US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for

Quantitative Method for Evaluating the Efficacy of Antimicrobial Products against *Candida auris* on Hard, Non-Porous Surfaces

SOP Number: MB-35-03

Date Revised: 10-12-21

SOP Number	MB-35-03
Title	Quantitative Method for Evaluating the Efficacy of Antimicrobial Products against *Candida auris* on Hard, Non-Porous Surfaces
Revisions Made	**Revisions to MB-35-00 docket version (03-21-17) → MB-35-01 (01-24-20)** • Minor editorial changes for clarification purposes. • Test microbe was changed from *Candida auris* AR-Bank#0381 to *Candida auris* AR-Bank#0385 (Section 11.1) • PBS-T with 0.1% (w/v) sodium thiosulfate was added for test system control neutralizer (Section 11.3.d) • The name of OECD soil load was changed to 3-part soil load (Section 11.3.e) • Replaced AISI 430 Stainless Steel carriers with AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side for carriers (Section 11.2.a) • Updates to the Apparatus Section (Section 11.2) • The overnight culture was changed from 18-24 hour culture to 24±2 hour culture (Section 12.2.e) • Use culture within 30 min to prepare the final test suspension (Section 12.2.g.i) • Specified rescreening each carrier prior to inoculation (Section 12.4.a) • Specified not to use carriers where the dried inoculum is near the edge of the carrier (Section 12.4.g) • Specified to record condition of inoculated carriers after approximately 10 min of drying (Section 12.4.g.i) • Filter the control carriers instead of direct plating the control carriers (Section 12.7.d) • Removed the use of magnet at the bottom of the vial to keep the carrier in place (Section 12.7.i) • Added sterility controls (Section 12.7.n) • Updated information pertaining to contamination and non-conforming data (Section 12.8)

	• Added to report non-conforming data (Section 12.8.d) • Specified that a new frozen stock culture may be initiated one time using and existing frozen stock culture as the source (Attachment 2.A.i) • Added record results of the streak isolation at the end of the incubation timeframe as pure or contaminated (Attachment 2.B.i) • Added within two to fourteen days of freezing, confirm antifungal susceptibility of the frozen stock cultures (Attachment 2.K) • Added examples of acceptable and unacceptable inoculated carriers (Attachment 3, Figure 3) • Test System Control for the OECD Quantitative Method for *Candida auris* was added (Attachment 4) **Revisions to MB-35-01 (01-24-20) → MB-35-02 (05-17-21)** • Minor editorial changes for clarification purposes. • Requirement to filter sterilize mucin (Section 11.d.ii) • Removed use of additional soil loads. (Section 11.d.v) • Renamed "test substance diluent" to "default diluent" (Section 11.f) • Removed laboratory grade sodium hypochlorite for preparation of test substance for test system control (was Section 11.j) • Added Fluconazole antifungal susceptibility tests (Section 11.j) • The three-part soil is specified as the soil load, with a prescribed order of preparation (Section 12.3). • Changed the time for carriers to dry to 45 to 60 minutes (Section 12.4.g) • Removed the test system control (was Section 12.5b) • Additional specifications for deposition of 50 µL of the test substance (Section 12.5.d) • Added thaw/refreeze the stock culture acquired from CDC to create additional stock cultures after the frozen stock culture expires (Attachment 2.A.i) • Removed Attachment 4 Tests System Control for the OECD

	Quantitative Method for *Candida auris* (was Attachment 4) **Revisions to MB-35-02 (05-17-21) → MB-35-03 (10-12-21)** • Minor editorial changes for clarification purposes. • Specified radio immunoassay grade of bovine serum albumin (Section 11.3.i.i). • The CAS # of mucin was added (Section 11.3.i.iii). • 375 ppm hard water listed as the test substance diluent (Section 11.3.k). • Specified vacuum pressure of vacuum source (Section 11.4.m). • Added figures for examples of typical acceptable and unacceptable carriers (Section 12.4.g). • Additional specifications for carrier inoculation (Section 12.4.g.iii). • Additional specifications for application of the antimicrobial test substance (Section 12.5.d.i). • Updated specifications for incubation (Section 12.7.o).

