



**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 against
Candida auris using the Quantitative Method**

SOP Number: MB-37-02

Date Revised: 07-06-21

SOP Number	MB-37-02
Title	Neutralization of Microbicidal Activity against <i>Candida auris</i> using the Quantitative Method
Revisions Made	Revisions to MB-37-00 (12-28-17) → MB-37-01 (03-27-20) <ul style="list-style-type: none">• Minor editorial changes for clarification purposes.• The name of the SOP changed from Neutralization Confirmation for the Efficacy Evaluation of Liquid Antimicrobials against <i>Candida auris</i> using the OECD Quantitative Method on Hard, Non-Porous Surfaces to Neutralization of Microbicidal Activity against <i>Candida auris</i> using the OECD Quantitative Method.• Removed the use of a magnet (Section 12.5.b.i)• Changed incubation to 72±4 hours from 48±4 hours (Section 12.5.d)
	Revisions to MB-37-01 (03-27-20) → MB37-02 (07-06-21) <ul style="list-style-type: none">• Minor editorial changes for clarification purposes.• Removed OECD from the title and entire document.

SOP Number	MB-37-02
Title	Neutralization of Microbicidal Activity against <i>Candida auris</i> using the Quantitative Method
Scope	This procedure describes a quantitative approach for assessing the effectiveness of the neutralization process associated with the Quantitative Method for testing <i>Candida auris</i> .
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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TABLE OF CONTENTS

<u>Contents</u>	<u>Page Number</u>
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	7
14. FORMS AND DATA SHEETS	7
15. REFERENCES	7

1. Definitions	<p>Additional abbreviations/definitions are provided in the text.</p> <ol style="list-style-type: none"> 1. Reaction vessel = vessel used to conduct the assay (vial or test tube). 2. <i>Test Suspension A</i> = suspension of the test microbe prior to the addition of the soil load 3. <i>Test Suspension B</i> = 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	<ol style="list-style-type: none"> 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	<ol style="list-style-type: none"> 1. Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	<ol style="list-style-type: none"> 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	<ol style="list-style-type: none"> 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	<ol style="list-style-type: none"> 1. For quality control purposes, document the required information on the appropriate form(s) (see section 14).
7. Interferences	<ol style="list-style-type: none"> 1. Prolonged exposure of cells to the neutralizer agent in excess of 30 minutes may result in erroneous values due to fungal replication; timely filtration will mitigate this potential interference.
8. Non-conforming Data	<ol style="list-style-type: none"> 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 colony forming units (CFU) in the Titer Control using <i>Test Suspension B</i> yields 20-200 CFU per vessel. 3. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.

9. Data Management	1. Archive data consistent with SOP ADM-03, Records and Archives.
10. Cautions	<ol style="list-style-type: none"> 1. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting. 2. 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 section 11 of SOP MB-35, Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against <i>Candida auris</i> on Hard, non-Porous Surfaces.
12. Procedure and Analysis	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 organism	<p>Refer to sections 12.2a-12.2i of SOP MB-35, Quantitative Method, for preparation of the test culture. Conduct preliminary tests as necessary to determine appropriate dilution(s) of <i>Test Suspension A</i> (used to prepare <i>Test Suspension B</i>) to achieve the target challenge of 20-200 CFU per 10 µL or per carrier.</p> <ol style="list-style-type: none"> a. Prepare <i>Test Suspension A (without soil load)</i>: Serially dilute the microbial test suspension with PBS (e.g., through 10⁻⁴ or 10⁻⁵). Select appropriate dilutions of <i>Test Suspension A</i> so that after the addition of the soil load, <i>Test Suspension B</i> will achieve an average challenge of 20-200 CFU per 10 µL (refer to the Neutralization Test Suspension Preparation Sheet in section 14). Use <i>Test Suspension A</i> within 30 min of preparation. b. Prepare <i>Test Suspension B (with soil load)</i>: 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 <i>Test Suspension A</i> and the 160 µL of the soil load (SL) and vortex-mix. c. Ensure <i>Test Suspension B</i> 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. <ol style="list-style-type: none"> i. If performing the assay with inoculated carriers, ensure an average challenge of 20-200 CFU per carrier after drying.

