



**US Environmental Protection Agency
Office of Pesticide Programs**

**Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD**

**Standard Operating Procedure for
Germicidal Spray Products as Disinfectants:
Staphylococcus aureus, Pseudomonas aeruginosa,
Salmonella enterica, and Mycobacterium bovis (BCG)**

SOP Number: MB-06-03

Date Revised: 09-04-08

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

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for
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Initiated By: _____ Date: ___/___/___
Print Name

Technical Review: _____ Date: ___/___/___
Print Name
Technical Staff

QA Review: _____ Date: ___/___/___
Print Name
QA Officer

Approved By: _____ Date: ___/___/___
Print Name
Branch Chief

Effective Date: _____/_____/_____

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the methodology used to determine the efficacy of germicidal spray products as disinfectants (AOAC Method 961.02, 15th Ed.) against three test organisms, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella enterica*. This SOP also includes an adaptation of AOAC Method 961.02 for determining the efficacy of spray products as hard surface disinfectants against *Mycobacterium bovis* (BCG).

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 ATCC = American Type Culture Collection

2.3 MPB = Modified Proskauer Beck medium

2.4 M7H9 = Middlebrook 7H9

2.5 M7H11 = Middlebrook 7H11

2.6 TSA = Trypticase soy agar

2.7 A carrier set for *S. aureus*, *P. aeruginosa*, or *S. enterica* is composed of the primary and secondary subculture tubes for each carrier. There are 60 glass slide carrier sets per lot of product sample tested per organism.

2.8 A carrier set for *M. bovis* (BCG) is composed of the primary MPB tube containing the slide along with duplicate tubes of two additional subculture media (5 tubes per carrier) inoculated from the corresponding neutralizer tube. There are 10 slide carrier sets (5 tubes per set).

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, Lab Biosafety.

3.2 Disinfectants may contain a number of different active ingredients, such as quaternary ammonium compounds, halogens, phenolics, aldehydes, peroxides, and heavy metals. Latex gloves and other personal protective clothing or devices are worn during the handling of these items. A chemical fume hood or other containment equipment is employed when performing tasks with products.

4.0 CAUTIONS:

- 4.1 Strict adherence to the protocol is necessary for the validity of the test results.
- 4.2 Do not allow the inoculum to contact the edge of the glass slide carriers during the inoculation process. Contamination of the sides of the carriers with the test microbe may lead to false positives.
- 4.3 The external surface of the micropipette 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 micropipette will be thoroughly wiped with 70% ethanol prior to removal from the BSC.

5.0 INTERFERENCES:

- 5.1 The carriers should be dry inside the Petri dishes. Moisture can interfere with the concentration and drying of the inoculum on the glass slide carrier.
- 5.2 Any carrier that is wet at the conclusion of the carrier drying period should not be used.

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 *Test organisms.* *Pseudomonas aeruginosa* (ATCC No. 15442), *Staphylococcus aureus* (ATCC No. 6538) and *Salmonella enterica* (ATCC No. 10708) obtained directly from a reputable supplier (e.g., ATCC). *Mycobacterium bovis* (BCG) obtained directly from Organon Teknika.
- 7.2 *Culture media* (e.g., nutrient broth, synthetic broth, nutrient agar).
Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Methods 955.15 and 964.02 may be substituted.
- 7.3 *Subculture media* (e.g., letheen broth, fluid thioglycollate medium).
Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Methods 955.15 and 964.02 may be substituted.

- 7.4 *Sterile water.* Use reagent-grade water. Reagent-grade water should be free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.
- 7.5 *Carriers.* Glass Slide Carriers, Bellco 25 mm × 25 mm (or comparable size) borosilicate glass cover slips with number 4 thickness (Bellco Glass, Inc., item number 1916-S0131).
- 7.6 *Glassware.* For cultures/subcultures, use autoclavable 38 × 100 mm medication tubes (Bellco Glass Inc., Vineland, NJ). For stock cultures, use 16 × 100 mm screw cap tubes. Cap tubes with closures before sterilizing. Sterilize all glassware in hot air oven at 180°C or steam sterilize for a minimum of 20 minutes at 121°C with drying cycle.
- 7.7 *Test tube racks.* Any convenient style.
- 7.8 *Pipettes.* Any micropipette with a volume range including 10µL (e.g., a volume range of 2-20 µL).
- 7.9 *Spectrophotometer.* Any spectrophotometer able to measure 650nm (e.g., Beckman DU Series 500).
- 7.10 *Spray Disinfectant Apparatus.* Refer to Attachment B or other suitable spray disinfectant apparatus.
- 8.0 INSTRUMENT OR METHOD CALIBRATION:
- 8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.
- 9.0 SAMPLE HANDLING AND STORAGE:
- 9.1 Disinfectants are stored according to the manufacturer's recommendations if stipulated, or at room temperature. Those disinfectants requiring activation or dilution prior to use are activated or diluted within three hours of testing unless test parameter specify otherwise.

