US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for Neutralization Confirmation Assay for Disinfectant Products Tested against *Mycobacterium bovis* (BCG)

SOP Number: MB-11-06

Date Revised: 07-08-19

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Title	Neutralization Confirmation Assay for Disinfectant Products Tested against *Mycobacterium bovis* (BCG)
Revisions Made	• Added requirement to document on the results sheet any observed interactions between a product's active ingredient(s) and the neutralizer can interfere with the reading of results. • Minor editorial changes.

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Title	Neutralization Confirmation Assay for Disinfectant Products Tested against *Mycobacterium bovis* (BCG)
Scope	Describes the methodology for determining the effectiveness of a neutralizer when testing the tuberculocidal activity of disinfectants against *Mycobacterium bovis* (BCG) on hard surfaces using liquid, sprays, or towelettes.
Application	For official product testing, a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, dilutions, neutralizers, etc.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. "Exposed" Neutralizer: Neutralizer solution which has been used to inactivate the product. 2. "Unexposed" Neutralizer: Neutralizer solution which has not been used to inactivate the product.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-04 (spectrophotometers), EQ-05 (timers), and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate forms (see section 14).
7. Interferences	1. An interaction between a product's active ingredient(s) and the neutralizer can interfere with the reading of results; if this is observed, record the observations on the results sheet (see section 14). 2. Presence of contamination will interfere with the interpretation of results and may necessitate repeat analysis.
8. Non-conforming Data	1. Management of non-conforming data will be consistent with SOP ADM-07, Non-Conformance Reports. 2. Media performance (Subculture Media Control) must be acceptable to interpret the neutralization results.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of *M. bovis* (BCG) is added to the subculture tubes. 2. There are time sensitive steps in this procedure including the use-period of the test chemical. 3. Verify the volume of dilution blanks, neutralizer tubes, and subculture

	tubes in advance of testing and adjust accordingly. 4. Interactions between the media and neutralizer may result in growth in some media and not others; if this occurs, a more suitable neutralizer should be selected.
11. Special Apparatus and Materials	1. For the neutralization assay, use carriers specified in section 11 (Special Apparatus and Materials) of the appropriate tuberculocidal activity of disinfectants SOP (MB-07, MB-23, or MB-24).
12. Procedure and Analysis	1. For an overview of the setup of this procedure, see Tables 1 and 2. 2. Sterile carriers are used for this assay. Sterile growth medium with or without soil load is applied to the test carriers (via carrier submersion or application using a pipette) in advance of testing to simulate inoculation. 3. Perform the neutralization assay in advance of or concurrently with product testing to verify that the prescribed neutralizer is suitable for the efficacy evaluation. It is preferable to use the same preparation of media to conduct the neutralization and efficacy assays. 4. The general procedure for conducting the assay is the same for liquid, spray, and towelette products. Follow the test parameters specified for product testing (e.g., H_2O hardness, use-dilution, soil load, neutralizer, contact time, temperature) for the neutralization confirmation assay. 5. Use the Neutralization Confirmation Assay for *M. bovis* (BCG): Processing Sheet (see section 14) for tracking testing activities.
12.1 Preparation of Inoculum	a. Prepare standardized inoculum per section 12.1 or 12.2 of MB-07, MB-23, or MB-24.
12.2 Preparation of inoculum dilutions and enumeration	a. Prepare serial ten-fold dilutions of the standardized inoculum in 9 mL of Modified Proskauer Beck medium (MPB) or phosphate buffered dilution water (PBDW). Use four dilutions (e.g., 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) to inoculate the subculture media described in sections 12.5-12.7. The target number of cells is 5-100 CFU/mL; this level should be seen in one of the two highest dilutions. b. To estimate CFU/mL, plate 0.1 mL aliquots of each of the four dilutions in duplicate on M7H11 agar using spread plating. Briefly mix each serial dilution tube prior to plating. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation.

	c. Record the dilution and plating information on the Neutralization Confirmation Assay for *M. bovis* (BCG): Inoculum Enumeration Form (see section 14). d. Incubate plates (inverted) concurrently with the neutralization test subculture tubes at 36±1°C for 17-21 days. An evaluation prior to 17 days may be made for plates with high colony counts. e. Count colonies. Record plates that have colony counts over 300 CFU as TNTC. Record the counts on the Neutralization Confirmation Assay for *M. bovis* (BCG): Inoculum Enumeration Form (see section 14).
12.3 Product Sample Preparation	a. Prepare the product according to the test parameters; follow guidelines provided in SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
12.4 Carrier Preparation	a. Use carrier type required for the specific test. b. Application of sterile medium with or without soil load. i. For CTB: Add 10 sterile carriers to a tube containing 15-20 mL of either MPB or Middlebrook 7H9 broth with 0.1% (v/v) polysorbate 80 (M7H9/P80); use the same sterile medium used to grow the test culture. Add soil load to the sterile medium as necessary per the test parameters. ii. For GSPT: Using a calibrated positive displacement pipette, transfer 10 µL of either MPB or M7H9/P80 onto approximately 1 square inch of the sterile test carrier in the Petri dish; use the same sterile medium used to grow the test culture. Add soil load to the sterile medium as necessary per the test parameters. Immediately spread the medium uniformly over the majority of the carrier surface using a sterile loop. Cover dish immediately. Prepare at least 10 carriers. iii. For DTT: Using a calibrated positive displacement pipette, transfer 10 µL of either MPB or M7H9/P80 onto the sterile test carrier in the Petri dish at one end of the slide; use the same sterile medium used to grow the test culture. Add soil load to the sterile medium as necessary per the test parameters. Immediately spread the medium uniformly over one third of the carrier surface using a sterile loop. Cover dish immediately. Prepare at least 10 carriers.

