# US Environmental Protection Agency
# Office of Pesticide Programs

**EPA MLB SOP MB-35-00:**

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

**Date: 03/21/17**

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

## I. Overview

A. This document describes a quantitative procedure for testing the fungicidal activity of liquid antimicrobial substances (disinfectants) against *Candida auris* designed for use on hard, non-porous surfaces.

B. This protocol is based on OECD Guidance Document dated June 21, 2013 (see reference A) and contains targeted revisions based on information and data collected by the EPA.

C. In brief, the OECD quantitative test method uses disks (1 cm in diameter) of brushed stainless steel as the carrier to represent a hard, non-porous surface. Each disk receives 10 µL of the test organism with a soil load incorporated into the inoculum. The inoculum is dried and exposed to 50 µL of the test substance; control carriers receive an equivalent volume of an innocuous control fluid. The contact time is allowed to elapse and a neutralizer is added at the end of the contact time. The neutralized carriers are vortexed and the resulting suspension is filtered to determine the presence of viable organisms. Based on mean $\log_{10}$ density values, the Log Reduction (LR) in the viability of the test organism on treated carriers is calculated in relation to the viability count on the control carriers. The LR value is used as the measure of product performance (i.e., product efficacy).

## II. Data Generation

A. For an acceptable test, the mean target test $\log_{10}$ density (*TestLD*) of the inoculated carriers is at least 5.0 (corresponding to a geometric mean density of $1.0\times10^5$) and not above 6.0 (corresponding to a geometric mean density of $1.0\times10^6$); a *TestLD* below 5.0 or above 6.0 invalidates the test.

## III. Special Apparatus and Materials

A. Test microbe: *Candida auris* (AR-BANK#0381) from the CDC. See **Attachment 1** for details.

B. Culture media. Purchase from a reputable source or prepare according to manufacturer's instructions.

1. *Sabouraud Dextrose Agar (SDA).*
2. *Sabouraud Dextrose Emmons Agar* (*SDEA*).
3. *Sabouraud Dextrose Broth* (*SDB*).
4. *Tryptic Soy Agar with 5% sheep blood/Blood Agar Plate (BAP).*
5. *Cryoprotectant solution (SDB with 15% (v/v) glycerol).*

C. Reagents

1. *Neutralizer.* Examples of neutralizers include Sabouraud Dextrose Broth, Letheen Broth, Sabouraud Dextrose Broth plus 0.07% lecithin, and 0.5% tween 80. If necessary, other ingredients may be added to the neutralizer (e.g., 0.1% (w/v) sodium thiosulfate for sodium hypochlorite-based

treatments).

2. *Phosphate buffered saline stock solution (*e.g., 10X*).* Used to prepare 1X phosphate buffered saline. Stock solution has a pH of 7.2±0.2.

3. *Phosphate buffered saline (PBS), 1X*. Used for dilution blanks and filtration. PBS with a pH of approximately 7.0±0.5 is desirable.

4. *Soil load.* The OECD soil load to be incorporated in the test suspension is a mixture of the following stock solutions in PBS:

   i. BSA: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot, and store at -20±2°C.

   ii. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter, aliquot, and store at -20±2°C.

   iii. Mucin: Add 0.04 g mucin (bovine or porcine) to 10 mL of PBS, mix thoroughly until dissolved, and autoclave (15 min at 121°C), aliquot, and store at -20±2°C.

   iv. The stock solutions of the soil load are single use only and should not be refrozen once thawed; store up to one year at -20±2°C.

   v. See section IV.C.2 for addition of soil load to inoculum.

   vi. Other soil loads may be used per the agency's guidance.

5. *Test substance.* Antimicrobial test solution. If dilution is required, see section III.C.6 for diluent.

