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

Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (*Bacillus* × porcelain component only)

SOP Number: MB-15-01

Date Revised: 11-30-06

Initiated By: _____ Date: ___/___/___

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Effective Date: ___/___/___

Controlled Copy No.: _____

Withdrawn By: _____ Date: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 As a result of EPA Homeland Security priorities, method 966.04 is being revised by the EPA. Several aspects of method 966.04 have been officially modified (editorial and procedural) to improve the quality and reproducibility of the efficacy data generated from the method. Modifications to the *Bacillus* × porcelain components have been completed and the data from the collaborative study are presented in: Tomasino, S.F. & Hamilton, M.A. (2006) *J.AOAC* Int. 89, 1373–1397. The revised method (Method II) appears in: Official Methods of Analysis (2006) 21st ED., AOAC INTERNATIONAL, Method 966.04, Gaithersburg, MD, Chapter 6.

1.2 This SOP is based on the revised AOAC Method 966.04, Sporicidal Activity of Disinfectants Test – Method II, and is suitable for determining the sporicidal efficacy of liquid sporicidal agents against *Bacillus* on hard surfaces (porcelain carriers). In most cases, *Bacillus subtilis* (ATCC #19659) will be the test microbe selected for sporicidal testing; however, if requested, other *Bacillus* species may also be used. Testing of suture loops and *Clostridium* is not addressed in this SOP

1.3 The revised method has been validated for products containing sodium hypochlorite, peracetic acid/hydrogen peroxide, and glutaraldehyde.

1.4 SOP MB-15 will be revised as additional modifications to method 966.04 are conducted and approved.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 CFU = Colony Forming Unit

2.3 TNTC = Too Numerous to Count

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

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in SOP MB-01.

3.2 Disinfectants may contain a number of different active ingredients, such as

heavy metals, aldehydes, peroxides, and phenol. Personal protective clothing or devices are recommended during the handling of these items for purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment may be employed when appropriate while 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 To ensure the stability of a diluted sporicidal agent, prepare the dilutions within three hours of the disinfectant treatment step unless specified otherwise.

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

4.3 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.

4.4 Detergents used in washing glassware may leave residues which are bacteriostatic. Test for inhibitory residues on glassware periodically according to SOP QC-03.

5.0 INTERFERENCES:

5.1 Touching the interior sides of the medication tube should be avoided while the carriers are being lowered into the sporicidal agent and the hook is being removed. Contact with the interior sides of the medication tube may cause adhesion of spores which are not in contact with the sporicidal agent. This may result in re-inoculation of the carriers with spores as they are being removed from the medication tube. Re-inoculation of the carriers with spores can lead to false positive results.

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.

7.0 APPARATUS AND MATERIALS:

7.1 *Culture Media.*–(1) *Nutrient broth*.–For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., 4410 S 102nd St., Omaha, NE 68127) to approximately 1 L water. Boil mixture for 20 minutes with constant stirring. Readjust volume to 1 L with water and allow cooling to around 50ºC. Adjust pH to 6.8 ± 0.2 with 1N HCL or 1N NaOH, if necessary. Filter through paper (e.g., Whatman filter paper No. 4). Dispense 10 mL portions into 20 × 150 mm culture tubes or 20 mL portions into 25 × 150 mm culture tubes. Dehydrated nutrient broth may be substituted – prepare according to the manufacturer's instructions. (2) *Nutrient agar*.–For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized nutrient broth. Boil mixture until agar is dissolved. Adjust pH to 7.2 ± 0.2 if necessary. Dispense 5 mL portions into 16 × 100 mm screw cap tubes. Larger tubes may be used as well. Autoclave for 20 minutes at 121ºC. Remove from autoclave and slant tubes to form agar slopes. (3) *Nutrient agar with 5µg/mL $MnSO_4$:H_20 (amended nutrient agar)*.–For spore production. Suspend 11.5 g nutrient agar in 495 mL water, add 5 mL 500 ppm $MnSO_4$:H_20. Dissolve by boiling. Adjust pH to 6.8 ± 0.2 if necessary. Autoclave for 15 minutes at 121ºC. Pour agar into plates. (4) *Trypticase soy agar (TSA)*.–Suspend 40 g dehydrated trypticase soy agar in 1 L water and heat gently while stirring. Boil one minute or until completely dissolved. Adjust pH to 7.3 ± 0.2. Autoclave 15 minutes at 121ºC. Pour agar into plates. (5) *Fluid thioglycollate medium (FTM)*.–Suspend 29.5 g of dehydrated fluid thioglycollate medium in 1 L water. Heat to boiling to dissolve completely. Adjust pH to 7.1 ± 0.2 if necessary. Dispense 10 mL portions into 20 ×150 mm culture tubes and autoclave for 15 minutes at 121ºC. Store at room temperature. Protect from light. Note: If after autoclaving the aerated portion of media consumes more than one third of tube, media must be re-boiled by placing tubes in beaker of boiling water. Media can only be re-boiled once. (6) *Fluid thioglycollate medium with 1M NaOH (modified FTM)*.– For subculturing spores exposed to 2.5 M HCl. Suspend 29.5 g of fluid thioglycollate medium in 1 L water. Heat boiling to dissolve completely. Cool and adjust pH to 7.1 ± 0.2 if necessary. Add 20 mL 1M NaOH, mix well. Check final pH and record (pH between 8 and 9 is typical). Dispense 10 mL into 20 × 150 mm culture tubes and autoclave for 15 minutes at 121ºC. Store at room temperature. Protect from light. Note: If after autoclaving the aerated portion of media consumes more than one third of tube, media must be re-boiled by placing tubes in beaker of boiling water. Media can only be re-boiled once. Note: Commercial dehydrated media made to conform to the specified recipes may be substituted. Media can be stored for up to two months.

