US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for
Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of Bacillus subtilis on a Hard Nonporous Surface

SOP Number: MB-21-00

Date Revised: 12-23-08

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

Standard Operating Procedure
For

Quantitative Three Step Method for Measuring the Efficacy of Liquid Sporicides against Spores of *Bacillus subtilis* on a Hard Nonporous Surface

SOP Number: MB-21-00

Date Revised: 12-23-08

Initiated By: _____ Date: ___/___/___

Print Name:_____

Technical Review: _____ Date: ___/___/___

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Technical Staff

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QA Officer

Approved By: _____ Date: ___/___/___

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Branch Chief

Effective Date: ___/___/___

Controlled Copy No.: _____

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the methodology for determining the sporicidal activity of liquid formulations against spores of *Bacillus subtilis* on a hard nonporous surface using the AOAC Method 2008.05.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 ATCC = American Type Culture Collection

2.3 CFU = Colony forming unit

2.4 TNTC = Too Numerous to Count

2.5 LB Broth = Luria-Bertani Broth

2.6 NA = Nutrient agar

2.7 NB = Nutrient broth

2.8 Amended NA = nutrient agar with 5 µg/mL $MnSO_4 \cdot H_2O$

2.9 Fraction A = Component from tubes that are exposed to disinfectant/control and are centrifuged

2.10 Fraction B = Component from tubes that are sonicated

2.11 Fraction C = Component from tubes that are rotated in the incubator

2.12 LD = Log_{10} density

2.13 LR = Log_{10} reduction

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, Lab Biosafety.

3.2 Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, phenol, etc. Also, hydrochloric acid (HCl) is

a highly corrosive liquid and considered hazardous. Personal protective clothing or devices are recommended during the handling of these items for the purpose of activation, dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products. The study analyst may wish to consult the Material Safety Data Sheet for the specific product/active ingredient to determine the best course of action.

4.0 CAUTIONS:

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

4.2 To ensure the stability of a diluted sporicidal agent, prepare the dilutions within three hours of the sporicidal treatment step unless specified otherwise.

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

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

4.5 Neutralization Confirmation, see section 10.7 for the procedure to determine suitability of the neutralizer for the test substance.

5.0 INTERFERENCES

5.1 Sporicidal products may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Personal protective equipment (PPE) or devices are recommended during the handling of these items.

5.2 To avoid cross-contamination, it is recommended that analyst should analyze chemical treatments from the most highly effective to the least effective, followed by the controls.

5.3 Touching the interior sides of the microcentrifuge tube should be avoided while the carriers are being lowered into the microcentrifuge tube and the forceps are being removed.

6.0 PERSONNEL QUALIFICATIONS:

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

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

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Culture media.

7.1.1 *Nutrient broth* (dehydrated). For use in rehydrating test organism and preparing nutrient agar.

7.1.2 *Nutrient agar*. 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 be used as well. Autoclave 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. Autoclave for 15 min at 121°C.

7.1.3 *Nutrient agar (NA) 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. Autoclave for 15 min at 121°C. Pour agar into plates.

7.1.4 *Trypticase soy agar*. Poured in plates for microbe isolation and spread plating.

7.1.5 *Luria-Bertani (LB) broth*. Dehydrated; suspend 25g in 1 L water, mix well, if necessary adjust pH to 7.0 ± 0.2, dispense in bottles, and autoclave for 15 min at 121°C; use as neutralizer.

7.1.6 *Modified Luria-Bertani broth*. Neutralizer in HCl resistance test. Add 20 mL 1 M NaOH to 1 L LB broth, mix well, dispense in bottles, and autoclave for 15 min at 121°C.

7.1.7 *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 autoclave for 15 min at 121°C.

7.2 Reagents.

7.2.1 *Manganese sulfate monohydrate (500 ppm)*. Add 0.25 g manganese sulfate to 500 mL water. Filter sterilize for use.

7.2.2 *Sodium thiosulfate*. Neutralizing agent for sodium hypochlorite.

7.2.3 *Water*. Sterile, reagent grade, 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.

