US Environmental Protection Agency
Office of Pesticide Programs

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

Standard Operating Procedure for
Germicidal and Detergent Sanitizing Action of Disinfectants Tests

SOP Number: MB-27-03

Date Revised: 11-19-19

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Title	Germicidal and Detergent Sanitizing Action of Disinfectants Test
Revisions Made	• Minor editorial changes for clarification purposes. • Updated References section. • Updated Attachment 2 (Culture Initiation and Stock Culture Generation) to be in alignment with MB-05.

SOP Number	MB-27-03
Title	Germicidal and Detergent Sanitizing Action of Disinfectants Test
Scope	This SOP describes the methodology used to determine the efficacy of food contact sanitizers against *Staphylococcus aureus* and *Escherichia coli*. The methodology is based on AOAC method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants – revision date 2013.
Application	For product evaluations, a study protocol is developed which identifies the specific test conditions for a test chemical sample including contact time, dilution, and neutralizer.

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

Data 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Date SOP withdrawn:	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 test chemicals.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	Refer to SOPs EQ-01 (pH meter), EQ-02 (Thermometer), EQ-03 (Weigh Balance), 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, Preparation and Sampling Procedures for Antimicrobial Test Substances, 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). It is critical to maintain the highest standards of good laboratory practices and aseptic technique during all manipulations.
7. Interferences	No contamination is acceptable in the treated or numbers control plates.
8. Non-conforming Data	1. Sterility and/or viability controls do not yield expected results. 2. The mean log density for numbers control plates falls outside the specified range of 7.0 – 8.0 $\log_{10}$/mL. 3. Manage non-conforming data as specified in the study protocol; procedures are consistent with SOP ADM-07, Non-Conformance Reports. 4. *Retesting guidance*: For tests where the test chemical meets the performance standard and the numbers control mean $\log_{10}$ density value is above 8.0, no retesting is necessary. For tests where the test chemical fails to meet the performance standard and the numbers control mean $\log_{10}$ density is below 7.0, no retesting is necessary.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Strict adherence to the protocol is necessary for the validity of the test results. 2. Plating should be completed within 1 hour after the initiation of serial dilutions. 3. For spread plating: ensure that the entire surface of the agar plate is dry before adding inoculum. If necessary, leave the agar plates uncovered in

	the biological safety cabinet (BSC) until the moisture has been completely absorbed into the medium. 4. Use diluted test chemical within three hours of preparation unless specified otherwise.
11. Special Apparatus and Materials	1. *Test organisms*. *Escherichia coli* (ATCC No. 11229) and *Staphylococcus aureus* (ATCC No. 6538) obtained directly from a reputable supplier (e.g., ATCC). 2. *Culture media*. Note: Commercial dehydrated media made to conform to the recipes provided in AOAC Method 960.09 may be substituted, unless indicated otherwise. a. *Trypticase Soy Agar (TSA).* Prepare according to manufacturer's instructions. Used for the generation of frozen stock cultures for *S. aureus.* b. *Tryptic Soy Broth (TSB).* Prepare according to manufacturer's instructions. Used for the generation of frozen stock cultures for *S. aureus.* c. *Nutrient broth*: Boil 5 g beef extract (powder), 5 g NaCl, and10 g peptone (anatone) in 1 L H_2O for 20 minutes and dilute to volume with de-ionized water; adjust to pH 6.8 ± 0.1. Filter through paper (Whatman No. 4, or equivalent). Steam sterilize 20 min at 121°C. Used for the preparation of Nutrient agar plates. d. *Nutrient agar (AOAC)*: Dissolve Bacto agar to 1.5% (w/v) in nutrient broth and adjust to pH 7.2-7.4. Steam sterilize for 20 min at 121°C. Dispense into plates. Used for the generation of frozen stock cultures for *E. coli.* e. *Nutrient agar – A (NA-A):* Boil 3 g beef extract, 5 g peptone and 15 g salt free agar in 1 L de-ionized water. Do not use premixed dehydrated medium. Dispense 10 mL portions in 20 × 150 mm tubes or 20 mL portions in 25 × 150 mm tubes and steam sterilize for 20 min at 121°C. Slant tubes after sterilization and let cool. Used for daily transfers of test cultures. f. *Nutrient agar – B (NA-B):* Boil 3 g beef extract, 5 g peptone and 30 g salt free agar in 1 L de-ionized water. Do not use premixed dehydrated medium. Steam sterilize for 20 min at 121°C. Temper medium prior to dispensing 20-30 mL portions into sterile Petri dishes. Used for development of the final test culture. 3. *Subculture media*: choose the appropriate recovery agar and neutralizer to inactivate the test chemical, for example:

a. *Tryptone glucose extract agar plus Neutralizer (TGEA-N):* Combine 24 g of dehydrated medium with 975 mL de-ionized water and 25 mL stock neutralizer if necessary. Steam sterilize for 15 min at 121°C. Used for the recovery of test organisms from treated samples.

b. *Tryptone glucose extract agar (TGEA):* Prepare according to the manufacturer's instructions. Used for the enumeration of numbers control samples.

4. *General Media and Reagents*:

a. *TSB with 15% (v/v) glycerol.* Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and autoclave for 15 min at 121°C. Store at room temperature for up to three months. Used for the preservation of frozen stock cultures as cryoprotectant solution.

b. *Neutralizer Stock Solution (NSS)*: Mix 40 g Lecithin, 280 mL polysorbate 80 and 1.25 mL 0.25 M phosphate buffer stock solution. Dilute with de-ionized water to 1 L and adjust pH to 7.2. Dispense in 100 mL portions and steam sterilize for 20 min at 121°C. Used for the preparation of the Neutralizer.

c. *Neutralizer blanks (NB)*: For use with ≤ 200 ppm quaternary ammonium compounds. Mix 100 mL neutralizer stock solution, 25 mL 0.25 M phosphate buffer stock solution, and 1675 mL of de-ionized water. Dispense into appropriate size vessel and steam sterilize for 20 min at 121°C. Alternate neutralizers may be used as necessary.

d. 0.25M *Phosphate buffer stock solution (PBSS)*: Dissolve 34 g KH_2PO4 in 500 mL de-ionized water in 1 L volumetric flask. Adjust pH to 7.2 with 1 N NaOH and dilute to 1 L volume mark. Sterilize by filtration. Used for the preparation of phosphate buffer solution.

e. *Phosphate buffer solution (PBS).* Add 1.25 mL of phosphate buffer stock solution and 8.75 g of NaCl to a volumetric flask; fill with de-ionized water to the 1000 mL mark and mix. A pH of approximately 7.0 is desirable. Sterilize by either filtration or steam sterilization at 121°C for 15-20 min. Alternative PBS formulations with the same pH may be used (e.g., dilute commercially prepared 10X PBS solution to 1X using de-ionized water). Used for the preparation of dilution blanks.

f. *Phosphate buffer dilution water stock solution (PBDW-SS)*. Dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL de-ionized water. Adjust pH to 7.2 ± 0.2 with 0.1 N NaOH or 0.1 N HCl