7.2 *Manganese Sulfate Monohydrate*.–500 ppm. Add 0.25 g of manganese sulfate to 500 mL water. Filter sterilize for use.

7.3 *Dilute hydrochloric acid*.–2.5M. Use to determine resistance of dried spores. Standardize and adjust to 2.5M as in AOAC method 936.15 or purchase certified 2.5M HCl.

7.4 *Sterile water*.–Use reagent-grade water. Reagent-grade water should be 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 for details on reagent-grade water.

7.5 *Triton X-100*

7.6 *Ethanol (40%)*

7.7 *Test organism.–Bacillus subtilis* (ATCC No. 19659) obtained directly from a reputable supplier (e.g., ATCC).

7.8 *Carriers*.–Penicylinders, porcelain, 8 ± 1 mm OD, 6 ± 1 mm ID, 10 ± 1 mm length (Available from CeramTec Ceramic, Laurens, SC, www.ceramtec.com, Cat. No. LE15819.)

7.9 *Glassware*.– For disinfectant, 25 × 150 mm or 25 × 100 mm culture tubes (Bellco Glass Inc., Vineland, NJ; reusable or disposable 20 × 150 mm (for cultures/subcultures); 16 × 100 mm screw cap tubes for stock cultures. Cap with closures before sterilizing. Sterilize all glassware 2 hr in hot air oven at 180^{o} C or steam sterilize for a minimum of 20 min at 121^{o}C with drying cycle.

7.10 *Sterile centrifuge tubes*.–Polypropylene, 15 mL conical tubes with conical bottoms (Corning), from Fisher, or equivalent.

7.11 *Water bath/chiller unit.*–Constant temperature for test chemical, capable of maintaining 20 ± 1ºC temperature or specified temperature for conducting the test.

7.12 *Petri dishes*.–Plastic (sterile)

7.13 *Filter paper*.–Whatman filter paper #2; placed in Petri dishes for storing carriers.

7.14 *Test tube racks*.–Any convenient style.

7.15 *Inoculating loop*.–Any convenient inoculation/transfer loop for culture transfer.

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

7.17 *Centrifuge*.–Non-refrigerated (e.g., Eppendorf 5804 R).

7.18 *Sonicator*.–Ultrasonic cleaner (e.g., Branson Model 1510).

7.19 *Orbital shaker*.–speed range from 25 to 500 rpm (e.g., VWR DS 500).

7.20 *Vacuum desiccator*.–For carrier storage. With adequate gauge for measuring 27" (69 cm) of Hg and fresh desiccant.

7.21 *Certified biosafety cabinet (Class I or II)*.–Recommended for use to maintain aseptic work environment.