- 9.2 Follow chain-of custody guidelines during testing as stipulated in SOP COC-01, Chain of Custody.

10.0 PROCEDURE AND ANALYSIS:

10.1 Carrier Preparation

- 10.1.1 Physical Screening and Cleaning: Visually screen glass slide carriers for scratches, chips or cracks and discard those which are damaged or defective.
- 10.1.2 Prior to carrier preparation for testing, rinse the carriers once with DI water, rinse three times with 95% ethyl alcohol, and finally rinse three times with DI water.
- 10.1.3 Drain and allow carriers to dry before use. Record screening results in the Physical Screening of Carriers Record form (see 16.1).
- 10.1.4 Place one glass slide carrier into a petri dish with 2 pieces of Whatman No. 2 filter paper. Fill out a media/reagent preparation sheet to assign a preparation number to a set of carriers (see SOP QC-15, Media Prep and Sterilization Run Numbers).
- 10.1.5 Autoclave for 45 minutes at 121°C with a 30 minute dry cycle or sterilize for 2 hours in a hot air oven at 180°C .
- 10.1.6 All glass slide carriers used in testing are discarded.

10.2 Test Culture Preparation for *S. aureus*, *P. aeruginosa*, and *S. enterica*

- 10.2.1 Initiate test culture by inoculating a 10 mL tube (20 × 150 mm) of nutrient broth or synthetic broth from a stock slant or stab culture. Transfer one 4 mm ID loopful (or use a 10 µL certified transfer loop) of inoculum from the stock culture into the broth. Refer to SOP MB-02, Test Microbes: Initiation, Maintenance and Quality Control, for stock culture preparation for *S. aureus* and *P. aeruginosa*. The test culture maintenance and preparation for *S. enterica* is described in AOAC Method 955.14A.
- 10.2.2 Two sets of cultures (one set as a backup) 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 actual testing.

Select set with typical growth.

- 10.2.3 Make at least 3 consecutive 24 ± 2 hour transfers (use one 4 mm ID loopful, or a 10 μ L certified transfer loop, or a calibrated micro volume pipet to deliver 10 μ L) in 10 mL nutrient broth or synthetic broth incubated at $36 \pm 1^\circ\text{C}$. Up to 30 ± 2 total transfers are allowed. If only one of the consecutive 24 hour transfers has been missed, it is not necessary to repeat the previous 3-day sequence prior to the inoculation of the 48-54 hour test culture.
- 10.2.4 For the final subculture step, inoculate for the test procedure, a sufficient number of 25×150 mm tubes (e.g., six to eight) containing 20 mL nutrient or synthetic broth; incubate 48-54 hours at $36 \pm 1^\circ\text{C}$.
- 10.2.5 A minimum of 5 days are required to obtain the culture for inoculating carriers. For example, the culture sequence must begin on Thursday for testing to commence on the following Tuesday.
- 10.2.6 Record all culture transfers on the Organism Culture Tracking form (see SOP MB-02, Test Microbes).
- 10.2.7 For *S. aureus* and *S. enterica*, using a vortex-style mixer, mix 48-54 hour nutrient broth test cultures 3-4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture (e.g., upper $\frac{3}{4}$ or 15 mL), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot 20 mL portions into sterile 25×150 mm test tubes. Prepare at least 2 tubes.
- 10.2.8 For *P. aeruginosa*, do not shake 48-54 hour test culture. The pellicle from the 48-54 hour cultures must be removed from the broth before mixing on a vortex mixer either by decanting the liquid aseptically into a sterile tube or by gently aspirating the broth away from the pellicle using a pipette. 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 efficacy test. Once the pellicle is removed, using a vortex-style mixer, mix nutrient broth test cultures 3-4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture (e.g., upper $\frac{3}{4}$ or 15 mL), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot 20 mL

portions into sterile 25 × 150 mm test tubes. Prepare at least 2 tubes.

