US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants

SOP Number: MB-05-07

Date Revised: 08-18-09

EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

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for
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Initiated By: ____ Date: ___/___/___

Print Name:______________________________

Technical Review: ____ Date: ___/___/___

Print Name:______________________________
Technical Staff

QA Review: ____ Date: ___/___/___

Print Name:______________________________
QA Officer

Approved By: ____ Date: ___/___/___

Print Name:______________________________
Branch Chief

Date Issued: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the Use-dilution methodology used to determine the efficacy of disinfectants against three organisms, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella enterica*, on hard surfaces. The methodology is based on AOAC methods 955.15 (Testing Disinfectants against *Staphylococcus aureus*), 964.02 (Testing Disinfectants against *Pseudomonas aeruginosa*), and 955.14 (Testing Disinfectants against *Salmonella cholerasuis*) which have been officially modified (editorial modifications) – see references 15.1 and 15.2.

1.2 For product evaluations under the Antimicrobial Testing Program (ATP), a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, dilutions, neutralizers, etc.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 ATCC = American Type Culture Collection

2.3 TSA = trypticase soy agar

2.4 TSB = trypticase soy broth

2.5 NB = nutrient broth

2.6 NA = nutrient agar

2.7 MSA = mannitol salt agar

2.8 CTA = cystine trypticase agar

2.9 OD = outside diameter

2.10 ID = inside diameter

2.11 CFU = colony forming unit

2.12 References to water mean reagent-grade water, except where otherwise specified.

2.13 Dilution blanks = tubes of phosphate buffered dilution water (PBDW)

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms are required to be performed in accordance to biosafety practices stipulated in SOP MB-01, Lab Biosafety.

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, etc. Personal protective clothing or devices are recommended during the handling of these items for the purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine the best course of action.

4.0 CAUTIONS:

4.1 Use aseptic techniques to prevent contamination.

4.2 Media indicated in sections 10.1.1.2 and 10.1.1.3 for rehydrating lyophilized cultures are specified on the ATCC Product Information Sheet that accompanies each organism. Upon purchase of new organisms, verify that media requirements have not changed by checking the new ATCC Product Information Sheet.

4.3 The volume of dilution blanks, neutralizer tubes, and subculture tubes will be verified in advance and adjusted accordingly.

4.4 Strict adherence to the protocol is necessary for the validity of the test results.

4.5 Use inoculated carriers for determining carrier counts and performing efficacy testing as soon as possible after drying on the day of preparation to avoid a reduction in microbial titer. Overnight or long term storage of inoculated carriers is not allowed.

4.6 Complete dilution plating within 2 hours after the completion of carrier sonication or vortexing. If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done.

4.7 For spread plating: ensure that the entire surface of the agar plate is dry before adding inoculum. If necessary, leave the agar plates uncovered in the biological safety cabinet (BSC) until the moisture has been completely absorbed into the medium.

4.8 To ensure the stability of a diluted product, prepare the dilutions within three hours of the disinfectant treatment step unless specified otherwise.

4.9 Use appropriate aseptic techniques for all test procedures involving the manipulation of the test organisms and associated test components.

4.10 These microbiological methods are very technique-sensitive and technique-oriented; thus, exact adherence to the method, good laboratory practices, and quality control are required for proficiency and validity of the results.

4.11 Detergents used in washing glassware may leave residues which are bacteriostatic. Test for inhibitory residues on glassware periodically according to SOP QC-03, Glass Washing and Detergent Residues Test.

4.12 The primary subculture medium should serve as a suitable neutralizer for the test substance as well as an adequate growth medium which must be confirmed in advance or concurrently with the use dilution test.

4.12.1 See SOP MB-17, Neutralization Confirmation, for the procedure to determine suitability of the neutralizer (primary subculture tube) for the test substance.

4.12.2 See SOP QC-11, Performance Assessment and Sterility Verification, to determine whether or not the subculture medium is an adequate growth medium.

5.0 INTERFERENCES:

5.1 Contamination of stock cultures will negatively impact disinfectant efficacy testing. It is critical to maintain the highest standards of good laboratory practices and aseptic technique during all manipulations and handling of stock cultures.

5.2 Avoid touching the interior sides of the medication tube while the carriers are being lowered into the disinfectant agent and the hook is being removed. Contact with the interior sides of the medication tube may cause adhesion of bacterial cells which are not in contact with the disinfectant. This may result in re-inoculation of the carriers with organism as they are being removed from the medication tube. Re-inoculation of the carriers with organism can lead to false positive results.

5.3 Contaminated plates will interfere with the recording of carrier count results. Visually inspect all agar plates prior to use – discard any plates with evidence of

contamination. For contamination following the incubation phase, if atypical colonies or contamination are evident that interfere with the enumeration of the test organism, record as a contaminant(s). Data from other dilutions, if the CFUs result in a countable range, may be used to calculate the final CFU/carrier.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

6.2 The laboratory staff shall confirm (i.e., documentation in the training file of familiarization with the SOP) that they can properly perform the procedure before commencing work. If the standard AOAC method changes, confirmation shall be repeated.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 *Test organisms*. *Pseudomonas aeruginosa* (ATCC No. 15442), *Staphylococcus aureus* (ATCC No. 6538) and *Salmonella enterica* (ATCC No. 10708) obtained directly from a reputable supplier (e.g., ATCC).

