EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Disinfectant Towelette Test Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

SOP Number: MB-09-02

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1.0 SCOPE AND APPLICATION:

1.1 This Standard Operating Procedure (SOP) describes the method to determine the efficacy of pre-saturated or impregnated towelettes as hard surface disinfectants against two test organisms, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This method, referred to as the Disinfectant Towelette Test, evaluates wiped slides for the presence/absence of viable microorganisms.

1.2 This SOP includes a neutralization confirmation step as part of the protocol.

1.3 As per internal Antimicrobials Division (AD) guidance (Towelettes: A. General Efficacy Data Requirements – Disinfectants for Use on Hard Surfaces Using Pre-Saturated or Impregnated Towelettes), one towelette is used to wipe multiple slides. Antimicrobials Division policy allows one towelette to be used to wipe ten carriers/slides. The area of the towelette used for wiping will be rotated so as to expose a new surface of the towelette for each carrier.

1.4 The method may be altered to accommodate fewer or greater than ten carriers per towelette.

1.5 Brief Summary: The Disinfectant Towelette Test is a carrier-based test based on the AOAC Germicidal Spray Products Test (see ref. 15.4). Carriers (glass slides) are inoculated with a test organism and dried. Following drying, a predetermined number of glass slides (typically ten glass slides) are wiped with one towelette. The used towelette is discarded. The ten wiped glass slides are held for the product-specified contact time. After the specified contact time, the glass slides are subcultured to assess the survival of the bacteria.

2.0 DEFINITIONS:

2.1 AOAC = AOAC International

2.2 TSA = Trypticase Soy Agar/Tryptic Soy Agar

2.3 Carrier set for *Staphylococcus aureus* or *Pseudomonas aeruginosa* = The primary and secondary subculture tubes for each carrier represent a carrier set. There are 60 slide carrier sets (2 tubes per set) per lot of product sample tested per organism.

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms are required to be performed in accordance to biosafety practices stipulated in SOP MB-01, Biosafety in the Laboratory.

3.2 Pre-saturated or impregnated towelettes may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, etc. Gloves and other personal protective clothing or devices are worn during the handling of these items for the purpose of efficacy testing.

3.3 The external surface of the eppendorf pipette used to inoculate the glass slide carriers with the test organisms may be contaminated during the inoculation process. Thus, after completion of the inoculation of the glass slide carriers, the eppendorf pipette will be thoroughly wiped with 70% ethanol prior to removal from the BSC.

4.0 CAUTIONS:

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

4.2 The analyst holding the towelette will avoid the direct contact of glove surfaces and the inoculated slides during wiping. A new pair of sterile gloves will be worn if the gloves are determined to be compromised during testing. In addition, the towelette will be folded so as to expose a new surface of the towelette for each carrier, as per Antimicrobials Division guidance (see section 1.3).

5.0 INTERFERENCES:

5.1 Prior to inoculation, carriers should be dry inside the Petri dishes. Moisture on the carrier can interfere with the concentration and drying of the inoculum.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 *Pseudomonas aeruginosa* (ATCC #15442)

7.2 *Staphylococcus aureus* (ATCC #6538)

7.3 Glass Slide Carriers, Bellco 25 H 75 mm (or comparable size) borosilicate glass cover slips with number 4 thickness (Bellco Glass, Inc., item number: 1916-SO134)

7.4 Sterile glass Petri dishes

7.5 Forceps

7.6 Eppendorf pipettes and tips (10 µL, 100 uL)

7.7 Sterile surgical gloves

7.8 38 H 100 mm medication tubes (Bellco) for neutralization and subculture media

7.9 Trypticase Soy Agar

7.10 VITEK® System for the automated identification of microorganisms

7.11 PCS 2 Pipette Calibration System for the calibration of Eppendorf pipettes

7.12 Neutralizer

7.13 Subculture medium

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the instructions stipulated in SOP QC-19, Calibration of Eppendorf Pipettes, for instrument calibration of the PCS 2 Pipette Calibration System.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Towelettes are stored according to the manufacturer's recommendations if stipulated, or at room temperature.