10.2.9 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 × 150 mm test tubes. Prepare at least 2 tubes.

10.2.10 Carrier Inoculation with *S. aureus*, *P. aeruginosa*, and *S. enterica*:

10.2.10.1 During the inoculation of the glass slide carriers, occasionally vortex the test culture to ensure the inoculum remains in suspension. Transfer 0.01 mL of the test culture with a sterile capillary pipette or micropipette with sterilized tips onto the 25 mm × 25 mm sterile dry glass slide. Spread the inoculum uniformly on the glass slide immediately using a sterile 4 mm loop; do not allow the inoculum to contact the edge of the glass slide carriers during the inoculation process.

10.2.10.2 After completion of all slide inoculations, thoroughly wipe the micropipette with 70% ethanol prior to removal from the BSC.

10.2.10.3 Dry the slides for 40 minutes at 36±1°C for testing of *S. aureus*, *P. aeruginosa*, or *S. enterica*.

10.2.10.4 Any carrier that is wet at the conclusion of the carrier drying period should not be used.

10.2.10.5 Record timed carrier inoculation activities on the Time Recording Sheet for Carrier Inoculation (see 16.2).

10.3 Test Culture Preparation for *Mycobacterium bovis* BCG:

10.3.1 Initiate test culture by inoculating several 20 mL tubes (25 × 150 mm) of MPB from a M7H9 stock agar slant by transferring one 4 mm id loopful or equivalent inoculum from the stock culture onto the surface of the broth. Typically, 2-4 M7H9 stock agar slants are used to inoculate 10-20 tubes of MPB. Record all transfers on the Organism Culture Tracking Form (culture notation = –SL).

10.3.2 Incubate the tubes 21–25 days undisturbed at 36 ± 1°C preferably in a

slanted position to increase surface area.

Note: The test cultures must be carefully managed. Over-inoculation of MPB may lead to reduced viability due to excessive growth after 21–25 days, and the resulting carrier counts may be negatively impacted. Inoculation of MPB with a smaller amount of inoculum (i.e., a partial loopful) may lead to higher quality cultures.

- 10.3.3 These tubes may be used as test culture or to conduct another set of transfers. If conducting a second series of transfers, use the 10-20, 21-25 day old cultures grown in MPB and inoculate an additional 25 × 150 mm tubes, each containing 20 mL of MPB. Incubate in a slanted position without disturbing for 21-25 days at $36 \pm 1^\circ\text{C}$. Record all transfers on the Organism Culture Tracking Form (culture notation = –LL).
- 10.3.4 Depending on the amount of growth from each 21-25 day old culture, 10-20 of the cultures may be required to generate enough standardized inoculum (approx. 75 mL) for a “typical” test day. A typical test day will require 15 inoculated carriers, 1 carrier per dish. These carriers are used for the following:
- Test of 1 product sample (15 total carriers; 10 for testing, 3 for carrier counts, 2 are extras).
- 10.3.5 One the day of the test: Using a transfer loop, transfer culture to a heat-sterilized glass tissue grinder, add 1.0 mL 0.1% polysorbate 80 in saline solution, grind to break up large clumps or aggregates of the test organism.
- 10.3.6 Dilute the homogenized culture with 9 mL MPB and transfer the suspension from the tissue grinder to a sterile test tube.
- 10.3.7 Allow the suspension to settle for 10-15 min.
- 10.3.8 Remove the upper portion of each culture, leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix.
- 10.3.9 Dilute the pooled culture with MPB to achieve $20.0 \pm 1\%$ T at 650 nm.

Note: Unless using a 1.5 mL semimicrocuvette with appropriate cap to

measure the transmittance, wear half face respirators with HEPA filter cartridges during this process.

