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

Standard Operating Procedure for
Neutralization Confirmation Procedure for Products Evaluated with the AOAC Use Dilution Method (UDM), the AOAC Germicidal Spray Products as Disinfectants Test (GSPT), and the Disinfectant Towelette Test (DTT)

SOP Number: MB-17-04

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Title	Neutralization Confirmation Procedure for Products Evaluated with the AOAC Use Dilution Method (UDM), the AOAC Germicidal Spray Products as Disinfectants Test (GSPT), and the Disinfectant Towelette Test (DTT)
Scope	This SOP describes methodology used to determine the effectiveness of neutralizers specified for disinfectant testing (UDM, GSPT, and DTT). A quantitative approach is used to assess the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer/disinfectant interactions across a range of microbe concentrations. This SOP can be modified to accommodate other test methods.
Application	This assay is designed to simulate the conditions of the UDM, GSPT, and DTT; however, sterile carriers are used instead of inoculated carriers. The test conditions specified for product testing (e.g., water hardness, use-dilution, pH, organic soil, neutralizer, contact time, temperature) are used.

	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Date SOP withdrawn:	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. Bacteriostatic = Capable of inhibiting or controlling the growth or reproduction of bacteria without killing the cells 2. CFU = Colony Forming Unit
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-02 (thermometers), EQ-03 (weigh balances), EQ-04 (spectrophotometer), 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, Preparation and Sampling Procedures for Antimicrobial Test Substances, 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	For each neutralizer and subculture medium tested per study, use one batch (preparation) of neutralizer and medium for all treatment and control groups. Differences in performance (quality) between batches of media may lead to misleading neutralization results.
8. Non-conforming Data	Management of non-conforming data will be specified; 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	1. To ensure the stability of the test disinfectant solution, perform testing within 3 hours of preparation. 2. Strict adherence to the procedure is necessary for validity of test results. 3. Use appropriate aseptic techniques for all test procedures involving the manipulation of test organisms and associated test components.
11. Special Apparatus and Materials	Refer to section 11 of MB-05 (UDM), MB-06 (GSPT), and MB-09 (DTT) for required apparatus and materials.

12. Procedure and Analysis	a. Sterile carriers are used for this assay (see section 12.4). b. Perform the neutralization assay in advance of product testing to verify that the prescribed neutralizer is suitable for the efficacy evaluation. Concurrently conduct two test scenarios to determine an appropriate approach for performing the product efficacy evaluation: i. First Scenario: expose carriers to the disinfectant and transfer them into the neutralizer subculture medium (primary tube). No secondary subculture medium transfers are conducted. Inoculate the neutralizer tubes containing the carrier with a test organism suspension to deliver 5-100 CFU/mL. ii. Second Scenario: expose carriers to the disinfectant and transfer them into the neutralizer subculture medium (primary tube); in addition, subsequently transfer the carriers to a secondary subculture medium (secondary tube). Inoculate tubes with a test organism suspension to deliver 5-100 CFU/mL. c. The purpose of the two scenario approach is to determine if the prescribed neutralizer for the disinfectant is sufficient to support growth.
12.1 Inoculum Preparation	a. Prepare the inoculum according to SOP MB-05, AOAC UDM, sections 12.1 through 12.2b.
12.2 Inoculum Enumeration	a. Prepare serial ten-fold dilutions of the inoculum by pipetting 1 mL of the final test culture into 9 mL of PBDW (see section 12.1). Use four dilutions, (e.g., 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) to inoculate the neutralizer (primary tubes) and subculture medium (secondary tubes). 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 of each of the four dilutions in duplicate on TSA or blood agar plates (BAP). Briefly vortex each dilution tube prior to plating. Plates must be dry prior to incubation. c. Record the dilution and plating information on the Neutralization Confirmation Assay: Enumeration Form (see section 14). d. Incubate plates (inverted) at 36±1ºC for up to 48±2 h and record colony counts. Plates that have colony counts over 300 are labeled as too numerous to count (TNTC). Record the counts on

