Interim Quantitative Method for Evaluating the Efficacy of Antimicrobial Test Substances on Porous Surfaces Against Bacteria

(12/12/2022)

Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this interim method to support efficacy criteria for the registration of products bearing claims for use on soft, porous surface claims. The method provides a quantitative assessment of the performance of antimicrobial substances against *Pseudomonas aeruginosa* and *Staphylococcus aureus* on soft-porous surfaces.

This method provides log reduction (LR) as the quantitative measure of efficacy for disinfectants against the test microbes on a soft-porous surface.

Method Overview

In brief, the method uses 1 cm diameter discs (carriers) of a set of representative soft-porous surface materials. Each disc receives 10 µL of microbial inoculum (with a three-part organic and inorganic soil load) deposited in the center of each carrier. The inoculum is allowed to dry and is then exposed to 50 µL of the antimicrobial treatment; control carriers receive an equivalent volume of an innocuous fluid (e.g., phosphate buffered saline). The exposure time is allowed to elapse; a liquid neutralizer is then added to the vial to halt the antimicrobial action. Each vial with the carrier is vortexed, serially diluted, and the contents are filtered to recover viable microorganisms. Based on the difference between the mean $\log_{10}$ density values of the untreated control and treated carriers, a mean $\log_{10}$ reduction (LR) in viable bacteria is calculated. The LR value is used as the measure of product effectiveness.

Appropriate safety procedures should always be used when working with laboratory test systems which include human pathogenic microorganisms. Laboratory safety is discussed in the current edition of "Biosafety in Microbiological and Biomedical Laboratories (BMBL)" 6th edition, from the subject matter experts within the U.S. Department of Health and Human Services (HHS), including experts from the Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH).

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1) Special Apparatus and Materials

a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538).

i. Additional bacteria may be tested (for an additional label claim) per the Agency's guidance.

b. Culture media

i. *Tryptic Soy Broth (TSB).* Use to rehydrate lyophilized cultures. Purchase broth from a reputable source or prepare according to manufacturer's instructions.

ii. *Synthetic broth (SB).* Growth medium for test cultures. Commercial media (HIMEDIA, Synthetic Broth, AOAC, #M334-500G). Suspend 16.9 g in 1000 mL DI water. Heat if necessary, to dissolve the medium completely. Final pH at 25°C should be 7.1±02. Medium may be dispensed in 10 mL amounts in 20×150 mm culture tubes or alternatively in 500 mL volumes in a 1 L bottle; steam sterilize at 15 lbs pressure (121°C) for 15 minutes. Cool to room temperature and just before use, aseptically add 0.1 mL of 10% sterile dextrose solution. Store prepared SB at 2-8°C.

1. Alternatively, SB made in-house per the recipe provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

iii. *10% dextrose solution.* Add 5.0 g dextrose to 50 mL de-ionized water and mix by stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution at 2-5°C for up to 30 days.

iv. *TSB with 15% (v/v) glycerol.* Use as a cryoprotectant. 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 steam sterilize for 15 min at 121°C.

v. *Tryptic soy agar (TSA)* and *TSA with 5% sheep blood.* Use for culturing, isolation, and characterization of the test microbes. Purchase plates from a reputable source or prepare according to manufacturer's instructions.

vi. *Selective media. (optional)* Mannitol salt agar and Cetrimide agar. Use for quality control of test microbes listed in this procedure. Purchase plates or prepare according to manufacturer's instructions.

c. Reagents

i. *Neutralizer.* A liquid reagent used to inactivate and/or dilute the antimicrobial treatment to end the contact time.

ii. *Phosphate buffered saline stock solution (e.g., 10X).* To prepare 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.

iii. *Phosphate buffered saline (PBS), 1X.* Dilution blanks and filtration. PBS with a pH of approximately 7.0±0.5 is desirable.

iv. *Soil load, 3-part.* Use as the soiling agent. Add to the test suspension in the following manner:

1. BSA: Add 0.5 g bovine serum albumin (BSA, radio immunoassay (RIA) grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 50 µL), and store at -20±2°C.

2. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 70 µL), and store at -20±2°C.

3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland, CAS # 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2 µm pore diameter (polyethersulfone) membrane filter, aliquot, and store at -20±2°C.