7.2 *Culture media* (e.g., nutrient agar). Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

NOTE: The use of synthetic broth is stipulated in the official AOAC methods for test culture preparation; however, it is rarely used by the laboratory and only used upon request.

7.2.1 *Nutrient broth*: Boil 5 g beef extract (paste or powder), 5 g NaCl, and10 g peptone (anatone) in 1 L H_2O for 20 minutes and dilute to volume with H_2O; adjust to pH 6.8 ± 0.1. Filter through paper (Whatman No. 4, or equivalent), place 10 mL portions in 20 × 150 mm test tubes, and steam sterilize 20 min at 121°C. Use this broth for daily transfers of test cultures.

7.3 *Subculture media* (e.g., letheen broth, fluid thioglycollate medium). Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

7.4 *Trypticase soy agar (TSA)*. Plating medium for carrier enumeration.

7.5 *Sterile 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 quality can be met. Reverse osmosis, distillation, and deionization in various combinations all can produce reagent-grade water when used in the proper arrangement. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

7.6 *Carriers*. Polished stainless steel cylinders, 8 ± 1 mm OD, 6 ± 1 mm ID, 10 ± 1 mm length; type 304 stainless steel, SS 18-8 (S & L Aerospace Metals, Maspeth, NY or Fisher Scientific catalog number 07-907-5Q as of December 2008).

7.7 *Glassware.* For disinfectant, use autoclavable 25 × 100 mm tubes (Bellco Glass Inc., Vineland, NJ). For cultures/subcultures, use autoclavable reusable or disposable 20 × 150 mm tubes. For stock cultures, use 16 × 100 mm screw cap tubes. Cap tubes with closures before sterilizing. Sterilize all glassware in hot air oven at 180°C or steam sterilize for a minimum of 20 minutes at 121°C with drying cycle.

7.8 *Water bath/chiller unit*. Constant temperature for test chemical, capable of maintaining 20 ± 1°C temperature or specified temperature for conducting the test.

7.9 *Test tube racks.* Any convenient style.

7.10 *Transfer loops*. Make 4 mm ID single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA 19380, USA). Fit other end in suitable holder. Bend loop at 30° angle with stem. Volumetric transfer devices may be used instead of transfer loops (e.g., micro volume pipet).

7.11 *Wire Hook*. For carrier transfer. Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Place other end in suitable holder.

7.12 *Timer*. For managing timed activities, any certified timer that can display time in seconds.

7.13 *Sonicator* (ultrasonic cleaner). For carrier counts.

7.14 *Gram stain kit*.

7.15 *VITEK 2 Compact*. For the automated identification of microorganisms.

7.16 *VITEK 2 Compact Identification Cards*. Gram negative (GN) and Gram positive (GP).

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Follow appropriate chain-of-custody (COC) guidelines during testing as stipulated in SOP COC-01, Sample Log-in and Tracking.

9.2 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 Culture Initiation, Maintenance and Quality Control.

10.1.1 Culture Initiation.

10.1.1.1 Every 12 months (or sooner if the quality of the stock culture is compromised) initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Salmonella enterica* (ATCC 10708) from ATCC or other reputable supplier.

10.1.1.2 Open ampule of freeze dried organism as indicated by ATCC.

10.1.1.3 Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Using a tube containing 5-6 mL of NB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *Salmonella enterica*.

10.1.1.4 Aseptically transfer the entire rehydrated pellet back into the original tube of broth (TSB for *S. aureus* and *P. aeruginosa*, NB for *S. enterica*) designated as "TUBE A," (see Attachment 2). Mix well.

10.1.1.5 Streak for isolation using a loopful of rehydrated suspension on duplicate plates – use TSA for *S. aureus* and *P. aeruginosa*, use NA for *S. enterica*.

10.1.1.5.1 In addition for *S. aureus* and *P. aeruginosa*, streak a loopful of rehydrated suspension onto both MSA and Cetrimide agar. Selective media is not used for *S. enterica*.

10.1.1.6 Incubate broth culture (TUBE A) and plate cultures at 36 ± 1ºC for 24 ± 2 hours for *S. aureus*, *P. aeruginosa*, and *S. enterica*.

10.1.1.7 Record all manipulations on the Organism Culture Tracking Form (see 16.1).

10.1.2 Culture Identification and Quality Control.

10.1.2.1 Initial confirmation testing for quality control (QC) will be performed using the 24 ± 2 hour NA or TSA plates from step 10.1.1.5.

10.1.2.2 Following the incubation period (as stated in 10.1.1.6), record the colony morphology as observed on the NA or TSA plates and selective media plates (including the absence of growth) and stain reaction. See Attachment 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

10.1.2.2.1 For *S. aureus*, note the organism's growth characteristics on MSA (colony size, color, texture, etc.) and Cetrimide (absence of growth). For *P. aeruginosa*, note the organism's growth characteristics on Cetrimide (colony size, color, texture, etc.) and MSA (absence of growth). Check for consistency with the genus and species of

the organism to be tested (round, shiny, and yellow for *S. aureus* on MSA, and flat, greenish-yellow, and opaque for *P. aeruginosa* on Cetrimide).