SOP Number	MB-35-03
Title	Quantitative Method for Evaluating the Efficacy of Antimicrobial Products against *Candida auris* on Hard, Non-Porous Surfaces
Scope	The method provides a quantitative assessment of the performance of liquid antimicrobial test substances including sprays (aerosol, pressurized spray, trigger, and pump dispensers) and water-soluble powders against a drug-resistant strain of *Candida auris* designed for use on hard, non-porous surfaces. Towelettes may be evaluated using liquid directly expressed from the towelette for the efficacy evaluation.
Application	This method provides $\log_{10}$ reduction (LR) as the quantitative measure of efficacy for these antimicrobial test substances against the test microbes on a hard, non-porous surface.

	Approval	Date
SOP Developer:	Lisa Smith Print Name: Lisa Smith	10/12/2021
SOP Reviewer	Stephen Tomasino Print Name: Stephen Tomasino	10/12/2021
Quality Assurance Unit	Kiran Verma Print Name: Kiran Verma	10/12/2021
Branch Chief	Rebecca Pines Print Name: Rebecca Pines (Acting Branch Chief)	10/12/2021

Data SOP issued:	10/12/2021
Controlled copy number:	0
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. LR = $\log_{10}$ reduction 2. Eluent = any liquid that is harmless to the test organism(s) and that is added to a vial containing the carrier to recover the test organism. 3. Eluate = recovered eluent that contains the test organism 4. Stock culture = frozen culture used to prepare the test culture 5. Treated carriers = five inoculated carriers exposed to the antimicrobial test substance 6. Control carriers = three inoculated carriers exposed to the control substance 7. Final test suspension = the harvested test culture with the addition of the three-part soil load 8. CFU = colony forming unit
2. Health and Safety	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	1. Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	1. Refer to SOPs 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	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	1. For quality control purposes, the required information is documented on the appropriate form(s) (see section 14). 2. Refer to SOP MB-10, Media and Reagents Used in Microbiological Assays, for QC of media and reagents.
7. Interferences	1. Inadequate neutralization may lead to errors in the measurement of test substance efficacy. Prior to efficacy testing, verify neutralizer effectiveness using the procedure outlined in SOP MB-37 (Neutralization of Microbicidal Activity against *Candida auris* using the Quantitative Method). 2. During testing, do not process carriers where the test substance runs off of the carrier; replace and retest with new inoculated carrier(s) and vial(s). 3. Avoid touching the carrier surface with a pipette tip during the application of

	the test substance or the control substance. 4. Transparent vials are more desirable to facilitate the application of 50 µL test substance or control substance on inoculated carriers. 5. Gently apply the disinfectant to the inoculated carrier; do not forcefully deposit the disinfectant.
8. Non-conforming Data	1. The outcome of the antifungal susceptibility test must conform with the anticipated profile (see Attachment 1). 2. For a valid test, the mean $\log_{10}$ density (LD) for control carriers is 5.0-6.0 CFU/carrier, with each control carrier exhibiting a LD of 5.0 to 6.0. 3. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.
9. Data Management	1. Data will be archived consistent with SOP ADM-03, Records and Archives. 2. See Section 13 for appropriate calculations and default values. 3. Use approved spreadsheets for calculations.
10. Cautions	1. Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting. 2. For storage, ensure carriers are completely dry following sterilization. 3. Ensure quality of media (sterility and performance) is adequate.
11. Special Apparatus and Materials	1. Test microbe: *Candida auris* (AR-Bank#0385*)* from the Centers for Disease Control and Prevention (CDC). See Attachment 1 for details. 2. Culture media. Purchase from a reputable source or prepare according to manufacturer's instructions. a. *Sabouraud Dextrose Agar (SDA).* Use for the preparation of frozen stock cultures, Attachment 2. b. *Sabouraud Dextrose Emmon's Agar (SDEA*). Use for culturing *C. auris*. c. *Sabouraud Dextrose Broth (SDB).* Use to propagate *C. auris.* d. *Tryptic soy agar with 5% sheep blood (BAP).* Use for isolation and characterization of *C. auris*. e. *SDB with 15% (v/v) glycerol*. Use as a cryoprotectant solution. 3. Reagents a. *Neutralizer.* A liquid reagent used to inactivate and/or dilute the antimicrobial treatment to end the contact time. Confirm neutralizer toxicity and effectiveness using the procedure outlined in EPA MLB SOP MB-37 (Neutralization Confirmation for the Efficacy Evaluation

