US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for Testing of *Mycobacterium bovis* (BCG) Using the Germicidal Spray Products as Disinfectants Test

SOP Number: MB-24-04

Date Revised: 05-21-19

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Title	Testing of *Mycobacterium bovis* (BCG) Using the Germicidal Spray Products as Disinfectants Test
Revisions Made	• Clarifications made to Section 11 (Special Apparatus and Materials). • Clarifications made to Section 12.3 (Carrier inoculation). • Clarifications made to Section 12.4 (Enumeration of viable bacteria from carriers (control carrier counts)). • References updated. • Minor editorial changes.

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Title	Testing of *Mycobacterium bovis* (BCG) Using the Germicidal Spray Products as Disinfectants Test
Scope	Describes the methodology to determine tuberculocidal activity of spray disinfectants labeled to treat hard non-porous surfaces against *Mycobacterium bovis* (BCG), see 15.1.
Application	For official product testing, a study protocol is developed which identifies the specific test conditions for a product sample such as contact time, dilutions, neutralizers, etc.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. Carrier Set = One "carrier set" is defined as the primary MPB tube containing the carrier and duplicate tubes of the two additional subculture media (e.g., M7H9 broth, Kirchner's medium, or TB broth) inoculated from the carrier's corresponding neutralizer tube for a total of 5 tubes per carrier. There are 10 carrier sets per disinfectant tested.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-04 (spectrophotometers), EQ-05 (timers), and QC-19 (pipettes) for details on method and frequency of calibration.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. The carriers inside the Petri dishes should be dry prior to inoculation. Moisture can interfere with the concentration and drying of the inoculum on the glass slide carrier. 2. Do not use any inoculated carrier that is wet at the conclusion of the carrier drying period.
8. Non-conforming Data	1. An assessment of media quality (performance) is necessary to ensure the validity of the tuberculocidal efficacy results; tests will be invalidated if any media exhibit unsatisfactory performance. The media assessment may be conducted in advance of or concurrently with efficacy testing; refer to SOP MB-10, Media and Reagents: Preparation and Quality Evaluation. 2. Sterility and/or viability controls do not yield expected results. 3. The mean log density for control carriers falls outside the specified range. Note: The prescribed minimum and maximum carrier counts also account for the addition of 5% organic soil to the inoculum. a. The mean *TestLD* must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0

	(corresponding to a geometric mean density of 1.0×10^6); a mean *TestLD* below 4.0 or above 6.0 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 4. Management of non-conforming data will be consistent with SOP ADM-07, Non-Conformance Reports.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. There are time sensitive steps in this procedure including the use periods of the inoculated carriers and the test chemical. 2. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly.
11. Special Apparatus and Materials	1. Culture media. a. *Modified Proskauer-Beck medium*. Dissolve 2.5 g KH_2PO_4, 5.0 g asparagine, 0.6 g $MgSO_4\times7H_2O$, 2.5 g magnesium citrate, 20.0 mL glycerol, 0.0046 g $FeCl_3$, and 0.001 g $ZnSO_4\cdot7H_2O$ in 1 L H_2O. Adjust to pH 7.2-7.4 with 1 N NaOH. Filter through Whatman No. 4 (or equivalent) filter paper, place 20 mL portions in separate 25×150 mm tubes, and steam sterilize 20 min at 121°C. Use this broth for propagating test cultures grown statically (25×150 mm tubes) and for recovery of test organism from treated carriers (38×100 mm tubes). b. *Middlebrook 7H9 broth (dehydrated M7H9 medium) with 0.1% (v/v) polysorbate 80.* Dissolve 4.7 g in 900 mL H_2O containing 1 mL polysorbate 80, 2 mL glycerol, and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions and mix thoroughly. Store prepared medium at 2-5°C. Use this broth for propagating test cultures grown with agitation. c. *Middlebrook 7H11 agar (dehydrated M7H11 medium)*. Dissolve 21 g dehydrated M7H11 agar medium in 900 mL H_2O containing 5 mL glycerol. Swirl to obtain a smooth suspension; boil if necessary to completely dissolve the powder. Steam sterilize 15 min at 121°C. Cool sterile medium to 50-55°C, add 100 mL OADC enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25×150 mm screw-capped tubes and slant or dispense a minimum of 30 mL into sterile Petri plates. Alternatively, pre-made M7H11 agar plates may be purchased. Use slants to maintain stock culture and plates for

inoculum isolation and enumeration.