9.2 Follow chain-of custody guidelines as stipulated in SOP COC-01, Disinfectant Sample Login and Tracking.

10.0 PROCEDURE AND ANALYSIS:

10.1 Carriers: A total of 72 inoculated carriers are prepared per organism. Sixty are used in the test, six are required to estimate the carrier load, and six are extras.

10.2 Carrier Preparation:

10.2.1 Visually screen glass slide carriers (25 H 75 mm) for scratches, chips or cracks and discard those which are damaged or defective.

10.2.2 Prior to carrier preparation for testing, rinse the carriers once with deionized water, rinse three times with 95% ethyl alcohol, and finally rinse three times with DI water.

10.2.3 Drain and allow carriers to dry before use. Record screening results in the Physical Screening of Carriers Record form of SOP MB-03, Screening Carriers Used in Disinfectant Efficacy Testing.

10.2.4 Place one glass slide carrier into a glass Petri dish, directly onto the glass surface of the dish (no filter paper will be added to the Petri dishes). Fill out a media/reagent preparation sheet to assign a preparation number to a set of carriers (see SOP QC-15, Media and Reagent Preparation: Assigning Prep and Sterilization Run Numbers).

10.2.5 Autoclave for 45 minutes at 121EC with a 30 minute dry cycle; cool; store at room temperature.

10.2.6 The 25 H 75 mm glass slide carriers may be reused provided that they are treated as described in this section (section 10.2) prior to reuse. Slides giving a positive result in a test must not be reused.

10.2.7 Sterility of carriers must be confirmed either in advance or concurrently with testing by adding the carrier to a 38 H 100 mm medication tube containing 20 mL subculture medium and incubating at 36±1ºC for 5-7 days.

10.3 Test Culture Preparation for *S. aureus* and *P. aeruginosa*:

10.3.1 Record all culture transfers on the Organism Culture Tracking form (see SOP MB-02, Test Microbes for the AOAC Use-Dilution Method, AOAC Germicidal Spray Products Test, AOAC Confirmatory Tuberculocidal Test, AOAC Sporicidal Activity Test method, and the Quantitative Suspension Test Method: Culture Initiation, Culture Maintenance and Quality Control).

10.3.2 Initiate test culture by inoculating a 10 mL tube (20 H 150 mm) of nutrient broth or synthetic broth from a stock slant culture. Transfer a loopful of inoculum from the stock slant into the broth. Refer to SOP MB-02 for stock culture preparation.

10.3.3 Two sets of cultures of the same organism may be initiated in parallel from the same stock culture and subcultured; however, only one set of the final cultures is used for product testing.

10.3.4 The test culture is serially subcultured for at least three consecutive 24∀2 hour periods in 10 mL of nutrient broth or synthetic broth at 36∀1EC.

10.3.5 The test culture is subcultured once again in nutrient broth or synthetic broth and incubated at 36∀1EC for 48 to 54 hours. For this final subculture step, inoculate four to six 25 H 150 mm tubes each containing 20 mL of nutrient broth or synthetic broth.

10.3.6 The culturing schedule must be considered when selecting a date for product testing. For example, the culture sequence must begin on Thursday for testing to commence on the following Tuesday.

10.3.7 Pool the four to six tubes of each 48-54 hour test culture in a sterile flask.

10.3.8 The pellicle from the 48-54 hour *Pseudomonas* cultures must be removed from the broth before pooling the culture either by decanting the liquid culture aseptically into a sterile tube or by gently aspirating the broth culture away from the pellicle using a sterile 10 mL pipette. In either case the pellicle must not be broken or fragmented or the culture is not usable.

10.3.9 Swirl to mix.

10.3.10 If no organic soil is required, aliquot 20 mL portions of the culture into sterile 25 H 150 mm test tubes.

10.3.11 If an organic soil load is to be added to the culture, measure the pooled culture and add the appropriate amount of soil to the flask. Swirl to mix. Aliquot 20 mL portions into sterile 25 H 150 mm test tubes.

