culture prior to transfer. For this final subculture step, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL synthetic broth with 10 µL per tube of the 24 h synthetic broth culture; incubate 48 to 54 h at 36 ± 1°C. Using a Vortex-style mixer, mix synthetic broth test cultures 3 to 4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask or tube; pool cultures in the flask and swirl to mix. Aliquot a sufficient volume of culture into a sterile test tube.

9.1.1.1 For each bacterium, one daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be subcultured for up to 5 d; each daily transfer may be used to generate a test culture. For the purpose of achieving the carrier count range, final cultures may be adjusted by dilution in growth medium or by concentration using centrifugation (for example, 5000 g for 20 min) resuspending the pellet in the appropriate volume of sterile test culture medium.

9.1.2 For *P. aeruginosa*, defrost a single cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 µL of the thawed frozen stock to a tube containing 10 mL broth (synthetic or nutrient broth) and then vortex to mix. Incubate at 36 ± 1ºC for 24 ± 2 h. Do not vortex the 24 h culture prior to transfer. For this final subculture step, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL broth (synthetic or nutrient) with 10 µL per tube of the 24 h broth culture; incubate 48 to 54 h at 36 ± 1°C. Do not shake 48 to 54 h test culture. The pellicle from the 48 to 54 h cultures must be removed from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipet, or by removal with a vacuum. Avoid harvesting pellicle from the bottom of the tube.

9.1.2.1 Any disruption of the pellicle resulting in dropping, or breaking up of the pellicle in culture before or during its removal renders that culture unusable in the test. This is extremely critical because any pellicle fragment remaining will result in uneven clumping and layering of organism, allowing for biased exposure to disinfectant and causing false-positive results. Pool the test culture from each tube and visually inspect culture for pellicle fragments. Presence of pellicle in the final culture makes it unusable for test. Using a Vortex-style mixer, mix test cultures 3 to 4 s and let stand 10 min at room temperature before continuing. Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask or tube; pool cultures from tubes in the flask and swirl to mix. Aliquot a sufficient volume of culture into a sterile test tube.

9.1.2.2 One daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be subcultured for up to 5 days; each daily transfer may be used to generate a test culture. For the purpose of achieving the carrier count range, final cultures may be adjusted by dilution in growth medium or by concentration using centrifugation (for example, 5000 g for 20 min) resuspending the pellet in the appropriate volume of sterile test culture medium.

9.2 *Mycobacteria*—Maintain a stock culture of *Mycobacterium* organisms on Middlebrook 7H11 or 7H9 agar slants by monthly transfer and incubation for 15 to 20 days at 36 ± 1 °C. Slants may be stored at 5 ± 3 °C for up to six weeks.[5]

9.2.1 *Mycobacteria Inoculum Preparation*—From stock culture, inoculate Modified Proskauer-Beck (MPB) Broth tubes and incubate 21 to 2 days at 36 ± 1 °C. Using a sterile transfer loop, transfer culture to a sterile glass tissue grinder.

[6] AOAC Official Method 961.02 Germicidal Spray Products as Disinfectants. AOAC International, Chapter 6.

Add 1.0 mL of 0.1% polysorbate (Tween) 80. Grind to break up large clumps or aggregates. Dilute the culture with 9 mL of Modified Proskauer-Beck Broth. Transfer the suspension to a sterile test tube and allow to settle for 10 to 15 min. Remove the upper portion to a sterile tube or flask, leaving behind any debris or clumps. Pool cultures, as applicable, and swirl to mix. Dilute the culture to achieve 20±1% T at 650 nm using Modified Proskauer-Beck Broth.[5]

9.3 *Fungi*—Maintain a stock culture of *Trichophyton mentagrophytes* on Sabouraud Dextrose Agar (SDA) or Glucose agar slants by transferring at less than or equal to 3 month intervals and incubate 10 d at 27.5± 2.5°C, followed by storage at 5±3°C.

9.3.1 *Conidial Suspension Preparation*—For *T. mentagrophytes*, prepare Petri dish cultures (≥5 plates) by planting inoculum from a stock culture at the center of the glucose agar or SDA plate and incubating culture at 27.5 ± 2.5°C for 10 to 15 d. Remove mycelial mats from surface of the agar plate cultures, using a sterile spatula or similar device. Transfer growth to a heat-sterilized glass tissue grinder and macerate with 25 mL sterile physiological saline solution (0.85% NaCl) or 0.85% saline with 0.05% Triton X-100, or to sterile Erlenmeyer flask containing 25 mL sterile saline solution with glass beads and shake thoroughly. (Maintain the ratio of 25 mL solution per 5 plates harvested.) Filter the suspension through sterile absorbent cotton or equivalent to remove hyphal elements. Estimate the density of the conidial suspension by counting in a hemacytometer or by direct plate count using glucose agar or SDA. Store suspension at 5 ± 3°C. This represents the stock spore suspension; it should contain approximately 10^7 - 10^8 conidia/mL. Use for up to 4 weeks for preparing test suspensions of conidia. Standardize test conidial suspension as needed by diluting (using sterile saline solution) or concentrating the stock spore suspension so that it contains a minimum of 5×10^6 conidia/mL. Add 0.02 mL Triton X-100/10 mL suspension to facilitate spreading, if previously not incorporated.

9.4 *Inocula used for Testing Pre-Cleaned Surfaces*—No organic soil load is added.

9.5 *Inocula used for Testing Formulations as Disinfectants on Soiled Surfaces*—Transfer an aliquot of the suspension into a sterile tube and add an appropriate volume of blood serum (soil) to yield a 5 % organic soil load (for example, 19 mL of the test organism suspension plus 1 mL fetal bovine serum). Perform a sterility control of the blood serum by adding 1.0 mL of serum to a tube of appropriate recovery broth, (for example, Fluid thioglycollate medium) and incubate with the test.

9.6 *Organism Purity*—Subculture each test organism to the appropriate agar, incubate with the test, and examine for purity.

10. Procedure

10.1 *Preparation of Carriers:*

10.1.1 Test carriers should be submerged in 70 to 95 % ethyl or isopropyl alcohol, rinsed with deionized or distilled water.

10.1.2 Place the test carriers into a large glass dish and sterilize in a hot air oven for ≥2 h at ≥180°C.

10.1.3 After sterilization, place each carrier horizontally into separate glass or plastic Petri dishes containing 2 pieces of sterile filter paper. Transfer at a minimum the required number of carriers for testing including a minimum of 6 carriers for the population control, 1 to 3 carriers for each viability control, and 1 carrier for the carrier sterility control.

10.2 *Inoculation of Carriers:*

10.2.1 Using a pipette or 4.0 mm inside diameter (i.d.) loop, transfer 0.01 mL (10 µL) of the test organism to the nonporous carrier which has been placed horizontally inside the above mentioned Petri dish.

10.2.2 Spread the inoculum suspension evenly over the designated test area (an approximate 1 in. by 1 in. area, approximately 25 mm by 25 mm on the end of the slide) and within 3 mm of the edge using the sterile pipette tip or 4.0 mm id loop used for inoculation and recover with the Petri dish lid.

10.3 *Carrier Drying:*

10.3.1 Place all Petri dishes containing inoculated carriers into an incubator equilibrated at 36 ± 1°C, for 30 to 40 min., until dry.

10.4 *Carrier Treatment/Application of Product:*

10.4.1 Wipe the inoculated test area according to label instructions or the procedure under test. The wiping procedure should closely simulate the direction for intended use.

NOTE 1—The carrier treatment phase is most easily performed with more than one technician. The technician performing the wiping (treatment) procedure must wear sterile gloves prior to handling the towelette under test and must not touch anything except the towelette under test and inoculated carriers. Aseptic technique is critical in minimizing potential environmental contamination of the carrier subcultures.

