



Standard Practice for Evaluation of Pre-saturated or Impregnated Towelettes for Hard Surface Disinfection¹

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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 *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 Standards³

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

¹ This practice is under the jurisdiction of ASTM Committee E35 on Pesticides and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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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

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 Practice⁴

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, 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. The used towelette, after the contact time, is also cultured for surviving test organism.

5. Significance and Use

This test method may be used to determine if a pre-saturated or impregnated towelette demonstrates antimicrobial effectiveness as a disinfectant on hard surfaces.

6. Apparatus

6.1 *Incubator*—any calibrated incubator that maintains a temperature specific for propagation of organisms. (for example, bacteria and mycobacteria at $35 \pm 2^\circ\text{C}$ and fungi at $25 \pm 2^\circ\text{C}$).

⁴ United States Environmental Protection Agency, Efficacy Data Requirements, "Pre-Saturated or Impregnated Towelettes for Hard Surface Disinfection" Standard Operating Procedure for Testing of Towelette Disinfectants against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, EPA/OPP Microbiology Laboratory, Ft. Meade, MD. SOP# MB09-02, Revised 12/31/06.

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

6.3 *Test Towlettes*—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 × 75 × 2 mm slides, pre-cleaned (or other hard surfaces and sizes as appropriate).

6.9 *Glass Culture Tubes*—20 × 150 or 25 × 150 mm without lip or equivalent.

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

6.11 *Petri Dishes*—100 × 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 forceps.

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 *Hemocytometer*—calibrated hemacytometer.

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

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

6.28 *Tissue grinder*—sterile disposable or sterilizable glass.

6.29 *Orbital Shaker*—calibrated shaker.

7. Reagents

7.1 Culture Media—Bacteria

7.1.1 *Nutrient Broth*—*Pseudomonas aeruginosa*,

7.1.2 *Cystine Trypticase Agar*—*Pseudomonas aeruginosa*,

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

7.1.4 *Nutrient Agar*.

7.1.5 *Fluid Thioglycollate Broth*.

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/Potato Dextrose Agar*.

7.3.2 *Sabouraud Dextrose 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.

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*—Fungi.

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

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

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

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

8. Test Organisms

8.1 Bacteria Test Organisms:

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

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

8.2 Mycobacteria Test Organisms:

8.2.1 *Mycobacterium chelonae* (ATCC 35752).

8.2.2 *Mycobacterium bovis* (ATCC 35743)

8.2.3 Other mycobacteria strains may be tested using appropriate culture and subculture procedures.

8.3 Fungi Test Organisms:

8.3.1 *Trichophyton mentagrophytes* (ATCC 9533)

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

9. Preparation of Organism

9.1 *Bacteria*—Maintain stock cultures of *S. aureus* and *S. enterica* on Nutrient Agar slants. Maintain stock cultures of *P. aeruginosa* on Cystine Trypticase Agar. Incubate freshly subcultured stock cultures for 48 ± 4 h at 35 ± 2 °C, then refrigerate cultures at 2 to 8 °C for up to one month. Stock cultures used for inoculation of broth cultures should not undergo more than 5 passages from the first subculture from the ATCC frozen stock.

9.1.1 *Bacteria Inoculum Preparation*—From stock cultures, inoculate tubes containing 10 mL of the appropriate fresh culture broth and incubate for 24 ± 4 h at 35 ± 2 °C. Using a 4 mm inside diameter transfer loop, transfer one loopful of the culture into fresh culture broth. Make at least 3 but less than 30 consecutive daily transfers prior to use as inoculum for testing. Incubate the final transfer for 48 ± 4 h, and use these cultures in the test. Aseptically remove the pellicle from the *P. aeruginosa* culture before use in the test.

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 30 days at 35 ± 2 °C followed by storage at 2 to 8°C.

