US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for Disinfectant Towelette Test: Testing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*

SOP Number: MB-09-07

Date Revised: 03-04-19

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Title	Disinfectant Towelette Test: Testing of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*
Scope	Describes the methodology to determine the efficacy of towelette-based disinfectants against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica* on hard surfaces. The test is based on AOAC Method 961.02 (Germicidal Spray Products as Disinfectants). 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, 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Date SOP withdrawn:	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**	Abbreviations/definitions are provided in the text.
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. Any disruption of the *Pseudomonas aeruginosa* pellicle resulting in the dropping or breaking of the pellicle in culture before or during its removal renders that culture unusable. 2. Prior to inoculation, ensure that the carriers are dry (inside Petri dishes). Moisture can interfere with the concentration and drying of the inoculum on the glass slide carrier. 3. Do not use any inoculated carrier that is wet at the conclusion of the carrier drying period. 4. For neutralizers/subculture media that do not result in turbidity as the outcome of growth, such as Dey/Engley broth, assess the interpretation of a positive tube in advance of the test (see section 12.7.e).
8. **Non-conforming Data**	1. Sterility and/or viability controls do not yield expected results. 2. 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* for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.5 (corresponding to a geometric mean density of 3.2×10^6); a mean *TestLD* below 5.0 and above 6.5 invalidates the test, except for two retesting scenarios (outlined in the study protocol).

	b. The mean *TestLD* for carriers inoculated with *S. enterica* must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 5.5 (corresponding to a geometric mean density of 3.2×10^5); a mean *TestLD* below 4.0 and above 5.5 invalidates the test, except for two retesting scenarios (outlined in the study protocol). 3. No contamination is acceptable in the test system. 4. Management of non-conforming data will be consistent with SOP ADM-07, Non-Conformance Reports.
9. **Data Management**	Data will be archived per 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. *Subculture media*; use 20 mL aliquots (e.g., letheen broth, fluid thioglycollate medium, and Dey/Engley broth). Note: Commercial media made to conform to the recipes provided in AOAC Method 961.02 may be substituted. 2. *Test organisms*. *Pseudomonas aeruginosa* (ATCC No. 15442), *Staphylococcus aureus* (ATCC No. 6538) and *Salmonella enterica* (ATCC No. 10708) obtained directly from ATCC. 3. *Culture media*. Note: Commercial media (e.g., synthetic broth) made to conform to the recipes provided in AOAC Method 961.02 may be substituted. a. *Synthetic broth*. Use for (10 mL) daily transfers and (10 mL) final test cultures of *S. aureus*, *P. aeruginosa* and *S. enterica.* b. *Nutrient broth*. Alternatively, use for (10 mL) daily transfers and (10 mL) final test cultures of *P. aeruginosa.* 4. *Trypticase soy agar (TSA)*. For use in propagation of the test organism to generate frozen cultures and as a plating medium for carrier enumeration. Alternately, TSA with 5% sheep blood (BAP) may be used. 5. *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. 6. *Carriers*. Glass Slide Carriers, 25 mm × 75 mm (or comparable size) borosilicate glass cover slips with number 4 thickness or Fisherfinest® Premium Frosted Microscope Slides (Fisher Scientific, Catalog number 12-544-2). Refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. 7. *Specialized glassware.* For primary and secondary subculture media, use autoclavable 38 × 100 mm glass tubes (Bellco Glass Inc., Vineland, NJ). Cap tubes with closures before sterilizing. 8. *Sterile surgical gloves*. For handling the towelette. 9. *Forceps*. For manipulating glass slides. 10. *Micropipettes*. For performing culture transfers and serial dilutions. 11. *Positive displacement pipette.* With corresponding sterile tips able to deliver 10 µL. 12. *Timer*. For managing timed activities, any certified timer that can display time in seconds. 13. *3M™ Petrifilm™ Aerobic Count Plates*. 3M Food Safety, St. Paul, MN, USA, Cat. No. 6400. 14. *Vitek 2 Compact*. Alternative for biochemical and antigenic analysis component of microbe confirmation.
12. **Procedure and Analysis**	One towelette is used to wipe ten carriers/slides. The area of the towelette used for wiping is folded and rotated so as to expose a new surface of the towelette for each carrier. The method may be altered to accommodate various towelette/carrier combinations (e.g., more than one towelette per set of ten slides). Prior to testing, perform the neutralization assay to determine if secondary subculture tubes are necessary. The Disinfectant Towelette Test Processing Sheet (see section 14) must be used for tracking testing activities.
12.1 Test Culture Preparation	Refer to SOP MB-02 for the test microbe culture transfer notation. Refer to Attachment 2 for culture initiation and generation of frozen stock cultures. a. Defrost a single cryovial (see Attachment 2) at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of growth medium.

	(Synthetic broth is used for *S. aureus*, *P. aeruginosa*, and *S. enterica*. Nutrient broth may be used for *P. aeruginosa*). Vortex, and incubate at 36 ± 1°C for 24 ± 2 h. One daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be subcultured for up to 5 days; each daily culture may be used to generate a test culture. For *S. aureus* and *S. enterica* only, briefly vortex the 24 h cultures prior to transfer. b. To generate test cultures, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL growth medium (e.g., synthetic broth or nutrient broth) with 10 µL per tube of the 24 h culture then vortex to mix. Incubate 48-54 h at 36 ± 1°C. Do not shake the 48-54 h *P. aeruginosa* test culture. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
12.2 Carrier Inoculation	a. Inoculate approximately 80 carriers; 60 carriers are required for testing, 6 for control carrier counts, and 1-2 for the viability control(s). Set aside 1-2 uninoculated carrier(s) for sterility control. b. For *P. aeruginosa*, remove the pellicle from the broth either by decanting the liquid aseptically into a sterile tube, by gently aspirating the broth away from the pellicle using a pipette, or by vacuum removal. Avoid harvesting pellicle from the bottom of the tube. Transfer test culture after pellicle removal into sterile 25 × 150 mm test tubes (up to approximately 20 mL per tube) and visually inspect for pellicle fragments. Presence of pellicle in the final culture makes it unusable for testing. Proceed as below in 12.2c. c. For *S. aureus*, *S. enterica*, and *P. aeruginosa* from 12.2.b, using a vortex-style mixer, mix 48-54 h test cultures 3-4 s and let stand 10 min at room temperature before continuing. 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. Measure and record the OD at 650 nm. Use sterile broth medium to calibrate the spectrophotometer. Use the test culture for carrier inoculation within 30 minutes. d. To achieve mean carrier counts within the appropriate range (see section 8), the final test culture may be diluted (e.g., one part culture plus one part sterile broth) prior to the addition of the OSL to the inoculum using the sterile culture medium used to generate the final test culture (e.g., synthetic broth). Use the diluted test culture for carrier inoculation within 30 minutes.

	Note: Concentration of the final test culture may be used in the event the bacterial titer in the final test cultures is too low. Concentration may be achieved using centrifugation (e.g., 5000g for 20 min) and resuspending the pellet in the appropriate volume of the sterile final test culture medium necessary to meet the carrier count range. Use the concentrated test culture for carrier inoculation within 30 min. e. Add appropriate amount of organic burden if required. Swirl to mix. f. Use a calibrated positive displacement pipette to transfer 0.01 mL of the test culture onto the sterile test carrier in the Petri dish, at one end of the slide. Do not place inoculum in the middle of the slide. Vortex-mix the inoculum periodically during the inoculation of carriers. Immediately spread the inoculum uniformly over one third of the carrier surface using a sterile loop. Do not allow the inoculum to contact the edge of the glass slide carriers during the inoculation process. Cover dish immediately. g. Dry carriers in incubator at 36 ± 1°C for 30-40 min. Record the timed carrier inoculation activities on the Disinfectant Towelette Test Processing Sheet (see section 14). Perform efficacy testing within two hours of drying. h. After completion of all slide inoculations, thoroughly wipe the micropipette with 70% ethanol prior to removal from the BSC.
12.3 Enumeration of viable bacteria from carriers (control carrier counts)	a. Assay dried carriers in 2 sets of three carriers, one set immediately prior to conducting the efficacy test and one set immediately following the test. Randomly select 6 inoculated carriers for carrier count analysis prior to efficacy testing. b. Place each of the inoculated, dried carriers in a 38 × 100 mm culture tube or sterile 50 mL polypropylene conical tube containing 20 mL of letheen broth. Vortex immediately – 60 ± 5 seconds for *P. aeruginosa* or 120 ± 5 seconds for *S. aureus* and *S. enterica*. Record the time of vortexing on the Disinfectant Towelette Test Processing Sheet (see section 14). c. After vortexing, briefly mix and make serial ten-fold dilutions in 9 mL dilution blanks of PBDW. Briefly vortex and plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA or BAP using spread plating. Plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate. Spread inoculum evenly over the surface of the agar. Plates must be dry

	prior to incubation. If the serial dilutions are not made and plated immediately, keep the tubes at 2-5°C until this step can be done. Complete the dilutions and plating within 2 h after vortexing. Alternatively, pool the letheen broth from the tubes with the carriers and briefly vortex. Serially dilute and plate 0.1 mL aliquots of the pooled media (60 mL). d. Incubate plates (inverted) at 36 ± 1°C for up to 48 ± 2 h. e. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the Disinfectant Towelette Test Carrier Counts Form and calculate the mean counts (see sections 13 and 14). f. Alternatively, Petrifilm may be used for enumeration of bacterial organisms. Follow manufacturer's instructions for preparation and incubation of Petrifilm cards. *Note*: At a minimum, conduct a culture purity check (isolation streak) using suspension from one dilution tube or letheen broth tube of one carrier or pooled set.
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22. b. Wipe the outside of the towelette packet or dispenser with 70% ethanol and allow to air dry prior to opening.
12.5 Test Procedure	a. Record timed events on the Disinfectant Towelette Test Time Recording Sheet for Carrier Transfers (see section 14). b. Aseptically remove several towelettes before aseptically removing a towelette to initiate testing. Fold towelette in half lengthwise one to two times depending on the size. Beginning at the bottom, fold up towards the top five times. The following steps in the "procedure" section are more conveniently done with two analysts – one to manage the Petri dishes and slides, and the other to perform the wiping procedure. c. Remove the lid from the Petri dish and aseptically remove the inoculated slide and hold it firmly against the rim of the Petri dish. d. Wipe the slide back and forth three times lengthwise with the towelette for a total of six passes across the inoculum or as specified by the study sponsor. Wiping should be done within ±5 seconds of specified time. Place slide in Petri dish, close the lid, and allow slide to sit undisturbed for the contact time. Maintain the wiped carriers in a horizontal position.

	e. Repeat with four additional slides, folding the used section of the towelette in such a way as to expose a new surface for wiping each slide. f. After the fifth slide, unfold the vertical fold in the towelette and reverse the towelette so that the used surface of the towelette faces inward. Continue wiping an additional five slides, folding the towelette between each slide to expose a new surface. g. After the last slide of a set (typically 10 slides) has been wiped and the exposure time is complete, sequentially transfer each slide into the primary subculture tube containing the appropriate neutralizer within the ±5 second time limit. Drain the excess disinfectant from each slide, without touching the Petri dish, and transfer into the neutralizer tube. Perform transfers with sterile forceps. Place the inoculated/wiped end of the slide into the primary subculture medium. h. After the slide is deposited, recap the subculture tube and shake thoroughly. i. If a secondary subculture tube is deemed necessary to achieve neutralization, then transfer the carrier from the primary tube to a secondary tube. Within 25-60 min of the initial transfer, transfer the carriers using sterile forceps to a second subculture tube. Move the carriers in order but the movements do not have to be timed. Thoroughly shake the subculture tubes after all of the carriers have been transferred. j. Incubate all subculture tubes 48 ± 2 h at 36 ± 1°C.
12.6 Sterility and viability controls	a. Viability controls. Place 1 (or 2) dried inoculated untreated carrier(s) into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Incubate tubes with the efficacy test. b. Sterility controls. Place 1 (or 2) sterile untreated carrier(s) into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Incubate tube(s) with the efficacy test.
12.7 Results	a. Gently shake each tube prior to recording results. Record results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity, on the Disinfectant Towelette Test Results Sheet (see section 14). b. Viability control. Growth should occur in all tubes.

	c. Sterility control. Growth should not occur in any of the tubes. d. If secondary subculture tubes are used, the primary and secondary subculture tubes for each carrier represent a "carrier set." A positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set. e. Specialized neutralizer/subculture medium such as Dey/Engley broth will not show turbidity; rather, the presence of pellicle at the surface of the medium (for *P. aeruginosa*) or a color change to the medium (yellow for growth of *S. aureus* or *S. enterica*) must be used to assess the results as a positive or negative outcome. i. Use viability controls for comparative determination of a positive tube. ii. If the product passes the performance standard, a minimum of 20% of the remaining negative tubes will be assayed for the presence of the test microbe using isolations streaks on TSA or BAP. Record preliminary results and conduct isolation streaks at 48 ± 2 h; however, continue to incubate negative tubes for up to an additional 24 hours to confirm the results.
12.8 Confirmatory Steps for Test Microbes	a. Confirm a minimum of three positive carrier sets per test. If there are less than three positive carriers, then confirm each carrier. If secondary subculture tubes are used and both tubes are positive in a carrier set, select only the tube with the carrier for confirmatory testing. b. For a test with greater than 20 positive carrier sets, confirm at least 20% by Gram staining, and a minimum of 4 positive carrier sets by Gram staining, solid media, and appropriate biochemical and antigenic analyses to ensure the identity of the organism. c. See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media. d. If confirmatory testing determines that the identity of the positive tube was not the test organism, annotate the positive entry (+) on the results sheet to indicate a contaminant was present. e. Alternatively, the Vitek 2 Compact may be used for confirmation in place of biochemical and antigenic analyses. Follow manufacturer's instructions for use of the Vitek 2 Compact.
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 *P. aeruginosa, S. aureus,* and *S. enterica.* 2. Attachment 2: Culture Initiation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica.* 3. 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 (Frozen Stock Cultures) — MB-06_F2.docx Test Microbe Confirmation Sheet (Quality Control) — MB-06_F3.docx Disinfectant Towelette Test Carrier Counts Form — MB-09-07_F1.docx Disinfectant Towelette Test Time Recording Sheet for Carrier Transfers — MB-09-07_F2.docx Disinfectant Towelette Test Information Sheet — MB-09-07_F3.docx Disinfectant Towelette Test Results Sheet (1°/2°) — MB-09-07_F4.docx Disinfectant Towelette Test Results Sheet (1°) — MB-09-07_F5.docx Test Microbe Confirmation Sheet — MB-09-07_F6.docx Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template DTT — MB-09-07_F7.xlsx Disinfectant Towelette Test Carrier Counts Form (Pooled Carriers) — MB-09-07_F8.docx Disinfectant Towelette Test Processing Sheet — MB-09-07_F9.docx
15. **References**	1. Official Methods of Analysis. Revised 2013. AOAC INTERNATIONAL, Gaithersburg, MD, (Method 961.02). 2. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164, *S. enterica* p. 447. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins,

	Baltimore, MD. *S. aureus* p. 1015.

Attachment 1

Typical Growth Characteristics of strains of *P. aeruginosa*, *S. aureus*, and *S. enterica* (see ref. 15.2 and 15.3).

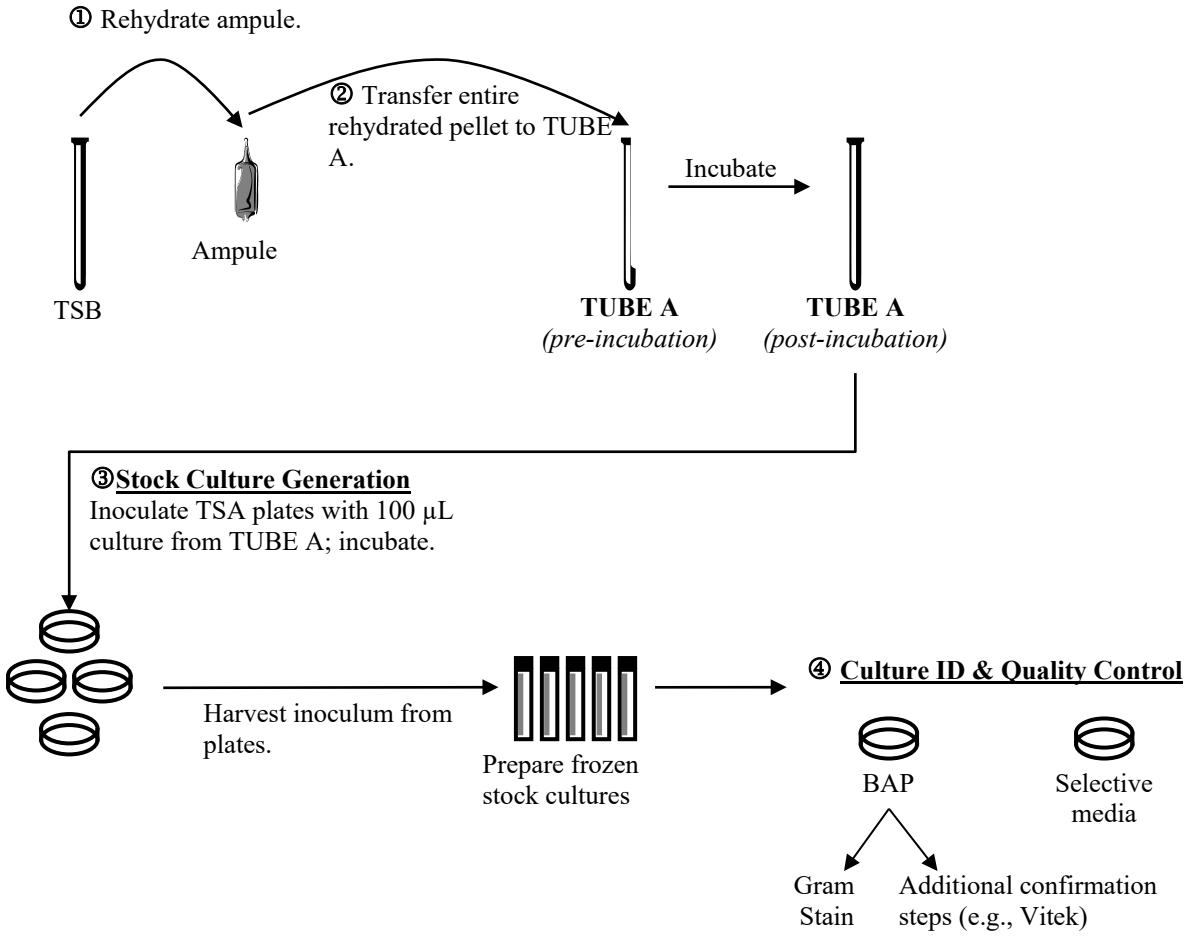
	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	(-)	(+)	(-)
Typical Growth Characteristics on Solid Media			
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A
Xylose lysine deoxycholate (XLD) agar	N/A	N/A	Round, clear red colonies with black centers
Blood agar (BAP)	flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	small, circular, yellow or white, glistening, beta hemolytic	entire, glistening, circular, smooth, translucent, low convex, non-hemolytic
Typical Microscopic Characteristics			
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*	0.5-1.5 µm in diameter*	0.7-1.5 µm in diameter by 2.0-5.0 µm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	straight rods, peritrichous flagella

