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 OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces

SOP Number: MB-26-01

Date Revised: 08-26-14

SOP Number	MB-26-01
Title	Neutralization of Microbicidal Activity using the OECD Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces
Scope	To verify the neutralization efficacy of a test substance (refer to reference 15.1).
Application	Identify a suitable neutralizer in advance of or concurrently with testing. Verify neutralization using the highest concentration of test substance if there are multiple concentrations being evaluated.

	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Date SOP withdrawn:	

TABLE OF CONTENTS

1. Definitions	Additional abbreviations/definitions are provided in the text. 1. Reaction vessel = vessel used to conduct the assay (vial or test tube). 2. Eluent = any liquid that is harmless to the test organism(s) and that is added to the reaction vessel to recover the test organism. 3. Eluate = recovered eluent that contains the test organism. 4. Test suspension = suspension of the test microbe prior to the addition of the soil load (*Test Suspension A*) 5. Final test suspension= test suspension with soil load (*Test Suspension B*) 6. Stock culture = frozen culture used to prepare the test culture 7. Test substance = a product or formulation that is under evaluation for its microbicidal activity
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material 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 SOP EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-04 (spectrophotometers) and EQ-05 (timers) 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 form(s) (see section 14).
7. Interferences	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	Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.

11. Special Apparatus and Materials	Refer to SOP MB-25, OECD Quantitative Method, section 11.
12. Procedure and Analysis	1. General description of the assay: a. 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/ sterilization of carriers	a. Refer to SOP MB-25, OECD Quantitative Method, section 12.1.
12.2 Preparation of test organisms	a. Refer to SOP MB-25, OECD Quantitative Method; section 12.2a through 12.2i for *P. aeruginosa* and *S. aureus*, Attachment 2 section A4.a through A4.e for *M. terrae*. b. Prepare *Test Suspension A (without soil load)*: Dilute the test microbial suspension with PBS to achieve an average challenge of 20-200 CFU per 10 µL (e.g., serially dilute cultures through 10^{-4} or 10^{-5}; refer to the Neutralization Test Suspension Preparation Sheet in section 14.0). *Test Suspension A* should be used within 4 hours of preparation. i. If performing the assay with dried carriers, prior testing may be required to account for differences in the loss of viability of the different test organisms upon drying. c. Prepare *Final Test Suspension B (with soil load)*: Prepare the soil load: vortex each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, and 100 µL of mucin, mix well. Combine 340 µL of *Test Suspension A* and 160 µL of the soil load (SL). The test microbial suspension with soil load should provide an average challenge of 20-200 CFU/tube. i. If performing the assay with dried carriers, ensure an average challenge of 20-200 CFU/carrier after drying. For example, serially dilute 10 mL cultures of *P. aeruginosa* through 10^{-3}, serially dilute 10 mL cultures of *S. aureus* through 10^{-4}, serially dilute 5 mL cultures of *M. terrae* through 10^{-2}. Note: Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Final Test*

		Suspension B to ensure at least one dilution with an average challenge of 20-200 CFU. If performing the assay with dried carriers, the use of two separate dilutions results in a total of 20 carriers to be processed; however, the dilutions may be evaluated separately. d. It is recommended that a calibration curve, using optical density (OD @ 650nm), be created to estimate the number of viable organisms in *Test Suspension A*.
12.3	Carrier inoculation (for alternative dried carrier-based assay)	a. Inoculate at least 13 carriers with *Final Test Suspension B* (per concentration of *Final Test Suspension B*) by adding 10 µL using a positive displacement pipette to each carrier. Refer to SOP MB-25, OECD Quantitative Method, sections 12.4c through 12.4d for carrier drying instructions. b. After drying, evaluate the dried carriers per section 12.5.
12.4	Suspension-based assay	a. ***Treatment 1: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl. After 10 s, gently add 10 µL *Final Test Suspension B* to each vessel and briefly vortex. Proceed with section 12.6. b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10 µL of *Final Test Suspension B* gently to each vessel and briefly vortex. Proceed with section 12.6. c. ***Treatment 3: Titer Control.*** Add 10 mL PBS to each of three reaction vessels. At timed intervals, add 10 µL of *Final Test Suspension B* gently to each vessel and briefly vortex. Proceed with section 12.6.
12.5	Alternative dried carrier-based assay	a. ***Treatment 1: Neutralizer Effectiveness.*** Add 50 µL of the test substance to each of three vials. At timed intervals, add 10 mL neutralizer to each vial and briefly swirl. After 10 s, gently add one dried carrier inoculated with *Final Test Suspension B* to each vessel and vortex for 30±2 s. Proceed with section 12.6. 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 *Final Test Suspension B* gently to each vessel and vortex for 30±2 s. Proceed with section 12.6. c. ***Treatment 3: Titer Control.*** Add 10 mL PBS to each of three reaction vessels. At timed intervals, add one dried carrier inoculated