7.2.4 *2.5 M hydrochloric acid*, e.g., certified HCl.

7.2.5 *Ethyl alcohol*. 40 and 95%.

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

7.4 Apparatus.

7.4.1 *Glass coupon*. Hard surface carrier, 5 × 5 × 1 mm (Erie Scientific Co.); custom order Part No. EPA-1101 (minimum order of 1000 pieces), single use.

7.4.2 *Microcentrifuge tubes*. Sterile, 1.5 mL.

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

7.4.4 *Dissecting forceps*.

7.4.5 *Micropipette*. Calibrated.

7.4.6 *Positive displacement pipette*.

7.4.7 *Desiccator*.

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

7.4.9 *Orbital shaker*.

7.4.10 *Microcentrifuge*.

7.4.11 *Microcentrifuge tube lid openers*.

7.4.12 *Sonicator* (ultrasonic cleaner).

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

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

7.4.15 *Vortex mixer*.

7.4.16 *Vortex adapters*.

7.4.17 *Certified timer*. Any certified timer that can display time in seconds.

7.4.18 *Test tubes*. 25× 150 mm.

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the pipette verification SOP (QC-19) and the laboratory equipment SOPs for details on method and frequency of calibration.

8.2 For the inoculation of glass carriers (coupon), the verification of micropipettes is required (refer to SOP QC-19).

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to the manufacturer's recommendations if stipulated, or at room temperature. Those disinfectants requiring activation or dilution prior to use are activated or diluted within three hours of testing unless test parameter specify otherwise.

9.2 Follow chain-of custody guidelines during testing as stipulated in SOP COC-01, Chain of Custody.

10.0 PROCEDURE AND ANALYSIS:

10.1 Culture Initiation.

10.1.1 Initiate *B. subtilis* culture (e.g., use NB to rehydrate a lyophilized culture, and incubate broth culture for 24±2 h at 36±1°C prior to streak inoculation).

10.1.2 Streak inoculate a set (e.g., 6) NA slants and incubate 24±2 h at 36±1°C. Perform purity and identification confirmation testing for QC (e.g., colony morphology on TSA, Gram stain, or use of other identification systems).

10.1.3 Following incubation, store at 2-5°C. Maintain stock culture on NA 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 NB and incubate tubes 24±2 h on an orbital shaker at approximately 150 rpm at 36±1°C.

10.2.2 Use this culture to inoculate amended NA plates. Inoculate each plate with 500 µL broth culture and spread inoculum with sterile bent glass rod or suitable spreading device.

10.2.3 Wrap each plate with parafilm or place in plastic bags. Incubate plates inverted for 12-14 days at 36±1°C.

10.2.4 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).

10.2.5 Centrifuge tubes at 5,000 rpm (4,500 × g) for approximately 10 min at room temperature.

10.2.6 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.

10.2.7 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.

10.2.8 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.

10.2.9 Spore suspension harvested from multiple plates can be combined and re-aliquoted into tubes for uniformity.

10.2.10 Prior to inoculation of carriers, determine spore titer of the concentrated spore suspension by plating serial dilutions (e.g., 1.0×10^{-6}–1.0×10^{-8}) onto TSA. Incubate plates for 24±2 h at 36±1°C 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.

10.3 Carrier preparation.

10.3.1 Visually screen glass coupons (carriers) for scratches, chips, or cracks. Discard those which are damaged or defective.

10.3.2 Rinse 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.

10.3.3 Steam sterilize 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).

10.4 Carrier inoculation.

10.4.1 Transfer 10 µL spore suspension with a micropipette using aerosol barrier tips or positive displacement pipette onto a 5 × 5 × 1 mm sterile, dry glass coupon. Apply to one central spot on each carrier. During carrier inoculation, mix inoculum frequently in Vortex mixer to ensure uniform distribution of spores.

10.4.2 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.

10.4.3 Inoculated glass carriers must be discarded after use.

10.4.4 Verify carrier counts (per the method for control carriers) prior to testing; mean counts must be 5.0×10^6 to 5.0×10^7 spores/carrier.