	c. Dry carriers in incubator at 36±1°C for 30±2 min.
12.5 Subculture Media + Exposed Neutralizer Treatment	a. Requires four dried carriers (with medium added). Each carrier will be associated with one dilution of inoculum. b. Apply the product to the carriers according to the efficacy method SOP and the specific instructions provided in the test parameters. Record the carrier transfer information on the Neutralization Confirmation Assay for *M. bovis* (BCG): Time Recording Sheet for Carrier Transfers (see section 14). c. After the last carrier of a set (4 total carriers) has been treated with the disinfectant and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Drain excess liquid from the carrier prior to the transfer. *Note: For spray and towelette products, use 20 mL neutralizer per 38×100 mm tube. For liquid products use 10 mL neutralizer per 25×100 mm tube.* d. Shake the tube containing the carrier in neutralizer thoroughly and transfer the carrier to the primary subculture medium tube containing 20 mL MPB broth within 5-10 minutes. e. For liquid products, sequentially transfer 2 mL aliquots from each of the four neutralizer tubes into one tube of each of the 2 additional subculture media (M7H9, K, or TB) specified by the test parameters. Do not add neutralizer to the MPB tube. This portion of the assay is not timed, but the aliquots should be transferred to the subculture media within approximately 30 minutes. Inoculate one tube of each medium with one of the four inoculum dilutions prepared in section 12.2. f. For spray and towelette products, sequentially transfer 2 mL aliquots from each of the four neutralizer tubes into two tubes of each of the 2 additional subculture media (M7H9, K, or TB) specified by the test parameters. Inoculate two tubes of each medium with one of the four inoculum dilutions prepared in section 12.2.
12.6 Subculture Media + Unexposed Neutralizer Treatment	a. Requires four dried carriers (with medium added). Each carrier will be associated with one dilution of inoculum. b. Expose four of the carriers to neutralizer, one carrier per tube of neutralizer (this portion of the assay is not timed).

	c. Shake the tube containing the carrier in neutralizer thoroughly and transfer the carrier to the primary subculture medium tube containing 20 mL MPB broth within 5-10 minutes. d. For liquid products, sequentially transfer 2 mL aliquots from each of the four neutralizer tubes into one tube of each of the 2 additional subculture media (M7H9, K, or TB) specified by the test parameters. Do not add neutralizer to the MPB tube. This portion of the assay is not timed, but the aliquots should be transferred to the subculture media within approximately 30 minutes. Inoculate one tube of each subculture media with one of the four inoculum dilutions prepared in section 12.2. e. For spray and towelette products, transfer 2 mL aliquots from each of the four neutralizer tubes into two tubes of each of the 2 additional subculture media (M7H9, K, or TB). Inoculate two tubes of each subculture media with one of the four inoculum dilutions prepared in section 12.2.
12.7 Subculture Media Controls	a. **Subculture Media Control**. This control contains four tubes of each preparation of subculture media used in the neutralization assay. Do not add neutralizer to the media. Inoculate each tube of each medium with one of the four inoculum dilutions prepared in section 12.2. b. **Negative** (uninoculated) **Media Control**. Incubate one uninoculated tube of each medium along with the assay.
12.8 Inoculating Subculture Media	a. Within 30 minutes after all carriers and neutralizer aliquots have been transferred, inoculate the **Subculture Media + Exposed Neutralizer** treatment, the **Subculture Media + Unexposed Neutralizer** treatment, and the **Subculture Media Control** with 0.1 mL of the diluted *M. bovis* (BCG) inoculum (dilution tubes 10^{-2} through 10^{-5}), beginning with the least concentrated dilution.
12.9 Incubation and Presumptive Confirmation Testing	a. Incubate tubes at 36±1°C for up to 60 days; tubes may be monitored for growth prior to 60 days. b. Record results between 45-60 days as positive (+) or negative (0) as indicated by the presence or absence of growth. c. For each medium in the **Subculture Media + Exposed Neutralizer** treatment, the **Subculture Media + Unexposed Neutralizer** treatment, and the **Subculture Media Control**, select the tube with growth from the highest dilution of inoculum (i.e., fewest CFU/mL delivered) and perform acid fast staining on