10.1.2.2.2 For each organism, perform a Gram stain from growth taken from the TSA or NA plates. Perform the Gram stain according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000× magnification (oil immersion).

10.1.2.3 Perform VITEK™ analysis according to the manufacturers' instructions.

10.1.2.4 Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see 16.2).

10.1.3 Generation of Stock Cultures.

10.1.3.1 Use the 24 ± 2 hour TUBE A (see Attachment 2) broth culture discussed in 10.1.1.4 to initiate stock cultures.

10.1.3.2 For *S. aureus* and *S. enterica*, streak six nutrient agar slants each. For *P. aeruginosa*, stab six CTA tubes.

10.1.3.3 Incubate the *S. aureus* and *S. enterica* slants and *P. aeruginosa* stabs at 36 ± 1ºC for 48 ± 2 hours.

10.1.3.4 Following incubation, store the cultures at 2-5ºC for 30 ± 2 days. These cultures are identified as the "stock cultures." Begin stock culture transfers as outlined in section 10.1.5. Repeat the cycle for a maximum of one year.

10.1.3.5 From a set of six stock cultures, one is used every 30 ± 2 days for QC and to generate new stock cultures, four may be used per month (one/week) for generation of test cultures, (see SOP MB-05, Use Dilution Method; and SOP MB-06, Testing Spray Disinfectants) and one is a back-up tube.

10.1.4 Monthly QC of Stock Cultures.

10.1.4.1 Conduct monthly QC of stock cultures. Use one refrigerated stock culture tube and streak a loopful on a plate of TSA. For *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide), as noted in section 10.1.1.5.

10.1.4.2 Incubate the plates at 36 ± 1ºC for 24 ± 2 hours (18-24 hours for use in the VITEK 2 Compact). Follow steps outlined in section 10.1.2.2 to confirm the identity of the organism.

10.1.5 Culture Maintenance.

10.1.5.1 Every 30 ± 2 days inoculate a new set of stock culture tubes from a current stock culture tube. Use the same refrigerated stock culture tube used for Monthly QC described in 10.1.4.1 to inoculate 6 new stock cultures tubes as outlined in 10.1.3.2.

10.1.5.2 Incubate the new stock cultures as indicated in 10.1.3.3.

10.1.5.3 Following the incubation period, store the stock cultures at 2-5ºC for 30 ± 2 days.

10.2 Test Culture Preparation:

10.2.1 Initiate test culture by inoculating a 10 mL tube (20 × 150 mm) of nutrient broth from a stock slant or stab culture. Transfer one 4 mm ID loopful (or use a 10 µL certified transfer loop) of inoculum from the stock culture into the broth.

10.2.2 Two sets of cultures (one set as a backup) of the same organism may be initiated in parallel from the same stock culture and subcultured; however, only one set of the final cultures is used for actual testing.

10.2.3 Make at least 3 consecutive 24 ± 2 hour transfers (use one 4 mm ID loopful, or a 10 µL certified transfer loop, or a calibrated micro volume pipet to deliver 10 µL) in 10 mL nutrient broth or synthetic broth incubated at 36 ± 1°C. For the purposes of conducting the Use-dilution method, the lab allows 5-7 daily transfers. If only one of the

consecutive 24 hour transfers has been missed, it is not necessary to repeat the previous 3-day sequence prior to the inoculation of the 48–54 hour test culture.

10.2.4 For the final subculture step, inoculate for the test procedure, a sufficient number of 25 × 150 mm tubes (e.g., eight to ten) containing 20 mL nutrient broth; incubate 48–54 hours at 36 ± 1°C.

10.2.5 A minimum of five days are required to obtain the culture for inoculating carriers. For example, the culture sequence must begin on Thursday for testing to commence on the following Tuesday.

10.2.6 Record all culture transfers on the Organism Culture Tracking Form (see 16.1).

10.3 Carrier Inoculation for *S. aureus*, *P. aeruginosa*, and *S. enterica*:

10.3.1 A single test involves the evaluation of 60 inoculated carriers (one organism) against one product sample. In addition to the 60 carriers, 6 carriers are required to estimate carrier bacterial load and a minimum of 6 more are included as extras. Thus, a minimum of 72 inoculated carriers are required to perform a single test.

10.3.2 For *S. aureus* and *S. enterica*, using a Vortex-style mixer, mix 48-54 hour nutrient broth test cultures 3–4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture (e.g., upper ¾ or approximately 15 mL), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot 20 mL portions into sterile 25 × 150 mm test tubes. Prepare a minimum of four tubes.

10.3.3 For *P. aeruginosa*, do not shake 48–54 hour test culture. The pellicle from the 48–54 hour cultures must be removed from the broth before mixing on a Vortex mixer by gently aspirating the broth away from the pellicle using a pipette. Any disruption of the pellicle resulting in dropping, or breaking up of the pellicle in culture before or during its removal renders that culture unusable in the use-dilution test. Visually inspect the culture for signs of pellicle. Discard tubes with fragments of pellicle. Using a vortex-style mixer, mix nutrient broth test cultures 3–4 seconds and let stand 10 minutes at room temperature before continuing. Remove the upper portion of each culture (e.g., upper ¾ or approximately 15 mL), leaving behind any debris or clumps, and

transfer to a sterile flask; pool cultures in the flask and swirl to mix. Aliquot 20 mL portions into sterile 25 × 150 mm test tubes. Prepare a minimum of four tubes.