d. *Middlebrook 7H9 broth (dehydrated M7H9 medium)*. Dissolve 4.7 g in 900 mL H_2O containing 2 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Distribute 18 mL portions in 25×150 mm tubes. Steam sterilize 10 min at 121°C, according to manufacturer's instructions. Cool sterile medium to approximately 40-45°C then add 2 mL Middlebrook ADC Enrichment to each tube under aseptic conditions and mix thoroughly. Store prepared medium at 2-5°C. Use for recovery of test organism from treated carriers.

e. *Kirchner's medium*. Dissolve 5 g asparagine, 2.5 g sodium citrate, 0.6 g magnesium sulfate (heptahydrate), 2.5 g monopotassium phosphate, and 1.5 g dipotassium phosphate, in 900 mL H_2O containing 20 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25×150 mm tubes. Use for recovery of test organism from treated carriers.

f. *TB broth base*. Dissolve 2.0 g yeast extract, 2.0 g proteose peptone No. 3, 2.0 g casitone, 1.0 g potassium phosphate monobasic, 2.5 g sodium phosphate dibasic, 1.5 g sodium citrate, and 0.6 g magnesium sulfate (heptahydrate) in 900 mL H_2O containing 50 mL glycerol and 1.0 g Bacto agar. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Dubos Medium Serum under aseptic conditions, and mix thoroughly. Distribute in 20 mL portions in sterile 25×150 mm tubes. Use for recovery of test organism from treated carriers.

g. *Middlebrook 7H10 agar.* Dissolve 19 g in 900 mL H_2O containing 5 mL glyerol. Heat to boiling to dissolve completely. Steam sterilize 15 min at 121°C. Cool sterile medium to 45°C, add 100 mL Middlebrook ADC Enrichment under aseptic conditions and mix thoroughly. Use for initiating stock cultures.

2. Test organism.

a. *Mycobacterium bovis* (BCG) (ATCC #35743). For stock culture, streak inoculate M7H11 agar slants. Incubate 15-20 days at 36±1°C. Following incubation, maintain at 2-5°C for up to 6 weeks.

3. Reagents

   a. *Sterile water*. Use reagent-grade water 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. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

   b. *0.1% polysorbate 80 in saline*. Add 0.1 mL polysorbate 80 to 100 mL sterile 0.85% aqueous saline (sodium chloride) solution, filter sterilize. Used in test culture preparation and dilution of culture grown with agitation.

4. Apparatus.

   a. *Specialized glassware*. For neutralizer/primary subcultures, use autoclavable 38×100 mm tubes (Bellco Glass Inc., Vineland, NJ). Cap tubes with closures before sterilizing. For glassware used to prepare test chemical, refer to SOP MB-22.

   b. *Tissue grinder*. Kimble glass tissue grinder (catalog number 885300-0015), for homogenization of the statically grown culture.

   c. *Spray Disinfectant Apparatus*. Refer to Attachment 3.

   d. *Inoculating loop*. For culture inoculation, 1 µL sterile disposable loops (Fisher Scientific). For culture harvest, 95% platinum, 3.5% rhodium alloy, 18 or 19 gauge, 4 mm loop with 75 mm shank (Baxter Scientific Products) or equivalent or disposable loops.

   e. *Carriers*. Glass Slide Carriers, 25×25 mm (or comparable size) borosilicate glass cover slips with number 4 thickness. Refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing.

   f. *Micropipettes*. For performing serial dilutions.

   g. *Positive displacement pipette.* With corresponding sterile tips able to deliver 10 µL.

   h. *Timer*. Any certified timer that can display time in seconds.

