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 Suspension Test Method for Determining Tuberculocidal Efficacy of Disinfectants Against Mycobacterium bovis (BCG)ts

SOP Number: MB-16-04

Date Revised: 03-12-21

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Revisions Made	• No changes made.

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Scope	This SOP describes the methodology used to determine the efficacy of disinfectants against *Mycobacterium bovis* (BCG) in suspension. This SOP is based on references 15.1 and 15.2.
Application	Use of this SOP is limited to disinfectants with certain active ingredients (e.g., glutaraldehyde).

	Approval	Date
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TABLE OF CONTENTS

1. Definitions	Additional abbreviations/definitions are provided in the text. 1. QSTM = Quantitative Suspension Test Method 2. CFU = Colony Forming Unit 3. MPB/Tween = Modified Proskauer Beck Medium with 0.1% (v/v) Tween 80
2. Health and Safety	1. Follow procedures specified in SOP MB-01, Laboratory Biosafety. 2. All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01, Lab Biosafety. 3. The Study Director and/or lead analyst should consult the Safety Data Sheets for specific hazards associated with products.
3. Personnel Qualifications and Training	1. Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	1. Refer to SOP EQ-02 (thermometers), EQ-04 (spectrophotometers), and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	1. Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	1. For quality control purposes, document the required information on the appropriate form(s) (see section 14).
7. Interferences	1. Filters with colonies greater than ~30 CFU can be difficult to count. Check filters regularly. Count filters with ≥30 CFU frequently (e.g., every other day) once growth is observed by indicating colonies with a marker on the lid of the Petri plate. At the end of the incubation period, record total counts on the appropriate form (see section 14).
8. Non-conforming Data	1. Refer to SOP ADM-07, Non-Conformance Reports.
9. Data Management	1. Archive data consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. To ensure the stability of the disinfectant, perform testing within 3 hours of preparation. 2. Strict adherence to the procedure is necessary for valid test results. 3. Use appropriate aseptic techniques for all test procedures involving the

manipulation of test organisms and associated test components.

11. Special Apparatus and Materials

1. *Filter units*: 47mm diameter filter membranes with 0.45 µm pore size. Use with appropriate filtration apparatus. For organism recovery.
2. *Tissue grinders with glass pestles* (15 mL glass). To homogenize test culture.
3. *Spectrophotometer.* To standardize test culture.
4. *Colony Counter*. To assist in counting filter membranes.

12. Procedure and Analysis

Table 1. Test Culture Preparation Summary

QSTM Test Culture Preparation		
Step	Description*	Culture Notation§
1. Stock M7H11 Slant used to inoculate several tubes of MPB (Sect. 12.1b)	**Solid→Liquid$_{stationary}$** – Incubate inoculated tubes in a slanted, stationary position until a pellicle forms	-QSTM-01
2. Use pellicle from Step 1 to inoculate several tubes of MPB/Tween (Sect. 12.1d)	**Liquid$_{stationary}$→Liquid$_{stationary}$** – Incubate the inoculated tubes of MPB/Tween upright in a stationary position until turbid	-QSTM-02
3. Use stationary MPB/Tween culture to inoculate flask of MPB/Tween (Sect. 12.1f)	**Liquid$_{stationary}$→Liquid$_{aerated}$** – Use 5 mL of the stationary MPB/Tween culture to inoculate 50 mL of MPB/Tween, incubate on orbital shaker (~150 rpm) for 5-7 days	-QSTM-03
4. Use aerated MPB/Tween culture to inoculate flask of MPB/Tween (Sect. 12.1h)	**Liquid$_{aerated}$→Liquid$_{aerated}$** – Use 10 or 15 mL of the aerated MPB/Tween culture to inoculate 100 or 150 mL of MPB/Tween, incubate on orbital shaker (~150 rpm) until OD_{500} is ~0.6	-QSTM-04
5. Add Tween 80 to culture -QSTM-04 (Sect. 12.1j)	One day prior to harvesting the aerated flask culture from step 4 (-QSTM-04), add Tween 80 (1 mL per liter of culture)	N/A
6. Culture Harvest (Sect. 12.1k)	Harvest cells by homogenization in a tissue grinder when OD_{500} is ~0.6	N/A
7. Frozen Test Culture (Sect. 12.1n)	Dispense pooled homogenized culture into cryovials and freeze at ≤ -80ºC	-QSTM-FTC

*All incubations are at 36±1ºC

	§ Culture notations should be added to the "Comments" section of the Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)
12.1 Frozen Test Culture Preparation	a. Record all transfers and manipulations on the Organism Culture Tracking Form for *Mycobacterium bovis* (BCG) (see section 14). b. Inoculate several 20 mL tubes of Modified Proskauer-Beck (MPB) medium with *Mycobacterium bovis* (BCG) from a stock Mycobacteria 7H11 (M7H11) slant culture (see SOP MB-07). c. Incubate in a slanted position at 36±1°C until a pellicle forms (approximately 19-23 days). d. Using a 10 µL loop, transfer a loopful of pellicle onto the surface of several 20 mL tubes of MPB/Tween 80. e. Incubate stationary at 36±1°C until cultures are turbid. Cultures will require agitation (by gentle shaking/vortexing) to assess turbidity. f. Transfer 5 mL of a stationary culture to 50 mL of MPB/Tween 80 in a 250 mL flask. g. Incubate for 5-7 days at 36±1°C with aeration (on a shaker at slow speed, approximately 150 rpm). h. Transfer 10 mL of the aerated culture to 100 mL of MPB/Tween 80 in a 500 mL flask. Alternately: Transfer 15 mL of the aerated culture to 150 mL of MPB/Tween 80 in a 500 mL flask. i. Incubate for 10-15 days at 36±1°C with aeration (on a shaker at 150 rpm) OR until the absorbance at 500 nm is about 0.6 (target stock culture titer: ~1-5×10^8 CFU/mL). j. One day prior to harvesting, add Tween 80 to the culture (1 mL per L of culture). k. Harvest cells when absorbance at 500 nm is approximately 0.6. l. Homogenize 10-20 mL aliquots in a tissue grinder. m. Pool homogenized culture. n. Dispense 1-2 mL aliquots of the homogenized suspension into cryotubes. o. Place in cryostorage at ≤ -80°C. Check the concentration of viable cells in the suspension by plating dilutions of the stock on M7H11 agar plates both before and after freezing. Check the frozen test culture stock by acid-fast staining and record results.
12.2 Suspension	Record culture preparation activities on QSTM: Processing Sheet (see section