10.3.12 Vortex the 20 mL cultures for 3-4 seconds and let stand 10 minutes at room temperature.

10.3.13 Withdraw approximately the top 3/4 of the culture from each tube with a sterile pipette and dispense a total of 20 mL into sterile 25 H 150 mm test tubes. Cultures may be combined from more than one tube to achieve the 20 mL total. Prepare one to two tubes for each organism this way. These cultures will be used to inoculate carriers.

10.4 Carrier Inoculation with *S. aureus* and *P. aeruginosa*:

10.4.1 Transfer 0.01 mL of the test culture, using an Eppendorf Pipette with sterilized tips, onto the sterile dry glass slide. Immediately spread the inoculum uniformly over one third of the slide (approximately 25 H 25 mm area) using a sterile loop. Do not allow inoculum to contact the edges of the glass slide.

10.4.2 After completion of all slide inoculations, thoroughly wipe the eppendorf pipette with 70% ethanol prior to removal from the biological safety cabinet (BSC).

10.4.3 Dry the slides for approximately 40 minutes at 36∀1EC.

10.4.4 Record timed carrier inoculation activities on the Time Recording Sheet for Carrier Inoculation Steps (see 16.1).

10.5 Disinfectant Sample Preparation:

10.5.1 Unless additional preparation is specified (e.g., addition of water to dry towelette), towelettes are ready-to-use.

10.6 General Test Procedure:

10.6.1 Wipe the outside of the towelette packet or dispenser with 70% ethanol and allow to air dry prior to opening.

10.6.2 All manipulations of towelettes must be performed aseptically. Towelettes are removed from the packet or dispenser using sterile surgical gloves. Flame sterilized forceps may be used to assist in the removal of the towelette from the dispenser.

10.6.3 Following wiping of the inoculated slides, the used towelette is discarded.

10.6.4 The bacterial carrier load is assayed as per section 10.2 of SOP MB-04, Determining Carrier Counts.

10.7 Towelette Tests with *S. aureus* or *P. aeruginosa*:

10.7.1 The wiping phase of the test requires two analysts – one to wipe carriers and the other (i.e., “second analyst”) to open the Petri dishes, lift up the carriers, and move the carriers.

10.7.2 Aseptically remove one towelette from the packet or dispenser using sterile surgical gloves.

10.7.3 The analyst handling the towelette must not touch any surface other than the towelette and the carrier to be wiped.

10.7.4 Folding/wiping directions: Fold the towelette in half lengthwise one to two times, depending upon its size. Grasp the towelette at the top and bottom. Starting from the bottom, fold the towelette up towards the top, for a total of four to five folds. At the appropriate wipe time, the analyst will manipulate the towelette to use one folded surface per slide to wipe the slide. In preparation for the next slide, the analyst will pull (using the hand holding the top of the towelette) the used section of towelette up and over the wiping finger, thereby exposing a new surface. Wipe subsequent slides, pulling the used section of towelette up and over the wiping hand. Once five slides have been wiped, unfold the vertical fold in the towelette and reverse it so that the inoculated section of towelette faces inward. Refold four to five times as before and wipe the next five slides.

10.7.5 At the appropriate time intervals, the second analyst removes the lid from the Petri dish, uses flame-sterilized forceps or sterile gloves to pick up the slide (avoid contact with the inoculated end), and holds it firmly against the sterile rim of the Petri dish so that the analyst with the towelette can wipe the glass slide within the specified time frame.

10.7.6 The analyst wiping the carriers will conform to a pre-determined pattern (e.g., wipe left to right three times).

10.7.7 The slide must be wiped within ±5 seconds of the specified time.

10.7.8 Immediately after wiping the carrier, the second analyst places the slide back into the Petri dish, replaces the lid, sets the dish aside, and takes up the next dish containing an inoculated carrier to be wiped.

10.7.9 The area of the towelette used for wiping will be rotated so as to expose a new surface of the towelette for each carrier.

10.7.10 After wiping ten inoculated carriers, the analyst discards the used towelette in a biohazard bin.

10.7.11 After the last slide of a set (typically ten slides) has been wiped and the contact time complete, transfer each slide, in order, into the tubes containing 20 mL of the appropriate neutralizer. Transfers will be made within the ±5 second time. Transfers are made with flame sterilized forceps. Place the inoculated/wiped end of the slide into the neutralizer.