6. *Test substance diluent.* The OECD test substance diluent is 375 ppm hard water. Adjust the recipe for volumes other than 1 L.

   i. Prepare Solution A by dissolving 19.84 g anhydrous magnesium chloride (or 42.36 g $MgCl_2 \cdot 6H_2O$) and 46.24 g anhydrous calcium chloride ($CaCl_2$) in de-ionized water and dilute to 1,000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator and use for up to one month.

   ii. Prepare Solution B by dissolving 35.02 g sodium bicarbonate ($NaHCO_3$) in water and dilute to 1,000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator and use for up to one month.

   iii. To prepare 1 L of 375 ppm hard water, place 600-700 mL of de-ionized water in a 1,000 mL volumetric flask and add 6.0 mL of Solution A and then 8.0 mL of Solution B. Mix and add water to the flask to reach 1,000 mL. The pH of the hard water should be 7.0±0.2 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl.

iv. Prepare the hard water under aseptic conditions and use within 5 days of preparation. Ensure sterility of hard water prior to use in efficacy testing.

v. On the day of the test, measure the hardness of the water using a water hardness test kit or other suitable titration method.

vi. The target hardness expressed as mg/L calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10% (338-394 ppm). Other levels of water hardness may be used as appropriate.

vii. Other diluents may be used per the agency's guidance.

7. *Water*. Either de-ionized distilled water or water with equivalent quality for making reagent solutions and culture media.

8. *Tween-80* (polysorbate 80).

9. *Lactophenol Cotton Blue Stain*. For presumptive identification.

D. Apparatus

1. Calibrated 10 µL positive displacement pipette with corresponding 10 µL tips, for carrier inoculation.

2. Calibrated micropipettes (e.g., 200 µL) with 10-100 or 20-200 µL tips, for deposition of test substance on carrier.

3. Carriers: Disks (1 cm in diameter) made from 0.7 mm thick sheets of brushed and magnetized stainless steel (AISI #430) (Pegen Industries, part #430-107). The top of the disk is brushed and has rounded edges; only the top is visually screened and inoculated. Carriers are single-use only. See **Attachment 2** for complete specifications.

4. Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes or other vessels, or pipettes to assist in the rinsing of vials and filters.

5. Forceps, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters, appropriate to pick up the carriers for placement in vials.

6. Magnet strong enough to hold the carrier in place in the vial while the liquid is being poured out of it for membrane filtration.

7. Membranes (polyethersulfone) for organism recovery, 47 mm diameter and 0.45 µm pore size. Filtration units (reusable or disposable) may be used.

8. Spectrophotometer; calibrated. Optional for use in culture standardization.

9. Sterile vials (plastic or comparable) to hold test carriers: flat bottom and wide-mouth to accommodate addition and removal of the carriers, for holding inoculated carriers to be exposed to the test substance and for accommodating neutralizer/eluent. Suitable vials should be at least 25 mm in neck diameter and capable of holding at least 20 mL of liquid.

Transparent vials are more desirable to facilitate application of 50 µL test substance or PBS and to allow for the viewing of the carriers for removal of inoculum.

10. Certified timer.

11. Desiccator with fresh desiccant (e.g., $CaCO_3$).

12. Vacuum source: in-house line or suitable vacuum pump.

13. Hach kit. For measuring water hardness.

    i. Total water hardness, 10 to 4,000 mg/L as $CaCO_3$ (Hach Digital Titrator Method 8213)

## IV. Procedure and Analysis

Verify neutralizer effectiveness using the procedure outlined in MLB SOP MB-37 (Neutralization Confirmation for the Efficacy Evaluation of Liquid Antimicrobials against *Candida auris* using the OECD Quantitative Method on Hard, Non-Porous Surfaces).

A. Preparation and sterilization of carriers

1. Visually check the brushed top surface of the carriers (with the rounded edge) for abnormalities (e.g., rust, chipping, deep striations) and discard if observed.

2. Soak visually screened carriers in a suitable detergent solution (e.g., 1 % Liquinox) for 2-4 h to degrease and then rinse thoroughly in distilled or deionized water. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.

