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Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for the AOAC
Sporicidal Activity of Disinfectants Test
(Bacillus subtilis × porcelain carrier)

SOP Number: MB-15-04

Date Revised: 01-03-18

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Title	Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (*Bacillus subtilis* × porcelain carrier)
Scope	This SOP describes the Sporicidal Activity of Disinfectants Test – Method II methodology used to determine the sporicidal efficacy of liquid sporicidal agents against *Bacillus subtilis* on hard surfaces (porcelain carriers).
Application	The method is based on AOAC method 966.04 (see 15.1). *B. subtilis* (ATCC #19659) is a test microbe used to support sporicidal claims. Testing of suture loops and *Clostridium sporogenes* is not addressed in this SOP.

	Approval Date
SOP Developer:	_____ Print Name: _____
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Quality Assurance Unit	_____ Print Name: _____
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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. AOAC = AOAC INTERNATIONAL 2. CFU = Colony Forming Unit 3. References to water mean reagent-grade water, except where otherwise specified.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-05 (timers), EQ-10 (orbital shakers) and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	Avoid touching the interior sides of the medication tube while the carrier is being lowered into the disinfectant and the hook is being removed as it may lead to false positive results.
8. Non-conforming Data	1. Sterility and/or viability controls do not yield expected results. 2. The mean spore counts per carrier falls outside the specified range of 1×10^5 to approximately 1.0×10^6 spores/carrier. 3. No contamination is acceptable in the test system. 4. Acceptable spore purity and HCl resistance test results must be achieved. 5. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. To ensure the stability of a diluted sporicidal agent, use the diluted product within three hours of preparation unless specified otherwise.

	2. Use appropriate aseptic techniques for all test procedures involving the manipulation of the test organisms and associated test components. 3. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. Culture Media. Note: Commercial dehydrated media made to conform to the specified recipes may be substituted. Media can be stored for up to two months. a. *Nutrient broth*. For use in preparing nutrient agar. Add 5 g beef extract (paste or powder), 5 g NaCl, and 10 g peptone (anatone) to approximately 1 L water. Boil mixture for 20 min 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. b. *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 min at 121ºC. Remove from autoclave and slant tubes to form agar slants. c. *Nutrient agar with 5µg/mL $MnSO_4 \cdot H_2O$ (amended nutrient agar)*. For spore production. Suspend 11.5 g nutrient agar in 495 mL water, add 5 mL 500 ppm $MnSO_4$:H_2O. Dissolve by boiling. Adjust pH to 6.8±0.2 if necessary. Autoclave for 15 min at 121ºC. Pour agar into plates. d. *Trypticase soy agar (TSA)*. Prepare according to manufacturer's instructions. e. *Fluid thioglycollate medium (FTM)*. For subculturing spores exposed to disinfectant. Prepare according to manufacturer's instructions. 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. f. *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 1 M 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 min at 121ºC. Store at room temperature. Protect from light. 2. *500 ppm Manganese sulfate monohydrate*. Add 0.25 g manganese sulfate monohydrate to 500 mL water. Filter sterilize. 3. *2.5 M Dilute hydrochloric acid*. Use to determine resistance of dried spores. Standardize and adjust to 2.5 M as in AOAC method 936.15 or purchase certified 2.5M HCl. 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. 5. *Triton X-100.* For washing used porcelain penicylinders. 6. *Ethanol (40%).* For preparing spore suspension used in the neutralization assay. 7. *3M™ Petrifilm™ Aerobic Count Plate*. For spore enumeration. 3M Food Safety (St. Paul, MD, USA; Cat. No. 6400). 8. *Test organism. Bacillus subtilis* (ATCC No. 19659) obtained directly from a reputable supplier (e.g., ATCC). 9. *Carriers.* Penicylinders, porcelain, 8±1 mm OD, 6±1 mm ID, 10±1 mm length. (Available from CeramTec Ceramic, Laurens, SC, www.ceramtec.com, SAP# 1010368) 10. *Glassware*. For disinfectant, 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 h in hot air oven at 180º C or steam sterilize for a minimum of 20 min at 121ºC with drying cycle. 11. *Sterile centrifuge tubes*. Polypropylene, 15 mL conical tubes with conical bottoms (Corning), or equivalent.