	and bring to 1000 mL with de-ionized water. Alternative phosphate buffers with the same pH may be used (e.g., commercially prepared 10X PBS solution). Used for the preparation of the phosphate buffer dilution water. g. *Phosphate buffer dilution water (PBDW)*: Add 1.25 mL of 0.25 M phosphate buffer stock solution to 1 L de-ionized water using a volumetric flask, mix thoroughly. Dispense into appropriate size vessel and steam sterilize for 20 min at 121°C. Used for numbers control assay and dilution blanks. h. *Tween-80 (polysorbate 80)*. Used for the preparation of PBS with 0.1% Tween 80. i. *PBS (1X) with 0.1% Tween 80 (PBS + T80)*: Add 100 mL PBS 10X solution and 1 mL Tween 80 to a volumetric flask; fill with de-ionized water to the 1000 mL mark and mix thoroughly. Sterilize by filtration. Used during test culture harvesting. j. *Sterile water*. De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media. k. *Blood Agar plate (BAP).* Commercially purchased Trypticase soy agar (TSA) with 5% sheep's blood agar plates. Used for the presumptive identification of the test microbes. l. *Mannitol Salt Agar (MSA).* Combine 111 g of dehydrated medium with 1 L de-ionized water and mix thoroughly. Steam sterilize for 15 min at 121°C. Used for the presumptive identification of the test microbes. m. *Xylose lysine deoxycholate agar (XLD).* Commercially purchased plates. Used for the presumptive identification of the test microbes. 5. *Equipment and Glassware.* For sanitizer efficacy and numbers control, use 250 mL wide mouth Erlenmeyer flasks. For measuring sanitizers and dilution blanks use 100 mL graduated cylinders. For glassware used to prepare test chemical, refer to SOP MB-22. a. *Sterile Petri dishes.* 20 mm × 100 mm in size. b. *Recirculating chiller unit*. For maintaining specified temperature of the test chemical (capable of maintaining 25 ± 1°C). c. *Test tube racks.* Any convenient style. d. *Transfer loops*. Make 4 mm ID 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. Commercially available 4 mm ID (i.e. 10 µL loops) transfer loops may also be used. Micropipettes may also be used instead of transfer loops. e. *Timer*. Certified timer for managing timed activities. f. *Micropipettes*. For performing culture transfers and serial dilutions. g. *Whatman No. 2 filter paper*. Sterile. h. *Gram stain kit*. i. *Vitek 2 Compact*. For microbe identification and confirmation.
12. Procedure and Analysis	Prior to testing, perform the neutralization assay to determine if the prescribed neutralizer is appropriate for the test chemical (see section 12.7).
12.1 Culture Preparation	Refer to Attachment 2 for preparation of the frozen stock cultures. Reinitiate new frozen stock cultures every 18 months with a new lyophilized culture. Note: A new frozen stock culture may be initiated <u>one</u> time using an existing, unexpired frozen stock culture as the source. . Refer to MB-02: Tracking of Test Microorganisms, Section 12 for the tracking and transfer notations for the test microbes a. For a daily culture, defrost a single cryovial of frozen stock culture at room temperature and briefly vortex to mix. Streak one loopful of the thawed frozen stock onto a NA – A slant and incubate at 36 ± 1°C for 24 ± 2 h. Only one daily transfer is required prior to the initiation of the final test culture. b. For the final test culture, add 5 mL of PBDW to a NA – A slant (daily culture). Using a sterile loop, dislodge growth from agar surface. Collect mixture and transfer to a flask containing 99 mL of PBDW. Mix throughly. Add 200 µL of the mixture to inoculate a minimum of 5 NA – B plates to create a bacterial lawn. Incubate at 36 ± 1°C for 24 ± 2 h. c. Record all culture transfers on the Organism Culture Tracking Form (see section 14).
12.2 Test Culture Harvesting	a. After incubation, add a minimum of 5 mL of PBS + Tween 80 to each plate. Using a sterile rod, gently dislodge culture from agar surface, avoid disrupting agar. Combine culture from all plates and mix thoroughly. b. Filter culture through sterile Whatman No. 2 filter paper using a