- 10.3.10 If an organic soil load is specified in the test parameters for the product test, measure the culture and add the appropriate amount of soil to the standardized culture. Swirl to mix.
- 10.3.11 Use standardized culture to inoculate porcelain cylinders.
- 10.3.12 Carrier Inoculation with *M. bovis* (BCG):
 - 10.3.12.1 Transfer 0.01 mL of the test culture with a sterile capillary pipette or micropipette with sterilized tips onto the 25 mm × 25 mm sterile dry glass slide. Spread the inoculum uniformly on the glass slide immediately using a sterile 4 mm loop; do not allow the inoculum to contact the edge of the glass slide carriers during the inoculation process. During the inoculation of the glass slide carriers, occasionally vortex the test culture to ensure the inoculum remains in suspension.
 - 10.3.12.2 After completion of all slide inoculations, thoroughly wipe the micropipette with 70% ethanol prior to removal from the BSC.
 - 10.3.12.3 Dry the slides for 30 minutes at 36±1°C when performing tuberculocidal testing with *M. bovis* (BCG).
 - 10.3.12.4 Any carrier that is wet at the conclusion of the carrier drying period should not be used.
 - 10.3.12.5 Record timed carrier inoculation activities on the Time Recording Sheet for Carrier Inoculation (see 16.2).

10.4 Disinfectant Sample Preparation:

- 10.4.1 Prepare disinfectant samples aseptically according to the test parameters. Use of the product, contact time, temperature, diluent, organic soil, hard water, and neutralizer will be specified. Record test parameter information on the Test Information Sheet (see 16.4).
- 10.4.2 Follow chain-of-custody guidelines for disinfectant samples as

stipulated in SOP COC-01, Chain of Custody.

- 10.4.3 For spray products which require preparation (e.g., preparing a use-dilution, use of a pump or trigger based sprayer) proceed as described in 10.4.4 through 10.4.8.
 - 10.4.4 To ensure stability, prepare the disinfectant dilutions within three hours of performing the assay unless test parameters specify otherwise.
 - 10.4.5 Prepare all dilutions with sterile standardized volumetric glassware. Record preparation of disinfectant on the Media and Reagent Prep Sheet.
 - 10.4.6 Prior to opening the container, gently shake the container and thoroughly clean the area around the cap and spout with 70% ethanol. Allow the surface to dry. Remove the cap. Do not touch the inside surface of the cap. If present, carefully remove the seal attached to the top of the spout with sterile instruments (i.e., razor blade, forceps).
 - 10.4.7 Pour an appropriate aliquot of the sample into a sterile beaker. Do not place a pipette or any other instrument inside the product container. Place the cap on the product container and secure tightly. From the beaker, dispense ready-to-use products directly into sterile medication tubes or initiate dilutions for diluted products.
 - 10.4.8 Use ≥ 1.0 mL of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to two decimal places toward a stronger product.
 - 10.4.9 For aerosol spray products, shake the can 25 times prior to use, unless otherwise specified by the manufacturer. The cans are immobilized in the Spray Disinfectant Apparatus (see Attachment B) or other suitable apparatus, and the distance from the nozzle to the inoculated carrier is measured to ensure the correct distance. Prior to testing, the spray nozzle is wiped with 70% ethanol and allowed to dry. Spray the product for 10-15 seconds prior to commencement of the test.
- 10.5 Spray Tests with *S. aureus*, *P. aeruginosa*, or *S. enterica*:
- 10.5.1 After the required drying time, slides are sprayed successively at timed intervals (typically 30 seconds) while in the Petri dish.

- 10.5.2 If a specific time is stipulated by the manufacturer other than a typical 10 minute exposure time, the interval is modified to accommodate the label claim.
- 10.5.3 The slide must be sprayed within ± 5 seconds of the specified time for a 10 minute contact time or within ± 3 seconds for contact times less than 1 minute. After spraying, maintain the Petri plates in a horizontal position. Treated carriers must be kept undisturbed during the contact time.
- 10.5.4 After the last slide of a set (typically 20 slides) has been sprayed with the disinfectant, and the exposure time is complete, transfer each slide in order into the primary subculture tube containing a neutralizer within the ± 5 second time limit. Drain the excess disinfectant from each slide prior to transfer into the neutralizer tube. Carriers should be drained without touching the Petri dish or filter paper. Transfers are made with flame sterilized forceps.
- 10.5.5 Each of the remaining slides is transferred into their corresponding subculture tubes at the appropriate time.
- 10.5.6 The slide can touch both the interior sides of the Petri dish and the subculture tube during the transfer, but this contact should be avoided as much as possible.
- 10.5.7 After the slide is deposited, the subculture tube is carefully recapped and shaken for a few seconds. Alternately, a set of tubes may be shaken after all primary transfers are completed.
- 10.5.8 After all the slides have been transferred, the subculture tubes are placed in a $36\pm 1^{\circ}\text{C}$ incubator.
- 10.5.9 A minimum of 30 minutes after the last slide was deposited into the neutralizer, transfer each slide to a secondary subculture tube containing 20 mL of the appropriate subculture medium.
- 10.5.10 Transfer the slides from the primary to secondary tubes in order but the movements do not have to be timed. Shake the tubes after all of the slides have been transferred.
- 10.5.11 Incubate the primary and secondary subculture tubes at $36\pm 1^{\circ}\text{C}$ for

48±2 hours. A total of 120 tubes will be incubated per sample tested per organism.