Note: 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.

10.5 Test chemical (e.g., sporicide, disinfectant) sample preparation.

10.5.1 Aseptically prepare test chemical samples as directed (refer to SOP MB-22, Disinfectant Preparation).

10.5.2 Place approximately 1.5 mL of each test chemical or control (sterile water) in microcentrifuge tubes. Allow to equilibrate to appropriate temperature, for approximately 10 min.

10.6 Test Procedure.

10.6.1 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.

10.6.2 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.

10.6.3 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.

10.6.4 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.

10.6.5 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.

10.6.6 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.

10.6.7 Place Fraction A tubes in microcentrifuge, centrifuge for 6 min ± 30 s at 13,000 rpm (15,500 × g).

10.6.8 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.

10.6.9 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.

10.6.10 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.

10.6.11 Sonicate Fraction B tubes 5 min ± 30 s using a floating microcentrifuge tube holder placed inside an ultrasonic cleaner.

10.6.12 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.

10.6.13 Place Fraction C tubes in a hematology rotator inside incubator for 30±2 min at 36±1°C.

10.6.14 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.

10.6.15 Mix on a Vortex mixer each microcentrifuge tube thoroughly prior to making dilutions.

10.6.16 For each fraction and control tube, remove 100 µL and serially dilute 10-fold in 900 µL ice-cold LB broth.

10.6.17 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.

10.6.18 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.

10.6.19 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.

10.6.20 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.

10.7 Neutralization confirmation.

10.7.1 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).

10.7.2 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.

10.7.3 Add 10 µL *B. subtilis* spore suspension (approximately 10^9 spores/mL) to each tube and mix in a Vortex mixer for approximately15 s.

10.7.4 Incubate tubes for 30±2 min at 20±1°C (or temperature specified by test chemical manufacturer).

10.7.5 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).

10.7.6 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.

10.7.7 Plate 100 µL of each dilution onto TSA. Incubate 24±2 h at 36±1°C. Count colonies on each plate.

10.7.8 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.

10.7.9 To be an effective neutralizer, LD in tubes 7–9 should be within 1 log of the LD in tubes 1–6. For this assay, produce a spore preparation according to the procedure for amended NA (refer to section 10.2). 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.

10.8 HCl resistance.

10.8.1 Perform on each preparation of inoculated carriers. Conduct TSM procedure with 2.5 M HCl.

10.8.2 Follow procedure as specified in section 10.6 with 2 and 5 min exposure periods with 3 inoculated carriers per time period.

10.8.3 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.

10.8.4 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.

11.0 DATA ANALYSIS/CALCULATIONS:

11.1 Use counts which fall within 0–300 CFU/plate for calculation of spore titer.

11.2 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.

11.3 Obtain the total number of spores per carrier by adding the total number of viable spores per fraction for Fractions A–C.

11.4 Determine LD of total number of viable spores per carrier by taking $\log_{10}$(total

number of spores per carrier).

11.5 Determine LR by subtracting the mean LD of test carriers from the mean LD of control carriers. Determine average LD and LR for each test chemical.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

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

13.0 QUALITY CONTROL:

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

15.0 REFERENCES:

15.1 Official Methods of Analysis of AOAC INTERNATIONAL (2008) 18th Ed., AOAC International, Gaithersburg, MD. Method 2008.05.

15.2 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.