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

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 Sporicidal agents are stored according to manufacturers' recommendations or at room temperature if the product label does not specify a storage temperature. Those sporicidal agents requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless label directions specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 *Culture initiation*.– Initiate *B. subtilis* culture (e.g., use nutrient broth to re-hydrate a lyophilized culture, and incubate the broth culture for 24 ± 2 hours at 36 ± 1ºC prior to streak inoculation). Streak inoculate a set (e.g., six) nutrient agar slopes and incubate 24 ± 2 hours at 36 ± 1ºC. Concurrently, perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or use of other identification systems). Following incubation, store

at 2-5ºC. Maintain stock culture on nutrient agar slants by monthly (30 ± 2 days) transfers.

10.2 *Production of B. subtilis spore suspension*.

10.2.1 Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of nutrient broth and incubate tubes on an orbital shaker for 24 ± 2 hours at approximately 150 rpm at 36 ± 1ºC. Use this culture to inoculate amended nutrient agar plates. Inoculate each plate with 500 µl of broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36 ± 1ºC.

10.2.2 Following incubation, harvest the spores by adding 10 mL cold sterile water to each plate. Using a spreader (e.g. bent glass rod), remove growth from plates and pipet suspensions into 15 mL sterile conical tubes (10 plates = 14 tubes, ~10 mL each). Centrifuge tubes at 5000 rpm for approximately 10 minutes at room temperature. Remove and discard supernatant. Re-suspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5000 rpm for approximately 10 minutes. Remove and discard supernatant. Repeat twice. Re-suspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5ºC.

10.2.3 Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of five fields and determine ratio of spores to vegetative cells (or sporangia). Percentage of spores versus vegetative cells should be at least 95%. Spore suspension from multiple plates can be combined and re-aliquoted into tubes for uniformity.

10.2.4 Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6} through 1.0×10^{-8}) using pour or spread plating on TSA plates. For pour plating, add molten TSA tempered to 45-55ºC to each plate, swirl, and allow agar to solidify. Incubate plates for 24 ± 2 hours at 36 ± 1ºC and determine titer. Note: When harvested and processed, ten plates of amended nutrient agar should provide 80-100 mL of concentrated spore suspension (approx. 10^9 CFU/mL). Diluting the suspension prior to carrier inoculation will be necessary; a titer of 1.0×10^8 to 5.0×10^8 CFU/mL should be adequate to achieve the target

carrier count.

10.3 *Preparation of porcelain carriers.*–Prior to use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. Rinse unused carriers gently in water three times to remove loose material and drain. Place rinsed carriers into Petri dishes matted with 2 layers of filter paper in groups of 15 carriers per Petri dish or place carriers into 25 × 150 mm tubes (10 carriers per tube). Sterilize 20 minutes at 121ºC. Cool and store at room temperature. Note: Handle porcelain carriers with care when placing in Petri dishes. Minimize carrier movement and avoid excessive contact between carriers that might result in chips and cracks. Wash carriers with Triton X-100 and rinse with water 4 times for reuse.

10.4 *Inoculation of Porcelain Carriers.*

10.4.1 Dilute the concentrated spore suspension as necessary with sterile water to achieve carrier counts between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Dispense 10 mL diluted spore suspension into an appropriate number of 25 × 150 mm tubes.

10.4.2 Add 10 sterile carriers to each tube containing 10 mL spore suspension, slightly agitate, and let stand 10-15 minutes. Remove each carrier with sterile hook and place upright in sterile Petri dish lined with two sheets of filter paper, no more than 30 carriers per Petri dish. Air dry in biological safety cabinet for approximately 30 ± 2 minutes. Place Petri dishes containing inoculated carriers in vacuum desiccator containing $CaCl_2$ and draw vacuum of 69 cm (27") Hg. Dry carriers under vacuum for 24 ± 2 hours before use in HCl resistance, efficacy testing or carrier counts. Maintain under vacuum for up to three months.

10.4.3 Carriers may be used after three months if they meet the acceptable HCl resistance and carrier count criteria. Inoculated carriers should not be used after one year of storage. Sterilize and reuse if necessary.

10.5 *Spore Enumeration (Carrier Counts).*

10.5.1 Prior to use, determine the carrier counts for each preparation of carriers. Assay 3 to 5 randomly selected carriers per preparation. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL of sterile water. Sonicate carriers for 5 minutes ± 30 seconds. Note: For sonication, place tubes

into an appropriately sized glass beaker with tap water to the level of sterile water in the tubes. Place beaker in sonicator so that water level in the beaker is even with water level fill line on sonicator tank. Fill tank with tap water to water level fill line. Suspend beaker in sonicator tank so it does not touch bottom of tank and so all three water levels (inside test tubes, inside beaker, and sonicator tank) are the same.