10.3.4 If organic burden is required for testing, the appropriate amount of organic burden is added to the pooled test culture prior to the inoculation of carriers. For a 5% v/v preparation, add 1 mL organic burden per 19 mL pooled test culture (e.g., add 5 mL organic burden to 95 mL pooled test culture). Swirl to mix. Aliquot 20 mL portions into sterile 25 × 150 mm test tubes. Prepare a minimum of four tubes.

10.3.5 Use only carriers that have been physically screened, have passed bioscreening, and have been appropriately prepared (see SOP MB-03, Screening Carriers).

10.3.6 Using a sterile hook, aseptically transfer 20 carriers prepared as described above into each of the tubes containing the test culture. Drain the water from the carriers by tapping them against the side of the tube before transferring. Multiple carriers may be transferred on a single wire hook. The test culture must completely cover the carriers. If a carrier is not covered, gently shake the tube, or reposition the carrier within the tube with a sterile wire hook. Be sure to inoculate a sufficient number of carriers for the test. (Alternately, the water may be siphoned off the carriers and the 20 mL test culture added directly to the carriers without transferring).

10.3.7 After 15 ± 2 min contact period, remove carriers using flamed nichrome wire hook; shake carrier vigorously against side of the tube to remove excess culture, and place on end in vertical position in sterile Petri dish matted with 2 layers of Whatman No. 2 (or equivalent) filter paper, making sure that carriers do not touch to prevent improper drying. Place no more than 12 carriers in a Petri dish. Carriers that touch or fall over cannot be used for testing and must be removed and recleaned. Once all of the carriers have been transferred, cover and place in incubator at 36 ± 1°C and let dry 40 ± 2 min.

10.3.8 Record the timed carrier inoculation activities on the Time Recording Sheet for Carrier Inoculation Steps (see 16.3).

10.4 Enumeration of bacterial inocula (carrier counts):

10.4.1 Synchronize the carrier count assay and efficacy testing. Assay carriers for carrier counts (sonicated) within 2 hours of drying. Record time of sonication on Serial Dilution/Plating Tracking Form (16.8).

10.4.2 One carrier is randomly extracted from each of 6 Petri dishes (12 carriers/dish).

10.4.3 Place each inoculated carrier into a tube containing 10 mL of letheen broth.

10.4.4 Place all tubes with carriers into an appropriately sized beaker and fill the beaker with tap water to the level of letheen broth in the tubes.

10.4.4.1 Hold the beaker in the sonicator so that the water level in the beaker is even with the water level fill line on the sonicator tank and fill the tank up with tap water to the water level fill line. Be sure that the water level in the tank never falls below one inch from the top of the tank.

10.4.4.2 Hold the beaker in the sonicator tank so that it is not touching the bottom and that all three liquid levels (inside the test tubes, inside the beaker and the sonicator tank) are the same.

10.4.4.3 Using an official timer, sonicate carriers for 60 ± 5 seconds.

10.4.5 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks (see 16.8).

10.4.6 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done. Complete the dilutions and plating within 2 hours after sonication.

10.4.7 Plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA using pour or surface spread plating. Briefly vortex (1-3 sec.) each serial dilution tube prior to plating.
Note: Dilutions 10^{-2} through 10^{-4} should produce plates with CFU in the appropriate range.

10.4.8 If the spread plate method is used for bacterial enumeration, TSA plates are prepared in advance and are refrigerated until needed.

10.4.8.1 Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the dry surface of the agar, use a glass, autoclavable or disposable spreading rod and plate spinner until the surface is completely dry.

10.4.9 If the pour plate method is used for bacterial enumeration, the TSA is prepared and tempered after autoclaving (approx. 1 hr) to 45-50°C in a water bath prior to use. Tempered agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.

10.4.10 Incubate plates at 36 ± 1°C for 24-48 hrs.

10.4.11 Colonies may be counted by hand or with the aid of a plate counter. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the Carrier Count Data Sheet (see 16.9). See section 11 for data analysis.

10.5 Disinfectant Sample Preparation.

10.5.1 Prepare disinfectant sample per SOP MB-22.

10.5.2 Equilibrate the water bath and allow it to come to 20 ± 1°C or the temperature specified (±1°C). Prepare the disinfectant dilutions within 3 hours of performing the assay unless test parameters specify otherwise. Ready-to-use products are tested as received; no dilution is required.

10.5.3 Dispense 10 mL aliquots of the diluted disinfectant or ready-to-use product into 25 × 100 mm test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 minutes to allow test solution to come to specified temperature. Record the temperature of the water bath and recirculating chiller before and after testing on the AOAC Use-Dilution Test Information Sheet (see 16.5).