	vacuum source; collect filtered test culture into a sterile vessel. Note: filter paper can be pre-cut and sterilized prior to use to fit on filter apparatus as necessary. c. Standardize the filtered test culture as necessary using PBDW to achieve a final test culture microbe titer between 1.0×10^9 CFU/mL and 1.0×10^{10} CFU/mL (9-10 $\log_{10}$/mL).
12.3 Test chemical Sample Preparation	a. Prepare test chemical sample per SOP MB-22. 1) Ready-to-use test chemicals are tested as received; no dilution is required. 2) If hard water is required as the diluent, prepare synthetic hard water as described in SOP MB-30. b. Equilibrate water bath and allow it to come to 25 ± 1°C or the temperature specified (±1°C). Prepare the test chemical dilutions within 3 h of performing the assay. c. Dispense 99 mL aliquots of the diluted test chemical or ready-to-use test chemical into sterile wide mouth Erlenmeyer flasks. Prepare one flask per test microbe for each test chemical to be tested. d. Place flasks in the equilibrated water bath for approximately 10 min to allow test chemical to come to specified temperature. e. Record the temperature of the water bath and recirculating chiller before and after testing on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Test Information and Culture Preparation Sheet (see section 14). f. In addition, prepare a similar flask containing 99 mL of PBDW to use for numbers control for each organism (see section 12.5).
12.4 Treated Sample Test Procedure	Flasks containing test culture × test chemical are referred to as the treated samples. a. Add 1 mL of test culture to the test flask as follows: b. Swirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at the point of contact with test sample. c. Add suspension midway between center and the inner edge of the flask with tip of pipette slightly immersed in test solution. Avoid touching the neck or side of flask during addition. Swirl flask to thoroughly mix contents. d. At 30 ± 3 seconds after addition of the test culture, transfer a 1 mL

	aliquot from the test flask (test culture × test chemical) to a tube containing 9 mL neutralizer blank and mix well. This corresponds to 10^{-1} dilution tube. Record timed events on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Time Recording Sheet for Transfers (see section 14). e. *Treated samples plating*. From 10^{-1} tube (i.e., 9 mL neutralizer tube inoculated with 1 mL of exposed culture), plate four 1 mL aliquots and four 0.1 mL aliquots onto TGEA - N plates, for a total of 8 plates per treated sample. This will result in 10^{-1} and 10^{-2} dilutions respectively. Incubate plates at 36±1ºC for 24-30 hours. f. Following incubation, count colonies on TGEA-N plates. Counts over 300 are recorded as TNTC. Record plate counts on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Results Sheet (see section 14).
12.5 Numbers Control Procedure	Flask containing 99 mL of PBDW is used for the numbers control procedure and referred to as the numbers control sample. a. Numbers control assay should be conducted within 5 minutes of completion of treated samples. b. In a sterile 250 mL wide mouth Erlenmeyer flask containing 99 mL sterile PBDW, add 1 mL of the test culture (same test culture used for the treated samples) as follows: c. Swirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at the point of contact with test sample. d. Add suspension midway between center and the inner edge of the flask with tip of pipette slightly immersed in test solution. Avoid touching to the neck or side of flask during addition. Swirl flask to thoroughly mix contents. e. *Numbers control plating*. Within 30 seconds of addition of test culture, transfer 1 mL aliquot from the numbers control test flask (test culture × PBDW) into a tube containing 9 mL of neutralizer and mix well. This corresponds to 10^{-1} dilution tube. Make serial 10-fold dilutions in 9 mL PBDW, out to 10^{-6}. f. Plate four 1 mL aliquots and four 0.1 mL aliquots from the 10^{-6} dilution tube onto TGEA plates, for a total of 8 plates per numbers control sample. This will result in 10^{-6} and 10^{-7} dilutions, respectively. g. Incubate plates at 36±1ºC for 24-30 hours.

