US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for
AOAC Use Dilution Method for Testing Disinfectants

SOP Number: MB-05-14

Date Revised: 08-11-16

SOP Number	MB-05-14
Title	AOAC Use Dilution Method for Testing Disinfectants
Scope	Describes the Use-dilution methodology used to determine the efficacy of disinfectants against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Salmonella enterica* on hard surfaces.
Application	The methodology described in this SOP is used to evaluate the performance of liquid disinfectants against the prescribed test microbes.

	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Date SOP withdrawn:	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) and EQ-05 (timers).
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 disrupting or breaking of the pellicle in culture before or during its removal renders that culture unusable in the use-dilution test. 2. Transferring the inoculated carriers into the disinfectant is a critical, technique-sensitive step. False positives can result from transfer of test microbe to sides of tubes due to contact or aerosol formation. 3. Viscous test chemicals may result in a substantial amount of product remaining on treated carriers following the contact time, which upon transfer to the primary subculture medium (neutralizer) produces cloudiness in the medium. This cloudiness may negatively impact the recording of results. 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.c.).
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. a. The mean *Test LD* for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6) and not above 7.0 (corresponding to a geometric mean density of 1.0×10^7). b. The mean *Test LD* for carriers inoculated with *S. enterica* must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5)

	and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6). 3. No contamination is acceptable in the test system. 4. Manage non-conforming data as specified in the study protocol; procedures are 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. Prior to testing, perform the neutralization assay to determine if secondary subculture tubes are necessary (refer to SOP MB-17, Neutralization Confirmation). 3. Verify the volume of dilution blanks, neutralizer tubes, and subculture tubes in advance and adjust accordingly. 4. When transferring inoculated carriers to disinfectant tubes during testing (see section 12.5.b), avoid intense swirling and agitation of the carriers.
11. Special Apparatus and Materials	1. *Subculture/neutralizer media* (e.g., letheen broth, fluid thioglycollate medium). Note: Commercial media made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 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., HiMedia synthetic broth) made to conform to the recipes provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted. a. *Synthetic broth (10 mL tubes)*. Use for daily transfers and final test cultures. 4. *Other media*. For example, Tryptic Soy Broth (TSB) and Nutrient Broth (NB) for rehydrating the lyophilized cultures. 5. *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. 6. *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. 7. *Carriers*. Polished stainless steel cylinders, 8 ± 1 mm outer diameter, 6 ± 1 mm inner diameter, 10 ± 1 mm length; type 304 stainless steel, SS 18-8 (S & L Aerospace Metals, Maspeth, NY or Fisher Scientific catalog number 07-907-5Q as of July 2012). Use only carriers that passed bioscreening; refer to SOP MB-03, Screening of Stainless Steel Cylinders, Porcelain Cylinders and Glass Slide Carriers Used in Disinfectant Efficacy Testing. 8. *Specialized glassware.* For disinfectant, use autoclavable 25 × 100 mm tubes (Bellco Glass Inc., Vineland, NJ). For glassware used to prepare test chemical, refer to SOP MB-22. 9. *Recirculating chiller unit*. For maintaining specified temperature of the test chemical. 10. *Transfer loops*. For performing culture transfers. Make 4 mm inner diameter single loop at end of 50–75 mm (2–3 in.) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3 in.) shaft (available from Johnson Matthey, West Chester, PA 19380, USA). Fit other end in suitable holder. Bend loop at 30° angle with stem. 11. *Micropipettes*. For performing culture transfers and serial dilutions. 12. *Wire Hook*. For carrier transfer. Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Place other end in suitable holder. 13. *Timer*. For managing timed activities, any certified timer that can display time in seconds. 14. *Sonicator* (ultrasonic cleaner). For conducting control carrier counts. 15. *Vitek 2 Compact*. For microbe identification.
12. Procedure and Analysis	
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 cryovial 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 synthetic broth, 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[1] the 24 h cultures prior to transfer. b. To generate test cultures, inoculate a sufficient number of 20 × 150 mm tubes containing 10 mL synthetic broth with 10 µL per tube of the 24 h culture then vortex[2] to mix. Incubate 48-54 h at 36 ± 1°C. Do not shake the 48-54 h test culture. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
12.2 Carrier Inoculation	Inoculate approximately 80 carriers; 60 carriers are required for testing, 6 for control carrier counts, and 1 for the viability control. a. For *P. aeruginosa*, remove the pellicle from the 48-54 h test culture 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 12.2b. b. For *S. aureus*, *S. enterica*, and *P. aeruginosa* (from 12.2.a), 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. *Note*: 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 organic soil 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 min. *Note*: Concentration of the final test culture may be used in the event the bacterial titer in the final test cultures is too low (OD ≤ 0.2). Concentration may be achieved using centrifugation (e.g., 5000 *g* for 20 min) and resuspending the pellet in the appropriate volume of the sterile final test culture medium necessary to meet the carrier count

