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 of Microbicidal Activity using the Quantitative Method for Evaluating Bactericidal and Mycobactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces

SOP Number: MB-26-03

Date Revised: 11-24-20

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Title	Neutralization of Microbicidal Activity using the Quantitative Method for Evaluating Bactericidal and Mycobactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces
Revisions Made	• Scope: This procedure describes a quantitative approach for assessing the effectiveness of the neutralization process associated with MB-25 for testing bacteria and mycobacteria. • Section 12.2: Reduced minimum carriers inoculated to 12. • Minor editorial changes.

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Scope	This procedure describes a quantitative approach for assessing the effectiveness of the neutralization process associated with MB-25 for testing bacteria and mycobacteria.
Application	A suspension-based assay and a carrier-based assay are provided. Identify a suitable neutralizer in advance of/or concurrently with product efficacy testing.

	Approval Date
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Date SOP issued:	
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TABLE OF CONTENTS

Contents	Page Number
1. DEFINITIONS	3
2. HEALTH AND SAFETY	3
3. PERSONNEL QUALIFICATIONS AND TRAINING	3
4. INSTRUMENT CALIBRATION	3
5. SAMPLE HANDLING AND STORAGE	3
6. QUALITY CONTROL	3
7. INTERFERENCES	3
8. NON-CONFORMING DATA	3
9. DATA MANAGEMENT	4
10. CAUTIONS	4
11. SPECIAL APPARATUS AND MATERIALS	4
12. PROCEDURE AND ANALYSIS	4
13. DATA ANALYSIS/CALCULATIONS	6
14. FORMS AND DATA SHEETS	7
15. REFERENCES	7

1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Reaction vessel = vessel used to conduct the assay (vial or test tube) 2. *Test Suspension A* = suspension of the test microbe prior to the addition of the soil load 3. *Test Suspension B* = test suspension with soil load 4. Stock culture = frozen culture used to prepare the test culture 5. Test substance = a product or formulation that is under evaluation for its microbicidal activity 6. CFU = colony forming unit
2. Health and Safety	1. Follow procedures specified in SOP MB-01, Laboratory Biosafety. 2. Consult the Safety Data Sheet for specific hazards associated with the test substance or other potentially hazardous materials.
3. Personnel Qualifications and Training	1. Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	1. Refer to SOP 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	1. Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	1. For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. Prolonged exposure of cells to the neutralizer agent in excess of 30 minutes may result in erroneous values due to bacterial replication; timely filtration will mitigate this potential interference.
8. Non-conforming Data	1. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports. 2. For the assay to be considered valid, ensure that the recovered number of CFU in the **Titer Control** using *Test Suspension B* yields 20-200 CFU per reaction vessel. 3. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.

9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Conduct steps (e.g., addition of organism and neutralizer) at timed intervals (e.g., 30 s intervals for suspension-based assay, 1 min intervals for dried carrier-based assay) to ensure consistent time of contact.
11. Special Apparatus and Materials	1. Refer to Special Apparatus and Materials section in SOP MB-25.
12. Procedure and Analysis	1. General description of the assay: The test substance is first mixed with a candidate neutralizer. A diluted suspension of the test organism is then added to the reaction mixture; if desired, additional evaluations may be conducted using the test organism as dried inoculum on a carrier. The neutralization process is deemed acceptable if the criteria outlined in section 13 are met.
12.1 Preparation of test organisms	Refer to the appropriate preparation of test cultures section of SOP MB-25, for preparation of the test cultures. Conduct preliminary tests as necessary to determine appropriate dilution(s) of *Test Suspension A* (used to prepare *Test Suspension B*) to achieve the target challenge of 20-200 CFU per 10 µL or per carrier. a. Prepare *Test Suspension A (without soil load)*. Serially dilute the microbial test suspension with PBS (e.g., through 10^{-4} or 10^{-5}). Select appropriate dilutions of *Test Suspension A* so that after the addition of the soil load, the *Test Suspension B* will achieve an average challenge of 20-200 CFU per 10 µL. Use *Test Suspension A* within 30 min of preparation. b. Prepare *Test Suspension B (with soil load)*. Prepare the 3-part soil load: using a vortex, mix each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, and 100 µL of mucin; then vortex-mix the solution. Combine 340 µL of diluted *Test Suspension A* and the 160 µL of the soil load (SL) and vortex-mix for 10 seconds. c. Ensure *Test Suspension B* provides an average challenge of 20-200 CFU per 10 µL. Other soil loads may be used per the Agency's guidance or research protocol. i. If performing the assay with inoculated carriers, ensure an average challenge of 20-200 CFU per carrier after drying. d. Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Test Suspension B* to ensure

