SOP Number	MB-21-05
Title	Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of *Bacillus subtilis* on Hard Non-porous and Porous Surfaces
Revisions Made	• Minor editorial changes for clarification purposes. • Updated Section 12.1 Culture Initiation, added instructions for rehydrating lyophilized cultures received on loops/swabs, as pellets, etc. • Updated Section 12.2 Production of *B. subtilis* spore suspension • Updated Section 13 Data Analysis/Calculations

SOP Number	MB-21-05
Title	Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of Bacillus subtilis on Hard Non-porous and Porous Surfaces
Scope	This quantitative method is used to evaluate the sporicidal efficacy of liquid disinfectants against spores of Bacillus subtilis (ATCC 19659) on hard, non-porous and porous surfaces. This SOP is based on the AOAC Method 2008.05 (see 15.1). Additional details for performing the method with spores of Clostridioides difficile are provided in Appendix 1.
Application	Data from this method are used to generate the log reduction (LR) values of spores as the quantitative measure of efficacy for liquid disinfectants on hard, non-porous and porous surfaces.

	Approval	Date
SOP Developer	LISA SMITH Digitally signed by LISA SMITH Date: 2023.06.27 09:06:12 -04'00'	06/27/23
	Print Name: Lisa Smith	
SOP Reviewer	REBECCA PINES Digitally signed by REBECCA PINES Date: 2023.06.27 09:11:05 -04'00'	06/27/23
	Print Name: Rebecca Pines	
Quality Assurance Unit	Kiran Verma Digitally signed by Kiran Verma Date: 2023.06.27 10:19:38 -04'00'	06/27/23
	Print Name: Kiran Verma	
Branch Chief	REBECCA PINES Digitally signed by REBECCA PINES Date: 2023.06.27 10:29:56 -04'00'	06/27/23
	Print Name: Rebecca Pines	

Date SOP issued:	06/27/23
Controlled copy number:	0
Date SOP withdrawn:	

TABLE OF CONTENTS

1. Definitions	Additional abbreviations/definitions are provided in the text. 1. ATCC = American Type Culture Collection
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. 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 SOP QC-19 (pipettes), EQ-02 (thermometers and hygrometers), and EQ-05 (timers) 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, document the required information on the appropriate form(s) (see section 14).
7. Interferences	1. During exposure of the carrier to the disinfectant, do not allow the pipette tip delivering the disinfectant to touch the inoculated carrier. 2. To avoid cross-contamination, it is recommended that analyst analyze chemical treatments from the most highly effective to the least effective, followed by the untreated controls. 3. Avoid touching the interior sides of the microcentrifuge tube while the carriers are being lowered into the microcentrifuge tube and the forceps are being removed. 4. Prior to testing, use the Neutralization Confirmation procedure (see section 12.8) to determine suitability of the neutralizer for the test substance.
8. Non-conforming Data	1. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports. 2. Due to the occurrence of statistical variability in the log reduction (LR) data, it is recommended that the analyst target carrier counts of 7-7.5 logs to ensure confidence in a 6 LR.
9. Data Management	Archive data consistent with SOP ADM-03, Records and Archives.
10. Cautions	To ensure the stability of a diluted sporicidal agent, prepare the diluted product within three hours of the sporicidal treatment step unless specified otherwise.

