US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for
OECD Quantitative Method for Evaluating Bactericidal and Mycobactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces

SOP Number: MB-25-04

Date Revised: 12-04-17

SOP Number	MB-25-04
Title	OECD Quantitative Method for Evaluating Bactericidal and Mycobactericidal Activity of Microbicides Used on Hard, Non-Porous Surfaces
Scope	The method provides a quantitative assessment of the performance of liquid antimicrobial substances against *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, and *Mycobacterium terrae* designed for use on hard, non-porous surfaces. This method is based on an Organization for Economic Co-operation and Development (OECD) Guidance Document, dated June 21, 2013 (see ref. 15.1); however, the SOP contains revisions based on information and data collected by the EPA since 2013.
Application	This method provides log reduction (LR) as the quantitative measure of efficacy for liquid disinfectants against the test microbes on a hard non-porous surface.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Data SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

TABLE OF CONTENTS

1. Definitions	Additional abbreviations/definitions are provided in the text. 1. OECD = Organization for Economic Co-operation and Development 2. LR = $\log_{10}$ reduction 3. 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. 4. Eluate = recovered eluent that contains the test organism 5. Stock culture = frozen culture used to prepare the test culture 6. Final test suspension = the harvested test culture with the addition of the OECD soil load 7. 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. 2. A test system control based on lab-grade sodium hypochlorite solutions may be used to verify the suitability of the test system and analyst proficiency.
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-26 (Neutralization of Microbicidal Activity using the OECD 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.
8. Non-conforming Data	1. For an acceptable test, achieve mean control carrier counts of 5.0-6.0 logs CFU/carrier for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Mycobacterium terrae,* and 4.5-5.5 logs CFU/carrier for *Salmonella enterica*; up to a one log difference between the three control carriers is permitted. 2. 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.
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.
11. Special Apparatus and Materials	1. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442*), Salmonella enterica* (ATCC #10708), *Staphylococcus aureus* (ATCC #6538), and *Mycobacterium terrae* (ATCC #15755). a. Additional bacteria may be evaluated using this method per the Agency's guidance or as identified in a research protocol. Record detailed information on the preparation of frozen stock cultures and the final test suspension. 2. Culture media for *P. aeruginosa*, *S. enterica* and *S. aureus.* a. *Tryptic Soy Broth (TSB).* Use to rehydrate lyophilized cultures. Purchase broth from a reputable source or prepare according to manufacturer's instructions. b. *TSB with 15% (v/v) glycerol.* Used as a cryoprotectant solution. Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and autoclave for 15 min at 121°C. c. *Tryptic soy agar (TSA)* and *TSA with 5% sheep blood.* Used for culturing, isolation, and characterization of the test microbes. Purchase plates from a reputable source or prepare according to manufacturer's instructions. d. *Selective media*. Used for quality control of test microbes listed in this procedure. Mannitol salt agar, Cetrimide agar, and Xylose lysine deoxycholate agar. See Table 1 for use. Purchase plates from a reputable source or prepare according to manufacturer's instructions.

3. Culture media for *M. terrae*.

   a. *Middlebrook 7H9 broth with 10% (v/v) ADC enrichment and 15% (v/v) glycerol (MADC)*. Used as the growth medium for test cultures. Combine 4.7 g Middlebrook 7H9 broth powder, 150 mL glycerol, 750 mL water and sterilize in autoclave per the manufacturer's instructions. After the medium has cooled to 40-45°C, aseptically add 100 mL Middlebrook ADC enrichment and then add sterilized water up to 1,000 mL. The pH of the medium should be 6.6±0.2.

   b. *Middlebrook 7H11 agar*. Used for recovery and enumeration of the test microbe. Purchase prepared agar from a reputable source or prepare according to manufacturer's instructions.

4. Reagents

   a. *Neutralizer*. Various neutralizers may be used including PBS with 0.1% (v/v) Tween-80 (PBS-T). If necessary, other ingredients may be added to PBS-T. Additionally, non-PBS based neutralizers may be used.

   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. *PBS-T with 0.1% (w/v) sodium thiosulfate*. Neutralizer for sodium hypochlorite-based test substances, including the test system control. A pH of approximately 7.2±0.2 is desirable.

   e. *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. Do not refreeze once thawed; store up to one year at -20±2°C.

v. See section 12.4 for addition of soil load to inoculum.

vi. Additional soil loads may be used per the Agency's guidance or research protocol.

f. *Test substance.* Antimicrobial test solution or product. If dilution is required, see section 11.4g for diluent.

g. *Test substance diluent.* Used for the preparation of dilutable products. 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 $MgCl \cdot 6H_2O$) 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 (2-8°C) 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 (2-8°C) 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 or research protocol.

h. *Water.* De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media.

i. *Tween-80* (polysorbate 80). To prepare PBS-T.

j. *Laboratory grade sodium hypochlorite (NaOCl) with total chlorine ≥4%.* For preparation of the test substance used in the test system

control.

k. *Liquinox (1% solution) or equivalent.* To clean carriers.

l. *Gram stain.* Used for diagnostic staining of *P. aeruginosa, S. enterica* and *S. aureus.*

m. *Acid fast stain.* Used for diagnostic staining of *M. terrae*.

5. Apparatus

a. *Carriers*: Disks (1 cm in diameter) made from 0.8 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 carrier specifications and photographs of screened carriers.

i. Additional carriers (e.g., carriers composed of AISI #304 stainless steel, carriers 2 cm in diameter) may be utilized per the Agency's guidance or as identified in a research protocol. 304 stainless steel is non-magnetized and must be managed in the efficacy assay without the use of a magnet. Carrier specifications for surface finishing will be retained according to **Attachment 2**.

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.

d. *Calibrated micropipettes* (e.g., 200 µL, 1 mL) with 10-100 or 20-200 µL tips, for deposition of test substance on carriers and preparing dilutions.

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

f. *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.

g. *Magnet*. To hold the carrier (430 stainless steel) in place in the vial while the liquid is being poured out of it for membrane filtration.

h. *Membranes (polyethersulfone)* for recovery of test microbe, 47 mm diameter and 0.2 µm pore size (for *P. aeruginosa, S. enterica,* and *S. aureus*) and or 0.45 µm pore size (for *M. terrae*). Filtration units (reusable or disposable) may be used.

	i. *Spectrophotometer*. For culture standardization. j. *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. i. 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., $CaCO_3$). For drying inoculated carriers. m. *Vacuum*. In-house line or suitable vacuum pump for drying inoculated carriers in desiccation unit and to aid in filtration. n. *Hach digital titrator kit*. For measuring total chlorine and water hardness. o. *Sterile Bijou bottles* (or equivalent). For *M. terrae* test culture preparation; glass (with aluminum caps) or plastic with capacity of approximately 5-7 mL, with 10 glass beads (3-5 mm in diameter). p. *Tissue grinder.* Any glass or plastic tissue grinder with a capacity of approximately 15 mL for homogenization of the *M. terrae* culture. q. *Vortex-style mixer*. For vortex mixing of various solutions. r. *Centrifuge* (with rotor capable of achieving 10,000g). For test culture preparation.
12. Procedure and Analysis	
12.1 Preparation and sterilization of carriers	a. Without magnification, 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. b. Soak visually screened carriers in a suitable detergent solution for 2-4 h to degrease and then rinse thoroughly in distilled or deionized water. c. Record physical screening of carriers on appropriate form (see section 14). d. Prior to sterilization, place up to 20 clean dry carriers on filter paper inside the bottom surface of a glass Petri dish (150 mm in diameter).

	Cover the Petri dish with its lid and sterilize at 121°C for 45 min. Ensure carriers are dry following sterilization. e. Use sterilized carriers for up to six months. After six months, re-sterilize any remaining unused carriers and assign a new preparation number.
12.2 Preparation of *P. aeruginosa*, *S. enterica*, and *S. aureus* test cultures	a. Refer to **Attachment 3** 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. Add 100 µL of defrosted stock culture to 10 mL TSB, briefly vortex mix and incubate for 18-24 h at 36±1°C. In addition, inoculate an agar plate (e.g., TSA or 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 for each organism. e. For *P. aeruginosa*, inspect culture prior to harvest; discard if pellicle has been disrupted (fragments in culture). Remove visible pellicle on surface of medium and around associated interior edges of the tube by pipetting or with vacuum suction. Using a serological pipette, withdraw the remaining broth culture (approx. 7-8 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL centrifuge tube. Alternatively, the culture may be removed by gently aspirating the broth away from the pellicle material. f. For *S. enterica* and *S. aureus*, briefly vortex the 18-24 h culture and transfer to a 15 mL centrifuge tube. g. Centrifuge the 18-24 h broth cultures at 5,000g_N for 20±5 min. h. 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 (5 mL) is permissible to concentrate culture. i. For *S. enterica* and *S. aureus*, disrupt the pellet using vortexing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in a maximum of 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation. ii. Further dilute the resuspended culture as necessary in PBS to achieve a mean control carrier count level of 5.0-6.0 logs CFU/carrier for *S. aureus* and *P. aeruginosa*, and 4.5-5.5 logs

	CFU/carrier for *S. enterica*. i. Use the diluted culture to prepare the final test suspension with the addition of the soil load per section 12.4. j. Optical density/absorbance (at 650 nm) may be used as a tool to monitor/adjust the diluted test suspension.
12.3 Preparation of *M. terrae* test cultures	a. Refer to **Attachment 4** for preparation of the frozen stock cultures for *M. terrae*. b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells. Each cryovial is for single use only. c. Add 1 mL thawed culture to a flask of 100 mL MADC and incubate at 36±1°C for 7-10 days under agitation at 150 rpm. Seal the flask (e.g., with foil and Parafilm) to reduce evaporation and inhibit contamination. d. In addition, inoculate an agar plate (e.g., M7H11) with a loopful from the inoculated flask and streak for isolation. Incubate plate for 17-21 days and examine for purity. e. Following incubation, aliquot 25 mL portions of the 7-10 day-old MADC broth culture into each of 2-50 mL conical screw cap tubes and centrifuge at 10,000g_N for 20±5 min. f. Carefully remove the supernatant and re-suspend each pellet in 25 mL sterile water. g. Centrifuge the tubes a second time at 10,000g_N for 20±5 min. After centrifuging, re-suspend the pellets in a total of 5 mL sterile DI water (1/10 of the starting volume), pool, and place in a tissue grinder. Homogenize the culture for approximately 1 min to eliminate visible clumps of bacteria. h. After homogenizing, transfer the culture to a bijou bottle (or equivalent) with 10 glass beads; vortex for 5 min. i. For preparation of the test suspension, dilution of the culture from the bijou bottle with sterile water may be required to achieve mean carrier counts within the range of 5.0-6.0 logs CFU/carrier. j. The approximate titer of test suspension may be estimated spectrophotometrically at 650 nm, based on a standard curve specific to the test organism. i. Prior to inoculation of carriers, aseptically add the OECD soil load per the instructions in 12.4.

12.4 Preparation of the final test suspension with OECD soil load	a. Vortex the test suspension for 10-30 s. b. 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 microbial test suspension c. Use final test suspension with soil load (at room temperature, 22±2°C) to inoculate carriers within 30 min of preparation.
12.5 Inoculation and drying of carriers	a. Vortex the final test suspension for 10 s following the addition of the soil load and immediately prior to use. b. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 3 treated) along with carrier(s) to serve as extras. If performing the test system control, refer to **Attachment 1**. c. 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); avoid contact of pipette tip with carrier and do not spread the final test suspension with the pipette tip. i. 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. d. 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. e. 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). f. Hold the inoculated carriers in the evacuated desiccation unit at 22±2°C for 30 to 50 min. Visually inspect inoculated carriers to verify that they have completely dried and remove from desiccation unit. Do not use carriers that are visibly wet for testing.

	i. If the carriers are not dry within the recommended time, continue to dry for up to an additional 10 min and record the time to dry on the paperwork. 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). g. Following the inoculation of carriers, streak inoculate an agar plate (e.g., TSA or TSA with 5% sheep blood for *P. aeruginosa*, *S. enterica* and *S. aureus*; M7H11 agar plate for *M. terrae*) with a loopful of the final test suspension (with OECD soil load). Incubate plate with the treated and 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. h. 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.
12.6 Exposure of the dried inoculum to the test substance or control substance	a. Evaluate 3 control carriers and 3 treated carriers for each test substance tested (one test organism and contact time/temperature combination) unless specified otherwise. Note: One set of control carriers may be used for evaluating multiple test substances against one organism on one test day (assuming the neutralizer is the same). b. See **Attachment 1** for conducting the test system control. Conduct the test system control per the Agency's guidance or as identified in a research protocol. 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. 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. During testing, do not process carriers where the test substance runs off of the carrier; 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 products, obtain the test substance by dispensing the product into a sterile vessel for collection. Cap the vessel. Using aseptic technique,

	remove 50 µL of the liquid from the vessel and deposit it on the inoculated carrier. ii. For foaming products, allow up to 5 min for the foam to break down and generate at least 1-2 mL for sample collection. iii. Use dispensed product within 30 min of collection. e. Hold the test carriers at 22±2°C (or other specified temperature) for the selected contact period. Use a certified timer to ensure that each carrier receives the required exposure time (OECD default exposure time is 5 min±5 s). f. Evaluate control carriers last. Each control carrier receives 50 µL PBS, equilibrated to 22±2°C, instead of the test substance. Hold the control carriers at 22±2°C for the contact period.
12.7 Neutralization of test substance and elution of test organisms	a. The neutralizer for the control carriers is the same as that for the treated carriers. b. Within ±5 s of the end of the contact period, add 10 mL of room temperature neutralizer to each vial in the specified order according to the predetermined schedule. Briefly vortex (2-3 s) each vial following the addition of the neutralizer. i. The neutralized vial with carrier is documented as the 10^0 dilution. c. Following the neutralization of the entire set of carriers, vortex each vial for 30±5 s at high speed to recover and disaggregate the inoculum; ensure that the liquid and carrier are fully vortexed. Do not remove the carrier from the vial.
12.8 Dilution and recovery	a. Initiate dilutions within 30 min after neutralization and vortexing. Initiate filtration within 30 min of preparing the dilutions. b. Dilute and filter samples from 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 carriers, dilute out to 10^{-1} and filter the entire contents of the 10^0 and 10^{-1} dilutions; additional dilutions may be prepared and filtered as necessary. e. For control carriers, dilute out to 10^{-4} and filter the entire contents of the 10^{-3} through 10^{-4} dilutions; additional dilutions may be prepared and filtered as necessary.

f. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS; apply vacuum to filter contents. Leave the vacuum on for the duration of the filtration process.

g. Use separate membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.

h. Filter samples through separate 0.2 µm PES membrane filters for *P. aeruginosa*, *S. enterica* and *S. aureus* or 0.45 µm PES membrane filters for *M. terrae*.

i. For eluates from treated carriers remaining in the vial (10^0 dilution), 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.

j. Rinse the treated vial with ~20 mL 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 PBS, briefly vortex, 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 ~40 mL PBS and filter the contents.

m. Aseptically remove the membrane filter and place on the appropriate recovery medium. Avoid trapping any air bubbles between the filter and the agar surface.

    i. Use TSA for recovery of *P. aeruginosa*, *S. enterica* and *S. aureus*.

    ii. Use M7H11 agar for recovery of *M. terrae*.

n. For *P. aeruginosa, S. enterica* and *S. aureus*, incubate plates at 36±1°C for 48±4 h for control carriers and for a minimum of 72±4 h for treated carriers. Following incubation, count the number of colonies.

    i. Monitor filters after 24 h of incubation to facilitate appropriate timing for counting the colonies.

    ii. If no growth appears on filters for the treated carrier after 72±4 h, continue to incubate for up to five days (total) and record the results.

o. For *M. terrae*, incubate all plates from treated and control carriers at 36±1°C for 17-21 days; however, monitor filters for growth and count

	the number of colonies beginning at 10-14 days.
12.9 Recording results	a. Count colonies and record results. 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. Inspect the growth on the filters for purity and typical characteristics of the test microbe i. See Table 1 for *P. aeruginosa, S. enterica* and *S. aureus*. ii. Colony morphology for *M. terrae* includes irregular margins, rough appearance, buff color, opaque and umbonate; *M. terrae* is acid fast positive. e. If isolated colonies are present, use the appropriate stain to assess one representative colony per 3-carrier set (treated and controls). Use Gram stain for *P. aeruginosa*, *S. enterica* and *S. aureus* and acid fast stain for *M. terrae.* f. If confluent growth is present, perform a streak isolation on the appropriate agar on growth taken from at least 1 carrier. i. For *P. aeruginosa*, *S. enterica* and *S. aureus*, use TSA or TSA with 5% sheep blood and incubate at 36±1°C for 24-48 h. ii. For *M. terrae* use M7H11 agar and incubate at 36±1°C for 17-21 days g. If additional verification of the test organism is required, perform further confirmatory analyses (e.g., Vitek and biochemical analyses for *P. aeruginosa, S. enterica* and *S. aureus*) and isolation streaks on selective media.

Table 1. Selective media and diagnostic characteristics for *P. aeruginosa*, *S. aureus*, and *S. enterica.* (see ref. 15.7 and 15.8)

Aspect	*P. aeruginosa**	*S. aureus*	*S. enterica*
Gram stain reaction	Negative	Positive	Negative
Mannitol Salt Agar	N/A	Circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetramide Agar	Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	N/A	N/A
Xylose lysine deoxycholate (XLD agar)	N/A	N/A	Round, clear red colonies with black centers
Blood agar (BAP)	Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	Small, circular, yellow or white, glistening, beta hemolytic	Entire, glistening, circular, smooth, translucent, low convex, non-hemolytic
	Typical Microscopic Characteristics		
Cell appearance	Straight or slightly curved rods, single polar flagella, rods formed in chains; 0.5 – 1.0 µm in diameter x 1.5 – 5.0 µm in length	Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters; 0.5 – 1.0 µm in diameter	Straight rods, peritrichous flagella; 0.7 – 1.5 µm in diameter x 2.0 – 5.0 µm in length

*After 24±2 h (1) *P. aeruginosa* may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.

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: $\left(\frac{CFU\ \ for\, 10^{-y} + CFU\ \ for\, 10^{-z}}{(a\times 10^{-y}) + (b\times 10^{-z})}\right)\times c$, 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. See examples below: 4. *Example 1*. When 10^0 dilution yields 130 CFU and the 10^{-1} dilution yields 20 CFU: $\left(\frac{130\ CFU + 20\ CFU}{(9\ mL\ \times 10^0) + (10\ mL\ \times 10^{-1})}\right) \times 10\ mL\ (vol.in\ vial) = 1.50 \times 10^2\ CFU/carrier$ 5. 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. 6. *Example 2*. When 10^0 dilution yields TNTC (>200) CFU and the10^{-1} dilution yields TNTC (>200) CFU: $\left(\frac{200\ CFU}{10\ mL \times 10^{-1}}\right) \times 10\ mL\ (vol.in\ vial) = 2.00 \times 10^3\ CFU/carrier$ 7. Calculate the log density of each carrier by taking the $\log_{10}$ of the density per carrier. 8. Calculate the mean $\log_{10}$ density across treated carriers. 9. Calculate the mean $\log_{10}$ density across control carriers. 10. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = the mean $\log_{10}$ density for control carriers minus the mean $\log_{10}$ density for treated carriers. 11. For a set of 3 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.
14. Forms and Data Sheets	1. Attachment 1: Test System Control 2. Attachment 2: Carrier Specifications 3. Attachment 3: Maintenance of Bacterial Cultures – Preparation of Frozen Stock Cultures 4. Attachment 4: Maintenance of *Mycobacterium terrae* – Preparation of Frozen Stock Culture 5. 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 OECD Method for Bactericidal Activity: Organism Culture Tracking Form — MB-25-04_F1.docx

	OECD Method for Bactericidal Activity: Test Microbe Confirmation Sheet (Quality Control)	MB-25-04_F2.docx
	OECD Method for Bactericidal Activity: Test Information Sheet	MB-25-04_F3.docx
	OECD Method for Bactericidal Activity: Serial Dilution Plating/Tracking Form	MB-25-04_F4.docx
	OECD Method for Bactericidal Activity: Results Sheet	MB-25-04_F5.docx
	OECD Method for Bactericidal Activity: Test Microbe Confirmation Sheet	MB-25-04_F6.docx
	OECD Method for Bactericidal Activity: Test Processing Sheet	MB-25-04_F7.docx
	OECD Method for Bactericidal Activity: Test Processing Sheet (*Mycobacteria*)	MB-25-04_F8.docx
15. References	1. OECD Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides Used on Hard Non-Porous Surfaces (June 21, 2013) 2. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015.	

Attachment 1

Test System Control for the OECD Quantitative Method for Bacteria and Mycobacteria

Purpose: Conduct the test system control periodically (per Agency guidance or research protocol) to verify the suitability of the overall test system using the test microbe to be evaluated against the antimicrobial product. It is recommended that the analyst responsible for conducting the product efficacy evaluation perform the test system control.

Process: Conduct the test system control per methodology in MLB SOP MB-25. Derive the final test suspension from the same set of frozen stock cultures used for the product efficacy test. Evaluate two concentrations of laboratory-grade sodium hypochlorite (NaOCl) using three carriers per NaOCl concentration and three control carriers per microbe. Use a 5 min contact time, the OECD 375 ppm hard water as the diluent, and the OECD three-part soil load included in the inoculum. See Table 1 for the concentrations of NaOCl and recommended dilutions for recovery. Use PBS with 0.1% (v/v) Tween-80 and 0.1% (w/v) sodium thiosulfate as the neutralizer. Verify the water hardness and concentration of the NaOCl solutions using an appropriate titration procedure (e.g., Hach digital titrator) prior to use. Evaluate the high NaOCl concentration (2000 ppm) first, followed by the low NaOCl concentration (e.g., 200 ppm) and then the control carriers. The three control carriers used in the product efficacy test may be used in the LR calculations for the test system control, assuming the OECD three-part soil load is incorporated into the inoculum.

Table 1. Test system control – concentrations of NaOCl and recommended dilutions to be assayed for treated carriers per test microbe

P. aeruginosa	***S. enterica***	***S. aureus***	***M. terrae***
2000 ppm (±5%)	2000 ppm (±5%)	2000 ppm (±5%)	4000 ppm (±5%)
(filter dilution 10^0)	(filter dilution 10^0)	(filter dilution 10^0)	(filter dilution 10^0)
……..	……	……	……
150 ppm (±5%)	100 ppm (±5%)	500 ppm (±5%)	1000 ppm (±5%)
(filter dilutions 10^0 to 10^{-2})	(filter dilutions 10^0 to 10^{-2})	(filter dilutions 10^{-1} to 10^{-3})	(filter dilutions 10^{-1} to 10^{-3})

Results: Count the number of colonies and calculate the mean control counts and the mean LR values per EPA MLB SOP MB-25, section 13.

Desired outcomes:

- Control carrier counts.
    - For *P. aeruginosa*, *S. aureus*, and *M. terrae*, mean control carrier counts to be within 5.0-6.0 logs per carrier. For *S. enterica*, the mean control carrier counts to be within 4.5-5.5 logs per carrier. Individual control carrier counts should be within one log of each other.

- Log reduction.
    - The high concentration NaOCl results in no recovery (i.e., complete kill of the test microbe) to very limited recovery (i.e., almost complete kill of the test microbe) for each of the three treated carriers:
        - Mean LR of ≥4.5 for *P. aeruginosa*, *S. aureus* and *M. terrae*
        - Mean LR ≥4.0 for *S. enterica*.
    - The low concentration of NaOCl results in a significant recovery of viable bacteria for each of the three treated carriers:
        - Mean LR between 0.0 to 3.5 for each microbe.

Note: Test system controls for additional microbes may be proposed per the Agency guidance or a research protocol.

Attachment 2

Carrier Specifications

(AISI Type 430 Stainless Steel Carriers)

General Description: 1 cm magnetic disc made of AISI Type 430 Stainless Steel (SS) with No.4 finish on one side and rounded edges on top side.

Material: AISI Type 430 Ferretic stainless steel consisting of 16% to 18% Chromium, a maximum of 0.5% Nickel and a maximum of 0.12% Carbon.

- European Specification X6Cr17 Number 1.4016
- Japanese Specification: JIS G4305; EN10088-2

Dimensions:

- Diameter: 1cm (0.39”) in diameter
- Thickness: 0.8 mm (22 gauge / 0.031”)
- Flatness: some concavity desired at edges

Finish

No. 4 Finish is produced with short, parallel polishing lines. The final finish can be anywhere between 120 and 320 grit.

Tumbling

To remove burrs from the edges of the discs they are tumble deburred in a vibratory tumbler using ceramic median and cleanser. Tumbling time is dependent on the extent to which burring occurs.

Passivation

Parts are passivated according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus based degreaser
- Rinse with tap water
- Passivate
    - 7% Citric Acid Solution
    - Minimum of 20 min at 20-50°C.
- Rinse with de-ionized water
- Air dry

Attachment 2 (continued)

Examples of Physically Screened Carriers[1]

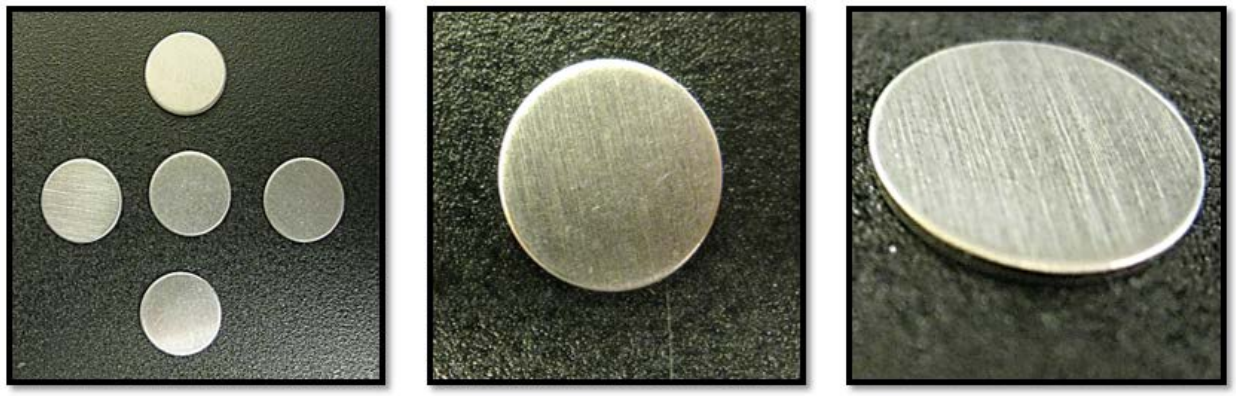

Fig. 1: Examples of typical acceptable 430 SS carriers.

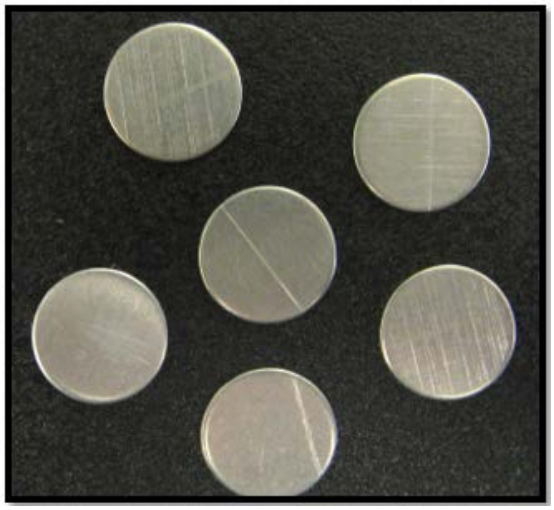

Fig. 2: Examples of typical unacceptable 430 SS carriers.

[1]The same acceptance criteria used to screen the 430 SS carriers are used to screen the 304 SS carriers. Carriers are screened without magnification.

Attachment 3

Maintenance of Bacterial Cultures – Preparation of Frozen Stock Cultures

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

I. Preparation of Frozen Stock Cultures

A. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa, Salmonella enterica* and S*taphylococcus aureus* from ATCC (or other reputable vendor) at least every 18 months.

B. Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36±1°C for 24±2 h.

C. After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plates for 18-24 h at 36±1°C. Refer to section II, below, for QC of stock cultures.