*After 24±2 hours

(1) Test organism may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent. Pyocyanin is not produced.

Attachment 2

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus*, *P. aeruginosa*, and *S. enterica*



Preparation of Frozen Stock Cultures. Refer to SOP MB-02 for establishment of the organism control number.

a. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Salmonella enterica* (ATCC 10708) from ATCC within 18 months.

b. Open ampule of freeze dried organism as indicated by ATCC. Using a tube containing 5-6 mL of TSB for *P. aeruginosa* and *S. aureus* and 5-6 mL of NB for *S. enterica*, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth designated as "TUBE A". Mix well.

i. Incubate broth culture (TUBE A) at 36±1ºC for 24±2 h. Record all manipulations on the Organism Culture Tracking Form (see section 14).

ii. For QC purposes, perform a streak isolation of the TUBE A culture on a BAP. In addition, for *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide); for *S. enterica*, streak a loopful onto XLD. Incubate all plates at 36±1°C for 24±2 h.

c. Following incubation, use a sterile spreader to inoculate a sufficient number of TSA plates (e.g., 5 to 10 plates per organism) with 100 µL each of the 24±2 h culture. Incubate plates at 36 ± 1°C for 24 ± 2 h.

d. Following incubation, add 5 mL cryoprotectant solution (TSB with 15% v/v glycerol for *S. aureus* and *P. aeruginosa* and NB with 15% v/v glycerol for *S. enterica*) to the surface of each agar plate. Resuspend the cells in this solution using a sterile spreader or a sterile swab and aspirate the cell suspension from the surface of the agar. Transfer the suspension into a sterile vessel. Repeat by adding another 5 mL of cryoprotectant to the agar plates, resuspend the cells, aspirate the suspension and pool with the initial cell suspension.

i. For QC purposes, use the pooled suspension to perform a streak isolation on a BAP. In addition, for *S. aureus* and *P. aeruginosa*, streak a loopful onto both selective media (MSA and Cetrimide); for *S. enterica*, streak a loopful onto XLD. Incubate all plates at 36±1°C for 24±2 h. Continue QC steps as per sections g through i.

e. Mix the pooled contents of the vessel thoroughly. Immediately after mixing, dispense approximately 0.5 to 1.0 mL aliquots into cryovials (e.g., 1.5 mL cyrovials).

f. Place and store the cryovials at -70°C or below; these are the frozen stock cultures. Stock cultures may be used up to 18 months; reinitiate using a new lyophilized culture. These cultures are single-use only.

g. Following the incubation period (see d.i.), record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth). See Attachment 1 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

h. For each organism, perform a Gram stain and Vitek from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

i. Record all confirmation results on the Test Microbe Confirmation Sheet (Quality Control) (see section 14).