11. Special Apparatus and Materials	1. Media. a. *Nutrient broth (NB) (dehydrated)*. For use in rehydrating test organism and preparing nutrient agar. b. *Nutrient agar (NA)*. For stock cultures slants. Add 1.5% (w/v) Bacto-agar to unsterilized NB. Boil mixture until agar is dissolved. If necessary, adjust pH to 7.2±0.2. Dispense 5 mL portions into 16×100 mm screw-cap tubes. Larger tubes may also be used with a proportional amount of agar/tube. Sterilize tubes by autoclaving for 20 min at 121°C. Remove from autoclave and slant tubes to form agar slopes. Dehydrated NA may be substituted; suspend 23 g NA/L water and dissolve by boiling. If necessary, adjust pH to 6.8±0.2. Steam sterilize for 15 min at 121°C. c. *Nutrient agar with 5 µg/mL manganese sulfate monohydrate (amended NA)*. For spore production. Suspend 11.5 g NA in 495 mL water and add 5 mL 500 ppm $MnSO_4 \cdot H_2O$. Dissolve by boiling. If necessary, adjust pH to 6.8±0.2. Steam sterilize for 15 min at 121°C. Pour agar into plates. d. *Trypticase soy agar (TSA)*. Poured in plates for microbe isolation and spread plating. e. *Luria-Bertani (LB) broth*. Dehydrated; suspend 25 g in 1 L water, mix well; if necessary, adjust pH to 7.0±0.2, dispense in bottles, and sterilize by autoclaving for 15 min at 121°C; use as neutralizer. f. *Modified Luria-Bertani broth*. Neutralizer for HCl resistance test. Add 20 mL 1 M NaOH to 1 L LB broth, mix well, dispense in bottles, and steam sterilize for 15 min at 121°C. g. *Luria-Bertani broth with 0.1% (w/v) sodium thiosulfate*. Neutralizer for sodium hypochlorite treatments. Add 1.0 g sodium thiosulfate to 1 L LB broth, mix well, dispense in bottles, and steam sterilize for 15 min at 121°C. 2. Reagents. a. *Manganese sulfate monohydrate (500 ppm)*. Add 0.25 g manganese sulfate to 500 mL water. Filter sterilize for use. b. *Sodium thiosulfate*. c. *Water*. Sterile, reagent grade. Either de-ionized distilled water or water with equivalent quality for making reagent solutions and culture media. d. *2.5 M hydrochloric acid*, e.g., certified HCl.

e. *Ethyl alcohol*. 40 and 95%.

3. Test organism.

   a. *Bacillus subtilis* (ATCC No. 19659) obtained directly from a commercial supplier.

4. Apparatus.

   a. *Hard non-porous carriers.*

      i. *Glass coupon*. 5×5×1 mm (borosilicate glass), single use.

      ii. *Stainless steel coupon*. Stainless steel (e.g., type 304) cut into 5×5×1 mm, single use.

   b. *Hard porous carriers.*

      i. *Ceramic tile*. Ceramic tile (unglazed porcelain) cut into 5×5×1 mm, use unglazed side for inoculation, single use.

      ii. *Untreated pine wood*. Non-treated select pine, No. 1 (Home Depot) cut into 5×5×1 mm, single use.

   c. *Microcentrifuge tubes*. Sterile, 1.5 mL.

   d. *Centrifuge tubes*. Sterile, polypropylene, 15 mL conical tubes with conical bottoms.

   e. *Dissecting forceps*.

   f. *Micropipettes*. Calibrated.

   g. *Positive displacement pipette*.

   h. *Desiccator*.

   i. *Water bath/chiller unit*. Constant temperature, capable of maintaining 20±1°C temperature or specified temperature.

   j. *Orbital shaker*.

   k. *Microcentrifuge*.

   l. *Microcentrifuge tube lid openers*.

   m. *Sonicator* (ultrasonic cleaner).

   n. *Floating microcentrifuge tube holder*. To hold the Fraction A tubes in a fixed upright position during sonication.

   o. *Hematology rotator*, or a suitable mixer/shaker to provide gentle agitation during incubation.

   p. *Vortex mixer*.

	q. *Vortex adapters*. r. *Certified timer*. Any certified timer that can display time in seconds. s. *Test tubes*. 25×150 mm. t. *Microscope*. With 10X eyepiece and 40X and 100X (oil) objectives with phase contrast option.
12. Procedure and Analysis	1. For non-porous and porous carriers, use the Three Step Method (TSM) described in section 12.6. 2. For non-porous carriers (glass and stainless steel), fractions B and C may be consolidated into fraction BC using the modified Three Step Method (mTSM) procedure outlined in section 12.7. 3. Use Three Step Method Processing Sheet (see section 14) to track testing activities. 4. Conduct the testing (e.g., addition of soil load) as specified by the study sponsor.
12.1 Culture Initiation	a. Initiate new stock cultures from lyophilized cultures of *Bacillus subtilis* (ATCC 19659) from a reputable supplier within 18 months. b. *Rehydrate lyophilized culture:* i. For lyophilized cultures received as ampules, open ampule of freeze-dried organism as indicated by the manufacturer. Using a tube containing 5-6 mL of NB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth designated as "TUBE A." Mix well. ii. <u>For lyophilized cultures received on loops/swabs (e.g., KWIK-STIK), as pellets, etc. ., open and rehydrate the lyophilized organism as indicated by the manufacturer. Transfer the rehydrated culture to a tube of 5-6 mL NB (e.g., swish inoculated swab/loop in the NB tube or transfer the rehydrated pellet to the NB tube); NB tube is designated as "TUBE A".[1]</u> c. <u>Incubate NB culture (TUBE A) at 36±1°C for 24±2 h at 150 rpm in an orbital shaker. Record all manipulations on the Organism Tracking Form.</u>