   i. *Spectrophotometer*. Calibrated; for preparing standardized test culture.

   j. *Semi-microcuvette with cap.* For measuring percent transmittance.

   k. *TB Stain Kit.* For presumptive identification of test microbe.

	l. *Rotary Shaker.* To provide rotation at 150 rpm for cultures grown with agitation.
12. Procedure and Analysis	The AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Processing Sheet (see section 14) must be used for tracking testing activities.
12.1 Test Culture Preparation: Agitated Culture	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Transfer a 10 µL loopful of *M. bovis* (BCG) from an M7H11 stock slant to a 25×150 mm tube containing 10 mL of Middlebrook 7H9 broth with 0.1% (v/v) polysorbate 80 (M7H9/P80), parafilm the cap to the tube, and briefly vortex. Incubate the tube at 36±1°C on a rotary shaker at 150 rpm for 5-8 days. *This represents a primary (1°) culture and is never used as a test culture.* b. After incubation, vortex the 1° tube well and transfer 1 mL to a 250 mL flask containing 50 mL of M7H9/P80. Incubate at 36±1°C on a rotary shaker at 150 rpm for 6-10 days. *This represents the secondary (2°) culture and is the test culture.* c. On the test day (following the 6-10 day incubation period), harvest the culture: i. Transfer the 2° culture to sterile 25×150 mm test tubes. Allow the suspension to settle for 10-15 min. ii. Remove the upper portion of each culture (e.g., upper ¾), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. iii. Dilute the pooled culture with sterile saline with 0.1% polysorbate 80 (saline/P80) to achieve 20±1% transmittance at 650 nm. Use a semi-microcuvette with cap while measuring transmittance. Blank the spectrophotometer with M7H9/P80. d. If an organic soil load is specified in the test parameters for the product test, add the appropriate amount of organic soil to the pooled test culture prior to the inoculation of carriers. Swirl to mix. e. Inoculate glass slide carriers with the standardized culture within 10 min of standardization. Briefly mix culture prior to use.
12.2 Test Culture Preparation: Static Culture (alternative	Refer to SOP MB-02 for the test microbe culture transfer notation. a. Initiate test culture by inoculating a sufficient number of 25×150 mm tubes containing 20 mL MPB (approximately 10) from stock culture slant(s) (M7H11 agar slants) by transferring 1-2 1 µL

culture preparation procedure)	loopfuls from the stock culture onto the surface of the broth. Record all transfers on the Organism Culture Tracking Form (culture notation = –SL, indicating a transfer from slant to liquid). b. Note: Over-inoculation of MPB may lead to reduced viability due to excessive growth after 21±2 days; the resulting carrier counts may be negatively impacted. c. Incubate the tubes 21±2 days undisturbed at 36±1°C in a slanted position to increase surface area. d. On the test day: using a transfer loop, transfer culture to a sterile glass tissue grinder, add 1.0 mL saline/P80, grind continuously for approximately 1 min to break up large clumps or aggregates of the test organism. e. Dilute the homogenized culture with 9 mL MPB broth and transfer the suspension from the tissue grinder to a sterile test tube. Harvest and homogenize culture from multiple MPB broth tubes. f. Repeat 12.2d-e as necessary to obtain enough concentrated culture. g. Note: Growth from multiple tubes may be harvested and combined to prepare the concentrated culture prior to standardization. i. Allow the suspension to settle for 10-15 min. ii. Remove the upper portion of each culture (e.g., upper ¾), leaving behind any debris or clumps, and transfer to a sterile flask; pool cultures in the flask and swirl to mix. iii. Dilute the pooled culture with MPB broth to achieve 20±1% T at 650 nm. Use a semi-microcuvette with cap while measuring transmittance. Blank the spectrophotometer with MPB. h. If an organic soil load is specified in the test parameters for the product test, add the appropriate amount of organic soil to the pooled test culture prior to the inoculation of carriers. Swirl to mix. i. Aliquot a sufficient volume of culture into a sterile test tube. j. Inoculate glass slide carriers with the standardized culture within 10 min of standardization. Briefly mix culture prior to use.
12.3 Carrier Inoculation	Inoculate approximately 20 carriers; 10 carriers are required for testing, 3 for control carrier counts, and 3 for the viability controls.