	with *Final Test Suspension B* gently to each vessel and vortex for 30±2 s. Proceed with section 12.6.
12.6 Processing and recovery	a. Hold the mixtures from 12.4 and 12.5 for 10±1 min at room temperature (22±2°C). Steps (e.g., addition of organism, neutralizer) should be conducted 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. b. At the conclusion of the holding period, briefly vortex each vessel (for the suspension-based assay) and pass each mixture through a separate, pre-wetted 0.2 or 0.45 µm polyethersulfone (PES) membrane filter. i. If performing the assay with dried carriers, vortex each vessel for 30±2 s at the conclusion of the holding period. Use a magnet to prevent carriers from falling onto the filter membrane. c. Wash each vessel with approximately 20 mL PBS and briefly vortex; filter the washes through the same filter membrane. Repeat once. Finish the filtering process by rinsing the inside of the funnel unit with about 40 mL of PBS and filtering the rinsing liquid through the same filter membrane. Note: Initiate filtration as soon as possible (e.g., within 30 minutes). Two analysts are recommended to perform vortexing and filtration steps to reduce holding time after vortexing. 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* and *S. aureus*, Middlebrook 7H11 agar for *M. terrae*). Avoid trapping air bubbles between the filter and the agar surface. Incubate the plates for 24-48 hours at 36±1°C for *P. aeruginosa* and *S. aureus*, 17-21 days at 36±1°C for *M. terrae*. e. Examine the plates after incubation and count colonies. Calculate the average CFU for each set of test conditions.
13. Data Analysis/ Calculations	1. For the assay to be considered valid, ensure that: a. The recovered number of CFU in the **Titer Control** (see section 12.4c) using *Final Test Suspension B* yields 20-200 CFU per tube. The same must also be true when using the dried carrier-based assay (see section 12.5c). 2. For determining and verifying the effectiveness of the neutralizer, ensure that: a. The recovered number of CFU in the **Neutralizer Toxicity Control**

	(see section 12.4b) is at least 50% of the **Titer Control** (see section 12.4c). A count lower than 50% indicates that the neutralizer is harmful to the test organism. Note: counts higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid. b. The recovered number of CFU in the **Neutralizer Effectiveness** treatment (see section 12.4a) is at least 50% of the **Titer Control**; this verifies effective neutralization. Note: counts higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid. 3. For both the suspension-based assay and the carrier-based assay, the criteria in sections 13.1 and 13.2 must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified. 4. For the suspension-based assay (see section 12.4), compare the average CFU of the **Titer Control** with the average CFU of the **Neutralizer Toxicity Control** and **Neutralizer Effectiveness** treatment. Compare results from the dried carrier-based assay (see section 12.5) in the same manner.
14. Forms and Data Sheets	1. Attachment 1: OECD Neutralization Assay Flow Chart 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: OECD Method for Bactericidal Activity: Neutralization Test Information Sheet — MB-26-01_F1.docx OECD Method for Bactericidal Activity: Neutralization Test Suspension Preparation Sheet — MB-26-01_F2.docx OECD Method for Bactericidal Activity: Neutralization Time Recording and Results Sheet — MB-26-01_F3.docx
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