	h. Following incubation, count colonies on TGEA plates. Counts over 300 are recorded as TNTC. Record plate counts on the Germicidal and Detergent Sanitizing Action of Disinfectants Method Results Sheet (see section 14).
12.6 Sterility controls	a. Neutralizer blank – plate 1 mL from a previously unopened neutralizer tube onto a TGEA plate. b. Test chemical – plate 1 mL of the test chemical used in the assay onto a TGEA plate. c. Diluent – plate 1 mL of the diluent used for the preparation of the test chemical if necessary, onto a TGEA plate. d. Incubate all plates at 36±1ºC for 24-30 hours, record results. e. To be considered valid, no growth should be observed on any of the sterility controls.
12.7 Neutralization Confirmation Test	a. Perform a neutralization confirmation test prior to or concurrently with the sanitizer evaluation assay. The neutralization assay must demonstrate the recovery of a low-level test organism population (e.g., 10-100 CFU/mL) on the recovery media (i.e. TGEA-N and TGEA). b. *Test Culture Titer (TCT).* Add 0.1 mL of the test organism, which has been serially diluted to target between 10-100 CFU/mL to 10 mL of PBDW and mix thoroughly. Dilutions 10^{-4} and 10^{-5} should provide the range of 10-100 CFU/mL. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto TGEA. Incubate plates at 36±1ºC for 24-30 hours and record number of colonies. c. *Neutralization Confirmation Treatment (NCT).* Add 1 mL of the test chemical to 9 mL of the prescribed neutralizer and mix thoroughly. Within 30 seconds, inoculate the sample with 0.1 mL of the test organism used for the TCT. Mix thoroughly. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto TGEA-N. Incubate plates at 36±1ºC for 24-30 hours and record number of colonies. d. *Neutralization Toxicity Treatment (NTT).* Add 0.1 mL of the test organism used for the TCT to 10 mL of the prescribed neutralizer and mix thoroughly. Hold the mixture for a minimum of two minutes. Plate 0.1 mL aliquots in duplicate onto TGEA-N. Incubate plates at 36±1ºC for 24-30 hours and record number of colonies. e. Plates that have colony counts over 300 are reported as TNTC.

	Record the counts on the Neutralization Confirmation Assay Results Sheet (see section 14). f. *Neutralization Results and Calculations.* In order to demonstrate effective neutralization of the sanitizer, differences between treatments should not exceed 1.0 log (e.g., TCT minus NCT). *To calculate CFU/mL for Neutralization Confirmation Test use the following equation*. $CFU/mL = \frac{\{(Mean\ CFU\ for\ 10^{-x}) + (Mean\ CFU\ for\ 10^{-y})\}}{10^{-x} + 10^{-y}}$ where 10^{-x} and 10^{-y} are the dilutions plated. Two plates per dilution are plated for all treatments. Alternatively, if only one dilution is plated, use the same equation for only one dilution. Use counts of 0 to 300 for calculation purposes. Score counts >300 as TNTC (too numerous to count). NOTE: A spreadsheet will be used for data analysis and calculations associated with the neutralization confirmation assay.
12.8 Results	a. For a valid test, numbers control counts must fall between 7.0 – 8.0 logs. b. For the test chemical to be considered efficacious, a mean LR ≥ 5 is required with a contact time of 30 seconds.
12.9 Confirmatory Steps for Test Microbes	a. Conduct presumptive identification of the test microbes when results are indicative of a failing efficacy evaluation or when results are inconclusive. b. Confirm representative growth from one plate per treatment sample by Gram stain and growth characteristics on general and selective media. c. Gram stains are performed on smears taken from the treatment sample plates. For the additional confirmatory tests, a smear of culture from each selected treatment sample plate is streaked onto BAP and selective media appropriate for the test organism and incubated for 18-24 hours at 36 ± 1°C. See Attachment 1 for Gram stain reactions, cell morphology, and colony characteristics on solid media. d. If characteristics on general and/or selective media are unusual, then further confirmation will be conducted by VITEK, see SOP-QC-22:

	VITEK 2 Compact: Use, Maintenance and Quality Control Procedures for details. e. If confirmatory testing determines that the identity of the organism was not the test organism, the entry on the results sheet must be annotated to indicate a contaminant was present.
13. Data Analysis/ Calculations	1. Calculations will be computed using a Microsoft Excel spreadsheet (see section 14). Both electronic and hard copies of the spreadsheet will be retained. 2. *To calculate CFU/mL, use the following equation.* $CFU/mL = \frac{\{(CFU\ for\ 10^{-x}) + (CFU\ for\ 10^{-y})\}}{10^{-x} + 10^{-y}}$ where 10^{-x} and 10^{-y} are the dilutions plated. Four plates per dilution are plated for treated samples and numbers control samples. Use counts of 0 to 300 for calculation purposes. Score counts >300 as TNTC (too numerous to count). a. Calculate the mean $\log_{10}$ density (LD) for numbers control plates. b. Calculate the mean $\log_{10}$ density (LD) for treated samples plates. c. Calculate the $\log_{10}$ reduction (LR) for treated samples: $\log_{10}$ reduction = mean $\log_{10}$ numbers control – mean $\log_{10}$ treated sample NOTE: If no growth is observed for treated sample, substitute 0.5 at the lowest (least dilute) dilution and account for the dilution factor in the calculations.
14. Forms and Data Sheets	1. Attachment 1: Typical Growth Characteristics of strains of *S. aureus* and *E. coli* 2. Attachment 2: Culture Initiation Flow Chart for *S. aureus* and *E. coli* 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Organism Culture Tracking Form — MB-27-03_F1.docx Test Microbe Confirmation Sheet (Quality Control) — MB-27-03_F2.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Information and Culture Preparation Sheet — MB-27-03_F3.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Serial Dilution/Plating — MB-27-03_F4.docx

	Tracking Form Germicidal and Detergent Sanitizing Action of Disinfectants Method: Titer of Final Test culture Form — MB-27-03_F5.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Results Sheet — MB-27-03_F6.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Time Recording Sheet for Transfers — MB-27-03_F7.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Test Microbe Confirmation Sheet — MB-27-03_F8.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay – Neutralization Confirmation Control — MB-27-03_F9.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Toxicity Control — MB-27-03_F10.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay – Test culture Control — MB-27-03_F11.docx Germicidal and Detergent Sanitizing Action of Disinfectants Method: Neutralization Confirmation Assay Results Sheet — MB-27-03_F12.docx
15. References	1. Official Methods of Analysis. 2013. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD. Method 960.09: Germicidal and Detergent Sanitizing Action of Disinfectants. Revised First Action 2013. 2. 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. 3. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. *Bergey's Manual of Systematic Bacteriology Volume 1*. Williams & Wilkins, Baltimore, MD. *E. coli* p. 420.