10.5.2 Following sonication, vortex tubes for 2 minutes ± 5 seconds. Dilute spore suspensions by transferring 1 mL aliquots to tubes containing 9 mL sterile water. Dilute spore suspensions out to 1.0×10^{-5} and plate dilutions 1.0×10^{-2} through 1.0×10^{-5}. Plate each dilution in duplicate using pour or surface spread plating with TSA. For pour plating, add molten TSA tempered to 45-55ºC to each plate. Swirl pour plates to distribute spores evenly and allow agar to solidify. Invert plates and incubate for 24-48 hours at 36 ± 1ºC.

10.5.3 Count colonies (by hand or with colony counter). Record all counts less than 300 and use those counts for enumeration. Report plates with colony counts over 300 as TNTC (Too Numerous to Count). Average spore counts per carrier should be between 1.0×10^5 and approximately 1.0×10^6 spores/carrier. Do not use carriers with counts outside this range.

10.6 *HCl resistance*.

10.6.1 Equilibrate water bath to 20 ± 1ºC. Pipet 10 mL of 2.5M HCl into two 25 × 100 mm tubes, place into water bath, and allow to equilibrate. Start timer and rapidly transfer 4 inoculated penicylinders into an acid tube (2.5 M HCl) with flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube.

10.6.2 Transfer individual carriers after 2, 5, 10, and 20 minutes of HCl exposure to a separate tube of modified FTM. Rotate each tube vigorously by hand for approximately 20 seconds and then transfer carrier to a second tube of modified FTM.

10.6.3 For viability control, place one unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use one tube of modified FTM.

10.6.4 Incubate all test and control tubes for 21 days at 36 ± 1ºC. Record

results as growth (+) or no growth (0) at each time period. Spores should resist HCl for ≥ 2 minutes to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described.

10.7 *Efficacy Test.*

10.7.1 Aseptically prepare disinfectant samples as directed. Prepare all dilutions with sterile standardized volumetric glassware. For diluted products, use 1.0 mL or 1.0 g of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. For a 30-carrier test, place 10 mL product at dilution recommended for use or under investigation into each of six 25 × 150 mm or 25 × 100 mm test tubes, or use appropriate number of tubes assuming 5 test carriers per tube of test chemical.

10.7.2 Place tubes in 20 ± 1ºC water bath and let equilibrate to temperature. Using a sterile hook (or forceps), transfer inoculated carriers sequentially at 2 minute intervals in groups of 5 from Petri dish to test tubes containing sporicidal agent. Use a certified timer to monitor time. Flame hook and allow cooling after each transfer. When lowering carriers into test tube, neither carriers nor wire hook may touch sides of tubes. If interior sides are touched, note tube number. Do not count carrier set if any carrier from that group of 5 yields a positive result; testing another set of five carriers is recommended. Carriers must be deposited into test tubes within ± 5 seconds of the prescribed drop time. Return tubes to water bath immediately after adding carriers.

10.7.3 After contact period has been achieved, transfer carriers in same sequential timed fashion into primary subculture tubes containing appropriate neutralizer (10 mL in 20 × 150 mm test tubes). Remove the carriers one at a time from the test tube with sterile hook, tap against interior side of tube to remove excess sporicidal agent, and transfer into neutralizer tube (primary tube). All five carriers must be transferred during each 2 minute interval. Flame hook between each carrier transfer. Move remaining carriers into their corresponding neutralizer tubes at appropriate time. Carriers may touch interior sides of neutralizer tube during transfer, but contact should be minimized.

10.7.4 After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. Within one

hour from when last carrier was deposited into primaries, transfer carriers using sterile wire hook to second subculture tube (secondary tube) containing 10 mL of appropriate recovery medium, one carrier per tube. Move carriers in order, but movements do not have to be timed. Gently shake entire rack of secondary tubes after all carriers have been transferred.

10.7.5 Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 36 ± 1ºC. Report results as growth (+) or no growth (0). A positive result is one in which medium appears turbid. A negative result is one in which medium appears clear. Shake each tube prior to recording results to determine presence or absence of growth/turbidity. Primary and secondary subculture tubes for each carrier represent a "carrier set". A positive result in either primary or secondary subculture tube is considered a positive result for the carrier set.