	a. Vortex-mix the inoculum periodically during the inoculation of carriers. Use a calibrated positive displacement pipette to transfer 10 µL of the test culture to the sterile test carrier in the Petri dish. Immediately spread the inoculum uniformly using a sterile loop. Do not allow the inoculum to contact the edge of the glass slide carriers. Cover dish immediately. b. Dry carriers in incubator at 36±1°C for 30±2 min. Record the timed carrier inoculation activities on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Processing Sheet (see section 14). Use inoculated carriers for testing within two hours of drying. c. After completion of all slide inoculations, thoroughly wipe the micropipette with 70% ethanol prior to removal from the BSC.
12.4 Enumeration of viable bacteria from carriers (control carrier counts)	a. After inoculated carriers have dried, randomly select 3 inoculated carriers for assay. Assay 1 carrier immediately prior to conducting the efficacy test and 2 carriers immediately following the test. b. Place each of the inoculated, dried carriers in a 38×100 mm tube or a sterile 50 mL polypropylene conical tube containing 20 mL of MPB broth and vortex each tube for 15 s. Record the time of vortexing on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Processing Sheet (see section 14). c. After vortexing, make serial ten-fold dilutions in 9 mL phosphate buffered dilution water. If the serial dilutions are not made and plated immediately, keep the vortexed tubes at 2-5°C until this step can be done; however, perform dilution and plating within 2 h of vortexing. d. Briefly mix each serial dilution tube prior to plating. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on M7H11 using surface spread plating. Serial dilution tubes 10^{-1} through 10^{-3} should produce plates with CFU in the countable range. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. e. Incubate plates (inverted) concurrently with the efficacy test subculture tubes at 36±1°C for 17-21 days. Place plates in sterile bags to reduce dehydration during the incubation period. f. Count colonies. Record plates that have colony counts over 300 as TNTC. Record counts on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Carrier Counts Form (see section 14). See section 13 for data analysis.

12.5 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22.
12.6 Test Procedure	a. Record timed events on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Time Recording Sheet for Carrier Transfers (see section 14). b. After the required carrier drying time, spray the slides sequentially for a specified time, distance, and number of pumps at timed intervals (typically 30 s) with the carriers in a horizontal position. Use a certified timer to time the spray interval. c. Spray the slide within ±5 s of the specified time for a contact time of 1-10 minutes or within ±3 s for contact times <1 min. After spraying, maintain the carriers in a horizontal position. Treated carriers must be kept undisturbed during the contact time. d. After the last slide of a set (typically 10 slides) has been sprayed with the disinfectant and the exposure time is complete, sequentially transfer each slide into the neutralizer tube within the ±5 s (or ±3 s) time limit. Drain the excess disinfectant from each slide prior to transfer into the neutralizer tube. Drain carriers without touching the Petri dish or filter paper. Perform transfers with flame sterilized or autoclaved forceps. e. The slide can touch both the interior sides of the Petri dish and the neutralizer tube during the transfer but avoid this contact as much as possible. f. After the slide is deposited, shake tube containing carrier in neutralizer thoroughly; transfer the carrier to the tube containing 20 mL MPB broth within 5-10 minutes. Sterilize forceps after each carrier transfer. g. Once all carriers have been transferred to the MPB broth tubes, sequentially transfer 2 mL aliquots from each neutralizer tube into duplicate tubes of 2 additional subculture media, M7H9 broth, Kirchner's medium, or TB broth, as specified. This portion of the assay is not timed, but the aliquots should be sequentially transferred to the subculture media within approximately 30±5 min. Repeat this with each tube of neutralizer. Shake each subculture tube thoroughly. Slightly loosen caps of growth media prior to incubation. h. Incubate 60 days at 36±1°C.