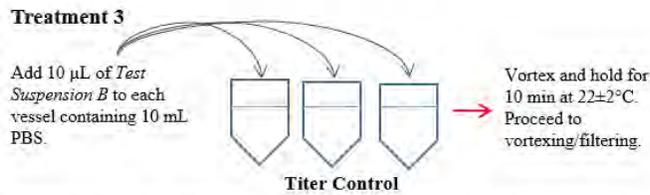
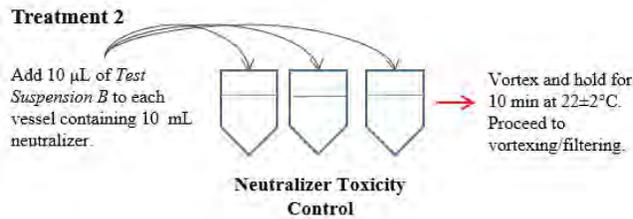
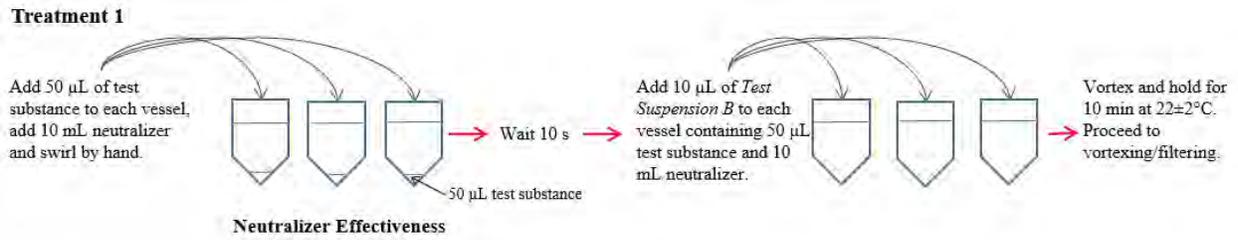
	<p>d. Two separate dilutions of <i>Test Suspension A</i> may be used to prepare two different concentrations of <i>Test Suspension B</i> to ensure at least one dilution with a challenge of 20-200 CFU per 10 μL.</p> <p>i. If performing the assay with inoculated carriers, the use of two separate dilutions results in a total of 20 carriers to be processed; however, the dilutions may be evaluated separately.</p> <p>ii. A calibration curve (OD @ 650nm) may be used to estimate the number of viable organisms in <i>Test Suspension A</i>.</p>
<p>12.2 Carrier inoculation for carrier-based assay</p>	<p>Refer to sections 12.1 and 12.4 of SOP MB-35, Quantitative Method for carrier preparation and carrier inoculation and drying, respectively.</p> <p>a. Inoculate at least 13 carriers with 10 μL of each <i>Test Suspension B</i> dilution (if evaluating two dilutions) using a positive displacement pipette</p> <p>b. After drying, evaluate the inoculated carriers per section 12.4 of this document.</p>
<p>12.3 Suspension-based assay</p>	<p>a. <i>Treatment 1: Neutralizer Effectiveness.</i> 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 <i>Test Suspension B</i> using a micropipette to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document.</p> <p>b. <i>Treatment 2: Neutralizer Toxicity Control.</i> Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10 μL of <i>Test Suspension B</i> using a micropipette gently to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document.</p> <p>c. <i>Treatment 3: Titer Control.</i> Add 10 mL PBS to each of three reaction vessels. At timed intervals, add 10 μL of <i>Test Suspension B</i> with a micropipette gently to each vessel and briefly vortex-mix. Proceed with section 12.5 of this document.</p>
<p>12.4 Carrier-based assay</p>	<p>a. <i>Treatment 1: Neutralizer Effectiveness.</i> Add 50 μL of the test substance to each of the 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 <i>Test Suspension B</i> to each vessel and vortex-mix for 30\pm2 s. Proceed with section 12.5 of this document.</p> <p>b. <i>Treatment 2: Neutralizer Toxicity Control.</i> Add 10 mL neutralizer to each of the three reaction vessels. At timed intervals, add one dried</p>

	<p>carrier inoculated with <i>Test Suspension B</i> gently to each vessel and vortex-mix for 30 ± 2 s. Proceed with section 12.5 of this document.</p> <p>c. <i>Treatment 3: Titer Control.</i> Add 10 mL PBS to each of the three reaction vessels. At timed intervals, add one dried carrier inoculated with <i>Test Suspension B</i> gently to each vessel and vortex-mix for 30 ± 2 s. Proceed with section 12.5 of this document.</p>
<p>12.5 Processing and recovery</p>	<p>a. Hold the mixtures from 12.3 and 12.4 for 10 ± 1 min at room temperature ($22 \pm 2^\circ\text{C}$).</p> <p>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 black $0.45 \mu\text{m}$ polyethersulfone (PES) membrane filter.</p> <p>i. If performing the assay with dried inoculated carriers, vortex-mix each vessel for 30 ± 2 s at the conclusion of the holding period, and then filter contents.</p> <p>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 at least 20 mL PBS and filter the rinsing liquid through the same filter membrane.</p> <p>i. Initiate filtration as soon as possible (e.g., within 30 minutes).</p> <p>ii. Two analysts are recommended to perform vortex-mixing and filtration steps to reduce holding time after vortex-mixing.</p> <p>d. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the Sabouraud Dextrose Emmon's Agar. Avoid trapping air bubbles between the filter and the agar surface. Incubate the plates for 72 ± 4 hours at $30 \pm 2^\circ\text{C}$.</p> <p>e. Proceed to section 13 of this document for data analysis and treatment assessment.</p>

<p>13. Data Analysis/ Calculations</p>	<ol style="list-style-type: none"> 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. <ol style="list-style-type: none"> 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. <ol style="list-style-type: none"> 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. 								
<p>14. Forms and Data Sheets</p>	<ol style="list-style-type: none"> 1. Attachment 1: Quantitative Method Neutralization Assay Flow Chart 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: <table border="0" style="margin-left: 40px;"> <tr> <td><i>C. auris</i> Neutralization Test Information Sheet</td> <td>MB-37-01_F1.docx</td> </tr> <tr> <td><i>C. auris</i> Neutralization Test Suspension Preparation Sheet</td> <td>MB-37-01_F2.docx</td> </tr> <tr> <td><i>C. auris</i> Neutralization Time Recording and Results Sheet</td> <td>MB-37-01_F3.docx</td> </tr> <tr> <td><i>C. auris</i> Neutralization Test Processing Sheet</td> <td>MB-37-01_F4.docx</td> </tr> </table> 	<i>C. auris</i> Neutralization Test Information Sheet	MB-37-01_F1.docx	<i>C. auris</i> Neutralization Test Suspension Preparation Sheet	MB-37-01_F2.docx	<i>C. auris</i> Neutralization Time Recording and Results Sheet	MB-37-01_F3.docx	<i>C. auris</i> Neutralization Test Processing Sheet	MB-37-01_F4.docx
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<p>15. References</p>	<ol style="list-style-type: none"> 1. EPA MLB SOP MB-35: Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against <i>Candida auris</i> on Hard, Non-Porous Surfaces. 								

Attachment 1

Quantitative Method Neutralization Assay Flow Chart



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