10.7.12 The slide can touch both the interior sides of the Petri dish and the neutralizer tube during the transfer, but this contact should be avoided as much as possible.

10.7.13 Each of the remaining slides are transferred into their corresponding neutralizer tubes at the appropriate time.

10.7.14 After the slides are deposited, the neutralizer tube is recapped and shaken briefly. Alternately, a set of tubes may be shaken after all primary transfers are completed.

10.7.15 Incubate the neutralizer tubes for a minimum of 30 minutes at 36±1°C.

10.7.16 Aseptically transfer slides to corresponding subculture tubes containing 20 mL of the appropriate subculture medium. Move the slides in order but the movements do not have to be timed. After the slides are deposited, the subculture tube is recapped and shaken briefly. Alternately, a set of tubes may be shaken after all primary transfers are completed.

10.7.17 Incubate the neutralizer and subculture tubes at 36±1ºC for 48±2 hr.

10.7.18 See Attachment A (Testing Footnotes and Explanations) for a list of footnotes which are used to indicate problematic events or observations that occur during testing.

10.7.19 Proceed with removal of a new towelette and wiping of the next set of carriers.

10.8 Results for Tests with *S. aureus* and *P. aeruginosa*:

10.8.1 Each tube is shaken prior to recording results to determine the presence or absence of turbidity. A positive result is one in which the broth culture appears turbid. A negative result is one in which the broth appears clear.

10.8.2 Report results as + (growth) or 0 (no growth) on the Disinfectant Towelette Test: Test Results Form (see 16.4).

10.9 Confirmation Procedures for Tests with *S. aureus* and *P. aeruginosa*:

10.9.1 If there are fewer than three positive carrier sets, one tube from each set will be tested by Gram staining and selective media, and confirmed by VITEK® analysis. If both tubes are positive in a carrier set, then only one tube is selected for confirmation.

10.9.2 If between three and twenty positive carrier sets occur, one tube from a minimum of three positive slide sets (set=1 primary and 1 secondary tube [with carrier]) should be tested using Gram staining and selective media, and confirmed by VITEK® analysis. If both tubes are positive in a carrier set, then only one tube is selected for confirmation.

10.9.3 For a test with greater than 20 positive carrier sets, Gram stain at least 20% (e.g., for 40 positive carrier sets, Gram stain at least eight positive sets) and confirm a minimum of 4 positive sets by selective media and VITEK® analysis. If both tubes are positive in a carrier set, then only one tube is selected for confirmation.

10.9.4 Gram stain reactions, cell morphology, and colony characteristics on selective media are given in SOP MB-02.

10.9.5 Initial Gram stains are performed on smears taken from the positive culture tubes.

10.9.6 For the additional confirmatory tests, a loopful of broth from each selected culture tube is streaked on both TSA and selective media appropriate for the test organism and incubated for 24±2 hours at 36±1°C.

10.9.7 The selective agar is checked for the presence of typical colonies and correct reaction (e.g., change in media color). The isolated colonies on the TSA plate are used for verifying the purity of culture and preparing the inoculum for the VITEK® system.

10.9.8 The VITEK® analysis should be conducted according to the manufacturer's instructions.

10.9.9 If confirmatory testing determines that the identity of the organism was not the test organism, the positive entry (+) on the results sheet must be annotated to indicate the presence of a contaminant. A footnote of "C" will be applied to the entry to indicate that the growth was determined not to be the test microbe (see Attachment A for list of footnotes).

10.10 Neutralization Confirmation – General Description:

10.10.1 Wiped slides will be evaluated to ensure that the neutralizing agent effectively neutralizes the active ingredients in the towelette product.

10.10.2 The neutralization of the active ingredients found in antimicrobial towelettes is an important step in efficacy testing. A neutralizing agent is used to inactivate the product's active ingredients, a process essential to achieving the desired contact time. In addition, the neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbes. Bacteriostatic activity may bias the outcome of an efficacy evaluation.

10.10.3 The test parameters specified for product testing (e.g., organic soil, neutralizer, contact time, temperature) must also be followed for the neutralization confirmation assay. The manipulation of carriers, towelettes, etc. will be identical to the efficacy evaluation.