10.7.6 Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended. For media controls, incubate 1-3 unopened subculture medium tubes with the test sample tubes for 21 days at 36 ± 1ºC. For system controls, use sterile forceps or needle hooks to transfer 3 sterile carriers into a tube of test chemical. Transfer system control carriers to neutralizer medium as follows: at start of sample test (prior to first tube), transfer 1 sterile carrier to tube of neutralizer medium. After one half of test carriers have been transferred to neutralizer tubes, transfer a second sterile carrier to tube of neutralizer medium. After all test carriers (last tube) have been transferred to neutralizer tubes, transfer third sterile carrier to tube of neutralizer medium. Transfer system control carriers to secondary subculture medium as follows: immediately prior to initiating transfer of test carriers into secondary subculture medium tubes, transfer first system control sterile carrier from neutralizer medium to tube of subculture medium. After one half of test carriers have been transferred to secondary subculture medium tubes, transfer second system control sterile carrier to tube of subculture medium. After all test carriers have been transferred to secondary subculture medium tubes, transfer third system control sterile carrier to tube of subculture medium. For each test, include a positive carrier control by placing one inoculated carrier into tube of secondary subculture medium. Incubate controls and test sample tubes together for 21 days at 36 ± 1ºC.

10.7.7 Perform identification confirmation on a minimum of three positive

carrier sets per test, if available, using Gram stain and/or plating on TSA. Additional confirmation may be performed using VITEK, API analysis or comparable method. If fewer than three positive carrier sets, confirm growth from each positive carrier set. If both tubes are positive in carrier set, select only one tube for confirmatory testing. For tests with 20 or more positive carrier sets, confirm at least 20% by Gram stain. If Gram stains are performed from growth taken directly from positive tubes, the staining should be performed within 5-7 days of conducting the efficacy test.

10.8 *Neutralization Confirmation Procedure.*

10.8.1 A neutralization confirmation test must be performed in advance or in conjunction with efficacy testing. This assay is designed to simulate the conditions (i.e., neutralizer, subculture medium, contact time, diluent, concentration of test substance) of the efficacy test and to demonstrate the recovery of a low level of spores (e.g., 5-100). Diluted inoculum (e.g., spores of *B. subtilis*) is added directly to the various sets of subculture media tubes (see Table 1). This assay provides for a quantitative approach to assessing the effectiveness of the neutralizer and any bacteriostatic action resulting from the neutralizer itself or neutralizer-disinfectant interactions.

10.8.2 Produce a spore preparation according to the procedure for amended nutrient agar. Harvest growth from plates (e.g., five plates) per the method, except re-suspend pellet after final centrifugation step in approximately 100 ml aqueous (40%) ethanol. Determine spore count by serial dilution and plating on TSA. Desirable target of the initial working suspension is 1.0×10^8 to 1.0×10^9 CFU/mL. The suspension may require adjustment to reach target titer. Prepare serial ten-fold dilutions of the inoculum in sterile water out to 10^{-8}. Use 10^{-6}, 10^{-7} and 10^{-8} dilutions to inoculate the neutralizer and subculture media tubes – the target number of spores to be delivered per tube in this assay is 5-100 per tube. Determine spore titer by plating (spread plate or pour plate) each of three dilutions in duplicate on TSA agar. Incubate plates inverted for 24-48 hours at 36 ± 1ºC. Count colonies (by hand or with colony counter). Report plates with colony counts over 300 as TNTC (Too Numerous to Count). Note: A standardized spore preparation adjusted to deliver 5-100 spores/mL may be substituted for the three dilutions of spore inoculum. In addition, spores sheared from inoculated carriers may be used as a working suspension.

10.8.3 Use 5 sterile porcelain carriers (only 3 to be used in the assay). Within 5 seconds, place a set of 5 carriers into a test tube (25 × 150 mm or 25 × 100 mm) containing test chemical; transfer carriers according to section 10.7.2. Allow carriers to remain in test chemical per the specified contact time and temperature. After the contact time is complete, aseptically transfer three of the five carriers individually into tubes containing the neutralizer per section 10.7.3. This set of tubes is the Neutralizer/Primary Subculture treatment. Following the transfer of the last carrier into neutralizer tube, transfer each carrier, in sequence, into tube containing secondary subculture medium. This portion of assay is not timed, but should be made as soon as possible. This set is the Secondary Subculture treatment.

