US Environmental Protection Agency
Office of Pesticide Programs

EPA MLB SOP MB-37-00:

Neutralization Confirmation for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* using the OECD Quantitative Method on Hard, Non-Porous Surfaces

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Please see EPA's companion interim guidance document for Candida auris under the Guidance tab.

Neutralization Confirmation for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* using the OECD Quantitative Method on Hard, Non-Porous Surfaces

I. Overview

A. This document describes a quantitative procedure for verifying the effectiveness of the neutralizer (refer to reference A). Verify neutralization using the highest concentration of test substance if there are multiple concentrations being evaluated.

B. In brief, 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 II of this document are met.

II. Measure of effectiveness

A. For the assay to be considered valid, ensure that:

1. The recovered number of Colony Forming Units (CFU) in the **Titer Control** (see section IV.D.3) using *Final Test Suspension B* yields 20-200 CFU per vessel.

B. For determining and verifying the effectiveness of the neutralizer, ensure that:

1. The recovered number of CFU in the **Neutralizer Toxicity Control** (see section IV.D.2) is at least 50% of the **Titer Control** (see section IV.D.3). 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.

2. The recovered number of CFU in the **Neutralizer Effectiveness** treatment (see section IV.D.1) 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.

C. For both the suspension-based assay and the carrier-based assay, meet the criteria in sections II.A and II. B. If the criteria are not met, identify and verify another neutralizer or mixture of neutralizers.

III. Special Apparatus and Materials

A. Refer to section III of EPA MLB SOP MB-35 (OECD Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* on Hard, Non-Porous Surfaces).

IV. Procedure and Analysis

A. Preparation and sterilization of carriers (if using the carrier-based approach)

1. Refer to section IV.A of EPA MLB SOP MB-35 (OECD Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* on Hard, Non-Porous Surfaces).

B. Preparation of test organisms

1. Refer to section IV.B of EPA MLB SOP MB-35 (OECD Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* on Hard, Non-Porous Surfaces).

2. Prepare *Test Suspension A (without soil load)*. Serially dilute the test microbial suspension with PBS (e.g., serially dilute cultures through 10^{-4} or 10^{-5}). Select appropriate dilutions of *Test Suspension A* so that after the addition of the soil load, *Final Test Suspension B* will achieve an average challenge of 20-200 CFU per 10 µL. Use *Test Suspension A* 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.

3. 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.

    ii. 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 a 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.

4. If desired, use optical density (OD @ 650 nm) to create a calibration curve to estimate the number of viable organisms in *Test Suspension A*.

C. Carrier inoculation (for carrier-based assay)

1. Inoculate at least 13 carriers with 10 µL of *Final Test Suspension B* (per concentration of *Final Test Suspension B*) using a positive displacement pipette with 10 µL tips. Refer to the section IV.D of EPA MLB SOP

MB-35 (OECD Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* on Hard, Non-Porous Surfaces) for carrier drying instructions.

2. After drying, evaluate the inoculated carriers per section IV.E of this document.

D. Suspension-based assay

1. ***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* using a micropipette to each vessel and briefly vortex. Proceed with section IV.F.

2. ***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* using a micropipette gently to each vessel and briefly vortex. Proceed with section IV.F.

3. ***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* with a micropipette gently to each vessel and briefly vortex. Proceed with section IV.F.

E. Alternative inoculated carrier-based assay

1. ***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. 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 IV.F.

2. ***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 IV.F.

3. ***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 IV.F.

F. Processing and recovery

1. Hold the mixtures from IV.D and IV.E for 10±1 min at room temperature (20-25°C). Steps (e.g., addition of organism and 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.

2. 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 0.45 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.

3. Wash each vessel with approximately 20 mL PBS and briefly vortex; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of the funnel unit with about 40 mL of PBS and filter the rinsing liquid through the same filter membrane.

   i. Note: Initiate filtration as soon as possible (e.g., within 30 min). Two analysts are recommended to perform vortexing and filtration steps to reduce holding time after vortexing.

4. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium (SDEA). Avoid trapping air bubbles between the filter and the agar surface. Incubate the plates for 48-72 h at 30±1°C.

5. Examine the plates beginning at 48±4 h after incubation and conduct final count at 72±4 h. Calculate the average CFU for each set of test conditions.

V. Data Analysis and Calculations

A. For the suspension-based assay (see section IV.D), 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 IV.E) in the same manner.

VI. Attachments

A. OECD Neutralization Assay Flow Chart

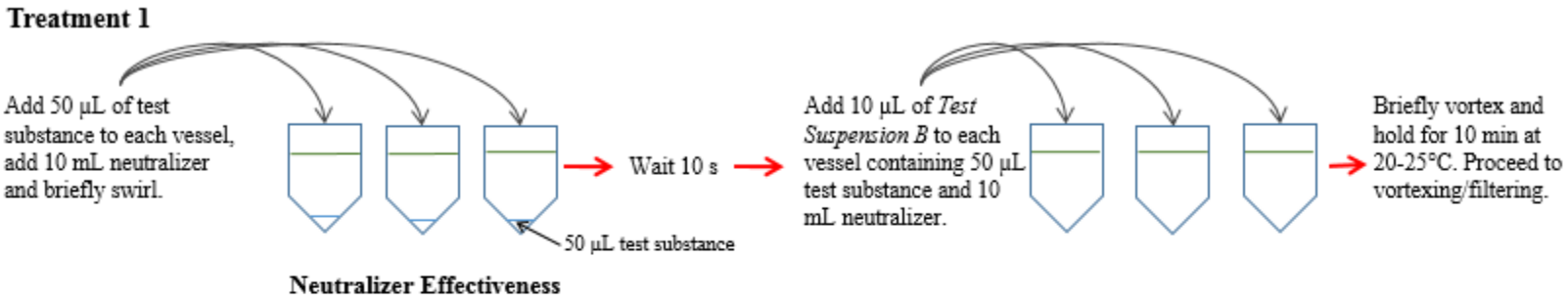
VII. References

A. OECD Guidance Document: Quantitative Method for Evaluating Bactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces (January 29, 2013).

B. EPA MLB SOP MB-35: OECD Quantitative Method for Evaluating the Efficacy of Liquid Antimicrobials against *Candida auris* on Hard, Non-Porous Surfaces

Attachment 1

OECD Neutralization Assay Flow Chart



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