	12. *Water bath/chiller unit.* Constant temperature for test chemical, capable of maintaining 20±1ºC temperature or specified temperature for conducting the test. 13. *Petri dishes*. Plastic (sterile). 14. *Filter paper*. Whatman filter paper #2; placed in Petri dishes for storing carriers. 15. *Test tube racks*. Any convenient style. 16. *Inoculating loop*. Any convenient inoculation/transfer loop for culture transfer. 17. *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. 18. *Centrifuge*. For preparing spore suspension. 19. *Sonicator* (ultrasonic cleaner). For conducting control carrier counts. 20. *Orbital shaker*. For preparing spore suspension. Speed range from 25 to 500 rpm. 21. *Vacuum desiccator*. For carrier storage. With gauge for measuring 27" (69 cm) of Hg and fresh desiccant. 22. *Certified Timer*. For managing timed activities, any certified timer that can display time in seconds.
12. Procedure and Analysis	
12.1 Culture Initiation	a. Every 12 months (or sooner if necessary) initiate a new stock culture from a lyophilized culture of *B. subtilis* (ATCC 19659). b. Open ampule of freeze dried organism as indicated by ATCC. c. Using a tube containing 5-6 mL of nutrient broth (NB), aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *B. subtilis*. d. Aseptically transfer the entire rehydrated pellet back into the original tube of nutrient broth designated as "TUBE A" (see Attachment 1). Mix well. e. Streak for isolation using a loopful of rehydrated suspension on duplicate trypticase soy agar (TSA) or nutrient agar (NA) plates. f. Incubate broth culture (TUBE A) and plate cultures at 30±1ºC for

	24±2 h. g. Record all manipulations on the Organism Culture Tracking Form (see section 14).
12.2 Culture Identification	a. Perform initial confirmation testing for quality control (QC) using the 24±2 h NA or TSA plates. b. Following the incubation period (as stated in section 12.1f), record the observed colony morphology on the NA or TSA plates and Gram stain reaction. See section 12.2d. for details on colony morphology and Gram stain reaction. c. 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 1000X magnification (oil immersion). d. *B. subtilis* is a Gram positive rod; 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. e. Perform VITEK™ analysis according to the manufacturer's instructions. f. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).
12.3 Generation of Stock Cultures	a. Use the 24±2 h TUBE A (see Attachment 1) broth culture discussed in section 12.1d to initiate stock cultures – streak a minimum of six NA slants with *B. subtilis* and incubate at 36±1ºC for 24±2 h. b. 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 12.1e. Repeat the cycle for a maximum of one year. c. 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, and one is a back-up tube.
12.4 Monthly QC of Stock Cultures	a. Perform monthly QC of stock cultures just prior to or concurrently with stock culture transfers. Use one refrigerated stock culture tube and streak a loopful on a plate of TSA.

	b. Incubate the plates at 36±1ºC for 24±2 h (18-24 h for use in the VITEK 2 Compact). Follow steps outlined in section 12.2b to confirm the identity of the organism.
12.5 Culture Maintenance	a. 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 section 12.4a to inoculate 6 new stock cultures tubes as outlined in section 12.3a. b. Incubate the new stock cultures as indicated in section 12.3a. c. Following the incubation period, store the stock cultures at 2-5ºC for 30±2 days.
12.6 Production of *B. subtilis* Spore Suspension	a. Use growth from a stock culture tube to inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of NB and incubate tubes on an orbital shaker for 24±2 h at approximately 150 rpm at 36±1ºC. Use this culture to inoculate amended NA plates. Inoculate each plate with 500 µL of broth culture and spread the inoculum with a sterile bent glass rod or suitable spreading device. In addition, verify the purity of this culture by streak isolating on amended NA (incubate at 36±1°C for 24±2 h). Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36±1ºC. b. Following incubation, harvest the spores by adding 10 mL chilled 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). c. Centrifuge tubes at 5,000 rpm (4,500×g) for approximately 10 min at room temperature. Remove and discard supernatant. Re-suspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5,000 rpm (4,500×g) for approximately 10 min. Remove and discard supernatant. Repeat twice. d. Re-suspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5ºC. e. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spore suspension. 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.