10.8.4 Following carrier transfer, inoculate each tube (Neutralizer/Primary and Secondary Subculture treatment tubes) with one mL of each of three inoculum dilutions (10^{-6}, 10^{-7} and 10^{-8}).

10.8.5 For controls, use three fresh unexposed tubes of neutralizer and three tubes of the secondary subculture medium; also inoculate each control tube with one mL of each of three inoculum dilutions. Include one uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls.

10.8.6 See Table 1 for tube inoculation scheme.

10.8.7 Incubate all tubes 5-7 days at 36 ± 1ºC.

10.8.8 Record results as growth (+) or no growth (0). Note: The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of inoculum (spores) is added to the subculture tubes.

Table 1. Neutralization confirmation procedure – inoculating treatment and control tubes with diluted spore suspension*

Neutralizer-Primary Subculture Treatment	Secondary Subculture Treatment (with Carrier)	Neutralizer-Primary Inoculated Control	Secondary Subculture Inoculated Control
1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3	1 mL of 10^{-6} → Tube 1 1 mL of 10^{-7} → Tube 2 1 mL of 10^{-8} → Tube 3

*1.0×10^{-6} through 1.0×10^{-8} based on an approx. starting suspension of 10^{8} spores/mL

10.8.9 Confirm a minimum of one positive per treatment and control (if available) using Gram staining and colony morphology on TSA. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered. *B. subtilis* is a Gram positive rod and colonies on TSA are opaque, rough, dull, round, with irregular margins, and low convex. Colonial variation may be observed and is typical for this strain.

10.8.10 Growth in the inoculated controls verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. Note: There may be cases when the neutralizer is significantly different from the secondary subculture media; in these cases, growth may not be comparable. The uninoculated control tubes are used to determine sterility, and must show no growth for the test to be valid.

10.8.11 The occurrence of growth in the Neutralizer/Primary Subculture and Secondary Subculture treatment tubes is used to assess the effectiveness of the neutralizer. No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions.

10.8.12 For a neutralizer to be deemed effective, growth must occur in the Secondary Subculture treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).

10.8.13 Growth in the Secondary Subculture inoculated Control verifies the presence of the spores, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.

10.8.14 Growth in the Neutralizer-Primary inoculated Control should be comparable to the Secondary Subculture inoculated Control if the neutralizer is the same as the secondary subculture media. There may be cases when the neutralizer is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

10.8.15 The Neutralizer-Primary and Secondary Subculture uninoculated Control tubes are used to determine sterility, and must show no growth for the test to be valid.

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Data will be recorded on data sheets (see 16.2). Calculations will be computed using a Microsoft Excel spreadsheet (see 16.3). Electronic copies of the spreadsheet as well as hard copies will be retained.

11.2 To calculate CFU/mL per carrier:

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

where 10^{-w}, 10^{-x}, 10^{-y}, and 10^{-z} are the dilutions plated. In the event that one or more dilutions yield plate counts greater than 300, those counts and their corresponding dilutions will not be used in the calculations. In the event that only one of two plates has counts yielding 300 CFU or less, that plate count and its corresponding dilution will be included but no average will be determined.

NOTE: Plate counts of 0 are to be included in all calculations.

11.3 To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication or vortexing. Numbers are rounded and only two significant figures are used in calculating averages.

NOTE: Numbers will be rounded upon determination of the CFU/carrier.

11.4 Calculate the average CFU/carrier for all carriers tested.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the forms indicated in section 16.0. Completed forms are archived in notebooks kept in secured file cabinets in 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 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 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 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious.