10.10.4 This assay is designed to simulate the conditions of the efficacy evaluation (i.e., towelette test) of the product; however, sterile glass slide carriers are used instead of inoculated carriers. Diluted inoculum (*S. aureus* or *P. aeruginosa*) is added directly to the various sets of neutralizer and subculture media tubes. The inoculum is quantified by plating on a suitable agar medium such as TSA. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer/subculture media/disinfectant carryover combinations.

10.11 Neutralization Confirmation – Preparation of Inoculum (*S. aureus* and *P. aeruginosa*):

10.11.1 Inoculum of both organisms is harvested according to the procedures outlined in section 10.3. Record harvest information (carrier inoculation information is not applicable to this study) on the form entitled Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Carrier Inoculation Steps.

10.11.2 If the product test conditions include the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum as per section 10.3.11. Otherwise, the inoculum should be prepared without the addition of an organic soil load.

10.12 Neutralization Confirmation – Determination of Inoculum Titer (*S. aureus* and *P. aeruginosa*):

10.12.1 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the inoculum into 9 mL of phosphate buffered dilution water (PBDW). 0.1 mL of three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) will be used to inoculate the neutralizer and subculture media tubes. The target number of cells to be delivered is 5-100 CFU/mL; this level should be seen in one of the two highest dilutions.

10.12.2 To estimate CFU/mL, plate the dilutions (0.1 mL aliquots of 10^{-5} through $10^{-7)}$ in duplicate on TSA agar. Briefly vortex each dilution tube prior to plating.

10.12.3 Record the dilution and plating information on the Neutralization Confirmation Assay of Towelette Products: Serial Dilution/Plating Form (see 16.11).

10.12.4 Incubate plates at 36±1°C for 24-48 hours. Count colonies with aid of a plate counter. Plates that have colony counts over 300 can be estimated or labeled Too Numerous To Count (TNTC). Record the counts on the Neutralization Confirmation Assay of Towelette Products: Serial Dilution/Plating Form (see 16.11).

10.13 Neutralization Confirmation – Product Sample Preparation:

10.13.1 Follow sections 10.6.1 and 10.6.2 for guidelines concerning handling disinfectant towelettes.

10.14 Neutralization Confirmation - Performing the Assay. The following instructions apply to the analysis of one neutralizer and one test organism:

10.14.1 The assay will require three sterile, uninoculated glass slide carriers per test organism. The three carriers will be wiped by one towelette (only 1 towlette used). Carriers are prepared according to section 10.2 but not dried for 40 minutes at 36±1ºC.

10.14.2 Treatments:

10.14.2.1 Per test, one test per organism, three carriers are wiped with one towelette and exposed to the disinfectant for the specified contact time in the same manner as product efficacy testing (see section 10.7). Record the carrier transfer information on the Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Transfers (see 16.7).

10.14.2.2 Wipe the carriers aseptically and discard the towelette.

10.14.2.3 As the contact time for each slide is completed, transfer the slide to 20 mL of neutralizer. Incubate the three tubes for a minimum of 30 minutes at 36±1ºC. This set represents the **Neutralizer Treatment.**

10.14.2.4 After a minimum of 30 minutes of incubation, move the slides as described in section 10.7.16 into tubes of subculture media. The set of three tubes containing the carrier represents the **Subculture Treatment.**

10.14.2.5 Each tube of the Neutralizer Treatments and the Subculture Treatments will be inoculated with one of the three inoculum dilutions (section 10.12.1) as indicated in Table 1 and section 10.14.5.

10.14.2.6 Repeat the assay with the second test organism, if required.

10.14.3 Inoculated Controls:

10.14.3.1 Inoculate three tubes of neutralizer and three tubes of subculture media with one of the three inoculum dilutions as indicated in Table 1. These tubes represent the **Neutralizer and Subculture Positive (or inoculated) Controls.**

10.14.3.2 It is highly desirable that the preparation (media preparation number) of each medium be the same as used in the treatments.

10.14.4 Sterility Controls:

10.14.4.1 **Neutralizer Negative** and **Subculture Negative Controls**. One tube each of neutralizer and subculture media are placed with the other tubes. They are not inoculated with organism.

10.14.5 Inoculating the Tubes:

10.14.5.1 Inoculate each treatment and positive control tube with 0.1 mL of the diluted inoculum as indicated in Table 1. Inoculate the media following the transfer of all carriers into the subculture media.

10.14.5.2 Incubate all tubes for 48±2 hours at 36±1ºC, including the sterility controls.

Table 1. Inoculation of Treatment and Control Groups with Dilutions of the Test Organism*

Neutralizer Treatment	Subculture Treatment	Neutralizer Positive Control	Subculture Positive Control	Neutralizer & Subculture Negative Controls
0.1 mL of 10^{-5} □ Tube 1 0.1 mL of 10^{-6} □ Tube 2 0.1 mL of 10^{-7} □ Tube 3	0.1 mL of 10^{-5} □ Tube 1 0.1 mL of 10^{-6} □ Tube 2 0.1 mL of 10^{-7} □ Tube 3	0.1 mL of 10^{-5} □ Tube 1 0.1 mL of 10^{-6} □ Tube 2 0.1 mL of 10^{-7} □ Tube 3	0.1 mL of 10^{-5} □ Tube 1 0.1 mL of 10^{-6} □ Tube 2 0.1 mL of 10^{-7} □ Tube 3	Not inoculated 1 Tube of Neutralizer 1 Tube of Subculture Media

*Based on an approx. starting suspension of 10^{7} to 10^{8} CFU/mL

10.15 Neutralization Confirmation – Results.

10.15.1 Each tube is shaken prior to recording results to determine the presence or absence of turbidity. A positive result is one in which the broth culture appears turbid. A negative result is one in which the broth appears clear. Report results as + (growth) or 0 (no growth) on the Neutralization Confirmation Assay of Towelette Products: Test Results Form (see 16.9).

10.16 Neutralization Confirmation - Confirmation Procedures for Tests with *S. aureus* and *P. aeruginosa*:

10.16.1 A minimum of one positive tube per treatment and control, if available, should be tested using Gram staining. If confirmation is deemed necessary, selective media and VITEK® analysis may be used.

10.16.2 For each treatment and control group, select the tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered) and conduct confirmation testing on a sample of the growth. Record confirmation results on the Neutralization Confirmation Assay of Towelette Products: Confirmation Results (see 16.10).

10.17 Neutralization Confirmation – Interpretation of Results:

10.17.1 Plate count data. One of the three dilutions plated should provide counts within the approximate target range, 5-100 CFU/mL.

10.17.1.1 Note: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum is added to the subculture tubes/filters.

10.17.2 Growth in the **Subculture Positive** Control verifies the presence of the test microbe, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.* Growth in the **Neutralizer Positive** Control should be comparable to the Secondary Positive Control if the neutralizer is the same as the subculture media.

10.17.3 There may be cases when the neutralizer is significantly different from the subculture media. In these cases, growth may not be comparable to the Secondary Positive Control.

10.17.4 The **Neutralizer** and **Subculture Negative** Control tubes are used to determine sterility, and must show no growth for the test to be valid.

10.17.5 The occurrence of growth in the **Neutralizer and Subculture Treatment** tubes is used to assess the effectiveness of the neutralizer to neutralize residual disinfectant present on the carrier. The neutralizer itself or in combination with the subculture medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the **Subculture** treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).*

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Plate counts are enumerated and CFU/mL is calculated based on the average of countable plates.

11.2 The evaluation of the data to determine whether a bacteriostatic effect is present is described in section 10.17.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in secure file cabinets in D217. Only authorized personnel have access to the secure files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocol must be brought to the study director's attention and recorded in the raw data, and an explanation for the deviation given. The deviation and reason for it must be documented on the GLP Compliance form in the final report.

15.0 REFERENCES:

15.1 bioMe!rieux VITEK, Inc. 2004. Vitek 2 Compact Online Software User Manual. Part Number: 510773-1EN1.

15.2 bioMe!rieux VITEK, Inc. 2004. Vitek 2 Compact Hardware User Manual. Part Number: 510773-1EN1.