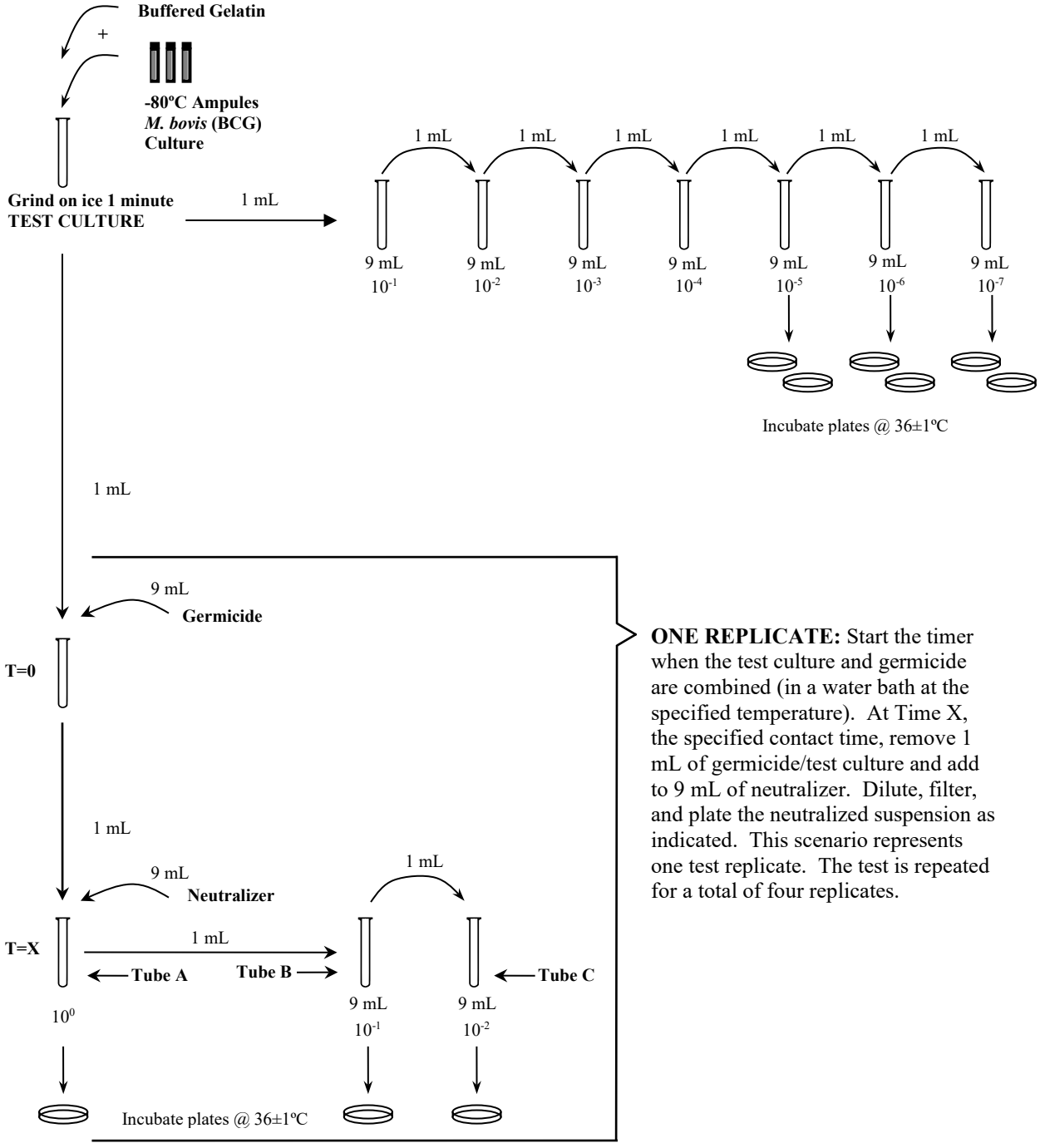
Test Culture Preparation	14). a. To prepare the suspension of *M. bovis* (BCG), remove the necessary number of vials of frozen stock culture and place on ice prior to thawing. b. Quickly thaw the frozen vials in a 36±1°C water bath then place the thawed vials back on ice. A vial of ~1.8 mL of frozen test culture requires ~90-120 s to thaw completely. c. Add an equal volume of buffered gelatin to the suspension and homogenize with a sterile tissue grinder for 1 min while keeping the culture at 0-4°C in an ice bath. d. Dilute the homogenate with sterile saline plus 0.1% Tween 80 to achieve the target density of approximately 1-5×10^7 CFU/mL. e. If organic soil is specified in the test parameters for the product test, measure the culture and add the appropriate volume of soil to the diluted homogenate. Swirl to mix.
12.3 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Equilibrate the water bath and allow it to come to 20±1ºC or the temperature specified (±1ºC). Record the temperature on the QSTM: Information Sheet (see section 14). c. After preparation, dispense 9 mL of the disinfectant into each of 4 sterile 20×150 mm tubes. Equilibrate tubes in water bath for 10 min.
12.4 Test Procedure	a. Suspension Test Procedure (see Attachment 1, Study Design for QSTM Disinfectant Efficacy Evaluation): i. In a timed step, add 1 mL of the test culture to each tube of disinfectant and lightly vortex. Repeat this step 3 additional times for a total of four replicates. ii. Following the specified exposure period, remove a 1 mL aliquot of the disinfectant-organism mixture and transfer directly to a 9 mL tube of neutralizer (the 10^0 dilution designated Tube A) and mix thoroughly. iii. Within 5 min of transfer to the neutralizer tube, make two additional ten-fold dilutions of Tube A in saline blanks to achieve 10^{-1} and 10^{-2} dilutions (designated Tube B and Tube C, respectively); mix thoroughly between dilutions. iv. Filter the three dilutions (tubes A, B, and C) separately. Pre-wet each filter with ~20 mL saline and add 1 mL from Tube A (10^0 dilution). Briefly swirl and filter. Rinse each filter

	with ~50 mL saline. v. Repeat for Tube B (10^{-1}) and Tube C (10^{-2}). vi. Place each filter (12 filters total) on the surface of an M7H11 agar plate. Incubate at 36±1ºC for 17-21 days (bag or parafilm plates to prevent desiccation). b. Enumeration of Inoculum (see Attachment 2, Study Design for QSTM Culture Titer and Controls): i. Transfer 1 mL of the test culture (with soil if specified) to a 9 mL saline blank and vortex. ii. Serially dilute in saline: 10^{-1} through 10^{-7}. iii. Pre-wet each filter with ~20 mL saline. Filter 1 mL aliquots of 10^{-5} through 10^{-7} dilutions in duplicate (6 filters total). iv. Briefly swirl and filter. Rinse each filter with ~50 mL saline. v. Place each filter on the surface of an M7H11 agar plate. Incubate at 36±1ºC for 17-21 days (bag or parafilm plates to prevent desiccation).
12.5 Quality Control	a. Static Control: The Static Control is designed to confirm the neutralization of the test substance (see Attachment 2, Experimental Design for QSTM Culture Titer and Controls). i. Allow 0.9 mL of disinfectant to come to the specified test temperature in a water bath. ii. Add 9 mL of neutralizer and mix by vortexing. iii. After 5 min, add 0.1 mL of the test culture and mix by vortexing. iv. Serially dilute in saline: 10^{-1} through 10^{-5}. v. Filter dilutions 10^{-3} through 10^{-5} in duplicate as indicated in Sections 12.4b, iii – 12.4b, v (6 filters total). vi. Incubate at 36±1ºC for 17-21 days (bag or parafilm plates to prevent desiccation). b. Neutralizer Toxicity Control: The Neutralizer Toxicity Control must demonstrate that the neutralizer does not impact the recovery of the test organism (see Attachment 2, Experimental Design for QSTM Culture Titer and Controls). i. Add 1.0 mL of the standardized test culture to a tube containing 9 mL of saline at room temperature.

	ii. Remove 1 mL of the saline/test culture mixture and add to a tube containing 9 mL neutralizer and mix. iii. After 5 min, serially dilute in saline 10^{-1} through 10^{-5}. iv. Filter dilutions 10^{-3} through 10^{-5} in duplicate as indicated in sections 12.4b, iii – 12.4b, v (6 filters total). v. Incubate at 36±1ºC for 17-21 days (bag or parafilm plates to prevent desiccation).
12.6 Reading Filters and Recording Results	a. Examine filters after approximately 10 days and frequently thereafter (see section 7). Record results after 17-21 days of incubation. b. Colonies appear initially as small buff colored accretions with irregular borders. Record colony counts at the end of the incubation period on appropriate test sheets.
12.7 Confirmation Procedures and Presumptive Identification of *M. bovis* (BCG)	a. Presumptively confirm the identification of *M. bovis* (BCG) using acid fast staining and plating on selective media (e.g., M7H11). b. Take a smear for acid fast staining from a representative colony from selected filters with growth on the day that final results are recorded. For each set of filters from the Product Test, Enumeration of Inoculum, Static Control, and Neutralizer Toxicity Control, choose the filter with growth from the highest dilution (i.e., the smallest number of colonies). c. Acid fast rods are typical for *M. bovis* (BCG). d. In addition, streak the representative growth from the colony that was used for acid fast staining over the surface of an M7H11 agar plate and incubate for 17-21 days at 36±1ºC. e. Following the incubation period, evaluate and record the colony morphology of the organism on M7H11 agar. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H11 agar. f. Record results on the Test Microbe Confirmation Sheet (see section 14)
13. Data Analysis/ Calculations	1. See section 14, QSTM: Calculations Worksheet. a. The test substance must demonstrate ≥ 1.0×10^4 CFU kill of the test organism at the stated contact time (i.e., a ≥ 4 $\log_{10}$ reduction of test organism). b. The Static Control should demonstrate that the neutralizer adequately neutralized the test substance (i.e., ≤ 1 $\log_{10}$ difference between the

	Static Control and the Neutralizer Toxicity Control). c. The Neutralizer Toxicity Control must demonstrate that the neutralizer does not impact the recovery of test organism (i.e., ≤ 1 $\log_{10}$ difference between the Neutralizer Toxicity Control and the Organism Titer). 2. The Organism Titer must be ≥ 1×10^7 CFU/mL. 3. When TNTC values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and scale up accordingly for the calculations.
14. Forms and Data Sheets	1. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Attachment 1: Study Design for QSTM Efficacy Evaluation — MB-16-04_A1.docx Attachment 2: Study Design for QSTM Culture Titer and Controls — MB-16-04_A2.docx QSTM: Test Information Sheet — MB-16-04_F1.docx QSTM: Time Recording — MB-16-04_F2.docx QSTM: Efficacy Evaluation Results Form — MB-16-04_F3.docx QSTM: Test Suspension Titer Form — MB-16-04_F4.docx QSTM: Static Control Form — MB-16-04_F5.docx QSTM: Neutralizer Toxicity Control Form — MB-16-04_F6.docx QSTM: Test Microbe Confirmation Sheet — MB-16-04_F7.docx QSTM: Processing Sheet — MB-16-04_F8.docx QSTM: Calculations Spreadsheet — MB-16-04_F9.xlsx
15. References	1. New Quantitative Tuberculocidal Procedure – Attachment C of US EPA Data Call-in Notice for Tuberculocidal Effectiveness Data for all Antimicrobial Pesticides with Tuberculocidal Claims, dated June 13, 1986. 2. A More Accurate Method for Measurement of Tuberculocidal Activity of Disinfectants (Ascenzi, J.M., et. al., *Applied Environmental Microbiology*, Vol. 53, No. 9, 1987, pp. 2189-2192).

Attachment 1
Study Design for QSTM Disinfectant Efficacy Evaluation



ONE REPLICATE: Start the timer when the test culture and germicide are combined (in a water bath at the specified temperature). At Time X, the specified contact time, remove 1 mL of germicide/test culture and add to 9 mL of neutralizer. Dilute, filter, and plate the neutralized suspension as indicated. This scenario represents one test replicate. The test is repeated for a total of four replicates.

Attachment 2
Study Design for QSTM Culture Titer and Controls