9.2.1 *Mycobacteria Inoculum Preparation*—From stock culture, inoculate Modified Proskauer-Beck (MPB) Broth tubes and incubate 10 to 25 days at $35 \pm 2^\circ\text{C}$. Add 1.0 mL of 0.1 % Tween 80 in saline to this 10 to 25-day culture, transfer to a sterile tissue grinder and grind thoroughly. Finally transfer the appropriate volume of suspension from the grinder to a vessel appropriate for spectrophotometric determination. Calibrate a spectrophotometer to 650 nm using a MPB blank as the 100 % transmittance point. Measure transmittance of the culture suspension. Adjust culture suspension with MPB to a target of 8 to 12 % transmittance to achieve a $\geq 1.0 \times 10^4$ CFU/carrier concentration.

9.3 *Fungi*—Maintain a stock culture of *Trichophyton mentagrophytes* on Sabouraud Dextrose or Potato Dextrose agar slants by transferring at less than or equal to 3 month intervals and incubate 10 days at $25 \pm 2^\circ\text{C}$, followed by storage at 2 to 8°C . Alternatively, a water stock may be prepared and held at ambient temperature.

9.3.1 *Conidial Suspension Preparation*—From stock culture, inoculate Sabouraud Dextrose or Potato Dextrose agar plates and incubate at $25 \pm 2^\circ\text{C}$ for 10 to 15 days. Transfer growth, using a sterile swab, to a glass screw-top vessel containing 5 sterile glass beads and 5 mL of 0.85–0.90 % sterile saline or other appropriate diluent per culture plate. Vortex mix the suspension for one min. Filter the conidial suspension through sterile cotton gauze or glass wool to remove the hyphal fragments. Estimate the conidial concentration by counting in a hemacytometer. Stock conidial suspension can be stored at 2 – 8°C for one month. On the day of testing, standardize the conidial suspension using 0.85 to 0.9 % sterile saline to $\geq 1.0 \times 10^6$ conidia/mL. A 0.2 mL aliquot of Triton X-100 may be added to a 10 mL aliquot of conidial suspension to facilitate spreading.

9.4 *Inocula used for Testing Pre-Cleaned Surfaces*—Immediately prior to use, thoroughly vortex mix the prepared fungi suspension. Thoroughly vortex mix the prepared bacteria and mycobacteria suspension and allow to settle for ≥ 10 min prior to use.

9.5 *Inocula used for Testing Formulations as Disinfectants on Soiled Surfaces*—Immediately prior to use, thoroughly vortex mix the prepared fungi and mycobacterial suspension. Thoroughly vortex mix the prepared bacteria suspension and allow to settle for ≥ 10 min prior to use. 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 9.0 mL of appropriate recovery broth 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 $\geq 180^\circ\text{C}$.

10.1.3 After sterilization, place each carrier horizontally into separate glass or plastic Petri dishes containing 2 pieces of sterile filter paper. Transfer the required number of carriers for testing including a minimum of 2 carriers for the population control, 1 carrier 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 (id) loop, transfer 0.01 mL 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 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 $35 \pm 2^\circ\text{C}$ until dry, but no longer than 40 min.

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 carrier sets/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 vessel containing the appropriate amount of subculture/neutralizing media to completely cover the inoculated and treated area of the carrier and mix thoroughly.

10.5.2 Following the same staggered interval used in 10.4.4, transfer the remaining carriers as described in 10.5.1.

10.5.3 If necessary ≥ 30 min from original subculture, transfer carrier to a second vessel containing the appropriate subculture/neutralizer media and mix thoroughly for further test substance neutralization by dilution (see Test Methods E1054).

10.5.4 Incubate subcultures as described in 10.11.

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 Following completion of the test and using a sterile forceps, transfer untreated inoculated carriers into a jar containing the appropriate subculture media at a ratio of 1 carrier to 10 mL subculture media.

10.7.2 Agitate the jars, mixing by hand, vortex, or orbital shaker for 1 to 5 min.

10.7.3 Prepare 10-fold serial dilutions of the inoculated subculture media using PBDW, Butterfield's buffer, or other sterile diluent to yield a dilution expected to contain 25 to 250 CFU/mL.