[1]Step not contained in the AOAC standard method 2008.05.

	d. Following incubation, streak inoculate a set (e.g., 6 or more) of NA slants using the NB culture (TUBE A)and incubate 24±2 h at 36±1°C. i. For QC purposes, perform a streak isolation of the 24±2 h broth culture on a TSA plate. Incubate all plates at 36±1°C for 24±2 h. e. Following incubation, store NA slants at 2-5°C. Maintain stock culture on NA slants by monthly transfers (i.e., within 30 days). f. Record monthly transfers on the appropriate organism tracking and confirmations sheets.
12.2 Production of *B. subtilis* spore suspension	a. Using growth from a stock culture tube, inoculate 10 mL tubes (e.g., 2 tubes, depending on the amount of spore preparation desired) of NB and incubate tubes 24±2 h on an orbital shaker at approximately 150 rpm at 36±1°C. b. Use this culture to inoculate amended NA plates (8-10). Inoculate each plate with 500 µL broth culture and spread inoculum with sterile bent glass rod or suitable spreading device. i. For QC purposes, perform a streak isolation of the 24±2 h broth culture on a TSA plate. Incubate all plates at 36±1°C for 24±2 h. c. Wrap each plate with parafilm or place in sterile plastic bags. Incubate plates inverted for 12-14 days at 36±1°C. Spore quality checks can be performed throughout duration of incubation to determine spore growth progression (see section 12.2 h). d. Following incubation, harvest the spores by adding 10 mL cold (2-5°C) 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 (e.g., 10 plates = 14 tubes, ~10 mL each). e. Centrifuge tubes at 5,000 rpm (4,500×g) for approximately 10 min at room temperature. f. Remove and discard supernatant. Resuspend pellet in each tube with 10 mL cold sterile water and centrifuge at 5,000 rpm (4,500×g) for 10±1 min. g. Remove and discard supernatant. Repeat twice. Resuspend the pellet in each tube with 10 mL sterile water. Store the spore suspension at 2-5°C. h. Examine spore suspension with a phase contrast microscope or by staining to assess quality of the spores. Examine a minimum of 5

	fields and determine ratio of spores to vegetative cells (or sporangia). Spores versus vegetative cells should be at least 95%. Record results on the spore suspension preparation sheet. i. Spore suspension harvested from multiple plates can be combined and re-aliquoted into tubes for uniformity. j. Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 10^{-6}-10^{-8}) onto TSA. Incubate plates for 24±2 h at 36±1°C, count colonies after incubation, and determine titer. Note: When harvested and processed, 10 plates of amended NA should provide 80-100 mL concentrated spore suspension. Diluting the suspension prior to carrier inoculation will be necessary; a spore titer of approximately 1.0×10^9 CFU/mL in the suspension should be adequate to achieve the target carrier count.
12.3 Carrier preparation	a. Visually screen glass carriers for scratches, chips, or cracks. Discard those which are damaged or defective. Ensure pine wood carriers are free of sawdust after cutting; no rinsing is required. b. Rinse glass, stainless steel, and ceramic wall tile carriers once with water, 3 times with 95% ethyl alcohol, and finally 3 times with water. Allow carriers to dry. Place in glass tubes (25×150 mm), 40 carriers per tube. c. Steam sterilize all carrier types 45 min at 121°C with a 30 min dry cycle or sterilize for 2 h in hot air oven at 180°C. Cool. Transfer carriers to sterile plastic Petri dishes for inoculation (approximately 40 carriers per dish).
12.4 Carrier inoculation	a. Transfer 10 µL spore suspension with a micropipette using aerosol barrier tips or positive displacement pipette onto a 5×5×1 mm sterile carrier coupon. Apply to one central spot on each carrier. During carrier inoculation, mix inoculum frequently in Vortex mixer to ensure uniform distribution of spores. b. Allow carriers to dry for minimum of 1 h in open Petri dish in a biosafety cabinet, then for a minimum of 12±2 h in a desiccator. Store inoculated carriers under desiccation for up to 30 days. c. Inoculated carriers must be discarded after use. d. Verify carrier counts (per the method for control carriers) prior to testing; the mean log density for control counts must be 7-7.5 logs spores/carrier.