	i. Report results as + (growth) or 0 (no growth). j. Record results at 60 days. If the 60th day of incubation falls on a weekend or holiday, record the results on the first workday following the 60th day of incubation. i. Tubes may be monitored beginning at day 21 for evidence of typical mycobacterial growth. If multiple tubes show significant growth prior to the 60th day, confirmatory tests (e.g., acid fast staining and streak isolation) may be initiated prior to day 60. If the results of the confirmatory test are indicative of *M. bovis* (BCG), the results may be recorded at that point to expedite the reporting process. ii. Provide justification when recording results on days other than 60 in the comments section of the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet (see section 14). k. If no growth or occasional growth (insufficient for confirmatory tests) occurs within a set of tubes after 60 days, incubate the set an additional 30 days and record the results. After 30 days, if growth occurs check using standard confirmatory procedures (e.g., acid fast staining and growth on M7H11 agar) to ensure that no contamination is present. l. Record results at 90 days. If the 90th day of incubation falls on a weekend or holiday, record the results on the first workday following the 90th day of incubation. Recording of results beyond the 90th day should be notated in the Comments section on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet (see section 14).
12.7 Sterility and viability controls	a. Sterility controls. Place one sterile, uninoculated carrier into a tube of MPB broth. In addition, incubate 1 tube of each subculture medium with 2 mL sterile neutralizer for quality control purposes. Shake each tube thoroughly and incubate all tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should not occur in any tube. Record results on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet (see section 14). b. Viability controls. On the day of testing, place a dried inoculated carrier into a tube of MPB broth and a tube of each subculture medium. Incubate tubes as in the efficacy test. Report results as +

		(growth) or 0 (no growth) as determined by presence or absence of turbidity or presence of culture growth. Growth should occur in all tubes. Record results on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet (see section 14).
12.8	Test microbe identification	a. Presumptively confirm at least one positive subculture tube for each carrier set with growth. The maximum number of tubes subjected to confirmatory tests per disinfectant tested is 10. b. If more than one subculture tube for a carrier set is positive, confirm a minimum of one tube using acid fast staining and isolation on selective media (M7H11 agar plates). c. If the MPB in the set is positive, it is the representative subculture tube used for identification. If MPB is not positive, any of the other subculture media may be used for identification. d. If growth is observed in only one carrier set, then all subculture tubes showing growth for that carrier are subject to confirmatory tests. e. Growth for acid fast staining is taken from the selected positive tubes on the day that results are read. Acid fast rods are typical for *M. bovis* (BCG). The acid fast staining results should be read promptly prior to assigning a + or 0 to the results. If acid fast rods are observed from the selected tubes then a + is assigned to the results. If no cells are observed for the acid fast stain, apply a 0 to the results. f. In addition, streak isolate growth from positive tubes on M7H11 agar and incubate for 17-21 days at 36±1ºC. g. Following the 17-21 day incubation period, evaluate the colony morphology on M7H11 agar. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H11 agar (see Attachment 1). h. If a satisfactory smear cannot be obtained directly from the tube, take the smear for acid fast staining from the 17-21 day old M7H11 agar plate that was inoculated with the growth from the tube. i. In the event that no cells were observed with acid fast staining initially but typical growth was observed on the M7H11, correct the 0 to read + on the test sheet. An entry error will be noted in the comments section of the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet (see section 14).

	j. Record results on the AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Microbe Confirmation Sheet (see section 14).
13. Data Analysis/ Calculations	Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts from 0 through 300 and their associated dilutions will be included in the calculations.
14. Forms and Data Sheets	1. Attachment 1: Typical Growth Characteristics of Strains of *M. bovis* (BCG) 2. Attachment 2: Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG) 3. Attachment 3: Photographs of spray apparatus 4. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Physical Screening of Carriers Record — MB-03_F1.docx Organism Culture Tracking Form for *Mycobacterium bovis* (BCG) — MB-07_F5.docx Test Microbe Confirmation Sheet (Quality Control) — MB-07_F6.docx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Time Recording Sheet for Carrier Transfers — MB-24-04_F1.docx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Information Sheet — MB-24-04_F2.docx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Results Sheet — MB-24-04_F3.docx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Test Microbe Confirmation Sheet — MB-24-04_F4.docx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Carrier Counts Form — MB-24-04_F5.docx Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template_CTBGSPT_v4 — MB-24-04_F6.xlsx AOAC Germicidal Spray Products Test for *M. bovis* (BCG) Processing Sheet — MB-24-04_F7.docx
15. References	1. Official Methods of Analysis. Revised 2013. AOAC