10.6 Test Procedure:

10.6.1 After the required drying time, the carriers are sequentially transferred from the Petri dish to the test tubes containing the disinfectant at 30 second intervals or at the pre-determined drop interval for products

with contact times less than 10 minutes. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook can touch the interior sides of the tube. (Individual manipulation of the carriers is required.) Use a certified timer to time the transfers. Modify intervals to accommodate exposure times other than 10 min.

Note: Above step is one of the most critical, technique-sensitive areas of method. False positives can result from transfer of live organisms to sides of tubes due to contact or aerosol formation. If the side is touched, mark or note the tube; the tube is not counted if it yields a positive result.

10.6.2 One carrier is added per tube. Immediately after placing carrier in the test tube, briefly swirl tube before placing it back in the bath. For a contact time of ten minutes, the carrier must be deposited in the tube within ± 5 seconds of the prescribed drop time. For contact times of less than ten minutes, the analyst will work with the team leader and senior scientist to identify an appropriate (i.e., shorter) drop interval. Using alternating hooks, flame-sterilize the hook and allow it to cool after each carrier transfer.

10.6.3 After the carriers have been deposited into the disinfectant, and the exposure time is complete, the carriers are then transferred in a sequentially timed fashion into the primary subculture tubes containing the appropriate neutralizer (10 mL in 20 × 150 mm tubes). As with the transfers to the disinfectant tubes, transfers into subculture tubes are to be done within ± 5 seconds (see section 10.6.2) of the actual transfer. The carrier is removed from the disinfectant tube with a sterile hook, tapped against the interior sides of the tube to remove the excess disinfectant and transferred into the subculture tube. Avoid contact of the carrier to the interior sides of the subculture tube during transfer. Flame-sterilize the hook after each carrier transfer.

10.6.4 After the carrier is deposited in the subculture tube, recap the subculture tube and shake thoroughly. Place subculture tubes into 36 ± 1°C incubator.

10.6.5 After a minimum of 30 ± 5 minutes from the end of the transfer into primary subculture tubes, remove tubes from the incubator and transfer carrier from the primary tube to a secondary tube of sterile medium.

10.6.5.1 Transfer the carriers using a sterile wire hook to a second subculture tube containing 10 mL of the appropriate subculture medium which may contain a suitable neutralizer. Move the carriers in order but the movements do not have to be timed. Thoroughly shake the subculture tubes after all of the carriers have been transferred.

10.6.6 Check all test tube racks for proper transfer of carriers (i.e., carriers are in the correct tubes, no tubes have two carriers) before completing the testing day.

10.6.7 Incubate both the primary and secondary subculture tubes 48 ± 2 hours at 36 ± 1°C.

10.6.8 Record timed events on the Time Recording Sheet for Carrier Transfer Form (see 16.4).

10.7 Viability controls. On testing day, place a dried inoculated carrier into a tube containing 10 mL primary subculture medium and a second dried, inoculated carrier into a tube containing 10 mL secondary subculture medium. Incubate tubes for 48 ± 2 hours at 36 ± 1°C. Positive growth in both tubes validates the test system. Failure to have growth in either of the tubes invalidates the test.

10.8 Results:

10.8.1 Report results as + (growth), or 0 (no growth) as determined by presence or absence of turbidity, on the AOAC Use-dilution Results Sheet (see 16.6). A positive result is one in which the broth culture appears turbid. A negative result is one in which the broth appears clear. Each tube is shaken prior to recording results to determine the presence or absence of turbidity. The primary and secondary subculture tubes for each carrier represent a "carrier set."

10.8.2 A positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set.

10.8.3 In the event that there are positive carriers present in the test, the test may be repeated in order to confirm the outcome.

10.8.4 Once the results are recorded, it is important that the carriers be reprocessed before use in another study.

10.9 Confirmation Steps:

10.9.1 Confirm a minimum of three positive carrier sets per test, if available, using Gram staining, solid media, and VITEK™ analysis. If there are less than three positive carrier sets, then each carrier set will be confirmed. If both tubes are positive in a carrier set, only one tube is selected for confirmatory testing (preferably the secondary subculture tube with carrier).

10.9.2 For a test with greater than 20 positive carrier sets, confirm at least 20% by Gram stain, and a minimum of 4 positive carrier sets by Gram staining, solid media, and VITEK™ analysis (see SOP QC-22, VITEK 2 Compact) to ensure the identity of the organism. Again, if both tubes are positive in a carrier set, only one tube (preferably the secondary subculture tube with carrier) is selected for confirmatory testing.

10.9.3 See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media.

10.9.4 Gram stains are performed on smears taken from the positive culture tubes. For the additional confirmatory tests, a loopful of broth from each selected culture tube is streaked on both TSA and selective media appropriate for the test organism and incubated for 18-24 hours at 36 ± 1°C. The selective agar is checked for the correct reaction and the culture on the TSA plate is used for preparing the inoculum for the VITEK™ analysis.

10.9.5 Perform the VITEK™ analysis according to the manufacturer's instructions.

10.9.6 If confirmatory testing determines that the identity of the organism was not the test organism, the positive entry (+) on the results sheet must be annotated to indicate a contaminant was present.