10.4.2 The area of the towelette used for wiping should be rotated so as to expose a new surface of the towelette surface in the course of each carrier treatment. One towelette may be used to treat multiple carriers (typically 10 carriers/wipe).

10.4.3 The wiping procedure should be performed at staggered intervals so as to allow for the prescribed exposure time before subculture.

NOTE 2—The technician should allow enough time between each carrier wiping (for example, 30 s) to allow for the exact exposure time prior to subculturing. The exposure time starts immediately following the completion of treatment.

10.4.4 *Optional*—Following the treatment of the last carrier in the set, the towelette or liquid expressed from the towelette is aseptically placed in a separate sterile Petri dish and held for the prescribed exposure time starting when treatment of the last carrier ended.

10.5 *Carrier Subculture:*

10.5.1 Following the prescribed exposure time and using separate sterile forceps, transfer each carrier into a sterile 38 mm diameter tube (or other appropriate vessel) containing 20 mL of neutralizer to completely cover the inoculated and treated area of the carrier and mix thoroughly.

10.5.2 Following the same staggered interval used in 10.4.3, transfer the remaining carriers as described in 10.5.1.

10.5.3 If necessary to achieve adequate neutralization, for all organisms except *Mycobacteria* transfer each carrier to a second vessel containing the appropriate subculture medium

within 25-60 min from original subculture, and mix thoroughly for further test substance neutralization by dilution (see Test Methods E1054). All neutralizing subculture media (primary and secondary, if used) must support growth of the test organism.

10.5.4 For *Mycobacteria*—After primary neutralization, transfer each carrier to 20 mL subcultures of Modified Proskauer. Beck broth. Within approximately 30 minutes of neutralization, transfer 2 mL aliquots from each neutralizer tube to 2 additional subculture media, Middlebrook 7H9 Broth and Kirchner's Medium or TB broth. Each subculture medium (excluding the neutralizer tube) is incubated for mycobacteria.

10.6 *Towelette Subculture (OPTIONAL):*

10.6.1 Following the prescribed contact time, aseptically transfer the entire towelette or a 0.1 mL volume of liquid expressed from the towelette into the same neutralizer/subculture media at the same volume as the carrier or another appropriate volume to completely cover if the entire towelette is subcultured.

10.6.2 Incubate subcultures as described in 10.11.

10.7 *Inoculated Carrier Population Control:*

10.7.1 After the carriers have dried, assay carriers in two sets of three carriers, one set prior to conducting the tests and one set following the test. Place each of the inoculated, dried carriers in a 38 × 100 mm culture tube, sterile 50 mL polypropylene conical tube, or other appropriate vessel containing 20 mL of appropriate neutralizing subculture broth as used in testing. Utilize Modified Proskauer-Beck broth for mycobacteria.

10.7.2 Sufficiently vortex mix the carriers immediately. For *P. aeruginosa*, vortex mix for 60 ± 5 s. For *S. enterica* and *S. aureus*, vortex mix for 120 ± 5 s, specifically.

10.7.3 After vortexing, make serial 10-fold dilutions in 9 mL of PBDW, Butterfield's Buffer or other sterile diluent. If the serial dilutions are not made and plated immediately, keep the vortexed tubes at 5 ± 3°C until this step can be done; however, dilution and plating should be performed within 2 h of vortexing. The broth tubes may be pooled after vortexing for each set of 3 carriers. An aliquot of the pooled media (60 mL) will be serially diluted and plated, and the average carrier count per set will be calculated. For bacterial organisms, plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA or TSA with 5% sheep blood using pour- or surface-spread plating; dilutions of 10^{-1} through 10^{-3} should result in plates with a countable range of colonies. For fungi, use Sabouraud Dextrose or Glucose Agar. For mycobacteria, use Middlebrook 7H11 agar.[6]

10.7.4 Following incubation visually confirm that the colonies present are typical of the test organism. Visually enumerate the colonies on each plate.