- 10.5.12 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.
 - 10.5.13 Determine the carrier counts (bacterial carrier load) on six carriers selected at random. Enumeration will be performed as stipulated in SOP MB-04: Enumeration of Bacterial Inocula on Carriers (Carrier Counts).
 - 10.5.14 Positive controls. On the day of testing, place a dried inoculated carrier into a tube containing 20 mL primary subculture medium and a second dried, inoculated carrier into a tube containing 20 mL secondary subculture medium. Incubate tubes for 48±2 hours at 36±1°C. Growth in both tubes validates the test system. Failure to have growth in either of the tubes invalidates the test.
 - 10.5.15 Negative controls. On the day of testing, spray 1 sterile glass slide carrier with the disinfectant and hold for the appropriate contact time (i.e. the contact time used in the efficacy test). After the contact time, drain the excess disinfectant from the slide, transfer it into a tube of neutralizer using flame sterilized forceps, and place the tube in a 36±1°C incubator. After a minimum of 30 minutes in the incubator, transfer the slide to a secondary subculture tube containing 20 mL of the appropriate subculture medium. Growth observed in either of the tubes invalidates the test. Record any observations of interactions between the disinfectant and the subculture media (including neutralizers) on the Test Results Sheet (see 16.4).
- 10.6 Results for *P. aeruginosa*, *S. aureus*, and *S. enterica*:
- 10.6.1 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.
 - 10.6.2 Report results as + (growth) or 0 (no growth) on the Results Sheet (see 16.5).
 - 10.6.3 A positive result in either the primary and/or secondary subculture tube is considered a positive result for a carrier set.

- 10.7 Confirmation Procedures for Spray Tests with *P. aeruginosa*, *S. aureus*, and *S. enterica*:
- 10.7.1 A minimum of three positive carrier sets per test, if available, should be confirmed using Gram staining, solid media, and VITEK™ analysis. If there are less than three positive carrier sets, then each carrier set will be confirmed. If both tubes are positive in a carrier set, only one tube is selected for confirmatory testing (preferably the secondary subculture tube with carrier).
 - 10.7.2 For a test with greater than 20 positive carrier sets, confirm at least 20% by Gram stain, and a minimum of 4 positive carrier sets by Gram staining, solid media, and VITEK™ analysis (see SOP QC-16, VITEK: Culture Identification Numbers and SOP QC-22, VITEK 2 Compact) to ensure the identity of the organism. Again, if both tubes are positive in a carrier set, only one tube (preferably the secondary subculture tube with carrier) is selected for confirmatory testing.
 - 10.7.3 Gram stain reactions, cell morphology, and colony characteristics on solid media are given in SOP MB-02, Test Microbes.
 - 10.7.4 Gram stains are performed on smears taken from the positive culture tubes. 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 18-24 hours at 36±1°C. The selective agar is checked for the correct reaction and the culture on the TSA plate is used for preparing the inoculum for the VITEK™ analysis.
 - 10.7.5 The VITEK™ analysis should always be performed according to the manufacture's instructions,
 - 10.7.6 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 a contaminant was present. 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).
 - 10.7.7 Record confirmation results on the Test Microbe Confirmation Sheet (see 16.9).