[1] Step not contained in the AOAC standard methods 955.14 and 955.15.
[2]Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

	range. Use the concentrated test culture for carrier inoculation within 30 min. c. Add appropriate amount of organic soil if required. Swirl to mix. d. Aliquot 20 mL portions into sterile 25 x 150 mm test tubes. e. Drain the water from the carriers. Aseptically transfer 20 carriers into each of the tubes containing the test culture. The test culture must completely cover the carriers; reposition carriers as necessary to ensure coverage. Alternatively, siphon off the water from the carriers and add 20 mL test culture directly to the carriers without transferring. f. Allow carriers to remain in the inoculum for 15 ± 2 min. g. Following the carrier exposure period, remove carriers individually from the inoculum using a flamed nichrome wire hook, briefly tap each carrier against the side of the tube to remove excess culture, and place on end in vertical position in sterile Petri dish matted with 2 layers of Whatman No. 2 (or equivalent) sterile filter paper. Do not remove inoculum from the tube in advance of removing carriers.[3] Ensure that carriers do not touch or fall over in the Petri dish. Place no more than 12 carriers in a Petri dish. Place lid on Petri dish. h. Dry carriers in incubator at 36 ± 1°C for 40 ± 2 min. Record the timed carrier inoculation activities on the AOAC Use-Dilution Test Processing Sheet (see section 14). Expose all carriers to disinfectant within two hours of drying.
12.3 Enumeration of viable bacteria from carriers (control carrier counts)	a. Select one carrier from each of 6 Petri dishes, 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. b. Place each inoculated dried carrier into a tube containing 10 mL of letheen broth and sonicate in an ultrasonic cleaner for 1 min ± 5 s. Record the time of sonication on the AOAC Use-Dilution Test Processing Sheet (see section 14). c. For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of the letheen broth in the tubes. Place the beaker in an ultrasonic cleaner so that the water level in the beaker is even with the water level fill-line on the tank. Fill the tank with tap water to the water level fill-line. Hold the beaker so that it does not touch the bottom of the tank and all 3 liquid levels (inside the test

[3]Note: Draining of inoculum with a pipette after contact time is currently provided in the AOAC standard methods 955.14, 955.15, and 964.02.

	tubes, inside the beaker, and inside the tank) are approximately the same. d. After sonication, 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 sonicated tubes at 2-5°C until this step can be done. Complete the dilutions and plating within 2 h after sonication. Alternatively, pool the letheen broth from the tubes with the carriers and briefly vortex for each set of three carriers. Serially dilute and plate 0.1 mL aliquots of the pooled media (30 mL). e. Incubate plates (inverted) at 36 ± 1°C for up to 48 ± 2 h. f. Count colonies. Plates that have colony counts over 300 will be reported as TNTC. Record counts on the AOAC Use-Dilution Test Carrier Counts Form and calculate the mean counts (see sections 13 and 14). g. 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 of one carrier or pooled set.
12.4 Disinfectant Sample Preparation	a. Prepare disinfectant sample per SOP MB-22, Disinfectant Product Preparation. b. Equilibrate the water bath and allow it to come to 20 ± 1°C or the temperature specified (±1°C). Prepare the disinfectant dilutions within 3 hours of performing the assay unless test parameters specify otherwise. Record the time of disinfectant preparation on the AOAC Use-Dilution Test Processing Sheet (see section 14). c. Dispense 10 mL aliquots of the disinfectant into 25 × 100 mm test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 min to allow disinfectant to come to specified temperature. Record the temperature of the water bath and recirculating chiller before and after testing on the AOAC Use-Dilution Test Information Sheet (see section 14).

12.5 Test Procedure	a. Sequentially transfer the carriers from the Petri dish to the test tubes containing the disinfectant at appropriate intervals (e.g., 30 second intervals). b. Add one carrier per tube and swirl the tube using 2-3 gentle rotations before placing it back in the water bath Add carrier within ±5 seconds of the specified time for a contact time of 1-10 minutes or within ±3 seconds for contact times <1 minute. c. Using alternating hooks, flame-sterilize the hook and allow it to cool after each carrier transfer. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook can touch the interior sides of the tube. If the interior sides of the tube are touched, repeat the carrier. d. Following the exposure time, sequentially transfer the carriers into subculture/neutralizer media. Remove the carrier from the disinfectant with a sterile hook, tap it against the interior sides of the tube to remove the excess disinfectant, and transfer it into the subculture tube within ±5 s. Avoid tapping the carrier against the upper third of the tube. Avoid contact of the carrier to the interior sides of the subculture tube during transfer. e. Recap the subculture tube and shake thoroughly. Incubate at 36 ± 1°C for 48 ± 2 h. f. If a secondary subculture tube is deemed necessary to achieve neutralization, then transfer the carrier from the primary tube to a secondary tube of sterile medium after a minimum of 30 ± 5 min at room temperature from the end of the initial transfer. Within 25-60 min of the initial transfer, transfer the carriers using a sterile wire hook 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. Incubate both the primary and secondary subculture tubes 48 ± 2 h at 36 ± 1°C. g. Record timed events on the AOAC Use-Dilution Test Time Recording Sheet for Carrier Transfers (see section 14).
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. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity. Growth should occur in both tubes. Record results on AOAC Use-Dilution Test Results Sheet (see

	section 14). b. Sterility controls. Place one sterile, uninoculated carrier into a tube of neutralizing subculture broth. Incubate tube with the efficacy test. Report results as + (growth), or 0 (no growth) as determined by presence or absence of turbidity. Growth should not occur in the tube. Record results on AOAC Use-Dilution Test Results Sheet (see section 14).
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 AOAC Use-Dilution Test Results Sheet (see section 14). b. 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. c. 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.[4]
12.8 Confirmatory Steps for Test Microbes[5]	a. For *S. aureus*, confirm a minimum of four positive carriers per test. For *P. aeruginosa* confirm a minimum of seven positive carriers. For tests with fewer positives, confirm each positive carrier accordingly. For any *S. aureus* or *P. aeruginosa* test with ≥20 positive carrier sets, confirm a minimum of 50% of the positives. b. For *S. enterica*, confirm a minimum of three positive carrier sets per test. If there are less than three positive carriers, then confirm each carrier. For any test with ≥20 positive carrier sets, confirm a

[4]Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.
[5]Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

	minimum of 20% of the positives. c. If secondary subculture tubes are used and both tubes are positive in a carrier set, select only the secondary tube for confirmatory testing. For confirmatory testing, use Gram staining, solid media, and Vitek 2 Compact or appropriate biochemical and antigenic analyses to ensure the identity of the organism. Follow manufacturer's instructions for use of the Vitek 2 Compact. i. Growth from the subculture medium should be examined for the test organism by inoculating onto TSA or TSA with 5% sheep blood, and selective media. Incubate media plates 18–24 h at 36 ± 1°C and record the results. Examine colonies on plates for morphology and characteristics of the test organism (conforming to the morphology in Bergey's Manual). d. See Attachment 1 for Gram stain reactions, cell morphology, results of biochemical and antigenic analyses, and colony characteristics on solid media, e. If confirmatory testing determines that the identity of the unknown was not the test organism, annotate the positive entry (+) on the results sheet to indicate a contaminant was present.
12.9 Performance Standard	a. The performance standard for *S. aureus* is 0-3 positive carriers out of sixty. b. The performance standard for *P. aeruginosa* is 0-6 positive carriers out of sixty. c. The performance standard for *S. enterica* is 0-1 positive carriers out of sixty. d. If replicated testing is required for any microbe, conduct testing with that microbe on independent test days.
12.10 Re-use of Stainless Steel Carriers	a. After use, autoclave all carriers. Carriers for which test results were negative may be reused after cleaning. Carriers that are positive are re-cleaned and screened biologically (see SOP MB-03, Screening Carriers) before re-use. These carriers may be reused if the biological screening test results in no growth. The extra inoculated carriers, positive control, and those used for carrier counts may be autoclaved, re-cleaned, and used again.[6]
13. Data Analysis/	Calculations will be computed using a Microsoft Excel spreadsheet (see

[6]Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

Calculations	section 14). Both electronic and hard copies of the spreadsheet will be retained. Counts up to 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*, and Preparation of Frozen Stocks 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Organism Culture Tracking Form — MB-05-14_F1.docx Test Microbe Confirmation Sheet (Quality Control) — MB-05-14_F2.docx AOAC Use-Dilution Test Time Recording Sheet for Carrier Transfers — MB-05-14_F3.docx AOAC Use-Dilution Test Information Sheet — MB-05-14_F4.docx AOAC Use-Dilution Test Results Sheet (1°) — MB-05-14_F5.docx AOAC Use-Dilution Test Results Sheet (1°/2°) — MB-05-14_F6.docx Test Microbe Confirmation Sheet — MB-05-14_F7.docx AOAC Use-Dilution Test Carrier Counts Form — MB-05-14_F8.docx AOAC Use-Dilution Test Processing Sheet — MB-05-14_F9.docx Carrier Count Spreadsheet MS Excel spreadsheet: Carrier Count Template_UDT_v4 — MB-05-14_F10.xlsx AOAC Use-Dilution Test Carrier Counts Form (Pooled Carriers) — MB-05-14_F11.docx
15. References	1. Official Methods of Analysis. Method 955.14 – *Salmonella enterica*. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD. 2. Official Methods of Analysis. Methods 955.15 – *Staphylococcus aureus*. Posted September 2013. AOAC INTERNATIONAL, Gaithersburg, MD. 3. Official Methods of Analysis. Method 964.02 – *Pseudomonas aeruginosa*. Posted September 2013. AOAC INTERNATIONAL, Gaithersburg, MD. 4. 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. 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. *S. aureus* p. 1015. 6. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 08/2014. 7. Package Insert – Catalase Reagent Droppers. Becton, Dickinson and Company. Part no. L001237. Revision 06/2010. 8. Package Insert – Staphaurex Plus. Remel. Part no. R30950102. Revised 11/23/07. 9. Package Insert – Oxidase Reagent Droppers. Becton, Dickinson and Company. Part no. L001133. Revision 06/2010. 10. Package Insert – Wellcolex Colour Salmonella. Remel. Part no. R30858301. Revised 10/17/07.

Attachment 1

Typical Characteristics of strains of *P. aeruginosa, S. aureus,* and *S. enterica* (see ref. 15.4 through 15.10).

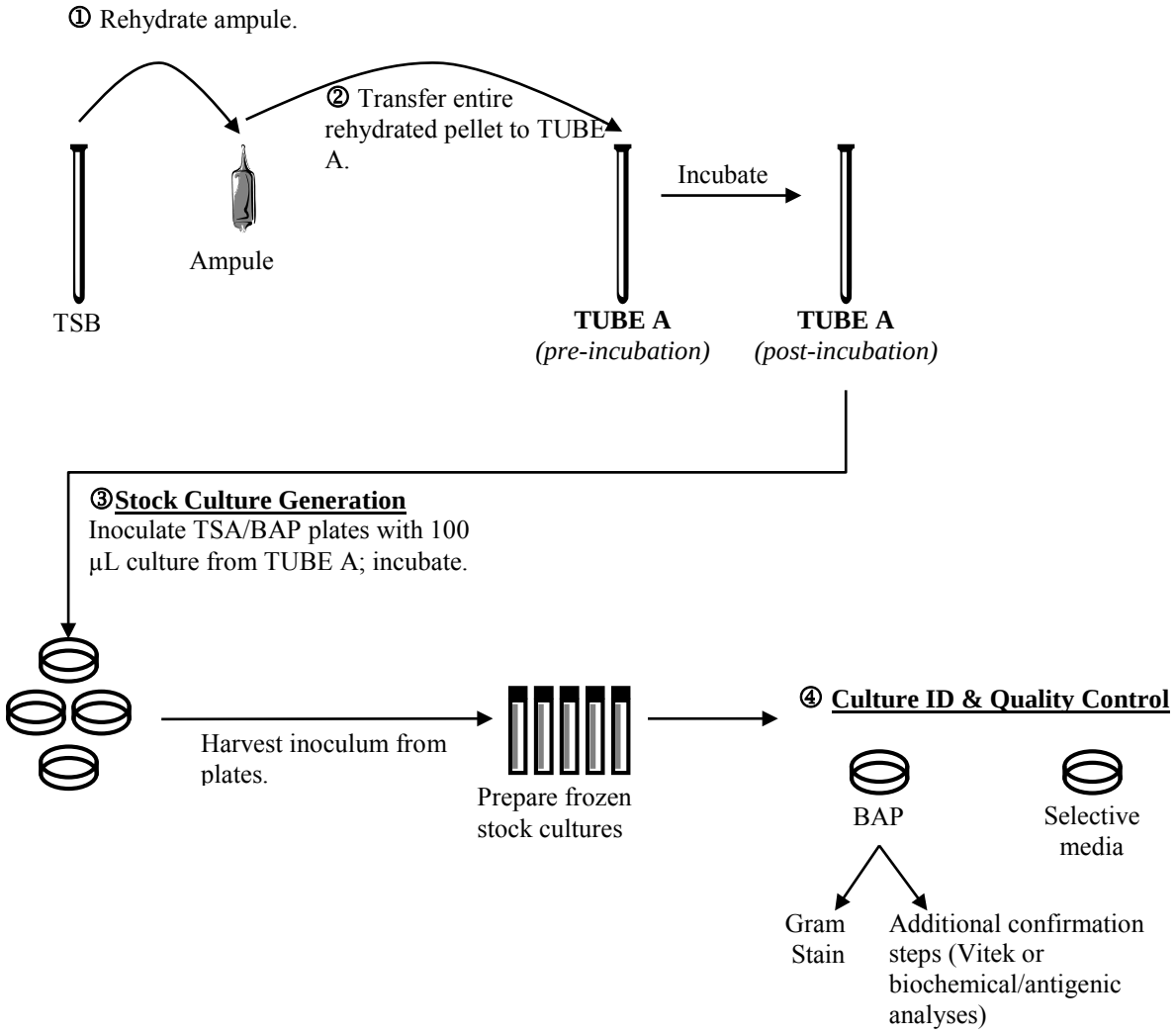
	*P. aeruginosa**	*S. aureus**	*S. enterica**
Gram stain reaction	(-)	(+)	(-)
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow	N/A
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth	N/A
Xylose 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
Biochemical and Antigenic Analyses	Oxidase Test (+)	Catalase Test (+) Staphaurex Test (+)	Wellcolex Color Salmonella Test (+)
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*



Attachment 2 continued.

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 hours. Record all manipulations on the Organism Culture Tracking Form (see section 14).

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 hour culture. Incubate plates at 36 ± 1°C for 24 ± 2 h.

   i. For QC purposes, perform a streak isolation of the 24 ± 2 hour broth 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.

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. Re-suspend 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, re-suspend 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.[7] These cultures are single-use only.

g. Following the incubation period (see d.i), record the colony morphology as observed on

[7] Step not contained in the AOAC standard methods 955.14, 955.15, and 964.02.

the BAPs and selective media plates (including the absence of growth). See Attachment 1 for details on cell and colony morphology, results of biochemical and antigenic analyses, 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).