3. Prior to sterilization, place up to 20 clean dry carriers on a piece of filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter). Cover the Petri dish with its lid and sterilize. After sterilization, transfer carriers top-side up to sterile Petri dishes without filter paper for inoculation.

4. Use sterilized carriers for up to six months.

B. Preparation of test organism:

1. Refer to **Attachment 3** for preparation of the frozen stock cultures for *C. auris*.

2. Defrost a cryovial; defrost rapidly (e.g., use a 37°C water bath) to avoid loss in the viability of the preserved cells. Each cryovial is single use only.

3. Add 100 µL of defrosted stock culture to 10 mL SDB, briefly vortex mix and incubate for 18-24 h at 30±1°C. Inoculate two tubes of (one as a backup).

4. In addition, inoculate an agar plate (e.g., TSA with 5% sheep blood, BAP) with a loopful from the inoculated tube and streak for isolation. Incubate plate with the test culture and examine for purity.

5. Following incubation, use the broth culture to prepare a test suspension for each organism.

6. Briefly vortex the 18-24 h culture and transfer to a sterile 15 mL centrifuge tube.

7. Centrifuge the 18-24 h broth culture at ~10,000 $g_N$ for 10±5 min. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in a maximum of 10 mL PBS.

    i. Disrupt the pellet using vortexing or if necessary, use repetitive tapping/striking against a hard surface to completely disaggregate the pellet.

8. The target control carrier count level is 5.0-6.0 logs per carrier. Dilute the resuspended culture as necessary to achieve the carrier counts.

9. Use the diluted culture to prepare the final test suspension with the soil load.

10. Optical density/absorbance (at 650 nm) may be used as a tool to monitor/adjust the re-suspended test suspension.

C. Preparation of final test suspension with soil load

1. Vortex the diluted culture for 10-30 s.

2. To obtain 500 µL of the final test suspension with the OECD soil load, vortex each component and combine the following (or appropriate ratio):

    i. 25 µL BSA stock

    ii. 35 µL yeast extract stock

    iii. 100 µL mucin stock

    iv. 340 µL test suspension

3. Within 30 min of preparation, use final test suspension with soil load (held at room temperature, 22±2°C) to inoculate carriers.

4. Other soil loads may be used per the agency's guidance.

D. Inoculation and drying of carriers

1. Vortex the final test suspension for 10 s following the addition of the soil load and immediately prior to use.

2. Inoculate the number of carriers required for the test; 3 controls and 5 treated plus at least 2 extras.

3. Using a calibrated positive displacement pipette with a 10 µL tip, deposit 10 µL of the final test suspension to the center of a carrier (a maximum of

20 carriers per Petri dish); avoid contact of pipette tip with carrier and do not spread the test suspension with the pipette tip. For consistency, vortex the inoculum frequently during inoculation of the carrier set. The same pipette tip may be used to inoculate all carriers (unless the tip is compromised). Discard any inoculated carrier where the final test suspension has run over the edge.

4. Transfer the Petri dish with the inoculated carriers into a desiccator and completely remove the lid of the Petri dish. Close the desiccator and check that it is properly sealed. Evacuate the desiccator using a vacuum source to achieve 20-25 inches mercury (508-635 torr; 677-847 mbar; 68000-85000 Pascal).

5. Hold the inoculated carriers in the evacuated desiccator (with vacuum on) at 22±2°C for 50±5 min. If carriers are not dry within the specified time, check the desiccator system (replace the desiccant if necessary). Do not use carriers that are visibly moist.

6. Following the inoculation of carriers, streak inoculate an agar plate (e.g., TSA with 5% sheep blood, Blood Agar Plate) with a loopful of the final test suspension. Incubate plate with the treated and control carrier plates and examine for purity.

7. Use inoculated carriers for testing within one hour after drying held at room temperature (22±2°C).

E. Exposure of the dried inoculum to the test substance or PBS (control counts)

Note: During testing, do not process carriers where the test substance runs off of the carrier; replace with new carrier(s) and vial(s). Evaluate 3 control carriers and 5 treated carriers for each test substance tested (one test organism and contact time/temperature combination) unless specified otherwise. Use a certified timer to ensure that each carrier receives the required exposure time.

1. Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred.

2. In a timed fashion, deposit 50 µL of the test substance (equilibrated to 22±2°C) with a calibrated micropipette (or positive displacement pipette) over the dried inoculum on each test carrier, ensuring complete coverage, at predetermined staggered intervals. Use a new tip for each carrier; do not touch the pipette tip to the carrier surface. Do not cap the vials.

3. Hold the test carriers at 22±2°C (or other specified temperature) for the selected contact period.

4. Treat control carriers last – each control carrier receives 50 µL phosphate buffered saline (PBS), equilibrated to 22±2°C, instead of the test substance. Hold the control carriers at 22±2°C for the contact period.

F. Neutralization of test substance

1. The neutralizer for the control carriers is the same as that for the treated carriers.

2. Within 5-10 s of the end of the contact period, add 10 mL of neutralizer (at room temperature) to each vial in the specified order, including controls, according to the predetermined schedule. The neutralized vial with carrier is documented as the $10^0$ dilution. Briefly (2-3 s) vortex each vial following the addition of the neutralizer.

3. Following the neutralization of the entire set of carriers, vortex each vial for 30±5 s at high speed to recover the inoculum; ensure that the liquid and carrier are fully vortexed. Do not remove the carrier from the vial.

G. Dilution and recovery

1. Initiate dilutions within 30 min after the neutralization and vortexing steps. Initiate filtration and plating within 30 min of preparing the dilutions.

2. Serially dilute the eluate from the vial with the carrier ($10^0$ dilution) prior to filtration by transferring 1 mL into 9 mL PBS in a dilution tube. Dilute out to $10^{-1}$ and filter entire contents of the $10^0$ and $10^{-1}$ dilutions.

3. Prior to filtration, pre-wet each membrane filter with approximately 10 mL of sterile PBS; apply vacuum to filter contents. The vacuum should be left on for the duration of the filtration process.

4. Use separate membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given treated carrier set starting with the most dilute sample ($10^{-1}$) first.

5. Filter the entire contents of the vial and associated dilution tube(s) of the treated carrier samples) through separate 0.45 µm PES membrane filters.

6. For the eluate remaining in the vial, vortex the vial for ~5 s and holding a magnet at the bottom of the vial to keep the carrier in place, pour the eluate into the filter unit.

7. Rinse the treated vial with ~20 mL of PBS, vortex for ~5 s and keeping magnet in place, pour the wash into the same filter unit. For dilution tubes, rinse tube once with ~10 mL of PBS, briefly vortex, and pour into filter unit.

8. Swirl the contents of the filter unit. The filtering process should proceed quickly with limited pooling of liquid in the filter apparatus.

9. With the vacuum on, rinse the inside surface of the funnel unit with ~40 mL PBS.

i. Aseptically remove the membrane filter and place on SDEA plate. Avoid trapping any air bubbles between the filter and the agar surface.

10. Serially dilute the eluate from the control carrier vials out to at least the $10^{-3}$ dilution; direct plate 100 µL in duplicate on SDEA plates.

11. Incubate all plates from treated and control carriers at 30±1°C.

12. For controls, begin monitoring plates at 48±4 h and record final results at 72±4 h.

13. For treated carriers, begin monitoring filters on plates at 72±4 h and record final results at 120±4 h.

H. Recording results

1. Count colonies and record results. Colony counts on filters (treated) in excess of 200 and in excess of 300 on plates (controls) should be recorded as Too Numerous to Count (TNTC). If no colonies are present, record as zero.

2. Inspect the growth on the filters and plates for purity and typical characteristics of the test microbe.

3. If isolated colonies are present, stain (lactophenol cotton blue stain) a sample of growth for at least one treated and one control carrier.

i. If confluent growth is present, perform a streak isolation on TSA with 5% sheep blood and stain growth from an isolated colony.