Attachment 1

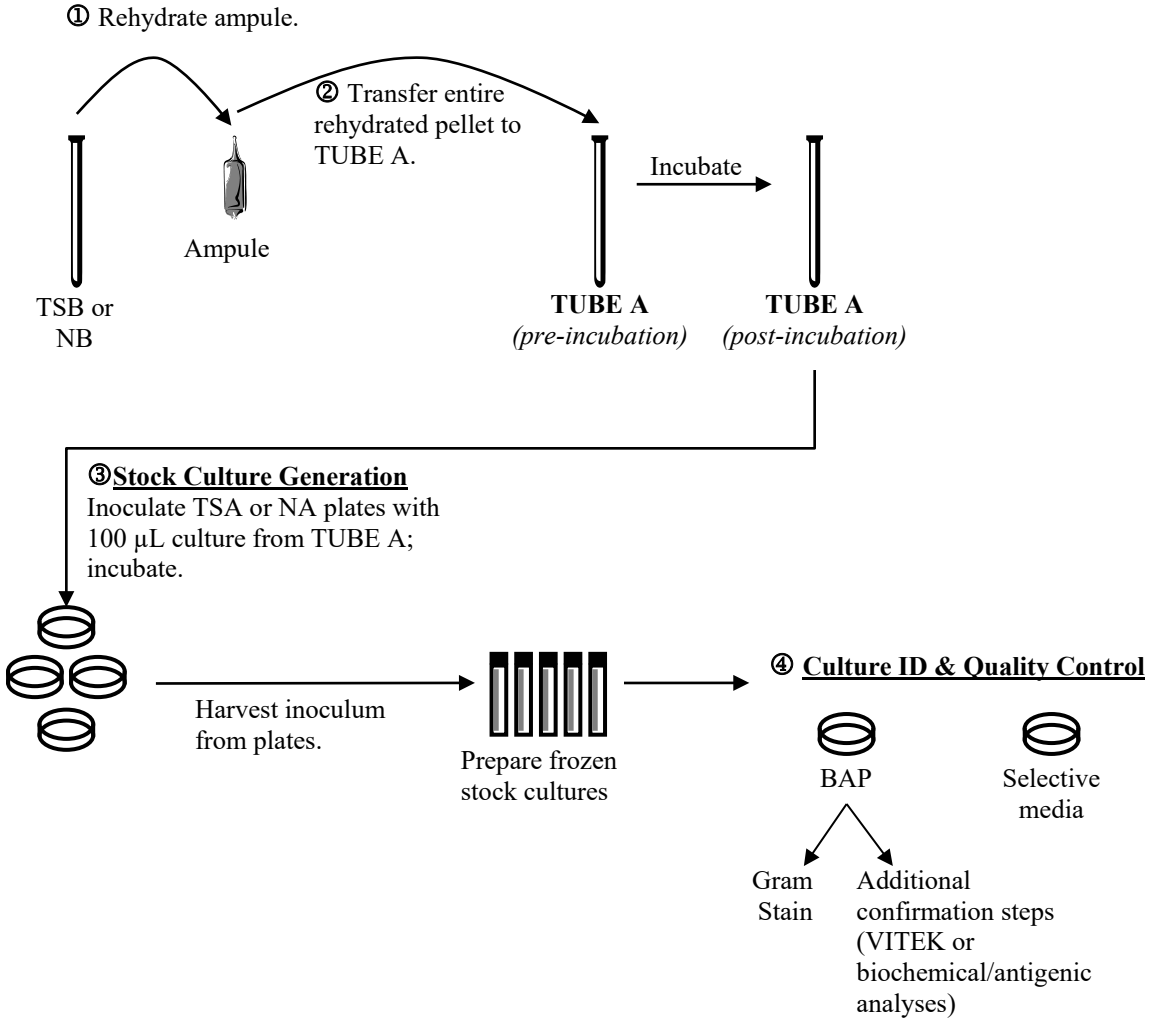
Typical Growth Characteristics of strains of *S. aureus* and *E. coli* (see references 15.2 for *S. aureus* and 15.3 for *E. coli)*

	*S. aureus**	*E. coli**
Gram stain reaction	(+)	(-)
Typical Growth Characteristics on Solid Media		
BAP	small, circular, yellow or white, glistening, beta hemolytic	large, round, white colonies, non-hemolytic.
Mannitol Salt	circular, small, yellow colonies, agar turning fluorescent yellow	No Growth
XLD agar	No growth	large, flat, yellow colonies with no black center.
Typical Microscopic Characteristics		
Cell dimensions	0.5-1.5 µm in diameter	0.5 × 1-3 µm in diameter
Cell appearance	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	varying from almost coccoid forms to long rods, occurring singly, in pairs and in short chairs. Motile or non-motile, if motile with flagella.

*After 24±2 hours

Attachment 2

Culture Initiation and Stock Culture Generation Flow Chart for *S. aureus* and *E. coli.*



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 *Staphylococcus aureus* (ATCC 6538), and *Escherichia coli* (ATCC No. 11229) from ATCC within 18 months.

   i. New frozen stock culture may be initiated <u>one</u> time using an existing, unexpired frozen stock culture as the source. Begin process at step C below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.

b. Open ampule of freeze dried organism as indicated by ATCC. Using a tube containing 5-6 mL of TSB for *S. aureus* and 5-6 mL of NB for *E. coli*, 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).

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.

i. For QC purposes, perform a streak isolation of the 24 ± 2 h broth culture, or frozen stock culture (a.i.), on a BAP. In addition, for *S. aureus*, streak a loopful onto selective media (MSA); for *E. coli*, 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) 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*, streak a loopful onto selective media (MSA); for *E. coli*, 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 cryovials).

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).