10.10 Re-use of Stainless Steel Carriers: After use, all carriers are autoclaved. Carriers for which test results were negative may be reused after cleaning. Carriers that are positive are re-cleaned and screened biologically (see SOP MB-03, Screening Carriers) before re-use. These carriers may be reused if the biological screening test results in no growth. The extra carriers that were inoculated but not used are autoclaved, re-cleaned, and used again.

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Calculations will be computed using a Microsoft Excel spreadsheet (see 16.10). Electronic copies of the spreadsheet as well as hard copies will be retained.

11.2 To calculate CFU/mL per carrier when 3 serial dilutions are plated, use the following calculation scheme where 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions plated:

$$\frac{(avg.\ CFU\ for 10^{-x}) + (avg.\ CFU\ for 10^{-y}) + (avg.\ CFU\ for 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

11.3 Counts from 0 through 300 and their associated dilutions will be included in the calculations.

11.3.1 Sample calculation: For average CFU of 115 at the 10^{-3} dilution, 15 at the 10^{-4} dilution, and 0 at the 10^{-5} dilution, the CFU/mL per carrier would be 1.2×10^{5} CFU/mL per carrier.

11.4 To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication (10 mL) and round numbers to 2 significant figures for reporting the final data.

11.4.1 Sample calculation: 1.2×10^{5} CFU/mL per carrier × 10 mL = 1.2×10^{6} CFU/carrier.

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

11.6 Calculate the mean log density across carriers for each test. Let M denote the mean log density. The 10^{M} is the geometric mean density for the test.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms (see 16.0). Completed forms are archived in notebooks kept in secured file cabinets in room D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the

appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 If the results of quality control do not verify the identity of the test organism, then the culture is discarded and a new culture is initiated. New stock cultures are established as outlined in section 10.0 of this SOP.

14.2 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocols must be recorded on the form and an explanation for the deviation given.

14.3 The mean log density for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6); a mean log density below 6.0 invalidates the test.

14.3.1 Values below 1.0×10^6 may be indicative of a dilution error, poor media quality, interference by environmental parameters (e.g., carrier drying and culture incubation conditions), contamination, or lack of adherence to the method.

14.3.2 The prescribed minimum will also account for the addition of 5% organic soil to the inoculum.

14.4 Carrier counts for *S. enterica* are expected to be comparable to counts for *S. aureus* and *P. aeruginosa*.

14.5 A product test exhibiting passing results (i.e., 0 or 1 positive carrier sets for the test microbe) will be repeated if contamination is present for more than one carrier set. For a failing product test (i.e., 2 or more positive carrier sets for the test microbe), no contamination is permissible in any of the carrier sets and the test is deemed invalid and must be repeated.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 2009. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Methods 955.15 and 964.02).

15.2 Official Methods of Analysis. 2006. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 955.14).

15.3 Holt, J., Krieg, N., Sneath, P., Staley, J. and Williams, S. eds. 1994. Bergey's

Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD.

15.4 Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD.

15.5 Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD.

16.0 FORMS AND DATA SHEETS:

16.1 Organism Culture Tracking Form

16.2 Test Microbe Confirmation Sheet (Quality Control)

16.3 AOAC Use-Dilution Test: Time Recording Sheet for Carrier Inoculation Steps

16.4 AOAC Use-Dilution Test: Time Recording Sheet for Carrier Transfers

16.5 AOAC Use-Dilution Test Information Sheet

16.6 AOAC Use-Dilution Test Results Sheet

16.7 Test Microbe Confirmation Sheet

16.8 AOAC Use-Dilution Test Serial Dilution/Plating Tracking Form

16.9 AOAC Use-Dilution Test Carrier Count Data Sheet

16.10 Sample Carrier Count Spreadsheet
MS Excel spreadsheet: Carrier Count Template_UDT_v2

Attachment 1: Typical Growth Characteristics of strains of *P. aeruginosa, S. aureus,* and *S. enterica*

Attachment 2: Culture Initiation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica*

16.1

ORGANISM CULTURE TRACKING FORM

OPP Microbiology Laboratory

Organism:		Supply Control Number:	
Source and Strain no.:		Lot Number:	
MRME Number:			

Date	Time	Init.	Subculture Source	Transfer*		Media Inoculated (and # inoc.)	Media Prep No.	Incubation Conditions	Comments
				Monthly	Daily				

* "Monthly" indicates the monthly transfers for culture and "Daily" indicates a 24/48 hr serial transfer (added to control number)
NR = None Required, TC = Test Culture, applied after daily transfer number
TSB = Tryptic soy broth, NB = nutrient broth

16.2

TEST MICROBE CONFIRMATION SHEET (Quality Control)

OPP Microbiology Laboratory

Organism:		MRME*** Number:	
Source and Strain no.:		Notes:	

Source: Tube/Plate ID	Date/ Initials	Staining Results*	Media Information			Results		
			Name	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek #**

* Record Gram stain results: GPC = Gram Positive Cocci; GNR = Gram Negative Rods
** Vitek tracking number
*** Use MRME notation for all organisms (refer to MB-02).
TSA = trypticase soy agar, MSA = mannitol salt agar, NA = nutrient agar

16.3

AOAC Use-Dilution Test: Time Recording Sheet for Carrier Inoculation Steps

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______	
Test Date	
Product Reg. No.	
Product Name	
Sample No.	
Organism	

Initials/Date	Test ID	Inoculum Settle Time*		Carrier Seeding Time*		Carrier Dry Time*	
		Start Time	End Time	Start Time	End Time	Start Time	End Time
		/	/	/	/	/	/
		/	/	/	/	/	/
		/	/	/	/	/	/

*Recorded from laboratory clock/and timer.