16.0 FORMS AND DATA SHEETS:

16.1 Three Step Method: Test Information Sheet

16.2 Three Step Method: Serial Dilution/ Plating Tracking Form

16.3 Three Step Method: Results Sheet

16.4 Three Step Method: Titer Dilution/ Plating and Results Form

16.5 Three Step Method: Media/ Reagent Sheet

16.6 Three Step Method Neutralization Test: Test Information Sheet

16.7 Three Step Method Neutralization Test: Time Recording Sheet

16.8 Three Step Method Neutralization Test: Serial Dilution / Plating Tracking Form

16.9 Three Step Method Neutralization Test: Results Sheet

16.10 Test Microbe Confirmation Sheet

16.11 Sample Spread Sheet

Attachment 1: Three Step Method Processing Sheet

16.1

Three Step Method: Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

TEST PARAMETERS			
H_2O Hardness ($CaCO_3$) ppm	Specified	Buret Titration/Date/Init	HACH Titration*/Date/Init
[redacted]		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init	
[redacted]		/ /	
Organic Soil	Specified	As Prepared/Date/Init	
[redacted]		/ /	
Neutralizer	Specified		
[redacted]			
Temperature (ºC)	Specified	Chiller Display	Thermometer
[redacted]		Before: After:	Before: After:
Contact Time	Specified	As Tested	
[redacted]			
Test Microbe			
Number of Carriers Analyzed			

*If applicable.

REAGENT/MEDIA INFORMATION			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Comments:

16.2

Three Step Method: Time Recording Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

TIME RECORDING – EXPOSURE TO DISINFECTANT/CONTROL (*FRACTION A*)			
Date/Initials:		Exposure Time:	
Start Time:*		Exposure Interval:	
Carrier Number	Exposure Start (min:sec)†	Exposure End (min:sec)†	

TIME RECORDING – INCUBATION/ROTATION (*FRACTION C*)			
Date/Initials:		Incubation Time:	30 minutes
Start Time:*			
Carrier Number	Incubation Start (min:sec)†	Incubation End (min:sec)†	

Comments:	*Time recorded from clock. †Time recorded from timer.

16.3

Three Step Method: Serial Dilution/Plating Tracking Form

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

General Dilution Scheme	Volume						
Overall Dilution in Dilution Tube	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Initial Volume in Dilution Tube	N/A	900µL	900µL	900µL	900µL	900µL	900µL
Volume Added to Dilution Tube	N/A	100µL	100µL	100µL	100µL	100µL	100µL

			Dilutions Plated	Volume Plated per Dilution	# Plates per Dilution
Test Chemical:		Fraction A			
Contact Time:		Fraction B			
Carrier #:		Fraction C			
Test Chemical:		Fraction A			
Contact Time:		Fraction B			
Carrier #:		Fraction C			

Comments:

16.4

Three Step Method: Results Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

RESULTS						
Date Recorded:						
	CFU/Plate					
Fraction / Carrier	*Dilution*	A Tubes	*Dilution*	B Tubes	*Dilution*	C Tubes

Comments:

16.5

Three Step Method: Titer Dilution, Plating, and Results Form

OPP Microbiology Laboratory

Titer Dilution Scheme	Volume						
Overall Dilution in Dilution Tube	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Initial Volume in Dilution Tube	990 µL	900 µL	900 µL	900 µL	900 µL	900 µL	900 µL
Volume Added to Dilution Tube	10µL	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
Volume Plated							
Overall Dilution on Plate							
Plates per Dilution							

Results			
Dilution			
CFU/Plate			

Comments:

16.6

Three Step Method Neutralization: Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

TEST PARAMETERS			
H_2O Hardness ($CaCO_3$) ppm	Specified	Buret Titration/Date/Init	HACH Titration*/Date/Init
		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init	
		/ /	
Organic Soil	Specified	As Prepared/Date/Init	
		/ /	
Neutralizer	Specified		
Temperature (ºC)	Specified	Chiller Display	Thermometer
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Test Microbe			
Number of Carriers Analyzed	N/A		

*If applicable.

REAGENT/MEDIA INFORMATION			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Comments:

16.7

Three Step Method Neutralization: Time Recording Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

NEUTRALIZATION TIME RECORDING – EXPOSURE			
Date/Initials:		Exposure Time:	
Start Time:*		Exposure Interval:	
Tube Number	Exposure Start (min:sec)†	Exposure End (min:sec)†	

Comments:	*Time recorded from clock. †Time recorded from timer.