	the Neutralization Confirmation Assay: Enumeration Form (see section 14).
12.3 Product Sample Preparation	a. Prepare the product according to the test parameters; follow guidelines for disinfectant sample preparation provided in SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances.
12.4 Carrier Preparation	a. Prepare carriers according to the applicable SOP: for the UDM, SOP MB-05 (stainless steel penicylinders), for the GSPT, SOP MB-06 (25×25 mm glass slide carriers), and for the DTT, SOP MB-09 (25×75 mm glass slide carriers). i. UDM: Follow carrier inoculation (SOP MB-05, section 12.2) except use sterile broth. Add organic soil to the sterile broth as necessary per the test parameters. ii. GSPT and DTT: Follow carrier inoculation (SOP MB-06 and SOP MB-09, section 12.2) except use sterile broth. Add organic soil to the sterile broth as necessary per the test parameters.
12.5 First Scenario: Neutralizer - Primary Subculture Treatment Only	a. Requires four dried carriers (with broth culture added) per organism. Use the carrier type required for the specific test. b. Apply the product to the carriers according to specific instruction provided in the test parameters (e.g., use dilution, spray distance, spray period, wipe pattern, and contact time). c. Per test, per one test organism, expose four of the carriers to the disinfectant for the specified contact time in the same manner as product efficacy testing. Record the carrier transfer information on the Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers (see section 14). d. 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. This set of neutralizer tubes (4 total tubes) represents the **Neutralizer-Primary Subculture Treatment**. Refer to section 12.8 for treatment inoculation (Table 1). Note: For GSPT and DTT, the amount of neutralizer is 20 mL per tube (38×100 mm tubes) compared to 10 mL (20×150 mm tubes) used in the UDM.

	e. Proceed immediately with the Second Scenario.
12.6 Second Scenario: Neutralizer Subculture Treatment Plus Secondary Subculture Treatment	a. Requires four dried carriers (with broth culture added) per organism. Use the carrier type required for the specific test. b. Apply the product to the carriers according to specific instruction provided in the test parameters (e.g., use dilution, spray distance, spray period, wipe pattern, and contact time). c. Per test, per one test organism, expose four of the carriers to the disinfectant for the specified contact time in the same manner as product efficacy testing. Record the carrier transfer information on the Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers. d. 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. This set of neutralizer tubes (4 total tubes) will represent the **Neutralizer-Primary Subculture Treatment**. Note: For GSPT and DTT, the amount of neutralizer is 20 mL per tube (38×100 mm tubes) compared to 10 mL (20×150 mm tubes) used in the test method for liquid products. e. Following the last carrier transfer into the neutralizer tube, incubate both First and Second Scenario neutralizer tubes at room temperature for 30-45 min. Then, for the Second Scenario, transfer each carrier in order into a culture tube containing the secondary subculture medium. This portion of the assay is not timed. This set of tubes (4 total tubes) represents the **Secondary Subculture Treatment**. Refer to section 12.8 for treatment inoculation (Table 2). f. Repeat the assay for the second test organism, if required.
12.7 Controls	a. Inoculated controls i. The **Neutralizer-Primary Inoculated Control** contains four tubes of fresh, unexposed (to disinfectant) neutralizer-primary media. ii. The **Secondary Subculture Inoculated Control** contains four tubes of secondary subculture media. iii. It is highly desirable that the preparation (media preparation number) of each medium be the same as used

in the treatments. Refer to section 12.8 for treatment inoculation (Table 3).

b. Uninoculated controls

i. **Neutralizer-Primary** and **Secondary Subculture Uninoculated Controls**. Incubate one tube each of uninoculated neutralizer and secondary subculture media with the other tubes.

c. Confirm sterility of carriers in advance or concurrently with testing: add an uninoculated carrier to a tube of 10-20 mL fluid thioglycollate medium or letheen broth and incubate at 36±1ºC for 3-10 days.

12.8 Treatment Inoculation

a. After step 12.6e, inoculate all tubes concurrently using Tables 1, 2, and 3.