15.3 bioMe!rieux VITEK, Inc. 2003. DensiChek User's Manual. Part Number : 93060 Version C.

15.4 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA, (Methods 955.15, 964.02, and 961.02).

16.0 FORMS AND DATA SHEETS:

16.1 Disinfectant Towelette Test: Time Recording Sheet for Carrier Inoculation Steps

16.2 Disinfectant Towelette Test: Time Recording Sheet for Transfers

16.3 Disinfectant Towelette Test: Test Information Sheet

16.4 Disinfectant Towelette Test: Test Results Form

16.5 Disinfectant Towelette Test: Confirmation Results

16.6 Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Carrier Inoculation Steps

16.7 Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Transfers

16.8 Neutralization Confirmation Assay of Towelette Products: Test Information Sheet

16.9 Neutralization Confirmation Assay of Towelette Products: Test Results Form

16.10 Neutralization Confirmation Assay of Towelette Products: Confirmation Results

16.11 Neutralization Confirmation Assay of Towelette Products: Serial Dilution/Plating Form

Attachment A: Testing Footnotes and Explanations

Disinfectant Towelette Test: Time Recording Sheet for Carrier Inoculation Steps

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______	
Test Date	
Product Reg. No.	
Product Name	
Sample No(s).	
Test Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Seeding Time*		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
Comments:							

* Recorded from laboratory clock/and timer.

Disinfectant Towelette Test: Time Recording Sheet for Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______	
Test Date	
Product Reg. No.	
Product Name	
Sample No(s).	
Test Organism	

Initials/date	Slide Set	Drop Interval	Carrier Wipe Start Time		Carrier Wipe End Time (last carrier into the neutralizer/ primary subculture)		Carrier Transfer (into secondary subculture)[1]
			Clock	Timer	Clock	Timer	
Comments:							

[1]Taken from clock.

Disinfectant Towelette Test: Test Information Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		SOP	
Product Name		Test Date	
Sample No.		Comments:	
Lot No.			
Expiration			

TEST PARAMETERS/Confirmed by:_______		
Organic Soil	Specified	As Prepared/Date/Init
		/ /
Neutralizer	Specified	
Temperature	Specified	Actual
		Before: After:
Contact Time	Specified	As Tested
Other Parameters	Specified	

TEST MICROBE INFORMATION/Confirmed by:________				
Test Microbe		48-54 Hour Culture		
Org. Control No.		Date/Time	Initiated	Harvested
Avg. CFU/Carrier				

REAGENT/MEDIA INFORMATION/Confirmed by:________			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Disinfectant Towelette Test: Test Results Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.			

CARRIER INFORMATION/Confirmed by:_________		
Carrier Wipe Time Interval	Carrier Set	Analysts Wiping/Transferring Slides

TEST RESULTS									
Date/Initials									
Primary Subculture / Secondary Subculture (slides)									
1	2	3	4	5	6	7	8	9	10
/	/	/	/	/	/	/	/	/	/
11	12	13	14	15	16	17	18	19	20
/	/	/	/	/	/	/	/	/	/
21	22	23	24	25	26	27	28	29	30
/	/	/	/	/	/	/	/	/	/
31	32	33	34	35	36	37	38	39	40
/	/	/	/	/	/	/	/	/	/
41	42	43	44	45	46	47	48	49	50
/	/	/	/	/	/	/	/	/	/
51	52	53	54	55	56	57	58	59	60
/	/	/	/	/	/	/	/	/	/
Comments:									

Disinfectant Towelette Test: Confirmation Results

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.		Comments	

Source: Tube/Plate ID	Date/Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)

[1]Record Gram Stain results as GPC=gram positive cocci or GNR=gram negative rods.

Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Carrier Inoculation Steps
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______	
Test Date	
Product Reg. No.	
Product Name	
Sample No(s).	
Test Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Seeding Time*		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/
Comments:							

* Recorded from laboratory clock/and timer.

Neutralization Confirmation Assay of Towelette Products: Time Recording Sheet for Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:________	
Test Date	
Product Reg. No.	
Product Name	
Sample No(s).	
Test Organism	

Initials/date	Slide #	Drop Interval	Carrier Wipe Start Time		Carrier Wipe End Time (carrier into the neutralizer/primary subculture)		Carrier Transfer (into secondary subculture)[1]
			Clock	Timer	Clock	Timer	
Comments:							

[1]Taken from the clock.