	INTERNATIONAL, Gaithersburg, MD, (Method 961.02). 2. Official Methods of Analysis. 2012. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Method 965.12 In vitro Test for Determining Tuberculocidal Activity). 3. Standard Methods for the Examination of Water and Wastewater. 23rd Ed. American Public Health Association, 1015 15th Street, NW, Washington, DC 4. Holt, J., Krieg, N., Sneath, P., Staley, J., and Williams, S. eds. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD. 5. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology. Volume 2. Williams & Wilkins, Baltimore, MD.

Attachment 1

Typical Growth Characteristics of strains of *M. bovis* (BCG) (see ref. 15.4 and 15.5)

	M. bovis (BCG)*
Gram stain reaction	weakly (+)
Acid Fast stain reaction	(+)
Typical Growth Characteristics on Solid Media	
Middlebrook 7H9	rough, raised, thick colonies with a nodular or wrinkled surface and an irregular thin margin, off-white to faint buff, or even yellow
Typical Microscopic Characteristics	
Cell dimensions	0.3-0.6 µm in diameter by 1-4 µm in length*
Cell appearance	rods, straight or slightly curved, occurring singly and in occasional threads

*After 15-20 days

Attachment 2

Culture Initiation and Stock Culture Generation for *Mycobacterium bovis* (BCG)

A1.	Culture initiation. Refer to SOP MB-02 for establishment of the organism control number.	
	a.	Initiate new stock cultures from lyophilized cultures of *Mycobacterium bovis* (BCG) from ATCC after no more than 18 stock culture transfers.
	b.	Open ampule of freeze-dried organism as indicated by ATCC. Using a tube containing 5-6 mL of M7H9 broth, 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. Mix well.
	c.	Use several drops of the suspension to inoculate two Middlebrook 7H10 agar plates and streak for isolation.
	d.	Incubate the tube of rehydrated culture and the plates at 36±1°C for 28±2 days.
A2.	Culture maintenance.	
	a.	Confirm the identity of a streak isolation plate and acid fast stain (see Attachment 1 for colony morphology and acid fast staining results).
	b.	Use an M7H10 streak isolation plate to streak M7H11 agar slants (stock slants). Based on anticipated use, streak approximately 10-20 stock slants.
	c.	Incubate the new stock transfers for 15-20 days at 36±1ºC. Store at 2-5ºC.
	d.	Every 6 weeks (42 days), generate an additional 10-20 M7H11 slants. Inoculate new M7H11 slants by streaking a loopful of *M. bovis* (BCG) growth from an established tube to each of the 10-20 tubes. Perform QC of stock cultures per section A3.
	e.	Incubate the stock culture slants at 36±1ºC for 15 to 20 days. Following incubation, maintain stock cultures at 2-5ºC for up to 6 weeks.
A3.	QC of stock cultures	
	a.	Up to every 6 weeks (42 days), streak a loopful of growth for isolation from the existing M7H11 stock slant used to inoculate new agar slants on a plate of M7H11 agar. Incubate the plate for 17-21 days at 36±1ºC.
	b.	Following the incubation period, record the colony morphology as observed on the M7H11 plate. See Attachment 1 for details on cell and colony morphology and stain reactions.
	c.	Perform an acid fast stain from growth taken from the M7H11 streak isolation plate according to the manufacturer's instructions. Observe the acid fast reaction by

using brightfield microscopy at 1000X magnification (oil immersion).

d. Record observations on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).

Attachment 3

Spray Apparatus