	a sample of the growth. Acid fast rods are typical for *M. bovis* (BCG). d. If necessary, conduct additional confirmation to include isolation streaks on selective media such as M7H11 agar plates. Following the 17-21 day incubation period, evaluate the colony morphology of the organism on M7H11 agar. On M7H11 agar, *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth. e. Record confirmation results on the Neutralization Confirmation Assay for *M. bovis* (BCG): Test Microbe Confirmation Sheet (see section 14).
12.10Interpretation of Results	a. Plate count data. One of the four dilutions plated should provide counts within the target range, 5-100 CFU/mL. b. Growth in the **Subculture Media Control** (media performance) verifies the presence of the test microbe, performance of the media, and provides a basis for comparing growth in the subculture tubes to growth in the **Subculture Media + Exposed Neutralizer** treatment and the **Subculture Media + Unexposed Neutralizer** treatment. c. The occurrence of growth in the **Subculture Media + Unexposed Neutralizer** treatment as compared to the **Subculture Media Control** tubes is used to assess any bacteriostatic effects attributable to possible interactions between the neutralizer and subculture media. d. The occurrence of growth in the **Subculture Media + Exposed Neutralizer** treatment tubes as compared to the **Subculture Media Control** tubes is used to determine the effectiveness of the neutralizer to inactivate the disinfectant when used under simulated test conditions. e. Verification of neutralizer effectiveness. i. Growth in tubes that were inoculated with dilutions yielding plate counts ranging from TNTC to 5-100 CFU; growth in at least one tube receiving a desired target of 5-100 CFU is required. ii. Growth in the **Subculture Media + Unexposed Neutralizer** treatment and **Subculture Media + Exposed Neutralizer** treatment should be comparable to the **Subculture Media Control**.

	f. Lack of verification of neutralizer effectiveness. i. Growth in tubes that were inoculated with dilutions yielding plate counts of TNTC and no growth in tubes that were inoculated with dilutions yielding plate counts with a desired target of 5-100 CFU. ii. No growth in any tubes. g. For the data to be deemed valid, each **Negative** (uninoculated) **Media Control** tube must show no microbial growth.
13. Data Analysis/ Calculations	None.
14. Forms and Data Sheets	Test Sheets. Test sheets are stored separately from the SOP under the following file names: Neutralization Confirmation Assay for *M. bovis* (BCG): Time Recording Sheet for Carrier Transfers — MB-11-06_F1.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Information Sheet — MB-11-06_F2.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Results Sheet – Liquid Products — MB-11-06_F3.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Results Sheet – Spray/Towelette Products — MB-11-06_F4.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Inoculum Enumeration Form — MB-11-06_F5.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Test Microbe Confirmation Sheet — MB-11-06_F6.docx Neutralization Confirmation Assay for *M. bovis* (BCG): Processing Sheet — MB-11-06_F7.docx
15. References	Official Methods of Analysis. Revised 2013. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 965.12 In vitro Test for Determining Tuberculocidal Activity).

Table 1: Components of the Neutralization Confirmation Assay for Liquid Products

Treatment/Control	***M. bovis* (BCG) Inoculum Dilution (0.1 mL added per tube)**	**Media (○ = Tube of Media)**		
		MPB (20 mL)	**M7H9 (20 mL)**	**K or TB (20 mL)**
Subculture Media + Exposed Neutralizer	10^{-2}	○/Carrier	○	○
	10^{-3}	○/Carrier	○	○
	10^{-4}	○/Carrier	○	○
	10^{-5}	○/Carrier	○	○
Subculture Media + Unexposed Neutralizer	10^{-2}	○/Carrier	○	○
	10^{-3}	○/Carrier	○	○
	10^{-4}	○/Carrier	○	○
	10^{-5}	○/Carrier	○	○
Subculture Media Control	10^{-2}	○	○	○
	10^{-3}	○	○	○
	10^{-4}	○	○	○
	10^{-5}	○	○	○
Negative Uninoculated Control	Not inoculated	○	○	○

Table 2: Components of the Neutralization Confirmation Assay for Spray/Towelette Products

Treatment/Control	***M. bovis* (BCG) Inoculum Dilution (0.1 mL added per tube)**	**Media (○ = Tube of Media)**		
		MPB (20 mL)	**M7H9 (20 mL)**	**K or TB (20 mL)**
Subculture Media + Exposed Neutralizer	10^{-2}	○/Carrier	○○	○○
	10^{-3}	○/Carrier	○○	○○
	10^{-4}	○/Carrier	○○	○○
	10^{-5}	○/Carrier	○○	○○
Subculture Media + Unexposed Neutralizer	10^{-2}	○/Carrier	○○	○○
	10^{-3}	○/Carrier	○○	○○
	10^{-4}	○/Carrier	○○	○○
	10^{-5}	○/Carrier	○○	○○
Subculture Media Control	10^{-2}	○	○	○
	10^{-3}	○	○	○
	10^{-4}	○	○	○
	10^{-5}	○	○	○
Negative Uninoculated Control	Not inoculated	○	○	○