12.5	Test chemical (e.g., sporicide, disinfectant) sample preparation	a. Aseptically prepare test chemical samples as directed (refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances). b. Place approximately 1.5 mL of each test chemical or control (sterile water) in microcentrifuge tubes. Allow to equilibrate to appropriate temperature, for approximately 15-30 min.
12.6	Test procedure for non-porous and porous carriers	a. A minimum of 3 carriers per test chemical and 3 carriers for the water control (control carriers) are required per product test. Use one pair of sterile forceps per fraction for each test chemical. Fractions may be refrigerated briefly to allow for processing of other fractions. It is recommended that 2 analysts perform this method so that dilution and plating of the multiple fractions may be conducted as soon as possible. b. Using sterile forceps, carefully transfer one inoculated carrier into each microcentrifuge tube labeled Fraction A. Avoid touching inoculated area of carrier and sides of microcentrifuge tube. Discard carrier and tube if carrier touches sides of tube. c. Place Fraction A tubes containing carriers and tubes containing test chemical(s) and sterile water (control) into chiller water bath at 20±1°C or use a labtop cooler to maintain temperature of the tubes. Equilibrate approximately 10 min. d. Add 400 µL test chemical (test carriers) or 400 µL sterile water (control carriers) at 15 or 30±5 s intervals to appropriate microcentrifuge tube (in triplicate). Allow contact of the carriers to the test chemical or water in Fraction A tubes for the appropriate exposure period. e. Following the exposure period, add 600 µL of appropriate ice-cold neutralizer (e.g., LB broth) to each test chemical Fraction A tube. Add 600 µL LB broth as neutralizer for water control Fraction A tubes. Slightly agitate tubes to thoroughly mix liquid components. f. Transfer each carrier using one pair of sterile forceps per carrier set (i.e., 3 carriers) from Fraction A tube to corresponding Fraction B tube. Fraction B tubes contain 400 µL ice-cold (0-5°C) sterile water. To prevent contamination, the use of a microcentrifuge tube cap opener is recommended. g. Place Fraction A tubes in microcentrifuge, centrifuge for 6 min ± 30 s at 13,000 rpm (15,500×g). h. Remove 900 µL from each tube without disturbing pellet. Discard

	supernatant. Carefully add 900 µL ice-cold LB broth to each tube. Repeat 2 additional times. i. After third centrifugation, remove 900 µL from each tube. Carefully add 100 µL ice-cold LB broth to each Fraction A tube and resuspend pellet by mixing in a Vortex mixer 5 min ± 30 s (use the Vortex adapter) at midrange speed. j. Add 800 µL ice-cold LB broth to each Fraction A tube. Proceed to dilution and plating if another analyst is available, or store Fraction A tubes in refrigerator. Note: Fluid remaining in the Fraction A tubes contains spores dislodged from carrier by exposure to the test chemical or water control. Consistent orientation of the microcentrifuge tubes in the microcentrifuge is important in locating the pellet. The pellet may range in size and be difficult to visualize depending on the treatment. Fractions B and C tubes can be evaluated while Fraction A tubes are being centrifuged. k. Sonicate Fraction B tubes 5 min ± 30 s using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner. l. After sonication is complete, add 600 µL ice-cold LB broth to Fraction B tubes. Mix on a Vortex mixer approximately 1 min. Transfer each carrier using one pair of sterile forceps per carrier set from Fraction B tube to corresponding Fraction C tube (Fraction C tubes contain 400 µL ice-cold LB broth). Proceed to dilution and plating if another analyst is available, or store Fraction B tubes at 2-5°C; storage should be limited to 2 h. Note: Fluid remaining in the Fraction B tubes contains spores dislodged from the carrier by sonication. m. Place Fraction C tubes in a hematology rotator inside incubator for 30±2 min at 36±1°C. n. Remove Fraction C tubes after 30±2 min rotation/incubation from incubator. Add 600 µL ice-cold LB broth to each tube. The carriers remain in the Fraction C tubes. Proceed to dilution and plating if another analyst is available, or store Fraction C tubes at 2-5°C; storage should be limited to 2 h. Note: Fluid remaining in Fraction C tubes contains spores dislodged from the carrier by gentle agitation for 30 min. o. Vortex each microcentrifuge tube thoroughly prior to making dilutions.