	of Liquid Antimicrobials against *Candida auris* using the Quantitative Method). b. *Phosphate buffered saline stock solution (e.g., 10X).* Use to prepare 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2. c. *Phosphate buffered saline (PBS), 1X.* Use for dilution blanks and filtration. PBS with a pH of approximately 7.0±0.5 is desirable. d. *Soil load.* Use as the soiling agent. Add to the test suspension in the following manner: i. BSA: Add 0.5 g bovine serum albumin (BSA) (radio immunoassay (RIA) grade or equivalent) 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 (from bovine submaxillary gland, CAS # 84195-52-8) to 10 mL of PBS, stir or vortex-mix thoroughly until dissolved, and pass through a 0.2 µm pore diameter membrane filter, aliquot, and store at -20±2°C. iv. The three stock solutions of the soil load are single use only. Do not refreeze once thawed; store up to one year at -20±2°C. v. See section 12.3 for addition of soil load to inoculum. e. *Antimicrobial test substance.* Ready-to-use, activated, or concentrated antimicrobial solution. For dilutable antimicrobial test substances, use within 3 hours of preparation or as otherwise instructed by the manufacturer. Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated antimicrobial test substance should be used when preparing use-dilutions for testing. Use v/v dilutions for liquids antimicrobial test substances and w/v dilutions for solid antimicrobial test substances. The use of a positive displacement pipette is recommended for viscous liquids. If dilution is required, see section 11.3.f for diluent. f. *375 ppm hard water (QM Water).* Use for the preparation of dilutable antimicrobial test substances. 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 $MgCl \cdot 6H_2O$) 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 (2-8ºC) for up to 30 days; do not use after that time.

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 (2-8ºC) for up to 30 days; do not use after that time.

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. Ensure sterility of hard water.

iv. Prepare the hard water under aseptic conditions and use within 5 days of preparation. On the day of the test, measure the hardness of the water using a water hardness test kit or other suitable titration method.

v. The target hardness expressed as mg/L calcium carbonate ($CaCO_3$) is 375 mg/L +5%/-10% (338-394 ppm).

vi. Additional diluents and levels of water hardness may be used per the Agency's guidance.

g. *Water*. Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite water quality can be met. See Section 15.2 for details on reagent-grade water.

h. *Lactophenol Cotton Blue Stain (LPCB).* Use for presumptive identification.

i. *Non-ionic detergent [Liquinox (1% solution)]*. Use to clean carriers.

j. *Fluconazole antifungal susceptibility tests [ETEST®] or equivalent*. To determine the Minimum Inhibitory Concentration (MIC) of antifungal agents. Other antifungal susceptibly tests for additional fungicides may be included.

2. Apparatus

a. *Carriers*: Discs (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side; refer to Attachment 3 for complete carrier specifications. Screen brushed surface of the disc. Carriers are single-use. See Attachment 3 for carrier specifications and photographs of screened carriers.

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

c. *Filter paper.* Whatman No. 2, to line glass Petri plates during carrier sterilization.

d. *Calibrated micropipettes or positive displacement pipettes* (e.g., 200 µL, 1 mL) with corresponding tips, use for deposition of antimicrobial test substance or control substance on carriers and preparing dilutions.

e. *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes, bottles, etc.* Use to rinse vials and filters.

f. *Forceps*, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters, and to transfer inoculated carriers into vials.

g. *Membranes (polyethersulfone), white or black PES membranes.* Use for recovery of test microbe, 47 mm diameter and 0.45 µm pore size.

   i. Use filter membranes in either a reusable or disposable filtration unit. Black membranes are preferrable for enumeration.

h. *Filter Sterilization Unit (with PES membrane, 0.2 µm pore size)*. Use to filter sterilize soil components.

i. *Spectrophotometer.* For antifungal susceptibility tests.

j. *Vials with lids (plastic or comparable).* Sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), for holding inoculated carriers and for accommodating neutralizer (e.g., 20 mL capacity).

   i. Highly transparent vials are more desirable to facilitate application of 50 µL test substance or control substance to inoculated carrier.

k. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals.

l. *Desiccation unit* (with gauge to measure vacuum) with fresh desiccant (e.g., anhydrous $CaCl_2$). Use to facilitate drying of inoculated carriers. Change desiccant when moisture absorption is evident (e.g., desiccant clumping).

m. *Vacuum source*. In-house line or suitable vacuum pump (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, 68000-85000, or 0.068 to 0.085 MPa) for drying inoculated carriers in desiccation unit and to aid in filtration.

n. *Titration kit* (e.g., Hach® digital titrator). For measuring water hardness.