Table 1. First Scenario: Inoculation of Treatment Group with Dilutions of the Test Organism*

Treatment	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 2. Second Scenario: Inoculation of Treatment Groups with Dilutions of the Test Organism*

Treatments	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Secondary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 3. Controls: Inoculation of Control Groups with Dilutions of the Test Organism*

Controls		Dilutions Added			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
Inoculated Controls (media performance)	Neutralizer-Primary	0.1 mL	0.1 mL	0.1 mL	0.1 mL
	Secondary Subculture	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Sterility Controls	Neutralizer-Primary	N/A	N/A	N/A	N/A
	Secondary Subculture	N/A	N/A	N/A	N/A

	*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL b. Shake tubes thoroughly. Incubate all tubes for up to 48±2 h at 36±1°C.
12.9 Recording Results and Confirmation Testing	a. Record results as + (growth/turbidity) or 0 (no growth) on the Neutralization Confirmation Assay Results Form (see section 14). b. For each treatment and control group, Gram stain a minimum of one positive tube per treatment. Select the tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered). c. Record confirmation results on the Neutralization Confirmation Assay: Microbe Confirmation Sheet (see section 14).
12.10 Interpretation of Results	a. <u>Plate count data</u>. One of the four dilutions plated should provide counts within the approximate target range, 5-100 CFU/mL. i. 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. b. <u>Controls</u>. Growth in the **Secondary Subculture Inoculated 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 TNTC) indicates poor media performance.* Growth in the **Neutralizer-Primary Inoculated Control** should be comparable to the **Secondary Subculture Inoculated Control** if the neutralizer is the same as the secondary subculture media. i. There may be cases when the neutralizer (primary tubes) is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control. ii. The **Neutralizer-Primary Uninoculated Control** and **Secondary Subculture Uninoculated Control** tubes are used to determine sterility and must show no growth for the test to be valid. c. <u>Treatments</u>. The occurrence of growth in the **Neutralizer-Primary Subculture** and **Secondary Subculture Treatment** tubes are used to assess the effectiveness of the neutralizer.

	i. First Scenario: The neutralizer itself may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high titer of inoculum (e.g., the dilution with plate counts which are TNTC) 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 **Neutralizer Primary Subculture Treatment** tubes which received a lower titer of inoculum (e.g., 5-100 CFU/mL).* ii. Second Scenario: The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high titer of inoculum (e.g., the dilution with plate counts which are TNTC) 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 **Secondary Subculture Treatment** tubes which received a lower titer of inoculum (e.g., 5-100 CFU/mL).*
12.11 Efficacy Evaluation based on Neutral-ization Results	a. If results from the First Scenario indicate effective neutralization, conduct the efficacy evaluation using only the neutralizer subculture tubes (i.e., primary tubes). b. If results from the First Scenario (Neutralizer-Primary Subculture Treatment only) are inconclusive and/or indicate that a bacteriostatic effect from the neutralizer or neutralizer-disinfectant interaction is present, evaluate results from the Second Scenario to determine if the Secondary Subculture tube provide appropriate neutralization. c. If the Second Scenario is deemed effective, conduct the efficacy evaluation using both subculture media tubes (i.e., primary and secondary tubes). d. If results from the Second Scenario (Neutralizer-Primary Subculture Treatment tubes and Secondary Subculture Treatment tubes) are inconclusive and/or indicate that a bacteriostatic effect from the neutralizer or neutralizer-disinfectant interaction is present, assay an alternative neutralizer prior to conducting the efficacy evaluation. The alternative neutralizer may not be specified in the test parameters.

13. Data Analysis/ Calculations	1. Enumerate plate counts and calculate CFU/mL added to each tube based on the average of countable plates. Apply TNTC for counts above 300 CFU. 2. To calculate the average CFU/mL per dilution added to each tube, add the plate counts for each plate within the dilution and divide by two. 3. Use counts from 0 through 300 in the calculations.
14. Forms and Data Sheets	Test Sheets. Test sheets are stored separately from the SOP under the following file names: Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers — MB-17-04_F1.docx Neutralization Confirmation Assay: Test Information Sheet — MB-17-04_F2.docx Neutralization Confirmation Assay: Results Form — MB-17-04_F3.docx Neutralization Confirmation Assay: Test Microbe Confirmation Sheet — MB-17-04_F4.docx Neutralization Confirmation Assay: Enumeration Form — MB-17-04_F5.docx Neutralization Confirmation Assay: Processing Sheet — MB-17-04_F6.docx
15. References	1. Official Methods of Analysis. Methods 955.14, 955.15, and 964.02. Posted September 2013. AOAC INTERNATIONAL, Gaithersburg, MD. 2. Official Methods of Analysis. Method 961.02. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD.