



**US Environmental Protection Agency  
Office of Pesticide Programs**

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

**Standard Operating Procedure for  
Enumeration of Bacterial Inocula on Carriers (Carrier Counts)  
for the Germicidal Spray Products as Disinfectants Test,  
Disinfectant Towelette Test, and the Tuberculocidal Activity  
of Disinfectants Test**

**SOP Number: MD-04-05**

**Date Revised: 01-13-09**

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

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1.0 SCOPE AND APPLICATION:

- 1.1 The monitoring of the titer of bacteria on inoculated carriers (e.g., porcelain and glass slides) used in antimicrobial product testing is required by the laboratory. This protocol describes the procedures for the enumeration of test microbes on the carriers (carrier counts) used in efficacy testing (see SOPs: Germicidal Spray Products as Disinfectants test (MB-06), Tuberculocidal Activity of Disinfectants test (MB-07), Disinfectant Towelette test (MB-09)).

## 2.0 DEFINITIONS:

- 2.1 CFU = Colony Forming Units
- 2.2 TNTC = Too Numerous to Count
- 2.3 AOAC = AOAC INTERNATIONAL
- 2.4 References to water mean reagent-grade water, except where otherwise specified.
- 2.5 PBDW = tubes of phosphate buffered dilution water, also referred to as dilution blanks.

## 3.0 HEALTH AND SAFETY:

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

## 4.0 CAUTIONS:

- 4.1 Assay carriers for carrier counts (sonicate/vortex) within 2 hours of drying.
- 4.2 Synchronize the carrier count assay and efficacy testing.
- 4.3 Use inoculated carriers for determining carrier counts and performing efficacy testing as soon as possible after drying on the day of preparation to avoid a reduction in microbial titer. Overnight or long term storage of inoculated carriers is not allowed.
- 4.4 The volume of dilution blanks and subculture tubes will be verified in advance and adjusted accordingly.
- 4.5 Complete dilution plating within 2 hours after the completion of carrier sonication or vortexing. If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done.

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

5.0 INTERFERENCES:

5.1 Contamination can interfere with the recording of results. Visually inspect all agar plates prior to use – discard any plates with evidence of contamination. For contamination following the incubation phase, if atypical colonies or contamination are evident that interfere with the enumeration of the test organism, record as a contaminant(s). Data from other dilutions, if the CFU result in a countable range, may be used to calculate the final CFU/carrier.

6.0 PERSONNEL QUALIFICATIONS:

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

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Branson Model 2200 Ultrasonic Cleaner (sonicator) or equivalent

7.2 A water bath set at 45-50°C

7.3 Incubator set at  $36 \pm 1^\circ\text{C}$  or another temperature suitable for growth of the target organism

7.4 Refrigerator set at 2-5°C

7.5 20 × 100 mm Petri dishes (total plating area of 58.1 cm<sup>2</sup>)

7.6 Plate spinner

7.7 Trypticase soy agar (TSA), Middlebrook 7H9 agar (M7H9), or Middlebrook 7H11 agar (M7H11)

7.8 Lethen broth or Modified Proskauer Beck medium (MPB)

7.9 Leica Darkfield Quebec Colony Counter (or equivalent)

8.0 INSTRUMENT OR METHOD CALIBRATION:

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

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Sonicated or vortexed tubes (see sections 10.3.1, 10.4.2 and 10.5.2) can be stored at 2-5°C until the dilution plating is performed. Complete plating within two hours of the sonication/vortexing step.

10.0 PROCEDURE AND ANALYSIS:

10.1 Assay carriers for carrier counts (sonicate/vortex) within 2 hours of drying.

10.2 Synchronize the carrier count assay and efficacy testing.

10.3 AOAC Germicidal Spray Products as Disinfectants Test with *S. aureus*, *P. aeruginosa*, and *S. enterica* (MB-06) and Disinfectant Towelette Test with *S. aureus* and *P. aeruginosa* (MB-09): Randomly select 6 inoculated carriers for carrier count analysis.

10.3.1 Place each of the inoculated, dried carriers in a 38 × 100 mm culture tube containing 20 mL of medium (e.g., letheen broth). Vortex immediately. Vortexing time is 60 ± 5 seconds for *P. aeruginosa*, and 120 ± 5 seconds for *S. aureus* and *S. enterica*. Record time of vortexing on Serial Dilution/Plating Tracking Form (16.1).

10.3.2 Serial ten-fold dilutions of the vortexed carrier tubes are made in 9 mL dilution blanks (see 16.1).

10.3.3 If the serial dilutions are not made and plated immediately, the tubes are kept at 2-5°C until this step can be performed. Complete the dilution and plating within 2 hours after vortexing of carriers.

10.3.4 Plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA using pour or surface spread plating. Briefly vortex (1-3 sec.) each serial dilution tube prior to plating.

Note: Dilutions 10<sup>-2</sup> through 10<sup>-4</sup> should produce plates with CFU in the appropriate range.

- 10.3.5 If the spread plate method is used for bacterial enumeration, TSA plates are prepared in advance and are refrigerated until needed.
- 10.3.5.1 Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the dry surface of the agar, use an autoclavable or disposable spreading rod and plate spinner until the surface is completely dry.
- 10.3.6 If the pour plate method is used for bacterial enumeration, the TSA is prepared and tempered after autoclaving (approx. 1 hr) to 45-50°C in a water bath prior to use. Tempered agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.
- 10.3.7 Incubate plates at  $36 \pm 1^\circ\text{C}$  for 24-48 hrs.
- 10.3.8 Colonies may be counted by hand or with the aid of a plate counter. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the Carrier Count Data Sheet (see 16.2). See section 11 for data analysis.
- 10.4 AOAC Confirmatory Tuberculocidal Test with *M. bovis* (BCG) (MB-07):  
Randomly select 3 inoculated carriers for carrier count analysis.
- 10.4.1 Place each inoculated carrier in 20 × 150 mm tube containing 10 mL of MPB. The tubes are then placed in a beaker containing tap water up to the level of media in the tubes. Likewise, the level of water in the sonicator bath is adjusted to the same level as the liquid in the tubes and beaker.
- 10.4.2 Sonicate carriers for 10 minutes. The sonicated tubes with the carriers are referred to as the “sonicated carrier tubes.” Record time of sonication on Serial Dilution/Plating Tracking Form (16.1).
- 10.4.3 Serial ten-fold dilutions of the sonicated carrier tubes are made in 9 mL dilution blanks.
- 10.4.4 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done (complete within 2 hours after sonication of carriers).
- 10.4.5 Plate 0.1 mL aliquots of appropriate dilutions in duplicate on M7H9 or

M7H11 using pour or surface spread plating. Briefly vortex (1-3 sec.) each serial dilution tube prior to plating.

Note: Dilutions  $10^{-1}$  through  $10^{-3}$  should produce plates with CFU in the appropriate range.

- 10.4.6 If the spread plate method is used for bacterial enumeration, M7H9 or M7H11 agar plates are prepared in advance and are refrigerated prior to use. Allow refrigerated plates to come to room temperature prior to use. To spread dilutions evenly over the surface of the agar, use an autoclavable or disposable spreading rod and plate spinner until the surface is completely dry.
  - 10.4.7 If the pour plate method is used for bacterial enumeration, the M7H9 or M7H11 agar is prepared and tempered (approx. 1 hr) to 45-50°C in a water bath prior to use. Tempered M7H9 or M7H11 agar is added to each plate after the addition of the appropriate dilution and swirled to uniformly disperse the inoculum.
  - 10.4.8 Incubate plates at  $36 \pm 1^\circ\text{C}$  for a minimum of 21 days (up to 25 days).
  - 10.4.9 Colonies may be counted by hand or with the aid of a plate counter. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the Carrier Count Data Sheet (see 16.2). See section 11 for data analysis.
- 10.5 AOAC Germicidal Spray Products Test with *M. bovis* (BCG) (MB-06):  
Randomly select 3 inoculated carriers for carrier count analysis.
- 10.5.1 Prior to testing, place each inoculated, dried carrier in a  $38 \times 100$  mm tube containing 20 mL of MPB broth.
  - 10.5.2 Vortex each tube for 15 seconds. These tubes are referred to as “vortexed carrier tubes.” Record time of vortexing on Serial Dilution/Plating Tracking Form (16.1).
  - 10.5.3 Serial ten-fold dilutions of the vortexed carrier tubes are made in 9 mL dilution blanks.
  - 10.5.4 If the serial dilutions are not made and plated immediately, the sonicated tubes are kept at 2-5°C until this step can be done (complete within 2 hours after vortexing of carriers).

10.5.5 Plate 0.1 mL aliquots of appropriate dilutions in duplicate on M7H9 or M7H11 using pour or surface spread plating. Briefly vortex (1-3 sec.) each serial dilution tube prior to plating.

Note: Dilutions  $10^{-1}$  through  $10^{-3}$  should produce plates with CFU in the appropriate range.

10.5.6 For plating methods and incubation conditions, refer to sections 10.3.6 through 10.3.9.

## 11.0 DATA ANALYSIS/CALCULATIONS:

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

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

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

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

11.3.1 Sample calculation: Adjust dilutions for volume plated (0.1 mL). For average CFU of 115 at the  $10^{-3}$  dilution, 15 at the  $10^{-4}$  dilution, and 0 at the  $10^{-5}$  dilution, the CFU/mL per carrier would be  $1.2 \times 10^5$  CFU/mL per carrier.

11.4 To calculate CFU/carrier, multiply the CFU/mL per carrier by the volume of media used to suspend carrier for sonication (10 mL) or vortexing (20 mL) and round numbers to 2 significant figures for reporting the final data.

11.4.1 Sample calculation:  $1.2 \times 10^5$  CFU/mL per carrier  $\times$  10 mL =  $1.2 \times 10^6$  CFU/carrier.

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

11.6 Calculate the mean log density across carriers for each test. Let M denote the

mean log density. The  $10^M$  is the geometric mean density for the test.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the Carrier Count Data Sheets. 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 as stipulated in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate forms (see 16.0).

13.2 If an unacceptable level of contamination occurs, the decision to repeat testing under this scenario is at the discretion of the Branch Chief or designee (Senior Science Advisor or Team Leader).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 For tests involving *S. aureus* and *P. aeruginosa*, the mean log density for inoculated carriers used in the AOAC Germicidal Spray Products as Disinfectants test and the Disinfectant Towelette test must be at least 5.0 (corresponding to a geometric mean density of  $1.0 \times 10^5$  CFU/carrier); a mean log density below 5.0 invalidates the test and retesting is required. For tests involving *S. enterica*, the mean log density for inoculated carriers used in the AOAC Germicidal Spray Products and Disinfectants test and the Disinfectant Towelette test must be at least 4.0 (corresponding to a geometric mean density of  $1.0 \times 10^4$  CFU/carrier); a mean log density below 4.0 invalidates the test and retesting is required.

14.1.1 A mean log density below 5 (for *S. aureus* and *P. aeruginosa*) or a mean log density below 4 (for *S. enterica*) may be indicative of a dilution error, poor media quality, interference by environmental parameters (e.g., carrier drying and culture incubation conditions), contamination, or lack of adherence to the method.

14.2 For tests involving *M. bovis* BCG, the mean log density for inoculated carriers used in the AOAC Germicidal Spray Products as Disinfectants test, the Disinfectant Towelette test and the Tuberculocidal Activity of Disinfectants test must be at least 4.0 (corresponding to a geometric mean density of  $1.0 \times 10^4$  CFU/carrier); a mean log density below 4.0 invalidates the test and retesting is

required.

14.2.1 A mean log density below 4.0 may be indicative of a dilution error, poor media quality, interference by environmental parameters (e.g., carrier drying and culture incubation conditions), contamination, or lack of adherence to the method.

14.3 The prescribed minimum carrier counts also account for the addition of 5% organic soil to the inoculum.

#### 15.0 REFERENCES:

15.1 SOP MB-05: AOAC Use Dilution Method for Testing Disinfectants

15.2 SOP MB-06: AOAC Germicidal Spray Products as Disinfectants Test Against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* and *Mycobacterium bovis* (BCG)

15.3 SOP MB-07: Tuberculocidal Activity of Disinfectants: II. Confirmative *in vitro* Test for Determining Tuberculocidal Activity

15.4 SOP MB-09: Disinfectant Towelette Test Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

15.5 SOP MB-15: Standard Operating Procedure for the AOAC Sporicidal Activity of Disinfectants Test (*Bacillus* × porcelain component only)

#### 16.0 FORMS AND DATA SHEETS:

16.1 Serial Dilution/Plating Tracking Form

16.2 Carrier Count Data Sheet

16.3 Sample Carrier Count Spreadsheet

The following MS Excel spreadsheets will be used:

Carrier Count Template\_CTB\_v2  
Carrier Count Template\_CTBGSPT\_v2  
Carrier Count Template\_GSPT\_v2

16.1  
 Serial Dilution/Plating Tracking Form  
 OPP Microbiology Laboratory

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

DILUTION/PLATING SCHEME/Confirmed by: _____					
Sonication/Vortex Start Time (clock): _____	Dilution Tube				
	10 <sup>0*</sup>	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>
Starting volume of diluent					
Volume added to serial dilution tube (1 mL)	N/A				
Volume plated (0.1 mL)					
Final dilution factor (used for calculations) <sup>1</sup>					
Number of plates per dilution					
Plating medium					
Number of carriers evaluated					
Comments: N/A = not applicable					
Dilution blank volume verified: <input type="checkbox"/> Yes <input type="checkbox"/> No      Subculture medium volume verified: <input type="checkbox"/> Yes <input type="checkbox"/> No					

\*Volume of medium in the tube with the carrier will be accounted for in the CFU/carrier calculation.

<sup>1</sup>Adjusted for volume plated.

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

16.2  
**Carrier Count Data Sheet**  
 OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by: _____	
EPA Reg. No.	
Name	
Sample No.	
Test Date	
Organism	
SOP	
Test Type	<input type="checkbox"/> AOAC Germicidal Spray Products Test <input type="checkbox"/> AOAC Confirmatory Tuberculocidal Test <input type="checkbox"/> AOAC Germicidal Towelette Products Test <input type="checkbox"/> Other – record test type in comments section

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

Sample Carrier Count Spreadsheet (v2)  
 OPP Microbiology Laboratory

Carrier Count Spreadsheet										
OPP Microbiology Laboratory										
TEST INFORMATION/Confirmed by:										
EPA Reg. No.										
Name										
Sample No.(s)										
Test Date										
Organism										
SOP										
Test Type										
Volume of media in tube with carrier (mL):										
Carrier No.		CFU per Plate			CFU/mL per carrier	CFU/carrier	LD/carrier			
Dilution										
1										
2										
3										
4										
5										
6										
Mean per carrier for all carriers tested:										
Comments:		LD = log density								