16.8

Three Step Method Neutralization: Serial Dilution/Plating Tracking Form

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-22-00
Sample No.			
Lot No.			
Expiration Date			

General Dilution Scheme	Volume						
Overall Dilution in Dilution Tube	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Initial Volume in Dilution Tube	1010µL	900µL	900µL	900µL	900µL	900µL	900µL
Volume Added to Dilution Tube	N/A	100µL	100µL	100µL	100µL	100µL	100µL

	Dilutions to be Plated	Volume Plated per Dilution	# Plates per Dilution
Tubes 1-3	$10^{-4} \rightarrow 10^{-5}$	100µL	1
Tubes 4-6	$10^{-4} \rightarrow 10^{-5}$	100µL	1
Tubes 7-9	$10^{-4} \rightarrow 10^{-5}$	100µL	1
Tubes 10-12	10^{0}	100µL	1

Comments:

16.9

Three Step Method Neutralization: Results Sheet

OPP Microbiology Laboratory

TEST INFORMATION			
EPA Reg. No.		Test Date	
Name		SOP	MB-21-00
Sample No.			
Lot No.			
Expiration Date			

RESULTS			
Date Recorded:			
[redacted]	CFU/Plate		
Dilution / Tube #	10^{0}	10^{-4}	10^{-5}
1	N/A		
2	N/A		
3	N/A		
4	N/A		
5	N/A		
6	N/A		
7	N/A		
8	N/A		
9	N/A		
10		N/A	N/A
11		N/A	N/A
12		N/A	N/A

Comments:

16.10

Test Microbe Confirmation Sheet

OPP Microbiology Laboratory

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

Source: Tube/Plate ID	Date/ Initials	Stain Results*	Media Information			Results		
			Name	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	VITEK ID** (if applicable)

* GPR = Gram positive rods
** VITEK numerical profile number

16.11

Sample Three Step Method Spreadsheet

OPP Microbiology Laboratory

1	**Laboratory:**							**Date of test:**		
2	**Lab Specialist:**							**Replicate:**		
3	**Disinfectant:**									
4	**Organism:**									
5										
6	*Spor-Klenz*	**CFU/Plate**								
7	Fraction	**A**	**B**	**C**						
8	Carrier #	*1.0E+00*	*1.0E+00*	*1.0E+00*						
9	1									
10	2									
11	3									
12										
13	**Carrier #**	**Total Surviving Cells**			**Tot. Surv.**	**Log Density**	**Average**	**STDEV**	**Log Reductio**	**Average**
14		**A**	**B**	**C**	**CFU/carrier**	**per Carrier**	**Log Density**	**Log Density**	**per Carrier**	**Log Red.**
15	1	0.0E+00	0.0E+00	0.0E+00	5.0E+00	0.70			#VALUE!	
16	2	0.0E+00	0.0E+00	0.0E+00	5.0E+00	0.70	**0.70**	0.00	#VALUE!	-
17	3	0.0E+00	0.0E+00	0.0E+00	5.0E+00	0.70			#VALUE!	
18										
19										
34	*Control*	**CFU/Plate**								
35	Fraction	**A**		**B**		**C**				
36	Carrier #	*1.0E-03*	*1.0E-04*	*1.0E-03*	*1.0E-04*	*1.0E-01*	*1.0E-02*			
37	4									
38	5									
39	6									
40										
41	**Carrier #**	**Total Surviving Cells**			**Tot. Surv.**	**Log Density**	**Average**	**STDEV**		
42		**A**	**B**	**C**	**CFU/carrier**	**per Carrier**	**Log Density**	**Log Density**		
43	4	0.0E+00	0.0E+00	0.0E+00	0.0E+00	-				
44	5	0.0E+00	0.0E+00	0.0E+00	0.0E+00	-	-	-		
45	6	0.0E+00	0.0E+00	0.0E+00	0.0E+00	-				
46										
47										
48	**Titer**	**CFU/Plate**			**CFU/mL**	**Log**				
49		*1.0E-06*	*1.0E-07*	*1.0E-08*		**CFU/mL**				
50	Titer				0.0E+00	#NUM!				

Attachment 1

Three Step Method Processing Sheet

OPP Microbiology Laboratory

Test Date:

Test Chemical(s):

Analyst(s):

NOTE: Carriers exposed to the disinfectant(s) and water control will be tested in triplicate. It is recommended that no more than three disinfectants plus the water control be tested during the same test period.