15.0 REFERENCES:

15.1 Official Methods of Analysis (2006) 21st ED., AOAC INTERNATIONAL, Method 966.04, Gaithersburg, MD, Chapter 6

15.2 Standard Methods for the Examination of Water and Wastewater. 21st Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC

15.3 Tomasino, S.F. & Hamilton, M.A. (2006) *J.AOAC* Int. 89, 1373–1397

16.0 FORMS AND DATA SHEETS:

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Sporicidal Activity of Disinfectants Test: Serial Dilution/Plating Tracking Form for Carrier Counts

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Carrier Type	

Confirmed by:_____	Dilution Tube				
	1	2	3	4	5

Vol. In Dilution Tube prior to Addition	10 mL	9 mL	9 mL	9 mL	9 mL
Volume Added to Dilution Tube	1 mL	1 mL	1 mL	1 mL	1 mL
Overall Dilution in Dilution Tube	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Volume Plated	N/A	1 mL	1 mL	1 mL	1 mL
Overall Dilution on Plate	N/A	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Number of Plates per Dilution	N/A	2	2	2	2
Media Plated Onto					
Number of Carriers Analyzed					
Comments: Dilution tube #1 contains the carrier and represents the 10^{-1} dilution.					

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

Sporicidal Activity of Disinfectants Test: Carrier Count Data Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Carrier Type	

RESULTS	
Date/Initials	

Plating Method				
Carrier No. / Dilution	CFU per Dilution Plate (2)			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1	/	/	/	/
2	/	/	/	/
3	/	/	/	/
4	/	/	/	/
5	/	/	/	/
Comments:				

Carrier Count Spreadsheet
OPP Microbiology Laboratory

Carrier Count Spreadsheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:	
EPA Reg. No.	
Name	
Sample No.(s)	
Test Date	
Organism	
SOP	MB-15
Test Type	Sporicidal Activity of Disinfectants Test

Carrier No. / Dilution	CFU per Plate								CFU/carrier
	1.E-02		1.E-03		1.E-04		1.E-05		
1									
2									
3									
4									
5									
Average CFU per carrier for all carriers tested:									
Comments:									

Sporicidal Activity of Disinfectants Test: Hydrochloric Acid Resistance Test Results Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	

Date/Initials:		Results (+ = Growth, 0 = No Growth)			
		2 Minutes	5 Minutes	10 Minutes	20 Minutes
Carrier Tracking. No.:	Primary				
	Secondary				
Carrier Viability Control Results*: Growth ____ No Growth _____					
Medium Control**: Growth ____ No Growth _____					
Comments:					

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

*Place one of each type of inoculated carrier in a separate tube of Modified FTM.
**One tube of uninoculated Modified FTM.

Sporicidal Activity of Disinfectants Test: Test Information Sheet
OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Comments:	
Lot No.			

TEST PARAMETERS/Confirmed by:_____		
Diluent	Specified	Diluent Used
[redacted]		
Neutralizer (1°)		
Recovery Medium (2°)		
Temperature (°C)	Specified	Chiller Water Bath Temperature
[redacted]		Before: After:
Contact Time (minutes)	Specified	As Tested
[redacted]		
Type of Carriers	Specified	Control or Lot #
[redacted]		

TEST MICROBE INFORMATION/Confirmed by:_____	
Test Microbe	
Avg. CFU/Carrier	

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

Sporicidal Activity of Disinfectants Test: Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Carrier Type	
Product Reg. No.	
Product Name	
Lot No.	
Contact Time(s)	
Organism	

Initials/date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time (into the disinfectant)		Carrier Drop End Time (into the neutralizer media)[1]		Carrier Transfer (into secondary)
			Clock	Timer	Clock	Timer	Start Time[2]
Comments:							

1 The time when the last carrier is dropped into neutralizer tube.
2 The time at which carriers are started to be transferred into secondary subculture; taken from clock.

Sporicidal Activity of Disinfectants Test: Results Form (1-30)
OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Carrier Type	
Lot No.		Comments:	

CARRIER INFORMATION (to be completed by Analyst)	
Carriers	Analyst Dropping Carriers
1-30	

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
/	/	/	/	/	/	/	/	/	/
Results Summary			Number of Carrier Sets with Growth						
			Number of Carrier Sets without Growth						
Modifications/Comments:									

Sporicidal Activity of Disinfectants Test: Results Form (31-60)
OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Carrier Type	
Lot No.		Comments:	

CARRIER INFORMATION (to be completed by Analyst)	
Carriers	Analyst Dropping Carriers
31-60	

TEST RESULTS									
Date Recorded/Initials									
Primary Subculture / Secondary Subculture (carrier)									
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						
Modifications/Comments:									

Sporicidal Activity of Disinfectants Test: Test Controls Results Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP	MB-15
Name		Test Date	
Sample No.		Comments:	
Lot No.			