Neutralization Confirmation Assay of Towelette Products: Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:________			
EPA Reg. No.		SOP	
Name		Test Date	
Sample No.		Comments:	
Lot No.			
Expiration			

TEST PARAMETERS/Confirmed by:_______		
Organic Soil	Specified	As Prepared/Date/Init
		/ /
Neutralizer	Specified	
Temperature	Specified	Actual
		Before: After:
Contact Time	Specified	As Tested
Other Parameters	Specified	

TEST MICROBE INFORMATION/Confirmed by:________				
Test Microbe		48-54 Hour Culture		
Org. Control No.		Date/Time	Initiated	Harvested
Avg. CFU/Carrier				

REAGENT/MEDIA INFORMATION/Confirmed by:________			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralization Confirmation Assay of Towelette Products: Test Results Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.			

CARRIER INFORMATION/Confirmed by:_________		
Carrier Wipe Time Interval	Carrier Number	Analysts Wiping/Transferring Slides

TEST RESULTS: Date Recorded/Initials: _________________			
Treatment/Controls	Dilutions of Organism		
	10^{-5}	10^{-6}	10^{-7}
Neutralizer Treatment			
Subculture Treatment			
Neutralizer Positive Control			
Subculture Positive Control			
Uninoculated:			
Neutralizer Negative Control			
Subculture Negative Control			

SUMMARY OF RESULTS: Date/Initials:____________________________	
Bacteriostatic Effect Observed?	Yes________ No_________
If yes, which treatment/media?	
Comments:	

Neutralization Confirmation Assay of Towelette Products: Confirmation Results

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		Test Date	
Product Name		Test Organism	
Sample No.		Comments	

Source: Tube/Plate ID	Date/Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)

[1]Record Gram Stain results as GPC=gram positive cocci or GNR=gram negative rods.

Neutralization Confirmation Assay of Towelette Products: Serial Dilution/Plating Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:________			
EPA Reg. No.		Test Date	
Product Name		Neutralizer(s)	
Sample No.		Organism Control #	

	Dilution Tube							
Confirmed by:_______	-1	-2	-3	-4	-5	-6	-7	-8
Vol. In Dil. Tube prior to Addition								
Volume Added to Dil. Tube								
Overall Dilution in Dil. Tube								
Volume Plated								
Overall Dilution on Plate								
Number of Plates per Dilution								
Media Plated Onto								
Comments:								

REAGENT/MEDIA INFORMATION/Confirmed by:_______			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

RESULTS: Date/Initials:_________________________			
Plating Method			
	CFU per Dilution Plate		Average CFU per Plate
Dilution*	Plate 1	Plate 2	
10^{-5}			
10^{-6}			
10^{-7}			
TNTC = Too Numerous To Count			
**Average CFU/mL =			

*Plated 0.1 mL of specified dilution. **Based on a range of 30-300 CFU/plate.

Attachment A:

Testing Footnotes and Explanations
OPP Microbiology Laboratory

Footnote	Description
A	Indicates that the seeded carrier, hook, or forceps hit the interior sides of the medication tube containing disinfectant as the carrier was being dropped.
B	Indicates that the carrier was lost (dropped) during a transfer and was not recovered.
C	Indicates that a tube of a positive carrier set (one showing growth) was later determined to be a contaminant and not the test microbe. In “Comments” refer to the confirmation information for details.
D	Indicates that the primary or secondary subculture tube containing the carrier broke during vortexing. In the “Comments” indicate if carrier was recovered or if the remaining broth was placed in another tube.
E	Indicates that the carrier was exposed to the disinfectant late or early, outside of the +/- 5 second drop, spray, or wipe interval. In “Comments” indicate the approximate number of seconds outside (+/-) of the 5 second interval.
F	Indicates that the carrier was placed in the neutralizer late or early, outside of the +/- 5 second drop interval. In “Comments” indicate the approximate number of seconds outside (+/-) of the 5 second interval.