D. Select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa* and the stock culture is representative of the phenotypes present on the streak isolation plate. Spread plate 0.1 mL of the suspension on each of 6-10 TSA plates. Incubate the plates for 18-24 h at 36±1°C.

E. Following the incubation of the agar plates from section I.D, above, place approximately 5 mL sterile cryoprotectant solution on the surface of each plate. Re-suspend the growth in the cryoprotectant solution 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 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). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

F. Immediately after mixing, dispense 0.5-1 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

G. Store the cryovials at -70°C or lower for a maximum of 18 months. Reinitiate cultures with a new lyophilized culture.

1. Note: New frozen stock culture may be initiated <u>one</u> time using an existing, unexpired frozen stock culture as the source.

II. QC of Frozen Stock Cultures

A. Conduct a purity check (isolation streak) throughout each step of the frozen stock culture generation process prior to freezing the culture.

1. For section I.B, above, conduct a streak isolation onto TSA with 5% sheep blood from the rehydrated lyophilized culture. In addition, streak a loopful onto MSA, Cetrimide and XLD agar as appropriate for each microbe.

2. For section I.C, above, conduct a streak isolation onto BAP from the TSA 18-24 h plate. In addition, streak a loopful onto MSA, Cetrimide or XLD agar as appropriate for each microbe.

3. For section I.D, above, conduct a streak isolation onto TSA with 5% sheep blood from the resuspended culture tube. In addition, streak a loopful onto MSA, Cetrimide or XLD agar as appropriate for each microbe.

B. Conduct QC of the pooled culture concurrently with freezing (section I.E, above). Streak a loopful on a plate of TSA with 5% sheep blood. In addition, streak a loopful onto MSA, Cetrimide or XLD agar as appropriate for each microbe. Incubate all plates at 36± 1°C for 24±2 h.

C. Following the incubation period, record the colony morphology as observed on the TSA with 5% sheep blood plates and selective media plates (including the absence of growth) and Gram stain. See Table 1 of SOP (section 12.9) for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

D. For each organism, perform a Gram stain from growth taken from the isolation plate according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 100X-1000X magnification (oil immersion).

E. Conduct additional biochemical and antigenic analyses as desired. Alternatively, Vitek 2 Compact may be used.

F. Record confirmation results.

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

Attachment 4

Maintenance of *Mycobacterium terrae* – Preparation of Frozen Stock Culture

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

I. Preparation of Frozen Stock Cultures

A. Initiate new stock cultures from a lyophilized culture of *Mycobacterium terrae* (ATCC #15755) from ATCC at least every 18 months. Aseptically add 1.0 mL of Middlebrook 7H9 Broth with 10% ADC enrichment (MADC) and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into 5 mL of MADC. Mix thoroughly.

B. Spread 0.1 mL of the test organism suspension onto approximately 6-10 M7H9 or M7H11 agar plates. Incubate for 20-22 days at 36±1°C. Refer to section II for QC of frozen stock cultures. Ensure sterility of the plates during the incubation period (e.g., place in sterile bags or wrap with Parafilm).

1. Note: Each plate will yield 10 mL of harvested suspension and consequently nine to ten cryovials, each containing 1-1.5 mL of frozen stock culture.

C. At the end of the incubation period, add 5 mL MADC to the surface of each agar plate. Re-suspend the cells in MADC using a sterile spreader without damaging the agar surface.

D. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL.

E. Repeat by adding another 5 mL of MADC to the agar plates, re-suspend the cells, aspirate the suspension and pool with the initial cell suspension.

F. 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).

G. Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting the culture.

H. While mixing continuously, dispense 1-1.5 mL aliquots of the harvested suspension into separate cryovials; these represent the frozen stock cultures.

I. Store the cryovials at -70°C or lower for a maximum of 18 months (from the date of harvesting/freezing). A titer of the frozen stock culture of approximately 1×10^9 CFU/mL is appropriate. New stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source.

II. QC of Frozen Stock Cultures

A. Conduct QC of the pooled culture concurrently with freezing. Streak a loopful on M7H11 agar and incubate at 36±1°C for 17-21 days. Following the incubation period, record the colony morphology as observed on the plates. Typical colony

morphology for *M. terrae* includes irregular margins, rough appearance, buff colored, opaque. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).