The contents of microcentrifuge tubes prior to processing fractions for 1 disinfectant and control.

Treatment	Tube	Contents
Disinfectant	A1-A3	400 µL disinfectant + 600 µL LB broth w/neutralizer (after exposure period)
	B1-B3	400 µL sterile water + 600 µL LB broth (after sonication)
	C1-C3	400 µL LB broth + 600 µL LB broth (after incubation/rotation)
Control	A4-A6	400 µL sterile water + 600 µL LB broth (after exposure period)
	B4-B6	400 µL sterile water + 600 µL LB broth (after sonication)
	C4-C6	400 µL LB broth + 600 µL LB broth (after incubation/rotation)

Prior to Testing (i.e. day before test); as you proceed, add initials to each step in the space provided.

____ Label *fraction **A**, **B** and **C*** microcentrifuge tubes with fraction letter (e.g. A, B, or C) and carrier number (e.g., 1, 2, 3, etc.).

- Fraction tube examples: A1, A2, A3, B1, B2, B3, C1, C2, C3 etc.

____ In advance of testing, prepare *fraction **B*** and *fraction **C*** microcentrifuge tubes:

- Add 400 µL of ice-cold sterile water to *fraction **B*** tubes.
- Add 400 µL of ice-cold LB broth to *fraction **C*** tubes.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Label dilution microcentrifuge tubes (serial dilution blanks) with fraction letter (e.g. A, B, or C), carrier number (e.g. 1, 2, 3, etc.), and dilution (e.g., 10^{-1}, 10^{-2}, 10^{-3}, etc.).

- Serial dilution blank examples: A1-10^{-1}, A1-10^{-2}, A1-10^{-3}, etc.

____ Prepare serial dilution blanks for **A**, **B**, and **C** fractions for all test carriers.
- Add 900 µL of ice-cold LB broth to each dilution blank for test carriers.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Prepare serial dilution blanks for **A**, **B**, and **C** fractions for all control carriers.
- Add 900 µL of ice-cold LB broth to each dilution blank for control carriers.
- Store these tubes in a refrigerator (2-5°C) until ready for use.

____ Sterilize forceps (two pair of forceps for each disinfectant/control tested plus 3 extra).

____ Prepare a 50 mL conical tube with the appropriate neutralizer (place it on ice on test day).

____ Turn on the recirculating chiller and water bath and allow them to reach 20 ± 1°C or the temperature specified.

____ On test day, prepare the disinfectant(s) and place ~1.5 mL of each disinfectant and water control into a microcentrifuge tube
- Be sure to prepare and use the disinfectant within its specified period.
- If the disinfectant requires a dilution, a minimum of 1 mL of the product must be used.
- Ready-to-use disinfectants are tested as received; no dilution is required.

……

Fraction A

____ Using sterile forceps, carefully place one inoculated carrier inside each microcentrifuge tube labeled *fraction **A***.
- Avoid touching the inoculated area of the carrier and the sides of the microcentrifuge tube. If the carrier touches the sides of the microcentrifuge tube, the carrier and tube must be discarded.
- These forceps should not be used for any additional transfers, unless sterilized appropriately.

____ Place the *fraction **A*** tubes containing the carriers and the tubes containing the disinfectant(s) and water control into a chiller water bath equilibrated at 20 ± 1°C.
- Allow the carriers, disinfectant(s), and water control to equilibrate for approx. 10 min.

____ Using a 1000 µL micropipette, add 400 µL of the disinfectant or 400 µL sterile water control at 15 or 30 ± 5 sec intervals to the appropriate microcentrifuge tubes (in triplicate).

____ Expose *fraction **A*** tubes in the chiller water bath for the appropriate exposure period.

____ After exposure is complete add 600 µL of the appropriate ice-cold neutralizer to each disinfectant and water control *fraction **A*** tube.
- Slight agitation (e.g., light tapping) may be necessary to thoroughly mix the liquid components.