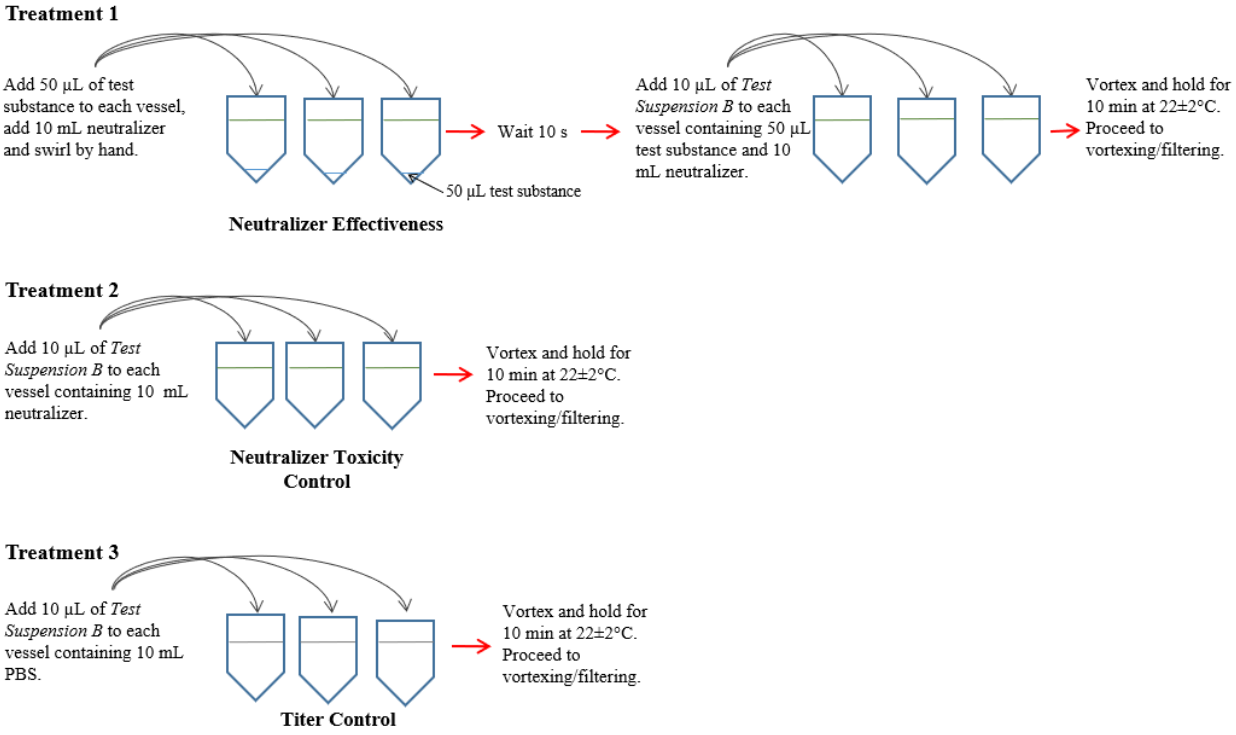
	at least one dilution with an average challenge of 20-200 CFU per 10 µL. i. If performing the assay with inoculated carriers, the use of two separate dilutions results in a total of 20 carriers to be processed; however, the dilutions may be evaluated separately. ii. A calibration curve (OD @ 650nm) may be used to estimate the number of viable organisms in *Test Suspension A*.
12.2 Carrier inoculation for carrier-based assay	Refer to the appropriate sections in SOP MB-25 for carrier preparation and carrier inoculation and drying. a. Inoculate at least 12 carriers with 10 µL of *Test Suspension B* (per concentration of *Test Suspension B*) using a positive displacement pipette. b. After drying, evaluate the dried carriers per section 12.4 of this document.
12.3 Suspension-based assay	a. ***Treatment 1: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s, gently add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document. b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document. c. ***Treatment 3: Titer Control.*** Add 10 mL PBS to each of three reaction vessels. At timed intervals, add 10 µL of *Test Suspension B* using a micropipette to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document.
12.4 Carrier-based assay	a. ***Treatment 1: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vial and briefly swirl (by hand). After 10 s, gently add one dried carrier inoculated with *Test Suspension B* to each vessel and vortex-mix for 30±2 s. Proceed with section 12.5 of this document. b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add one dried carrier