Comments:

16.4

AOAC Use-Dilution Test: Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______	
Test Date	
Product Reg. No.	
Product Name	
Sample No.	
Organism	

Initials/Date	Set	Drop Interval	Carrier Drop Start Time (into the disinfectant)		Carrier Drop End Time (into the primary subculture/neutralizer media)		Carrier Transfer (into secondary subculture)
			Clock	Timer	Clock	Timer	Start Time[1]
Comments:							

1 Carrier transfer into secondary subculture (time elapsed after last carrier dropped in primary); taken from clock

16.5
AOAC Use-Dilution Test Information Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_______			
EPA Reg. No.		SOP	
Name		Test Date	
Sample No.		Comments:	
Lot No.			
Expiration Date			

TEST PARAMETERS/Confirmed by:_______			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated (Buret)/Date/Init.	HACH/Date/Init.
Use Dilution	Specified	As Prepared/Date/Init.	
Organic Soil	Specified	As Prepared/Date/Init.	
Neutralizer	Specified		
Temperature (ºC)	Specified	Chiller Unit Display	Test Tube Water Bath
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Other Parameters	Specified		

TEST MICROBE INFORMATION/Confirmed by:_______				
Test Microbe		48-54 Hour Culture		
Org. Control No		Date/Time	Initiated	Harvested
Avg. CFU/Carrier				

REAGENT/MEDIA INFORMATION/Confirmed by:_______			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralizer volume verified: □ Yes □ No Subculture volume verified: □ Yes □ No

16.6

AOAC Use-Dilution Test Results Sheet

OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_______			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.			

CARRIER INFORMATION (to be completed by Analyst)		
Carrier Drop Time Interval	Carrier Set	Analyst

TEST RESULTS									
Date Recorded/Initials									
Primary Subculture / Secondary Subculture (carrier)									
1	2	3	4	5	6	7	8	9	10
/	/	/	/	/	/	/	/	/	/
11	12	13	14	15	16	17	18	19	20
/	/	/	/	/	/	/	/	/	/
21	22	23	24	25	26	27	28	29	30
/	/	/	/	/	/	/	/	/	/
31	32	33	34	35	36	37	38	39	40
/	/	/	/	/	/	/	/	/	/
41	42	43	44	45	46	47	48	49	50
/	/	/	/	/	/	/	/	/	/
51	52	53	54	55	56	57	58	59	60
/	/	/	/	/	/	/	/	/	/
Results Summary			Number of Carrier Sets with Growth						
			Number of Carrier Sets without Growth						
Viability Controls (Record growth as “+”, no growth as “0”): ___ Primary ___ Secondary Acceptable: __Yes __No									
Modifications/Comments:									

16.7

Test Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:__________			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Comments:	

Source: Tube/Plate ID	Date/ Initials	Stain Results[1]	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	Vitek ID (if applicable)

[1]Record Gram Stain results as GPC=Gram positive cocci or GNR=Gram negative rods.

16.8

AOAC Use-Dilution Test Serial Dilution/Plating Tracking Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: ________	
EPA Reg. No.	
Name	
Sample No.	
Test Date	
Organism	
SOP	

DILUTION/PLATING SCHEME/Confirmed by: _____________					
Sonication Start Time (clock): _________	Dilution Tube				
	10^{0}*	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Starting volume of diluent					
Volume added to serial dilution tube (1 mL)	N/A				
Volume plated (0.1 mL)					
Final dilution (used for calculations)[1]					
Number of plates per dilution					
Plating medium					
Number of carriers evaluated					
Comments: N/A = not applicable					
Dilution blank volume verified: □ Yes □ No Subculture medium volume verified: □ Yes □ No					

*Volume of medium in the tube with the carrier will be accounted for in the CFU/carrier calculation.
[1]Adjusted for volume plated.

REAGENT/MEDIA INFORMATION/Confirmed by:_______			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

16.9

AOAC Use-Dilution Test Carrier Count Data Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: ________	
EPA Reg. No.	
Name	
Sample No.	
Test Date	
Organism	
SOP	
Test Type	

RESULTS			
Date/Initials			
Plating method			
Volume of media in initial tube receiving carrier			
Carrier No. / Dilution	CFU per Dilution Plate (2)		
1	/	/	/
2	/	/	/
3	/	/	/
4	/	/	/
5	/	/	/
6	/	/	/
Comments: N/A = not applicable			

Sample Carrier Count Spreadsheet (v2)

OPP Microbiology Laboratory

Carrier Count Spreadsheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:	
EPA Reg. No.	
Name	
Sample No.(s)	
Test Date	
Organism	
SOP	
Test Type	

Volume of media in tube with carrier (mL): ______

Carrier No. / Dilution	CFU per Plate			CFU/mL per carrier	CFU/carrier	LD/carrier
1						
2						
3						
4						
5						
6						
Mean per carrier for all carriers tested:						
Comments:	LD = log density					