10.8 Spray Tests with *M. bovis* (BCG):

- 10.8.1 After the required drying time, slides are sprayed with the disinfectant at 30-60 second intervals.
- 10.8.2 If a specific time is stipulated by the manufacturer other than a 10 minute exposure time, the spray interval is modified to accommodate their claims.
- 10.8.3 The slide must be sprayed within ± 5 seconds of the specified time for a 10 minute contact time or within ± 3 seconds for contact times less than 1 minute. After spraying, maintain the Petri plates in a horizontal position. Treated carriers must be kept undisturbed during the contact time.
- 10.8.4 After one set of slides has been sprayed with the disinfectant and the exposure time is complete, the slides are then transferred in the same sequentially timed fashion into the neutralizer tubes containing the appropriate neutralizer (e.g., letheen neutralizer blank or horse serum).
- 10.8.5 Drain the excess disinfectant from each slide prior to transfer into the neutralizer tube. Carriers should be drained without touching the Petri dish or filter paper.
- 10.8.6 The slide is removed from the Petri dish with flame sterilized forceps.
- 10.8.7 Primary transfers should be within ± 5 seconds of the specified time of transfer.
- 10.8.8 The neutralizer tube is shaken and the slide is immediately removed from the neutralizer tube with flame sterilized forceps and transferred to MPB or the designated subculture tube.
- 10.8.9 The remaining slides are moved into their corresponding neutralizer and subculture tubes at the appropriate times. The lip of the subculture tube does not need to be flamed.
- 10.8.10 After the slide is deposited, the subculture tube is recapped and shaken for a few seconds. Alternately, the tubes may be shaken after all primary transfers are completed.

- 10.8.11 Once all slides have been transferred to the MPB medium, transfer 2 mL aliquots from the neutralizer tube into each of 2 tubes of the specified additional subculture media; repeat with all ten slides. Each of two tubes of two additional subculture media receives 2 mL of neutralizer for a total of 4 tubes of additional subculture media.
- 10.8.12 The transfers of neutralizer medium into subculture media are not timed.
- 10.8.13 Incubate all tubes for 60 days at $36\pm 1^{\circ}\text{C}$. If no growth or occasional (insufficient for confirmation purposes) growth occurs within a tube, incubate an additional 30 days and record the final results.
- 10.8.14 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.
- 10.8.15 Determine the carrier counts (bacterial carrier load) on three carriers selected at random. Enumeration will be performed as stipulated in SOP MB-04: Enumeration of Bacterial Inocula on Carriers (Carrier Counts).
- 10.8.16 Positive controls. On the day of testing, place a dried inoculated carrier into a tube of MPB and a tube of each subculture media. Incubate tubes as in the test. Growth in the tubes validates the test system viability.
- 10.8.17 Negative controls. On the day of testing, spray 1 sterile glass slide carrier with the disinfectant and hold for the appropriate contact time (i.e. the contact time used in the efficacy test). After the contact time, drain the excess disinfectant from the glass slide and transfer it into a tube with 20 mL neutralizer using flame sterilized forceps. Shake the tube containing the neutralizer thoroughly and immediately transfer the carrier to a tube containing 20 mL MPB. Transfer 2 mL aliquots from the neutralizer tube into 1 tube of each of the additional subculture media. Incubate tubes as in the test. Growth observed in any of the tubes invalidates the test. Record any observations of interactions between the disinfectant and the subculture media on the Test Results Sheet for *M. bovis* (BCG) (see 16.8).

10.9 Recording Results for *M. bovis* (BCG):

- 10.9.1 Results are recorded as positive (+) or negative (0) as indicated by the presence or absence of typical mycobacterial growth.
 - 10.9.2 Record results at 60 days. If the 60th day of incubation falls on a weekend or holiday, record the results on the first workday following the 60th day of incubation. Recording of results beyond the 60th day should be notated in the Comments section of the Results block.
 - 10.9.3 Record results at 90 days. If the 90th day of incubation falls on a weekend or holiday, record the results on the first workday following the 90th day of incubation. Recording of results beyond the 90th day should be notated in the Comments section of the Results block.
- 10.10 Confirmatory Procedures for Spray Tests with *M. bovis* (BCG):
- 10.10.1 To confirm the results of testing, representative positive subculture tubes are selected for further investigation.
 - 10.10.2 The maximum number of tubes that is confirmed per sample tested is 10.
 - 10.10.3 At least one positive subculture tube for each carrier set with growth is confirmed.
 - 10.10.4 If more than one subculture tube for a carrier set is positive, only growth in one subculture tube is confirmed. If the MPB in the set is positive, it is the representative subculture tube used for confirmation.
 - 10.10.5 If MPB is not positive, then the order of selecting the representative subculture tubes for confirmation is: M7H9, Kirchners, and TB.
 - 10.10.6 If growth is observed in only one carrier set (5 tubes per set), then all subculture tubes showing growth for that carrier are subject to confirmation.
- 10.11 Identification of *M. bovis* (BCG):
- 10.11.1 The confirmatory tests used to verify the identity of *M. bovis* (BCG) are acid fast staining and typical growth on selective media.
 - 10.11.2 Growth for acid fast staining is taken from the selected positive tubes (based on the hierarchy of media in Sect. 10.10.5) on the day that

results are read. Acid fast rods are typical for *M. bovis* (BCG). The acid fast staining results should be read promptly and prior to assigning a (+) or (0) to the results.