	o. *Vortex-style mixer*. For vortex mixing of various solutions. p. *15 mL conical centrifuge tubes.* For centrifugation of test cultures. q. *Centrifuge* (with rotor capable of achieving 10,000g). For test culture preparation.
12. Procedure and Analysis	
12.1 Preparation and sterilization of carriers	a. Use flat carriers for testing. Visually inspect the brushed top surface of the carriers for abnormalities (e.g., rust, chipping, deep striations) and discard if observed; refer to Figures 1 and 2 for examples of typical acceptable and unacceptable carriers, respectively. Magnification is not necessary for the visual inspection. Record outcome of physical screening and use only those deemed acceptable for testing. b. Soak visually screened carriers in a suitable non-ionic detergent solution for 2-4 h to degrease and then rinse thoroughly in distilled or deionized water (e.g., place carriers in large beaker and flush beaker with DI water for 5-10 min), ensuring complete removal of detergent. Allow carriers to dry. i. Incomplete removal of detergent may cause inoculum to run off the carrier during inoculation. c. Using gloved-hands or forceps, place up to 20 clean dry carriers on filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter) ensure carriers were not damaged (scratched) during processing. Cover the Petri dish with its lid and sterilize at 121°C for 45 min. Ensure carriers are dry following sterilization. i. Screened, cleaned carriers may be stored dry in a clean, covered vessel prior to sterilization. d. Use sterilized carriers for up to six months. After six months, re-sterilize any remaining unused carriers and assign a new tracking number.
12.2 Preparation of *C. auris* test cultures	a. Refer to Attachment 2 for preparation of the frozen stock cultures. b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only. c. Using a calibrated micropipette, add 100 µL of defrosted stock culture to 10 mL SDB, briefly vortex mix and incubate for 24±2 h at 30±2°C. In addition, inoculate an agar plate (e.g., TSA with 5% sheep blood) with a loopful from the inoculated tube and streak for isolation. Incubate plate with the test culture and examine for purity. d. Following incubation, use the broth cultures to prepare a test

	suspension. e. Briefly vortex-mix the 24±2 h culture and transfer to a 15 mL conical centrifuge tube. f. Within 15 minutes, centrifuge the 24±2 h harvested broth culture at ~10,000g_N for 10±1 min. g. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in a maximum of 10 mL PBS. Resuspension of the pellet in a smaller volume (e.g., 5 mL) is permissible to concentrate the culture. i. Use this culture within 30 min to prepare the final test suspension; record time of pellet resuspension. ii. Briefly vortex-mix to disrupt the pellet using vortex-mixing to disaggregate the pellet completely prior to re-suspending it in a maximum of 10 mL. iii. Dilute as necessary in PBS to achieve control carrier counts of 5.0-6.0 logs CFU carrier. h. Use the diluted culture to prepare the final test suspension with the addition of the soil load per section 12.3.
12.3 Preparation of the final test suspension with 3-part soil load	a. Vortex-mix the test suspension for 10-30 s. b. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette: i. 25 µL BSA stock ii. 35 µL yeast extract stock iii. 100 µL mucin stock iv. Vortex soil suspension for 10 s prior to adding microbial test suspension. v. 340 µL microbial test suspension c. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 22±2°C) and use to inoculate carriers within 30 min of preparation. Record time of final test suspension preparation.
12.4 Inoculation and drying of carriers	a. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to inoculation. Place carriers brushed-side up inside an empty, sterile plastic Petri dish (no more than 20 carriers/dish). b. Vortex-mix the final test suspension for 10 s immediately prior to use. c. Inoculate five (5) carriers to serve as treated carriers and three (3) carriers to serve as control carriers. Inoculate additional carriers to

serve as extras. Multiple antimicrobial test substances may be evaluated using the same set of control carriers provided the same neutralizer is used

d. Using a calibrated positive displacement pipette with a 10 µL tip, withdraw 10 µL of the final test suspension and deposit it at the center of each carrier (clean, screened, and sterile), keeping the pipette perpendicular to the carrier during deposition of the test suspension. Avoid contact of pipette tip with carrier and do not spread the final test suspension with the pipette tip.

    i. For consistency, vortex-mix the inoculum after every 10 carriers during inoculation of the carrier set.

    ii. The same pipette tip may be used to inoculate all carriers (unless aseptic technique is not maintained in which case the tip should be changed).

    iii. Discard any inoculated carrier where the final test suspension has run over the edge of the carrier.

e. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with desiccant) and completely remove the lid of the Petri dish. Close the desiccation unit door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit.

    i. Note: do not exceed 40 inoculated carriers per desiccator to ensure carriers dry within the prescribed time.

f. Maintain and monitor the vacuum level using a gauge. Achieve and maintain consistent level of vacuum (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000 Pascal) by leaving the vacuum on during the drying period with the desiccator stopcock opened or closed as necessary.

g. Hold the inoculated carriers in the evacuated desiccation unit at 22±2°C for 45 to 60 min. If the carriers are not dry after 60 minutes, then continue to dry for up to an additional 10 min and record the time to dry on the paperwork. Do not use carriers dried for longer than 70 minutes.

h. Visually inspect inoculated carriers to verify that the inoculation spot appears dry (i.e., no visible wetness) and remove from desiccation unit. Do not use carriers that are visibly wet for testing. Do not use carriers where the dried inoculum is near the edge of the carrier (see Attachment 3, Fig. 3). Do not use carriers whose dried inoculum spot cannot be completely covered by the 50 µL of disinfectant.

    i. Monitor inoculated carriers periodically to ensure proper pace of drying; by 30 minutes they should be showing signs of

	desiccation (see 12.4.g.iii) ii. If carriers dry very quickly (e.g., 5-10 min) or are not dry within 10 min beyond the specified time, check the desiccation unit and the vacuum system to ensure proper function (e.g., replace the desiccant if necessary and check the seal for leaks). i. Following the inoculation of carriers, streak inoculate TSA with 5% sheep blood with a loopful of the test culture plus 3-part soil load. Streak for isolation and incubate plates with the control carrier plates generated from the test day and examine for purity at the end of the incubation period. The purity plate should be free of contamination. j. Use dried inoculated carriers for testing within 30 min following removal from desiccation unit; hold carriers in closed Petri dish at room temperature (22±2°C) until use.
12.5 Exposure of the dried inoculum to the antimicrobial test substance and control substance	a. Conduct the test at room temperature (22±2˚C) for the selected contact time; record temperature. Use a certified timer to ensure that each carrier receives the required contact time. b. Use 5 carriers for each test substance evaluated (one test organism and contact time/temperature combination) unless specified otherwise. i. Use 3 control carriers per test condition. c. 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. d. Prepare the antimicrobial test substance as specified by the manufacturer and use antimicrobial test substance within 3 hours of preparation e. In a timed fashion with appropriate intervals, sequentially 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. i. Note: Gently apply the antimicrobial test substance at a perpendicular angle to the inoculated carrier; do not forcefully deposit the antimicrobial test substance f. Use a new pipette tip for each carrier. Do not touch the carrier surface with a pipette tip during the application of the antimicrobial test substance or the control substance; replace with new carrier(s) and vial(s) if this occurs. Do not cap the vials. i. For non-foaming aerosols and pump/trigger spray antimicrobial test substance, obtain the antimicrobial test substance by dispensing it through the nozzle into a sterile vessel for

	collection. Cap the vessel. ii. For foaming spray formulations, allow the foam to break down for at least 5-10 minutes for the generation of a 1-2 mL liquid sample. Cap the vessel. iii. For highly volatile liquids, use dispensed antimicrobial test substance for testing within 30 minutes. iv. For towelettes, use liquid directly expressed from the towelette for the efficacy evaluation. g. Do not process carriers where the antimicrobial test substance or control substance runs off the carrier or does not completely cover the inoculum spot; replace with new carrier(s) and vial(s) if this occurs. h. Treat control carriers last. Each control carrier receives 50 µL PBS, equilibrated to 22±2°C, instead of the antimicrobial test substance. Hold the control carriers for the same contact time as used for the antimicrobial test substance.
12.6 Neutralization of antimicrobial test substance and elution of test organisms	a. The neutralizer for the control carriers is the same as that for the treated carriers. i. Inadequate neutralization may lead to bias in the measurement of antimicrobial test substance efficacy. Prior to efficacy testing, verify neutralizer effectiveness using the procedure outlined in EPA MLB SOP MB-37 (Neutralization of Microbicidal Activity against *Candida auris* using the Quantitative Method). b. Within ±5 s of the end of the contact period, add 10 mL of neutralizer equilibrated to 22±2°C to each vial in the specified order according to the predetermined schedule. Briefly (2-3 s) vortex each vial following the addition of the neutralizer. i. For calculation purposes, the solution in the neutralized vial with carrier is deemed the 10^0 dilution. Following the neutralization of the entire set of carriers, vortex-mix each vial for 30±5 s at high speed to recover and disaggregate the inoculum. Ensure that the liquid and carrier are both spinning in the vial during vortex-mix. Do not remove the carrier from the vial.
12.7 Dilution and recovery	a. Initiate dilutions within 30 min after neutralization and vortex-mixing. Initiate filtration within 30 min of preparing the dilutions; record times for initiation of both dilutions and filtration. b. Dilute and filter samples for the treated and control carriers; process treated carriers first.