____ Using one pair of sterile forceps per set of 3 carriers (e.g., per disinfectant), transfer each carrier from its *fraction **A*** tube to its corresponding *fraction **B*** tube.

- Avoid touching the sides of the tube if possible.
- These forceps should not be used for any additional transfers.
  Note: Processing of the *fraction B* and *fraction C* tubes can be completed by a second analyst while the *fraction A* tubes are being centrifuged (e.g., sonication of the *fraction B* tubes, transfer of the carrier to the fraction C tubes, rotation of the *fraction C* tubes.

____ Place the *fraction* ***A*** tubes into the microcentrifuge for 6 min ± 30 sec at 13,000 rpm (15,500 × g).
- Centrifugation #1

____ After *fraction* ***A*** tubes complete centrifugation #1, carefully remove 900 µL from each *fraction* ***A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 900 µL of ice-cold LB broth into each *fraction* ***A*** tube and place the *fraction* ***A*** tubes into the microcentrifuge for 6 min ± 30 sec at 13,000 rpm (15,500 × g).
- Centrifugation #2

Note: The final volume in each *fraction A* tube is 1000 µL.

____ After *fraction* ***A*** tubes complete centrifugation #2, carefully remove 900 µL from each *fraction* ***A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 900 µL of ice-cold LB broth into each *fraction* ***A*** tube and place the *fraction* ***A*** *tubes* into the microcentrifuge for 6 min at 13,000 rpm (15,500 × g).
- Centrifugation #3

____ After *fraction* ***A*** tubes complete centrifugation #3, carefully remove 900 µL from each *fraction* ***A*** tube without disturbing the pellet. Discard the supernatant.

____ Carefully add 100 µL of ice-cold LB broth into each *fraction* ***A*** tube.

____ Resuspend the pellet in the *fraction* ***A*** tubes by vortex mixing for 5 min ± 30 sec at a midrange speed.
- The use of a vortex adapter is recommended.

____ Add 800 µL of ice-cold LB broth into each *fraction* ***A*** tube.

____ Proceed to the **Dilutions and Plating** section.
- Place *fraction* ***A*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

.....

Fraction B

____ Sonicate *fraction* ***B*** tubes for 5 min ± 30 sec using a floating microcentrifuge tube holder.

____ After sonication is complete, add 600 µL of ice-cold LB broth to the *fraction **B*** tubes and vortex for one minute.

____ Using one pair of sterile forceps per set of 3 carriers (e.g., per disinfectant), transfer each carrier from its *fraction **B*** tube to its corresponding *fraction **C*** tube.
- These forceps should not be used for any additional transfers, unless sterilized appropriately.

____ Proceed to the **Dilutions and Plating** section.
- Place *fraction **B*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

……

Fraction C

____ Place the *fraction **C*** tubes in a hematology rotator inside a 36 ± 1°C incubator for 30 ± 2 min.

____ After the 30 min ± 2 min rotation/incubation, remove the *fraction **C*** tubes from the incubator and add 600 µL of ice-cold LB broth to each tube.
- The carriers can remain in the *fraction **C*** tubes.

____ Proceed to the **Dilutions and Plating** section.
- Place *fraction **C*** tubes in a refrigerator if dilutions are not made immediately. Processing should proceed as quickly as possible.

…….

Dilutions and Plating

____ Remove dilution blanks from the refrigerator immediately prior to making dilutions.

____ Vortex mix each microcentrifuge tube thoroughly prior to making any dilutions.

____ Serially dilute *fractions **A**, **B**, and **C*** for test carriers.
- Vortex mix each tube prior to making the next dilution.
- Appropriate dilutions for test carriers will be determined via a screen prior to testing.

____ Serially dilute *fractions **A**, **B**, and **C*** for control carriers.
- Vortex mix each tube prior to making the next dilution.
- Appropriate dilutions for control carriers will be determined via a screen prior to testing.

____ Directly plate 100 µL of the dilutions that will yield counts within the target range (30-300) for each carrier.
- Spread using sterile spreader.

____ Incubate all plates at 36 ± 1°C for a minimum of 24 ± 2 hr