## V. Data Analysis and Calculations

A. Per test, colony counts are recorded and used in calculations to determine log reductions.

B. To calculate the Colony Forming Units (CFU)/treated carrier use the following equation: $\left(\frac{CFU\ \ for\ 10^{-y} + CFU\ \ for\ 10^{-z}}{(a\times 10^{-y}) + (b\times 10^{-z})}\right)\times c$, where $10^{-y}$ and $10^{-z}$ are the dilutions filtered, "a" and "b" are the volumes filtered at each dilution (typically 9 or 10 mL), and "c" is the volume of medium originally in the vial with the carrier (10 mL). Account for the volume filtered in the calculations.

1. When TNTC values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculations. For direct plating, substitute 300 for TNTC.

C. To calculate the CFU/control carrier when using agar plates, use the following equation:

$\left(\frac{Mean\ CFU\ \ for\ 10^{-y} + Mean\ CFU\ \ for\ 10^{-z}}{10^{-y} + 10^{-z}}\right) \times c \times 10$, where $10^{-y}$ and $10^{-z}$ are the dilutions plated, "c" is the volume of medium originally in the vial with the carrier (10 mL), and 10 accounts for the volume plated (100 µL).

D. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

E. Calculate the mean $\log_{10}$ density across treated carriers.

F. Calculate the mean $\log_{10}$ density across control carriers.

G. Calculate the $\log_{10}$ reduction (LR) for treated carriers:
$\log_{10}$ reduction = mean $\log_{10}$ density for control – mean $\log_{10}$ density for treated

H. For a set of five treated carriers: when the $10^0$ dilution (the contents of the vial with the carrier) is filtered either by itself or in addition to other dilutions and the data for each carrier result in zeros for each dilution filtered, report the LR as greater than or equal to the mean $\log_{10}$ density for the control carriers.

## VI. Attachments

A. Attachment 1: *Candida auris* test strain

B. Attachment 2: Carrier Specifications

C. Attachment 3: Preparation and Quality Control for Frozen Stock Cultures

## VII. References

A. Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides Used on Hard, Non-Porous Surfaces (June 21, 2013).

B. ASTM Standard E2197-11, 2011, "Standard Quantitative Disk Carrier Test Method for Determining the Bactericidal, Virucidal, Fungicidal, Mycobactericidal and Sporicidal Activities of Liquid Chemical Germicides," ASTM International, West Conshohocken, PA.

## Attachment 1 – *Candida auris* test strain

See web order form at: https://www.cdc.gov/drugresistance/resistance-bank/form/index.html
Email: ARbank@cdc.gov Phone: (404) 639-2180

FDA ANTIMICROBIAL RESISTANCE BANK CDC

STORAGE TEMPERATURE -70°C
BIOSAFETY LEVEL 2

### PROPAGATION

**MEDIUM**

Medium: Sabouraud Dextrose agar (SDA)

**GROWTH CONDITIONS**

Temperature: 30°C
Atmosphere: Aerobic

**PROPAGATION PROCEDURE**

Remove the sample vial to a container with dry ice or a freezer block. Keep vial on ice or block. (Do not let vial content thaw)

Open vial aseptically to avoid contamination

Using a sterile loop, remove a small amount of frozen isolate from the top of the vial

Aseptically transfer the loop to SDA

Use streak plate method to isolate single colonies

Incubate inverted plate at 30°C for 48 hrs.