	c. Serially dilute the eluate from the 10^0 dilution prior to filtration by transferring 1 mL into 9 mL PBS in a dilution tube. d. For the treated and control carriers, prepare additional 10-fold serial dilutions as necessary to achieve countable filters. Filter the entire contents. e. Turn on vacuum and leave on for the duration of the filtration process. f. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS; apply vacuum to filter contents. g. Use separate membrane filters for each eluate; however, the same filtration unit (i.e., the apparatus that holds the membrane filter) may be used for processing eluates from a given carrier set starting with the most dilute sample first. h. Filter samples through separate 0.45 µm PES membrane filters. i. For eluates from treated carriers remaining in the vial (10^0 dilution), vortex-mix the vial for ~5 s and pour the eluate into the filter unit. j. Rinse the treated vial with ~20 mL PBS, vortex-mix for ~5 s and pour the wash into the same filter unit. For dilution tubes, rinse tube once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit. k. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus. l. Rinse the inside surface of the funnel unit with at least 20 mL PBS and filter the contents. m. Aseptically remove the membrane filter and place on a SDEA plate. Avoid trapping any air bubbles between the filter and the agar surface. n. Sterility controls. i. On the day of the test, filter ~20 mL of neutralizer and ~20 mL of the PBS used in the test using two separate membrane filters and place on TSA. ii. Incubate these filters along with a plate of recovery medium (e.g., SDEA) for 72±4 h at 30±2°C; record sterility results. o. Incubate plates at 30±2°C for 72±4 h.
12.8 Recording results	a. Count colonies and record results. i. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data. ii. For example, contamination occurring on multiple filters within one set of serial dilutions and/or across multiple carriers is

	considered systemic and the test is deemed invalid. b. For colony counts on filters in excess of 200 record as Too Numerous to Count (TNTC). c. If no colonies are present, record as zero. d. Report non-conforming data. i. Systemic contamination. ii. Within a test day, differences in control counts >1 log/carrier. iii. Atypical serial dilution results (e.g., higher CFUs at more dilute levels). e. Inspect the growth on the filters for purity and typical characteristics of the test microbe. i. Colony morphology for *C. auris* includes colonies that are smooth and dull white to cream colored. f. If isolated colonies are present, use the lactophenol cotton blue (LPCB) stain to assess one representative colony per 5 carrier set (treated) and 3-carrier set (controls). See Attachment 2 Section I.iv for staining results. g. If confluent growth is present, perform a streak isolation on the appropriate agar on growth taken from at least 1 carrier. i. Use SDEA and incubate at 30±2°C for 72±4 h. h. If additional verification of the test organism is required when colonies appear atypical and/or there is a potential for contamination, perform further confirmatory analyses (e.g., biochemical analyses) and isolation streaks on selective media.
13. Data Analysis/ Calculations	1. Per test, use colony counts to determine log reductions. 2. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with two significant figures (e.g., round up to the nearest tenth). 3. Calculate the Colony Forming Units (CFU)/carrier using the following equation: $\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i \times D_i)}\right] \times V\right\}$ where: Y = CFU per filter, C = volume filtered, V = total volume of neutralizer,