	inoculated with *Test Suspension B* to each vessel and vortex-mix for 30±2 s. Proceed with section 12.5 of this document. c. ***Treatment 3: Titer Control.*** Add 10 mL PBS to each of three reaction vessels. At timed intervals, add one dried carrier inoculated with *Test Suspension B* to each vessel and vortex-mix for 30±2 s. Proceed with section 12.5 of this document.
12.5 Processing and recovery	a. Hold the mixtures from 12.3 and 12.4 for 10±1 min at room temperature (22±2°C). b. At the conclusion of the holding period, vortex-mix each reaction vessel (for the suspension-based assay) and filter each mixture through a separate, pre-wetted 0.2 or 0.45 µm polyethersulfone (PES) membrane filter. i. If performing the assay with dried inoculated carriers, vortex each vessel for 30±2 s at the conclusion of the holding period, and then filter contents. c. Wash each reaction vessel with ~20 mL PBS and vortex-mix; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of the funnel unit with ~20 mL PBS and filter the rinsing liquid through the same filter membrane. i. Initiate filtration as soon as possible (e.g., within 30 min). d. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium (trypticase soy agar for *P. aeruginosa*, *S. enterica*, and *S. aureus*, Middlebrook 7H11 agar for *M. terrae*). Avoid trapping air bubbles between the filter and the agar surface. e. For *P. aeruginosa*, *S. enterica* and *S. aureus*, incubate plates at 36±1°C for 48±4 h and count the colonies. i. Incubate an additional 24±4 h if no or few colonies are present at 48±4 h and re-count the colonies. f. For *M. terrae*, incubate all plates at 36±1°C for 17-21 days; however, monitor filters for growth and count the number of colonies beginning at 10-14 days.
13. Data Analysis/ Calculations	1. Compare the average CFU of the **Titer Control** with the average CFU of the **Neutralizer Toxicity Control** and **Neutralizer Effectiveness** treatment. Determine the percent difference in CFU.

	2. For determining the suitability of the neutralizer, ensure that the average CFU in the **Neutralizer Toxicity Control** is at least 50% of the **Titer Control**. A count lower than 50% indicates that the neutralizer is harmful to the test organism. a. Average CFU for the **Neutralizer Toxicity Control** that are higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid. 3. To verify effectiveness of the neutralization, the average number of CFU in the **Neutralizer Effectiveness** treatment is at least 50% of the **Titer Control**. a. Average CFU for the **Neutralizer Effectiveness** treatment that are higher than the **Titer Control** (e.g., 120% of the Titer Control) are also deemed valid. 4. If the criteria are not met, verify another neutralizer or mixture of neutralizers.
14. Forms and Data Sheets	1. Attachment 1: Neutralization Assay Flow Chart 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Neutralization Test Information Sheet MB-26-03_F1.docx Neutralization Test Suspension Preparation Sheet MB-26-03_F2.docx Neutralization Time Recording and Results Sheet MB-26-03_F3.docx Neutralization Test Processing Sheet MB-26-03_F4.docx Neutralization Test Spreadsheet MB-26-03_F5.xlsx
15. References	1. OECD Guidance Document: Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard Non-Porous Surfaces (January 29, 2013).

Attachment 1

OECD Neutralization Assay Flow Chart



Alternatively, perform the assay using dried-carriers in place of the liquid suspension.