## *Candida auris*

AR-BANK#0381

KNOWN ACQUIRED RESISTANCE:

**Unknown**

**MIC (µg/ml) RESULTS AND INTERPRETATION**

| DRUG | MIC | INT |
|---|---|---|
| Fluconazole | 4 | --- |
| Voriconazole | 0.03 | --- |
| Posaconazole | 0.06 | --- |
| Itraconazole | 0.125 | --- |
| Caspofungin | 0.125 | --- |
| Anidulafungin | 0.25 | --- |
| Micafungin | 0.125 | --- |
| Flucytosine | 2 | --- |
| Amphotericin B* | 0.38 | --- |

S – I –R Interpretation (INT) derived from *CLSI 2012 M27 S4*
SDD (Susceptible Dose Dependent)
*Measured using Etest

arbank@cdc.gov http://www.cdc.gov/drugresistance/resistance-bank/

## Attachment 2

Carrier Specifications

- Ferritic stainless steel: Consist of chromium (17%) and iron and essentially nickel-free.
- AISI Type 430 (European equivalent name X6Cr17 and number 1.4016) belongs to Group 2, which is the most widely used family of ferritic alloys.
- Dimensions: 1 cm (0.39370 inch) in diameter; 0.7 mm (0.027559 inch) thick.
- AISI 430 - ASTM A240; Japanese Industrial Standard (JIS) G4305; EN 10088-2
- No. 4 Finish (EN 10088-2 1J/2J): A ground unidirectional finish obtained with 150 grit abrasive (AISI).
- Passivation: A soak in a mild acid bath for a few minutes to remove any impurities and accumulated debris from the disk surface.
- Tumbling: To remove the punching burrs from the edges of the discs they are tumbled in a barrel together with ceramic chips and a cleanser.

## Attachment 3

Preparation and Quality Control for Frozen Stock Cultures

A. Initiate stock cultures of *Candida auris* at least every 18 months.

   a. Using a new culture from CDC, streak isolate from a slant or rehydrated lyophilized culture onto a Sabouraud Dextrose Agar (SDA) plate. Incubate for 48-72 h at 30±2°C.
   b. Following incubation, take a single representative colony and inoculate 10 mL of Sabouraud Dextrose Broth (SDB) and incubate for 24±2 h at 30±2°C.
   c. Vortex the sample, then spread 0.1 mL of the test organism suspension onto 5-10 Sabouraud Dextrose Emmons Agar (SDEA) plates. Incubate for 48-72 h at 30±2°C.
   d. At the end of the incubation period, add 5 mL 1X PBS with 0.1% (v/v) Tween 80 to the surface of each agar plate and re-suspend the cells using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat by adding another 5 mL of 1X PBS with 0.1% (v/v) Tween 80 to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension. Thus, each plate should yield ~10 mL of harvested suspension.
   e. Repeat the growth harvesting procedure with the remaining plates and continue pooling the suspension to the vessel (more than 1 vessel may be used if necessary).
      i. At a minimum, conduct QC on the pooled culture according to Section B below. Additional QC may be conducted at any step in the process to confirm purity.
   f. Centrifuge the pooled suspension at 10,000 x g for 10 minutes. Pipette supernatant off and resuspend in 10 mL SDB with 15% (v/v) glycerol. If two vessels are used, resuspend each pellet in 5 mL of SDB with 15% (v/v) glycerol. Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting the culture.
   g. While mixing continuously, dispense 0.5-1.0 mL aliquots of the harvested suspension into separate cryovials; these represent the frozen stock cultures.
   h. Store the cryovials at -70ºC or lower for a maximum of 18 months (from the date of harvesting/freezing).

B. Quality Control of Stock Cultures.

   a. Conduct a purity check of the pooled culture concurrently with freezing. Streak a loopful on TSA with 5% sheep blood and SDA and incubate at 30±2ºC for 48-72 hours.

b. Following the incubation period, record the colony morphology as observed on the plates and conduct stain with lactophenol cotton blue stain from a selected typical colony.

    i. Colonies on TSA with 5% blood are smooth, dull white to cream colored.

    ii. Observe the staining results by using bright field microscopy at 1000X magnification (oil immersion). Stained cells are dark blue, ovoid, ellipsoidal to elongate, (2.0–3.0) × (2.5–5.0) $\mu$m, single, in pairs, or in group showing globose budding yeast cells on microscopic examination

c. Record all confirmation results.