	D = 10^{-k}, k = dilution, n = number of dilutions, and i = lower limit of summation (the fewest number of dilutions). 4. When TNTC (Too Numerous To Count) 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. 5. Calculate the log density of each carrier by taking the $\log_{10}$ of the density per carrier. 6. Calculate the mean log density for five treated carriers and three control carriers as follows: i. Mean LD = [Log(carrier 1) + Log(carrier 2) + Log(carrier 3)]/3 ii. For the purpose of calculation, if no organism is recovered from a test carrier, the log density for that carrier is 0 provided that the entire contents of all dilutions (including the 10^0 dilution) were filtered. 7. Calculate the $\log_{10}$ reduction (LR) for one set of treated carriers: LR = Mean LD (Control Carriers) – Mean LD (Treated Carriers) 8. If no organism is recovered from each of the three treated carriers, the log reduction is greater than or equal to the mean control carrier log density.
14 **Attachments**	1. Attachment 1: Anticipated Drug-Resistant Profile Using Etest (see fluconazole) for AR-Bank#0385 2. Attachment 2: Maintenance of Bacterial Cultures – Preparation of Frozen Stock Cultures 3. Attachment 3: Carrier Specifications 4. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record Form — MB-03_F1.docx Quantitative Method for Fungicidal Activity: Organism Culture Tracking Form — MB-35-03_F1.docx Quantitative Method for Fungicidal Activity: Test Microbe Confirmation Sheet (Quality Control) — MB-35-03_F2.docx Quantitative Method for Fungicidal Activity: Test Information Sheet — MB-35-03_F3.docx Quantitative Method for Fungicidal Activity: — MB-35-03_F4.docx

	Serial Dilution Plating/Tracking Form Quantitative Method for Fungicidal Activity: Results Sheet MB-35-03_F5.docx Quantitative Method for Fungicidal Activity: Test Microbe Confirmation Sheet MB-35-03_F6.docx Quantitative Method for Fungicidal Activity: Test Processing Sheet MB-35-03_F7.docx
15 References	1. ASTM E2197-17: Standard Quantitative Disk Carrier Test Method for Determining Bactericidal, Virucidal, Fungicidal, Mycobactericidal, and Sporicidal Activities of Chemicals. 2. Standard Methods for the Examination of Water and Wastewater. 23rd Ed. American Public Health Association, Washington, DC.

Attachment 1

Anticipated Drug-Resistant Profile Using Etest (see fluconazole) for AR-Bank #0385

CDC & FDA Antibiotic Resistance Isolate Bank

ARISOLATEBANK

Storage Temperature: -70°C

Biosafety Level: 2

Candida auris Active

AR-Bank #0385 *Candida auris* (Active)

Biosample Accession #: SAMN05379620

PROPAGATION

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.

MIC (µg/ml) RESULTS AND INTERPRETATION

DRUG	MIC	INT	DRUG	MIC	INT
Amphotericin B [1]	0.5	—	Itraconazole	1	—
Anidulafungin	1	—	Micafungin	0.5	—
Caspofungin	0.5	—	Posaconazole	1	—
Fluconazole	>256	—	Voriconazole	16	—
Flucytosine	0.5	—			

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

[1] Measured using Etest

https://wwwn.cdc.gov/ARIsolateBank/Panel/IsolateDetail?IsolateID=385
CDC and FDA Antibiotic Resistance Isolate Bank. Atlanta (GA): CDC 1/7/2020

Attachment 2

Maintenance of Bacterial Cultures – Preparation of Frozen Stock Cultures

(Refer to SOP MB-02 for establishment of the organism control number.)

A. Initiate stock cultures of *Candida auris* A- Bank #0385 from CDC at least every 18 months. Record all microbe transfers on the Organism Culture Tracking Form, see section 14.

   i. Thaw/refreeze the stock culture acquired from CDC to create additional stock cultures after the frozen stock culture expires.

   ii. New frozen stock culture may be initiated up to three times using an existing, unexpired frozen stock culture as the source. Begin process at step B below by streaking a loopful of the frozen stock culture onto 2 SDA plates.

B. Using a new culture from CDC or an unexpired frozen stock culture, streak isolate onto a SDA plate. Incubate plates for 48-72 h at 30±2°C.

   i. Record results of the streak isolation at the end of the incubation timeframe as pure or contaminated on the Organism Culture Tracking Form (see section 14).

C. From the SDA plates (step B), use a single representative colony to inoculate 10 mL of SDB. Incubate broth for 24±2 h at 30±2°C. In addition, perform a streak isolation from the inoculated SDB tube onto an SDA or SDEA plate.

   i. Record results of the streak isolation at the end of the incubation timeframe as pure or contaminated on the Organism Culture Tracking Form (see section 14).

D. Vortex-mix the broth tube (step C) and spread plate 0.1 mL of the suspension onto 5-10 SDEA plates. Incubate the plates for 48-72 h at 30±2°C. In addition, perform a streak isolation onto a SDA or SDEA plate.

   i. Record results of the streak isolation at the end of the incubation timeframe as pure or contaminated on the Organism Culture Tracking Form (see section 14).