	f. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating 100 µL aliquots of serial dilutions (e.g., 10^{-5} through 10^{-7}) using spread plating on TSA plates or another comparable validated enumeration procedure. Incubate plates for 24±2 h at 36±1ºC and determine titer. i. 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.
12.7 Preparation of Porcelain Carriers	Preparation of porcelain carriers can also be found in MB-03, Screening of Polished Stainless Steel Penicylinders, Porcelain Penicylinders, and Glass Slide Carriers Used in Disinfectant Efficacy Testing. a. Prior to use, examine porcelain carriers individually and discard those with scratches, nicks, spurs, or discolorations. b. Rinse unused carriers gently in water three times to remove loose material and drain. c. 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). d. Sterilize 20 min 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.
12.8 Inoculation of Porcelain Carriers	a. 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. b. Add 10 sterile carriers to each tube containing 10 mL spore suspension, slightly agitate, and let stand 10-15 min. c. Remove each carrier with sterile hook and place upright in a sterile Petri dish lined with two sheets of filter paper, no more than 30 carriers per Petri dish. d. Air dry in biological safety cabinet for approximately 30±2 min. Place Petri dishes containing inoculated carriers in vacuum

	desiccator (with gauge) containing $CaCl_2$ and draw vacuum of 27" (69 cm) Hg. e. Dry carriers under vacuum for 24±2 h before use in HCl resistance testing, efficacy testing or carrier counts. Maintain in a sealed desiccation unit under vacuum (27" Hg) for up to three months. i. Inoculated carriers may be used after three months (within one year) if they meet the acceptable HCl resistance and carrier count criteria. Sterilize and reuse if necessary.
12.9 Spore Enumeration (carrier counts)	a. Prior to use, determine the carrier counts for each preparation of inoculated carriers. Assay 3 to 5 randomly selected carriers per preparation. b. Place each inoculated carrier into a 50 mL plastic, polypropylene conical centrifuge tube containing 10 mL of sterile water. c. Sonicate carriers for 5 min ± 30 s. 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. d. Following sonication, vortex tubes for 2 min ± 5 s. e. Dilute spore suspensions to 10^{-3} by transferring 1 mL aliquots to tubes containing 9 mL sterile water. i. Alternatively, pool the water from the tubes with the carriers and briefly vortex. Serially dilute and plate appropriate aliquots of the pooled water (30-50 mL) and calculate the average carrier count per set. f. Plate 100 µL of the 10^0 (tube with the carrier) through the 10^{-3} dilution in duplicate using spread plating with TSA. Invert plates and incubate for 24-48 h at 36±1ºC. i. Alternatively, use 3M™ Petrifilm™ AC Plates for enumeration of the test organism. Dilute the spore suspensions through 10^{-4} and plate 1 mL aliquots on the Petrifilm. Note: Conduct a culture purity check on one dilution of

	one carrier. g. Count colonies. 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. Average spore counts per carrier should be within one log of each other.
12.10 HCl Resistance	a. 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 a tube with 2.5 M HCl using flamed hooks or forceps. Do not allow carriers or transfer device to contact inside of wall of acid tube. b. 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 s and then transfer carrier to a second tube of modified FTM. c. For viability control, place one unexposed inoculated carrier in a separate tube of modified FTM. For media sterility, use one tube of modified FTM. d. 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 (i.e., remain viable) for ≥2 minutes to be qualified as resistant test spores. Discard carriers if not resistant (i.e., inactivated) and repeat inoculation of carriers as previously described.
12.11 Efficacy Test	a. Prepare disinfectant samples according to MB-22. For a 60-carrier test, place 10 mL product at dilution recommended for use or under investigation into each of twelve 25×150 mm or 25×100 mm test tubes, or use appropriate number of tubes assuming 5 test carriers per tube of test chemical. b. 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. i. Flame hook and allow cooling after each transfer. When lowering carriers into test tube, neither carriers nor wire

	hook may touch sides of tubes. ii. 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. iii. Deposit carriers into test tubes within ± 5 s of the prescribed drop time. Return tubes to water bath immediately after adding carriers. c. 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). i. 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). ii. 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. iii. Carriers may touch interior sides of neutralizer tube during transfer, but contact should be minimized. d. After each carrier is deposited, recap neutralizer tube and gently shake to facilitate adequate mixing and efficient neutralization. e. 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. i. 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. f. Incubate primary (neutralizer) and secondary subculture tubes for 21 days at 36±1ºC. Report results as growth (+) or no growth (0). i. 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. ii. 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.

g. Media sterility controls and system controls (check for aseptic technique during carrier transfer process) are recommended.

   i. For media controls, incubate 1-3 unopened subculture medium tubes with the test sample tubes for 21 days at 36±1ºC.

   ii. For system controls, use sterile forceps or hooks to transfer 3 sterile carriers into a tube of test chemical.