10.8 *Viability Control*—On the day of testing, place one (or two) dried inoculated carrier(s) into separate tubes of neutralizing subculture broth (if primary and secondary media are different). For mycobacteria, place an inoculated carrier into each of the three subculture media used in testing. Incubate as in 10.11.

10.9 *Carrier Sterility Control*—Perform a carrier sterility control by transferring one uninoculated carrier to subculture broth (or Modified Proskauer-Beck broth for mycobacteria), incubate as in 10.11 and observe for growth.

10.10 *Neutralization Confirmation Controls:*

10.10.1 A neutralization confirmation test must be performed in advance or in conjunction with the test. Historical use of neutralizer media for specific active ingredients may also be taken into consideration. A neutralization confirmation procedure must demonstrate the recovery of a low level (for example, 10–100 CFU) of the test organism in the subculture media. For example, in a separate assay to simulate actual test conditions, expose a sterile carrier to the test material and transfer to subculture medium (or both primary and secondary tubes if used in the efficacy test) as in the test procedure. Immediately following the transfer, inoculate the tube(s) with 10–100 CFU/tube of the specified culture and incubate as in the test. Confirm number of cells in the suspension using duplicate pour or spread plates. Count colonies on plates to determine inoculum level. Examine tubes for growth. Growth in tubes indicates effective neutralization.

10.10.1.1 For *Mycobacteria*—transfer the carriers to Modified Proskauer-Beck broth and 2 mL aliquots of neutralizer to the subculture medium as described in 10.5.1. Inoculate each of the 3 subculture media with the inoculum. Additional guidance for Mycobacteria is found in AOAC official method 965.12.[6,5]

10.11 *Incubation:*

10.11.1 Incubate all bacterial organism cultures/subcultures for 48 ± 2 h at 36 ± 1°C.

10.11.2 Incubate all fungal broth cultures/subcultures for 10 days at 27.5 ± 2.5°C. Incubate fungal agar plate cultures for 44 to 76 h at 27.5 ± 2.5°C.

10.11.3 Incubate all mycobacterial subculture plates for 17 to 21 days at 36 ± 1°C in an appropriate manner to prevent desiccation. Incubate subculture tubes for a minimum of 60 days at 36±1°C, and if no growth is observed in the test subcultures, incubator for a minimum of 30 additional days at 36±1°C.

10.12 *Examination of Subcultures:*

10.12.1 Following incubation, carefully visually observe the liquid subculture media for growth as indicated by turbidity or presence of growth typical for the test organism. Following incubation visually confirm that the colonies present on plates are typical of the test organism. Visually enumerate the colonies on each plate.

10.12.2 Positive test carriers are examined for test organism by inoculating onto the appropriate medium (for example, TSA, TSA with 5% sheep blood, glucose agar, SDA, Middlebrook 7H11 or selective media) for the test microbe. Incubate inoculated media as in the test until sufficient growth is present. Examine plates for colonial morphology characteristic to the test organism (conforming to the morphology described in Bergeys Manual). Bacterial growth from subculture media should be checked by Gram stain. In addition, any suitable confirmation/identification may be done. This may include, but not be limited to, selected biochemical testing, manual and/or

automated identification (for example, VITEK). *Mycobacteria* can be confirmed using acid-fast staining techniques and may be stained directly from positive tubes without subculture

11. Calculation or Interpretation of Results

11.1 *Number of Viable Organisms Tested per Carrier(Inoculated Carrier Population Control)*

11.1.1 Count the colonies by hand or with a colony counter. Use dilutions yielding counts up to 300 for enumeration; plate counts of 0 are to be included in the calculations. Calculate the CFU per carrier as follows:

$$\text{CFU/carrier} = \frac{[(\text{avg. CFU for } 10^{-x}) + (\text{avg. CFU for } 10^{-y}) + (\text{avg. CFU for } 10^{-z})] \times (\text{Vol. of broth})}{[10^{-x} + 10^{-y} + 10^{-z}] \times (\text{Vol. plated}) \times (\#\text{of carriers per set})} \quad (1)$$

where:

10^{-x}, 10^{-y}, 10^{-z} = are examples dilutions that may be used.