	p. For each fraction and control tube, serially dilute from the 10^0 dilution by transferring 100 µL into 900 µL ice-cold LB broth in a dilution tube. At a minimum for treated carriers, prepare at least one 10-fold dilution for each fraction. q. For each carrier, direct plate 100 µL of the sufficient dilutions onto TSA to ensure obtaining counts within the target range of 30-300 CFU/plate. i. For non-porous control carriers: the 10^{-3} and 10^{-4} dilutions for Fractions A and B, and 10^{-2} and 10^{-3} for Fraction C should result in countable plates. ii. For porous control carriers: the 10^{-2} and 10^{-3} dilutions for Fraction A, 10^{-3} and 10^{-4} dilutions for Fraction B, and 10^{-2} and 10^{-3} for Fraction C should result in countable plates. iii. For non-porous control carriers using the mTSM: the 10^{-3} and 10^{-4} dilutions for both Fractions A and BC should result in countable plates. r. Incubate plates a minimum of 24±2 h at 36±1°C. Record control counts at 24±2 h. Record treated carrier counts at 24±2 and at 48±2 h. s. Count colonies and record results. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data. For colony counts in excess of 300, record as Too Numerous to Count (TNTC). If no colonies are present, record as zero. t. Confirm the identity of a minimum of one representative colony taken from at least one plate per treatment level (if available) using Gram staining, general growth media (e.g., TSA), or other confirmative procedure (e.g., VITEK 2 Compact). *B. subtilis* is a large Gram-positive rod. On general growth media, *B. subtilis* colonies are opaque, rough, round, low convex colonies with irregular margins. u. After plating, dilution tubes may be stored at 2-5°C until the results are recorded; the tubes may be used for additional plating if initial plate counts are beyond the recommended target range.
12.7 Alternative test procedure (modified Three Step	a. Process Fraction A as in 12.6, b-j. b. Transfer each carrier using one pair of sterile forceps per carrier set (i.e., three carriers) from Fraction A tube to corresponding Fraction BC tube. Fraction BC tubes contain 400 µL ice-cold (0-5°C) sterile

	Method) for non-porous carriers	water. Fraction BC tubes can be processed while Fraction A tubes are centrifuged. c. Sonicate Fraction BC tubes for 5 min ± 30 s using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner. d. After sonication is complete, mix on a Vortex mixer approximately 1 min. e. Place Fraction BC tubes in a hematology rotator inside an incubator for 30±2 min at 36±1°C. f. Remove Fraction BC tubes from the incubator after 30±2 min rotation/ incubation. Add 600 µL ice-cold LB broth to each tube. The carriers remain in the Fraction BC tubes. Proceed to dilution and plating if another analyst is available, or store Fraction BC tubes at 2-5°C; storage should be limited to 2 h. Note: Fluid remaining in Fraction BC tubes contains spores dislodged from the carrier by sonication, vortexing, and gentle agitation for 30 min. g. Vortex each microcentrifuge tube thoroughly prior to making dilutions. h. Prepare serial dilutions and plate as in 12.6, p-q.
12.8	Neutralization confirmation	a. For this assay, produce a *B. subtilis* spore preparation according to the procedure for amended NA (refer to section 12.2). Harvest growth from plates (e.g., 5 plates) per the method, except resuspend pellet after final centrifugation step in approximately 100 mL aqueous (40%) ethanol. b. Use 12 microcentrifuge tubes. Add 400 µL sterile water to tubes 1-6 and 400 µL test chemical to tubes 7-12. Allow tubes to equilibrate approximately 10 min at 20±1°C (or other specified temperature). c. Add 600 µL neutralizer in ice-cold LB broth (or only LB broth depending on the product) to tubes 4-6 (neutralizer controls). Add 600 µL neutralizer in ice-cold LB broth to tubes 7-9 (ability of neutralizer to inactivate the test chemical). Gently mix. d. Add 10 µL *B. subtilis* spore suspension (approximately 10^9 spores/mL) to each tube and vortex for approximately 15 s. e. Incubate tubes for 30±2 min at 20±1°C (or temperature specified by test chemical manufacturer). f. After incubation, add 600 µL ice-cold LB broth to tubes 1-3 (survival controls). Add 600 µL ice-cold LB broth to tubes 10-12 (test