RESULTS			
Date Read/Initials:			
Testing Controls			
Type of Controls	Tube #1	Tube #2	Tube #3
Media Controls: Neutralizer Tubes			
Media Controls: FTM Tubes			
System Controls:	Beginning	Middle	End
	Primary: ____ Secondary: ____	Primary: ____ Secondary: ____	Primary: ____ Secondary: ____
Positive carrier control: Growth_____ No Growth_____			
Comments: Record results as growth (+) or no growth (0)			

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

Sporicidal Activity of Disinfectants Test: Microbe Confirmation Sheet
OPP Microbiology Laboratory

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

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)[2]

* GPR = gram positive rods, GPC = gram positive cocci, GNR = gram negative rods

Sporicidal Activity of Disinfectants Test: Neutralization Confirmation Assay Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP(s)	
Product Name		Test Date	
Product Sample No.		Neutralizer	
Product Lot No.		Comments:	
Expiration Date			

TEST PARAMETERS/Confirmed by:_____			
Diluent	Specified	Diluent Used	Hardness/Date/Init.
[redacted]			/ /
Organic Soil	Specified	As Prepared/Date/Init.	
[redacted]		/ /	
Neutralizer	Specified		
[redacted]			
Temperature (°C)	Specified	Chiller Unit Display	Test Tube Waterbath
[redacted]		Before: After:	Before: After:
Contact Time (minutes)	Specified	As Tested	
[redacted]			
Carriers (Unseeded)	Preparation #		
[redacted]	Type:		

TEST MICROBE INFORMATION/Confirmed by:_____	
Test Microbe	
Org. Control No.	

REGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Sporicidal Activity of Disinfectants Test: Neutralization Confirmation Assay Results Form

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments:	

TEST RESULTS*: Date Recorded/Initials:_____

Treatments/Controls	Inoculum Dilutions		
	1×10^{-6}	1×10^{-7}	1×10^{-8}
Neutralizer-Primary Subculture Treatment			
Secondary Subculture Media Treatment (with Carrier)			
Neutralizer Inoculated Control			
Subculture Media Inoculated Control			
Neutralizer Uninoculated Control Tube			
Subculture Media Uninoculated Control Tube			

*+ = growth, 0 = no growth

SUMMARY OF RESULTS: Date/Initials:_____	
Bacteriostatic Effect Observed?	Yes_____ No_____
Comments:	

Sporicidal Activity of Disinfectants Test: Neutralization Confirmation Assay Time Recording Sheet for Carrier Transfers

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
EPA Reg. No.	
Product Name	
Sample No(s).	
Organism(s)	
Neutralizer(s)	
Carrier Type	

Initials/date	Disinfectant Tube No.	Carrier No.	Carrier Drop Start Time for carriers (into the disinfectant)		Carrier Drop End Time for carriers (into the neutralizer)		Carrier Transfer (into secondary media)
			Clock	Timer*	Clock	Timer	Start Time[1]
	1						
	2						
	3						
Comments:							

* = ±5 seconds
1= Taken from clock

Sporicidal Activity of Disinfectants Test: Neutralization Confirmation Assay Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Carrier Type[3]	

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)[2]

1 Record Gram Stain results as GPC=gram positive cocci, GNR=gram negative rods, GPR=Gram positive rods.
2 Vitek Identification Number
3. Porcelain penicylinders (P) or Suture loops (SL)

Sporicidal Activity of Disinfectants Test: Neutralization Confirmation Assay
Serial Dilution/Plating Tracking Form
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Neutralizer(s)	
Sample No.		Organism Control #	

Confirmed by:_____	Dilution Tube							
	1	2	3	4	5	6	7	8
Vol. In Dil. Tube prior to Addition								
Volume Added to Dil. Tube								
Overall Dilution in Dil. Tube								
Volume Plated								
Overall Dilution on Plate								
Number of Plates per Dilution								
Media Plated Onto								
Comments:								

REGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.

Inoculum Enumeration Form
OPP Microbiology Laboratory

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

RESULTS: Date/Initials:_____			
Plating Method			
	CFU per Dilution Plate		Average CFU per mL
Dilution	Plate 1	Plate 2	
1×10^{-6}			
1×10^{-7}			
1×10^{-8}			
TNTC = Too Numerous To Count			
Comments:			

REGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep No.	Reagent/Media	Prep No.