E. At the end of the incubation period, harvest growth from SDEA plates by adding 5 mL 1X PBS with 0.1% (v/v) Tween 80 (PBS-T) to the surface of each plate. Re-suspend the cells in PBS-T 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.

F. Repeat by adding another 5 mL of 1X PBS-T to the 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.

G. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary).

H. Centrifuge the pooled suspension at 10,000×g for 10±1 min. Pipette supernatant off and resuspend pellet 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.

I. While mixing continuously, dispense 0.5-1.0 mL aliquots of the harvested suspension into separate cryovials; these represent the frozen stock cultures.

   i. For QC purposes, perform a streak isolation of the pooled culture onto BAP and SDA; incubate plates at 30±2ºC for 48-72 hours.

   ii. Following the incubation period, record the colony morphology as observed on the plates and stain a representative colony with lactophenol cotton blue stain.

   iii. Colonies on BAP are smooth, dull white to cream colored.

   iv. 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 µm, single, in pairs, or in a group showing globose budding yeast cells on microscopic examination.

   v. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

J. Store the cryovials at -70ºC or lower for a maximum of 18 months (from the date of harvesting/freezing). These cultures are single-use only.

K. Within two weeks of freezing, ensure the frozen stock culture (a minimum of one frozen vial) is consistent with the fluconazole antifungal susceptibility profile outlined in Attachment 1. Conduct antifungal susceptibility testing according to the manufacturer’s instructions. If the antifungal susceptibility is inconsistent with the organism, discard the cultures and re-initiate the process.

Attachment 3

Carrier Specifications

(AISI Type 304 Stainless Steel Carriers)

General Description: 1 cm non-magnetic disc made of AISI Type 304 Stainless Steel (SS) with 150 grit unidirectional brushed finish on one side.

Material: AISI Type 304 Austensic stainless steel consisting of 18% to 20% Chromium, 8% to 10.5% Nickel, and a maximum of 0.8% Carbon.

- European Specification X5CrNi18-10 Number 1.4301
- Japanese Specification: JIS 4303 SUS 304

Carrier Dimensions:

- Diameter: 1cm (±0.5 mm)
- Stainless Steel Sheet Thickness: 22 gauge; carrier manufacturer will provide thickness of the original stainless steel sheet (in mm).
- Flatness: Carrier height not to exceed 110% of the thickness of the uncut sheet of stainless steel from which the carriers are manufactured.

Finish: A ground unidirectional finish obtained with 150 grit abrasive (AISI) on the top side of the stainless steel sheet.

Burr Removal: Remove burrs from the edges of the discs on the bottom side of the carrier using a manual process.

Passivation: Parts are passivated by the carrier manufacturer according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus-based degreaser by soaking in the degrease solution for 1 hour
- Rinse with de-ionized water
- Passivate by soaking carriers:
    - 7% citric acid solution
    - 20-30 min at 35±5°C.
- Rinse with de-ionized water
- Air dry

Examples of Physically Screened Carriers[1]

Fig. 1: Examples of typical acceptable carriers.

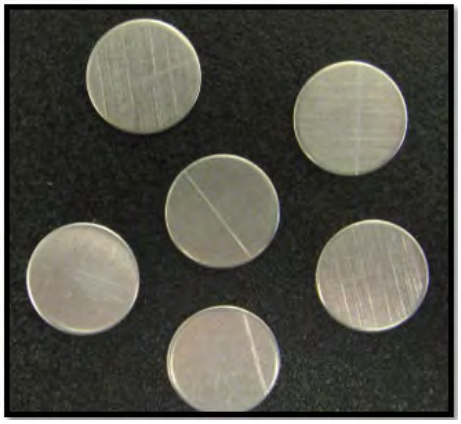

Fig. 2: Examples of typical unacceptable carriers.

A B

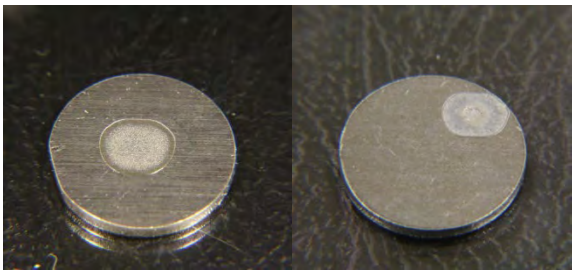

Fig. 3: Example of an acceptable (A) and unacceptable inoculated carrier (B).

[1] Carriers are screened without magnification.