11.1.2 Calculate the $\log_{10}$ density (LD) for each carrier by taking the $\log_{10}$ of the density (per carrier). The mean LD across carriers is the mean LD for the test.

11.1.3 The mean LD must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.5 (corresponding to a geometric mean density of 3.2×10^6) for *P. aeruginosa* and *S. aureus*; a mean LD below 5.0 or above 6.5 invalidates the test (see retesting guidance below).[6]

11.1.3.1 For *S. enterica*, the mean LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 5.5 (corresponding to a geometric mean density of 3.2×10^5); a mean LD below 4.0 or above 5.5 invalidates the test (see retesting guidance below).[6]

11.1.3.2 For *T. mentagrophytes*, the mean LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 5.0 (corresponding to a geometric mean density of 1.0×10^5); a mean LD below 4.0 or above 5.0 invalidates the test (see retesting guidance below).

11.1.3.3 For *M. bovis*, the mean LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean LD below 4.0 or above 6.0 invalidates the test (see retesting guidance below).[5]

11.1.4 *Retesting guidance*—For tests where the product passes and the mean LD value is above the upper limits described in 11.1.3, no retesting is necessary. For a test where the product fails and the mean LD is below the lower limits described in 11.1.3, no retesting is necessary. For tests where the product fails and the mean mean LD is above the upper limits described in 11.1.3, retesting may be conducted.[6,5]

11.2 The inoculated carrier population control must meet the requirements described in 11.1.3.

11.3 The viability control must show growth.

11.4 The sterility controls must show no growth.

11.5 The neutralization control subcultures must show growth in the final subculture medium, minimally following inoculation with ≤100 CFU.

11.6 Record the number of carriers positive

12. Report

12.1 Report the number of carriers tested and the number of carriers positive for the test organism (per substance/test organism).

12.2 Also report the following information:

12.2.1 Name of product(s) under test.

12.2.2 Chemical composition of product(s) under test.

12.2.3 Concentration(s) of active ingredient(s) tested.

12.2.4 Whether or not organic load (bovine serum in inoculum) was employed.

12.2.5 Organisms tested.

12.2.6 Neutralizer and neutralizer concentration employed. Subculture medium used (for Mycobacterium).

12.2.7 Mean LD in the inoculated carrier population control for each test organism.

12.2.8 Neutralization confirmation control results.

13. Precision and Bias

13.1 *Precision*—Precision will depend on each of the variables listed in Section 12, consequently no statement on precision can be made. Individual laboratories performing this test or encouraged to develop repeatability statistics based on the specific protocol(s) that they adopt from the method in order to determine the precision of that protocol.

13.2 *Bias*—Because there is no accepted reference materials suitable for the bias in this method, no statement of bias is made.

14. Keywords

14.1 Efficacy; glass carriers; *Mycobacterium bovis* ; presaturated; *Pseudomonas aeruginosa* ; *Salmonella enterica* ; *Staphylococcus aureus* ; towelette; *Trichophyton mentagrophytes*

ASTM International takes no position respecting the validity of any patent rights asserted in connection with any item mentioned in this standard. Users of this standard are expressly advised that determination of the validity of any such patent rights, and the risk of infringement of such rights, are entirely their own responsibility.

This standard is subject to revision at any time by the responsible technical committee and must be reviewed every five years and if not revised, either reapproved or withdrawn. Your comments are invited either for revision of this standard or for additional standards and should be addressed to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend. If you feel that your comments have not received a fair hearing you should make your views known to the ASTM Committee on Standards, at the address shown below.

This standard is copyrighted by ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States. Individual reprints (single or multiple copies) of this standard may be obtained by contacting ASTM at the above address or at 610-832-9585 (phone), 610-832-9555 (fax), or service@astm.org (e-mail); or through the ASTM website (www.astm.org). Permission rights to photocopy the standard may also be secured from the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, Tel: (978) 646-2600; http://www.copyright.com/