- 10.11.3 If acid fast rods are observed from the selected tubes then a (+) is assigned to the results. If no cells are observed for the acid fast stain then a (0) is applied to the results.
- 10.11.4 In addition, growth from these positive tubes is streaked over the surface of an M7H9 or M7H11 agar plate (selective media) and incubated for 21-25 days at 36±1°C.
- 10.11.5 If a satisfactory smear cannot be obtained directly from the tube, the smear for acid fast staining will be taken from the 21-25 day old M7H9 agar plate that was inoculated with the growth from the tube.
- 10.11.6 Following the 21-25 day incubation period, the colony morphology of the organism on M7H9 or M7H11 agar should be evaluated. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H9 or M7H11 agar (see SOP MB-02, Test Microbes: Initiation, Maintenance and Quality Control).
- 10.11.7 In the event that no cells were observed with acid fast staining initially but typical growth was observed on the M7H9, then the (0) will be corrected to read (+) on the test sheet. An entry error will be noted on the Results Sheet (16.8).
- 10.11.8 Record confirmation results on the Test Microbe Confirmation Sheet (see 16.9).

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

- 12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms (see 16.0). Completed forms are archived in notebooks kept in secured file cabinets in room D217. Only authorized personnel have access to the secured 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 forms (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 recorded on the form and an explanation for the deviation given.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA, (Method 961.02: Germicidal Spray Products as Disinfectants).

15.2 Official Methods of Analysis. 2006. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 955.14A: Testing Disinfectants against *Salmonella enterica*).

16.0 FORMS AND DATA SHEETS:

16.1 Physical Screening of Carriers Record

16.2 AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Inoculation Steps

16.3 AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Transfers

16.4 AOAC Germicidal Spray Test: Information Sheet

16.5 AOAC Germicidal Spray Test: Results Sheet

16.6 AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Inoculation Steps for *M. bovis* (BCG)

16.7 AOAC Germicidal Spray Test: Information Sheet For *M. bovis* (BCG)

16.8 AOAC Germicidal Spray Test: Results Sheet for *M. bovis* (BCG)

16.9 Test Microbe Confirmation Sheet

Attachment A: Testing Footnotes

Attachment B: Spray Apparatus

16.2

AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Inoculation Steps
 OPP Microbiology Laboratory

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

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

* Recorded from laboratory clock/and timer.

16.3

AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Transfers
 OPP Microbiology Laboratory

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

Initials/date	Set	Spray Interval	Carrier Spray Start Time (into the disinfectant)		Carrier Spray End Time (into the neutralizer/primary subculture) ¹		Carrier Transfer (into secondary subculture)
			Clock	Timer	Clock	Timer	Start Time ²
Comments: Carriers sprayed by: _____. Carriers transferred by: _____. For spray test with <i>M. bovis</i> (BCG), neutralizer transferred by: _____.							

¹For spray test with *M. bovis*, the slide end time is when the slide is transferred into the neutralizer tube. The slide is then immediately transferred into MPB.

²Carrier transfer into secondary subculture; taken from clock

16.4
AOAC Germicidal Spray Test: Information Sheet
 OPP Microbiology Laboratory

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

TEST PARAMETERS/Confirmed by: _____			
H ₂ O Hardness (CaCO ₃) ppm	Specified	Titrated (Buret)/Date/Init	HACH/Date/Init
Use Dilution	Specified	As Prepared/Date/Init	
Organic Soil	Specified	As Prepared/Date/Init	
Neutralizer	Specified		
Temperature (°C)	Specified	Temperature	Relative Humidity
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.