   iii. 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.

   iv. 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.

   v. 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.

h. Perform presumptive identification 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 or comparable method.

   i. If there are 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. See section 12.2d. for Gram stain reaction and colony characteristics.
12.12 Neutralization Confirmation Procedure	a. Perform a neutralization confirmation test 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. b. 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. i. 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. ii. Prepare serial ten-fold dilutions of the inoculum in sterile water out to 10^{-7}. Use 100 µL aliquots of the 10^{-5}, 10^{-6} and 10^{-7} 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. iii. Determine spore titer by plating each of three dilutions in duplicate on TSA agar. Incubate plates inverted for 24-48 h at 36±1ºC. Count colonies. Report plates with colony counts over 300 as TNTC. 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.

c. Use 5 sterile porcelain carriers (only 3 to be used in the assay). Within 5 s, 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 12.11b. Allow carriers to remain in test chemical per the specified contact time and temperature.

   i. After the contact time is complete, aseptically transfer three of the five carriers individually into tubes containing the neutralizer per section 12.11c. This set of tubes is the Neutralizer/Primary Subculture treatment.

   ii. 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.

d. Following carrier transfer, inoculate each tube (Neutralizer/ Primary and Secondary Subculture treatment tubes) with 100 µL of each of three inoculum dilutions (10^{-5}, 10^{-6} and 10^{-7}).

e. For controls, use three fresh unexposed tubes of neutralizer and three tubes of the secondary subculture medium; also inoculate each control tube with 100 µL of each of three inoculum dilutions. Include one uninoculated tube of neutralizer and secondary subculture media to serve as sterility controls.

f. See Table 1 for tube inoculation scheme.

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

h. Record results as growth (+) or no growth (0). 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*

Treatment	Dilution & Tube #
Neutralizer-Primary Subculture Treatment	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Secondary Subculture Treatment (with Carrier)	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Neutralizer-Primary Inoculated Control	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3
Secondary Subculture Inoculated Control	100 µL of 10^{-5} → Tube 1 100 µL of 10^{-6} → Tube 2 100 µL of 10^{-7} → Tube 3

*Use of 10^{-5} through 10^{-7} based on an approx. starting suspension of 10^{8} spores/mL

i. Confirm a minimum of one positive per treatment and control (if available) using Gram staining and colony morphology on TSA, see section 12.2d. For each treatment and control group, conduct confirmation testing on growth from tube with fewest spores delivered.

j. 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.

k. 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.

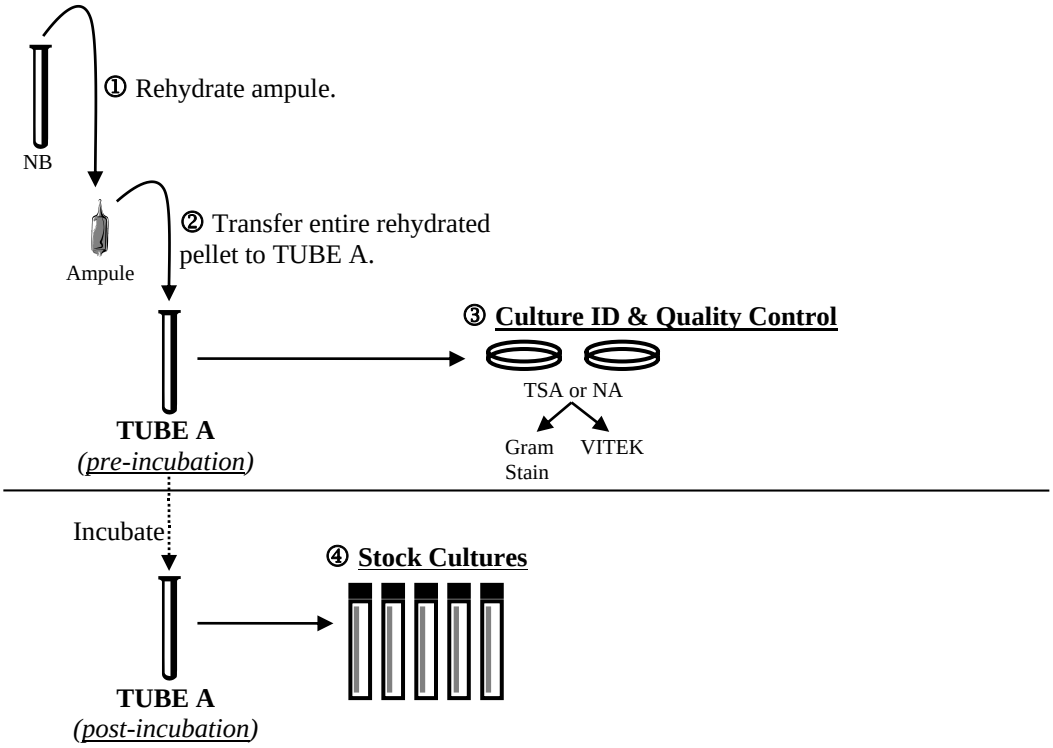
l. For a neutralizer to be deemed effective, growth must occur in the Secondary Subculture treatment tubes which received lower levels of inoculum (e.g., 5-100 CFU/mL).