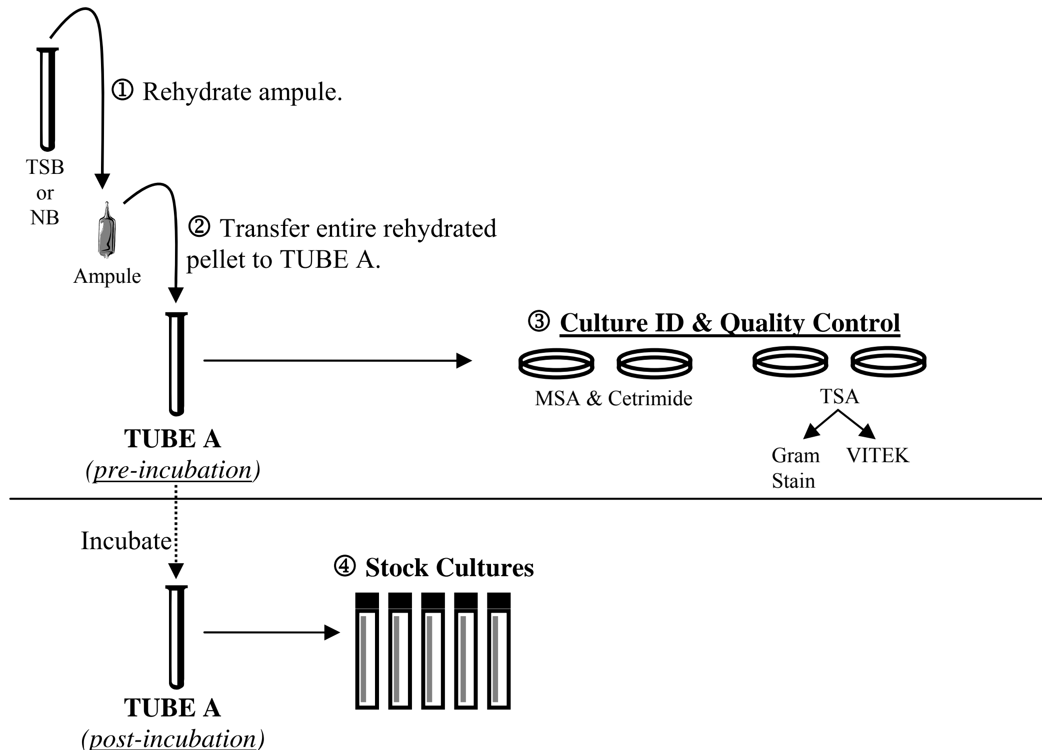
Typical Growth Characteristics of strains of *P. aeruginosa, S. aureus,* and *S. enterica* (see ref. 15.2, 15.3 and 15.4).

	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	(-)	(+)	(-)
Typical Growth Characteristics on Solid Media			
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A
TSA	flat, opaque to off-white, round spreading (1)	small, circular, yellow or white, glistening	entire, glistening, circular, smooth, translucent, low convex
Typical Microscopic Characteristics			
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*	0.5-1.5 µm in diameter*	0.7-1.5 µm in diameter by 2.0-5.0 µm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	straight rods, peritrichous flagella

*After 24±2 hours

(1) Plates from dilution plating. The agar plate may show three different 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. Colony c) reverts to colony type a) after 24 hour incubation. Pyocyanin is not produced.

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica*



CULTURE INITIATION

① Obtain lyophilized cultures annually from ATCC. Using a tube containing 5-6 mL of TSB aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *S. aureus* and *P. aeruginosa*. Using a tube containing 5-6 mL of NB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *S. enterica.*

② Aseptically transfer the entire rehydrated pellet back into the original tube of broth (TSB for *S. aureus* and *P. aeruginosa*, NB for *S. enterica*) designated as “TUBE A.” Mix well. Use suspension in TUBE A for CULTURE ID & QUALITY CONTROL. Incubate TUBE A for *S. aureus*, *P. aeruginosa*, and *S. enterica* for 24 hours at 36 ± 1°C.

CULTURE ID & QUALITY CONTROL

③ Using a loopful of rehydrated suspension from TUBE A, streak for isolation on duplicate plates (TSA for *S. aureus, P. aeruginosa*, and NA for *S. enterica*). In addition for *S. aureus* and *P. aeruginosa*, streak a loopful of rehydrated suspension onto both MSA and Cetrimide agar. Selective media is not used for *S. enterica*. Incubate plates for *S. aureus*, *P. aeruginosa*, and *S. enterica* for 24 hours at 36 ± 1°C. Record results on the Test Microbe Confirmation Sheet.

STOCK CULTURE GENERATION

④ Using the 24 ± 2 hour TUBE A broth culture: initiate stock cultures. For *S. aureus* and *S. enterica*, streak-inoculate six NA slants. For *P. aeruginosa*, stab-inoculate six CTA tubes. Incubate the *S. aureus* and *S. enterica* slants and *P. aeruginosa* stabs at 36 ± 1ºC for 48 ± 2 hours. Record all manipulations on the Organism Culture Tracking Form.