	chemical controls). g. Serially dilute each tube (e.g., 10 µL into 990 µL ice-cold LB broth or 100 µL into 900 µL ice-cold LB broth) to achieve plate counts of 30-300 CFU/plate. h. Plate 100 µL of each dilution onto TSA. Incubate 24±2 h at 36±1°C. Count colonies on each plate. i. LD (CFU/mL) in tubes 1-3 and 4-6 should reflect the original spore suspension titer and should be within 1 log of each other. If the difference in LD between tubes 1-3 and 4-6 is greater than 1 log, then the neutralizer has a sporicidal effect. If the test chemical is highly effective, LD in tubes 10-12 should be approximately 5-6 logs lower than LD in tubes 1-6. j. To be an effective neutralizer, LD in tubes 7-9 should be within 1 log of the LD in tubes 1-6.
12.9 HCl resistance.	a. Perform on each preparation of inoculated carriers. Conduct TSM procedure with 2.5 M HCl. b. Follow procedure as specified in section 12.6 or 12.7 with 2 and 5 min exposure periods with 3 inoculated carriers per time period. c. Include 3 control (sterile water) carriers to determine control carrier counts. Use LB broth modified with NaOH as the neutralizer (instead of LB broth) for HCl treatments. Perform test at 20±1°C. d. Calculate LR. Spores should resist HCl for ≥ 2 min (i.e., based on presence of viable spores after 2 min) to be qualified as resistant test spores. Discard carriers if not resistant and repeat preparation of carriers as previously described. Note: Compared to the water control, anticipate LR of 0-3 at 2 min exposure and LR of 2-6 following the 5 min exposure.
13. Data Analysis/ Calculations	1. Use counts which fall within 0-300 CFU/plate for calculation of spore titer. 2. Per test, use colony counts to determine log reductions. 3. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with two significant figures (e.g., round up to the nearest tenth). 4. Obtain the total number of spores per fraction by dividing the number of colonies counted in each fraction by its dilution, and account for volume plated. Calculate the Colony Forming Units (CFU)/carrier using the

	following equation: $\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(D_i)}\right] \div 0.1\right\}$ where: Y = CFU per plate, 0.1 = volume plated (0.1 mL), D = 10^{-k}, k = dilution, n = number of dilutions, and i = lower limit of summation (the fewest number of dilutions). 5. a. When TNTC values are observed for each dilution plated, substitute 300 for the TNTC at the highest (most dilute) dilution and scale up accordingly for the calculations. b. When zeroes are observed for each dilution plated, substitute 0.5 for the zero at the lowest (least dilute) dilution and scale up accordingly for the calculations. 6. Obtain the total number of spores per carrier by adding the total number of viable spores per fraction for Fractions A-C. For the modified Three Step Method, obtain the total number of spores per carrier by adding the total number of viable spores per fraction for Fractions A and BC. 7. Calculate the $\log_{10}$ density (LD) of each carrier by taking the $\log_{10}$ of the spores/carrier. 8. Calculate the mean $\log_{10}$ density across treated carriers. 9. Calculate the mean $\log_{10}$ density across control carriers. 10. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = mean $\log_{10}$ control – mean $\log_{10}$ treated c. If no spores are recovered for any treated carrier, report the LR as greater than or equal to the mean $\log_{10}$ density for the control carriers.
14. Forms and Data Sheets	1. Appendix 1: Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of *Clostridioides difficile* on Hard Non-porous and Porous Surfaces 2. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Three Step Method: Test Information Sheet MB-21-05_F1.docx

	Three Step Method: Time Recording Sheet	MB-21-05_F2.docx
	Three Step Method: Serial Dilution/Plating Tracking Form	MB-21-05_F3.docx
	Three Step Method: Results Sheet	MB-21-05_F4.docx
	Three Step Method Neutralization Test: Test Information Sheet	MB-21-05_F5.docx
	Three Step Method Neutralization Test: Time Recording Sheet	MB-21-05_F6.docx
	Three Step Method Neutralization Test: Serial Dilution/Plating Tracking Form	MB-21-05_F7.docx
	Three Step Method Neutralization Test: Results Sheet	MB-21-05_F8.docx
	Test Microbe Confirmation Sheet	MB-21-05_F9.docx
	Three Step Method Processing Sheet	MB-21-05_F10.docx
	Three Step Method Processing Sheet (mTSM)	MB-21-05_F11.docx
	Three Step Method Spreadsheet (3 Fractions)	MB-21-05_F12.xlsx
	Three Step Method Spreadsheet (2 Fractions)	MB-21-05_F13.xlsx
15. References	1. Official Methods of Analysis of AOAC INTERNATIONAL (Revised 2013) 18th Ed., AOAC International, Gaithersburg, MD. Method 2008.05. 2. Tomasino, S. et. al., "Determining the Efficacy of Liquid Sporicides Against Spores of *Bacillus subtilis* on a Hard Nonporous Surface Using the Quantitative Three Step Method: Collaborative Study." Journal of AOAC International Vol. 91, No. 4, 2008. 3. Tomasino, S. et. al., "Use of Alternate Carrier Materials in AOAC *Official Method*SM 2008.05, Efficacy of Liquid Sporicides Against Spores of Bacillus subtilis on a Hard, Nonporous Surface, Quantitative Three-Step Method." Journal of AOAC International Vol.93, No.1, 2010. 4. Rastogi, V. et. al., "Modified AOAC Three Step Method (*Official Method 2008.05*): Consolidation of Fractions B and C." Journal of AOAC International Vol.96, No.5, 2013.	

Appendix 1 – Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of *Clostridioides difficile* on Hard Non-porous Surfaces. The following steps are used when conducting efficacy evaluation of products with sporicidal claims against spores of *C. difficile* (ATCC 43598):

Spores of *Clostridioides difficile* are prepared according to MLB SOP MB-28.

Refer to MB-21 and substitute the following based on MB-28:

Section 7: Incubate the test organism (*C. difficile* ATCC 43598) under strict anaerobic conditions. The presence of oxygen will severely compromise the viability and growth of *C. difficile.*

Section 11.1: The recovery medium for *C. difficile* is brain-heart infusion agar with yeast extract, horse blood and sodium taurocholate (BHIY-HT).

Section 11.2: *Phosphate buffered saline (PBS) containing 0.1% Tween 80 (PBS-T).* Prepare per MB-28. Used for control carriers, Fraction B tubes, and serial dilutions.

Section 11.4: Use an anaerobic chamber and anaerobic incubator (supported by a compressed gas mixture containing at least 5% hydrogen with the balance comprising any inert gas such as CO_2, N_2, or Ar) to provide an anaerobic environment. Use sterile 1.5-mL low-retention (siliconized) microcentrifuge tubes for preparing dilutions.

Section 12.6, d: Add 400 µL PBS-T to all control carriers in place of sterile water.

Section 12.6, f: Fraction B tubes contain 400 µL ice-cold (0-5°C) PBS-T in place of sterile water.

Section 12.6, p: For each treated and control tube, serially dilute in 900 µL ice-cold PBS-T in place of LB broth.

Section 12.6, q: Use pre-reduced BHIY-HT recovery medium. Open each sealed package inside the BSC just prior to direct plating. Place BHIY-HT plates with under anaerobic conditions within 60 min of opening the sealed package of plates.

Section 12.6, r: Incubate BHIY-HT from control carriers and treated carriers at 36±1°C for 120±4 h. Record results as CFU per carrier after 120±4 h of incubation for control and treated carriers.

Section 12.6, s: See Table 2 for growth/diagnostic characteristics of *C. difficile* (ATCC 43598). Growth from a typical colony from one or two plates will be processed either for spore staining or to observe under phase contrast microscopy or using Vitek.

Section 12.7, b: Fraction BC tubes contain 400 µL ice-cold (0-5°C) PBS-T in place of sterile water.

Section 12.8, b: Add 400 µL PBS-T to tubes 1-6, in place of sterile water.

Section 12.8, g: Serially dilute in 900 µL ice-cold PBS-T in place of sterile water.

Section 12.8, h: Use pre-reduced BHIY-HT recovery medium. Open each sealed package inside the BSC just prior to plating. Place BHIY-HT plates under anaerobic conditions within 60 min of opening the package of plates. Incubate BHIY-HT from control carriers and treated carriers at 36±1°C for 120±4 h. Record results as CFU per carrier after 120±4 h of incubation for control and treated carriers.

Table 2. Growth/Microscopic Characteristics of *C. difficile* (ATCC 43598)

Typical Diagnostic Characteristics	
BHIY-HT plate	Growth circular, entire edge, convex, smooth and grey colonies*
Phase-contrast microscopy	Spores appear bright and ovular while vegetative cells appear dark and rod-shaped.
Spore staining	Spores appear green while vegetative cells appear red.

*After 120±4 h