m. 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. n. 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. o. 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.
13. Data Analysis/ Calculations	1. Data will be recorded on data sheets (see section 14). Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Electronic copies of the spreadsheet as well as hard copies will be retained. 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. If only one of two plates has counts yielding 300 CFU or less, forgo its use in calculations in place of counts from a subsequent dilution; if none exists, use only the countable plate for calculations (e.g., do not average the TNTC (as 300) and the countable plate). Note: Include plate counts of 0 in all calculations. 3. To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication or vortexing. Round numbers and use only two significant figures to calculate averages and CFU/carrier. 4. Calculate the average CFU/carrier for all carriers tested.
14. Forms and Data Sheets	1. Attachment 1: Culture Initiation and Stock Culture Generation Flow Chart for *B. subtilis* 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record MB-03_F1.docx

	SAT: Organism Culture Tracking Form	MB-15-04_F1.docx
	SAT: Test Microbe Confirmation Sheet (Quality Control)	MB-15-04_F2.docx
	SAT: Carrier Enumeration Form	MB-15-04_F3.docx
	SAT: Carrier Count Spreadsheet	MB-15-04_F4.xlsx
	SAT: HCl Resistance Test Data Sheet	MB-15-04_F5.docx
	SAT: Information Sheet	MB-15-04_F6.docx
	SAT: Time Recording Sheet for Carrier Transfers	MB-15-04_F7.docx
	SAT: Results Form (1-30)	MB-15-04_F8.docx
	SAT: Results Form (31-60)	MB-15-04_F9.docx
	SAT: Performance Controls Results Sheet	MB-15-04_F10.docx
	SAT: Test Microbe Confirmation Sheet	MB-15-04_F11.docx
	SAT: Neutralization Confirmation Assay Information Sheet	MB-15-04_F12.docx
	SAT: Neutralization Confirmation Assay Results Form	MB-15-04_F13.docx
	SAT: Neutralization Confirmation Assay Time Recording Sheet for Carrier Transfers	MB-15-04_F14.docx
	SAT: Neutralization Confirmation Assay Serial Dilution/Plating Tracking Form	MB-15-04_F15.docx
	SAT: Neutralization Confirmation Assay Inoculum Enumeration Form	MB-15-04_F16.docx
15. References	1. Official Methods of Analysis (Revised 2013) 21st ED., AOAC INTERNATIONAL, Method 966.04, Gaithersburg, MD, Chapter 6 2. Standard Methods for the Examination of Water and Wastewater. 23rd Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC 3. Tomasino, S.F. & Hamilton, M.A. (2006) *JAOAC* Int. 89, 1373-1397	

Attachment 1: Culture Initiation and Stock Culture Generation Flow Chart for *B. subtilis*



a. Obtain lyophilized cultures annually from ATCC. Using a tube containing 5-6 mL of NB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the pellet for *B. subtilis.*

b. Aseptically transfer the entire rehydrated pellet back into the original tube of nutrient broth designated as "TUBE A." Mix well. Use suspension in TUBE A for CULTURE ID & QUALITY CONTROL. Incubate TUBE A for *B. subtilis* for 24 h at 30±1°C.

c. Culture ID and Quality Control. Using a loopful of rehydrated suspension from TUBE A, streak for isolation on duplicate plates (NA or TSA). Incubate plates at 30±1°C for 24 h. Record results on the Test Microbe Confirmation Sheet.

d. Stock Culture Generation. Using the 24±2 h TUBE A broth culture: initiate stock cultures by streak-inoculating six NA slants. Incubate the slants at 36±1ºC for 24±2 h. Record all manipulations on the Organism Culture Tracking Form.