10.7.4 Plate, in duplicate, 1.0 mL of the appropriate dilution(s) from above onto the appropriate recovery media using standard surface/pour plating techniques.

10.7.5 Incubate subculture as described in 10.11.

10.7.6 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*—Perform a viability control by transferring one contaminated dried carrier to subculture broth, incubate with the test and observe for growth.

10.9 *Carrier Sterility Control*—Perform a carrier sterility control by transferring one uninoculated carrier to subculture broth, incubate with the test and observe for growth.

10.10 Neutralization Confirmation Controls:

10.10.1 When testing bacteria, following incubation, inoculate a minimum of 10 % of subculture jars demonstrating no growth with 1.0 mL aliquots of a suspension containing ≤ 100 CFU/mL of the representative test organism.

10.10.2 Alternatively, or when testing fungi or mycobacteria, neutralization confirmation may be performed the day of test by exposing sterile carriers to the test substance, and subculturing as in test. Inoculate the subcultures with a 1.0 mL aliquot of a suspension containing ≤ 100 CFU/mL of the test organism.

10.10.3 Verify the number of CFU in the suspension by plating 1.0 mL aliquots onto appropriate subculture agar plates in duplicate.

10.11 Incubation:

10.11.1 Incubate all bacterial organism cultures/subcultures for 48 ± 4 h at $35 \pm 2^\circ\text{C}$.

10.11.2 Incubate all fungal broth cultures/subcultures for 10 days at $25 \pm 2^\circ\text{C}$. Incubate fungal agar plate cultures for 44 to 76 h at $25 \pm 2^\circ\text{C}$.

10.11.3 Incubate all mycobacterial cultures/subcultures for 15 to 25 days at $35 \pm 2^\circ\text{C}$.

10.12 Examination of Subcultures:

10.12.1 Following incubation, carefully visually observe the liquid subculture media for growth as indicated by turbidity. 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 Confirm ≥ 20 % of all subcultures demonstrating growth by standard microbiological techniques including but not limited to macroscopic or microscopic examination, or both, and biochemical analysis.

11. Report

11.1 Number of Viable Organisms Tested per Carrier

11.1.1 Add the number of colonies from the duplicate countable plates. (Use plates with counts between 25 to 250 colonies for bacterial and 8 to 80 colonies for fungal organisms when possible). Divide this total by 2 to obtain the average number of colony forming units for dilution plated.

11.1.2 Multiply the average number of colonies by the reciprocal of the dilution factor corresponding to the data and the volume of neutralizer to obtain the number of colony forming units per mL of subculture media.

11.1.3 Divide this value by the number of carriers tested. This final value represents the number of viable test organisms per carrier.

11.1.4 The entire calculation can be summarized by equation:

$$\text{CFU/carrier} = \frac{(A)(R)(V)}{(N)} \quad (1)$$

Where:

A = avg. No. colonies found at dilution plated

R = reciprocal dilution factor used

V = volume neutralizer

N = No. of carriers tested

11.2 *Organisms per milliliter*—calculation is performed the same as above but disregard multiplying by volume neutralizer and dividing by the number of carriers.

$$\text{CFU/mL} = \frac{(A)(R)}{VP(\text{mL})} \quad (2)$$

Where:

A = avg. No. colonies found at dilution plated

R = reciprocal dilution factor

VP = Volume Plated

11.3 The inoculated carrier population control must show $\geq 1.0 \times 10^4$ CFU/carrier.

11.4 The viability control must show growth.

11.5 The carrier sterility control must show no growth.

11.6 The neutralization control subcultures must show growth following inoculation with ≤ 100 CFU.

12. Keywords

12.1 Efficacy; glass carriers; *Mycobacterium bovis*; *Mycobacterium chelonae*, pre-saturated; *Pseudomonas aeruginosa*; *Salmonella enterica*; *Staphylococcus aureus*; towelette; *Trichophyton (T.) mentagrophytes*

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