16.5
 AOAC Germicidal Spray Test: Results Sheet
 OPP Microbiology Laboratory

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

TEST RESULTS									
Date/Initials									
Primary Subculture/Secondary Subculture (carrier)									
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
/	/	/	/	/	/	/	/	/	/
Results Summary		Number of carrier sets with growth							
		Number of carrier sets without growth							
Controls Summary		Positive Controls*	/				Acceptable: ___Yes___ No		
		Negative Controls*	/				Acceptable: ___Yes___ No		
Comments:									

*Record growth as "+", no growth as "0"

16.6

AOAC Germicidal Spray Test: Time Recording Sheet for Carrier Inoculation Steps for *M. bovis* (BCG)
 OPP Microbiology Laboratory

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

Date/Initials						
Inoculum Settle Time*		Test Culture %T	Carrier Inoculation Time*		Carrier Dry Time*	
Start Time	End Time		Start Time	End Time	Start Time	End Time
/	/		/	/	/	/
/	/		/	/	/	/
Comments:						

*Recorded from laboratory clock/and timer.

16.7
 AOAC Germicidal Spray Test: Information Sheet for *M. bovis* (BCG)
 OPP Microbiology Laboratory

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

TEST PARAMETERS/Confirmed by: _____			
H ₂ O Hardness (CaCO ₃) ppm	Specified	Titrated (Buret)/Date/Init	HACH/Date/Init
Use Dilution	Specified	As Prepared/Date/Init	
Organic Soil	Specified	As Prepared/Date/Init	
Neutralizer	Specified		
Temperature (°C)	Specified	Temperature	Relative Humidity
Contact Time	Specified	As Tested	
Other Parameters	Specified		

TEST MICROBE INFORMATION/Confirmed by: _____			
Test Microbe		21-25 Day Culture	
Org. Control No.		Date/Time	Initiated
Avg. CFU/Carrier			Harvested

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

16.8

AOAC Germicidal Spray Test: Results Sheet for *M. bovis* (BCG)
 OPP Microbiology Laboratory

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

TEST RESULTS											
Date Recorded/Initials		60 Day: _____/90Day: _____									
60 Day Results and 90 Day Results ¹											
Media		Carrier									
		1	2	3	4	5	6	7	8	9	10
MPB	60 day										
	90 day										
M7H9 ³	60 day										
	90 day										
	60 day										
	90 day										
Kirchners TB Broth ^{2,3}	60 day										
	90 day										
	60 day										
	90 day										
Results Summary		Number of carrier sets with growth									
		Number of carrier sets without growth									

CONTROLS ¹	MPB	M7H9	Kirchners/TB Broth ²	Acceptable?
Positive Controls*	/	/	/	____ Yes ____ No
Negative Controls*	/	/	/	____ Yes ____ No
Comments:*60 day results/90 day results				

¹ Record presence or absence of typical *Mycobacterial* growth as positive (+) or negative (0).

² Circle the medium used in the test.

³ There are two subculture sets for this media. The upper row represents subculture set one and the lower row subculture set two.

16.9
 Test Microbe Confirmation Sheet
 OPP Microbiology Laboratory

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

Source: Tube/Plate ID	Date/ Initials	Stain Results*	Media Information			Results		
			Name	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	VITEK ID** (if applicable)

* GPC = gram positive cocci, GNR = gram negative rods, AFR = acid fast rods
 ** VITEK numerical profile number
 *** Use MRME notation for all organisms except *M. bovis* (BCG); use MR notation for *M. bovis* (BCG).

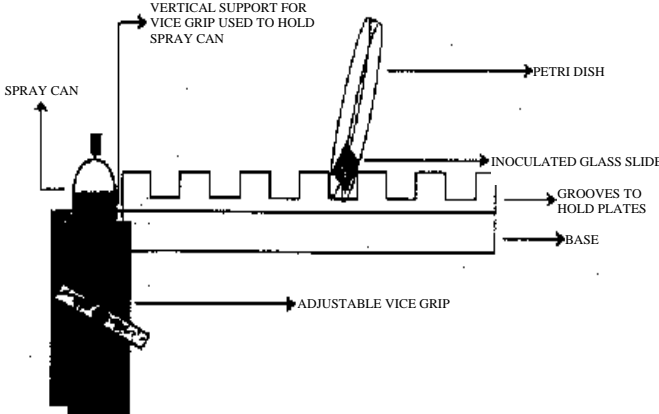
Attachment A:

Testing Footnotes and Explanations
OPP Microbiology Laboratory

Footnote	Description
A	Indicates that the inoculated 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 (\pm) 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 (\pm) of the 5 second interval.

Attachment B:

SIDE VIEW OF SPRAY CAN HOLDER USED FOR SPRAY DISINFECTANT TEST



FRONT VIEW OF SPRAY CAN HOLDER USED FOR SPRAY DISINFECTANT TEST