4. The three stock solutions of the soil load are single use only. Do not refreeze; store up to one year at -20±2°C.

v. *Antimicrobial Test substance.* Ready-to-use, activated, or concentrated antimicrobial. If the antimicrobial test substance is prepared by diluting a concentrate, adequately mix antimicrobial test substance with the appropriate diluent (e.g., hard water), then use prepared test substance within 3 hours of preparation or as otherwise instructed by the manufacturer. Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated antimicrobial test substance should be used when preparing use-dilutions for testing. Use v/v dilutions for liquids antimicrobial test substances and w/v dilutions for solid antimicrobial test substances. The use of a positive displacement pipette is recommended for viscous liquids.

vi. *1 N NaOH and 1 N HCl.* Used for pH adjustment of media/reagents.

vii. *Water.* De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media.

viii. *Tween-80* (polysorbate 80). Used to prepare PBS-T.

ix. *Gram stain.* Used for diagnostic staining.

d. Apparatus

i. *Carriers*: Discs (1 cm in diameter) cut from porous material. Carriers are single use only. See Section 2 for carrier specifications.

ii. *Hole punch:* If necessary, for use in the preparation of 1 cm disc from material. Model number: SKU# HP-MEI448R or equivalent

iii. *Calibrated 10 µL positive displacement pipette* with corresponding 10 µL tips, for carrier inoculation.

iv. *Filter paper.* Whatman No. 2, to line glass Petri plates.

v. *Calibrated micropipettes* (e.g., 200 µL, 1 mL) with appropriate corresponding

111 tips, for deposition of test substance on carriers and preparing dilutions.

vi. *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes, bottles, etc.* For rinsing vials and filters.

vii. *Forceps*, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters, appropriate to pick up the carriers for placement in vials.

viii. *Polyethersulfone (PES) membranes.* Use for recovery of test microbe, 47 mm diameter and 0.2 µm pore size.

1. Use filter membranes in either a reusable or disposable filtration unit.

ix. *Filter Sterilization Unit (with PES*, 0.2 µm pore size). Use to filter sterilize soil components.

x. *20 x 150 m glass culture tubes* with Morton closures for test culture preparation.

xi. *Spectrophotometer.* For culture standardization (if deemed necessary)

xii. *Vials with lids (plastic or comparable).* Sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), approximately 20 mL capacity, for holding inoculated carriers to be exposed to the test chemical and for accommodating neutralizer.

1. Transparent vials are more desirable to facilitate application of 50 µL test substance or control substance to inoculated carrier.

xiii. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals.

xiv. *Desiccation unit* (with gauge to measure vacuum level) with fresh desiccant (e.g., anhydrous $CaCO_3$). For drying inoculated carriers.

xv. *Vacuum source*. In-house line or suitable vacuum pump capable of achieving 0.068 to 0.085 MPa, for drying inoculated carriers in desiccation unit and to perform membrane filtration.

xvi. *Titration kit*. (ie., Hach digital titrator) Used for measuring water hardness.

xvii. *Vortex-style mixer*. Used for vortex-mixing of various solutions.

xviii. *15 mL conical centrifuge tubes*. Used for centrifugation of test cultures.

xix. *Centrifuge* (with rotor capable of achieving 5,000g). Used for test culture preparation.

2) <u>Carriers</u>

a. Carrier Materials

i. Privacy Curtain Fabric (PCF-03): 54% Polyester, 46% Fire Resistant (FR) Polyester. CF Stinson, LLC. Mambo MAM34 Nights.

ii. Non-PVC Fabric (NVF-01): Polyurethane Face made with Polycarbonate and Polyether Resins, Polyester Backing. CF Stinson, LLC. Kid BlueSky KID17.

iii. Vinyl Seating Fabric (VF-01): Vinyl Face, Polyester Backing. CF Stinson, LLC. Hopsack - HOP24 Fjord.

Figure 1: Examples of carrier materials cut into 1 cm discs; materials 2.a.i, 2.a.ii, and 2.a.iii (from left to right)

b. Carrier Preparation

i. Punch, or obtain, 1 cm round carriers or use comparable cutting procedure from fabric.

ii. Visually screen carriers to ensure consistent surface characteristics; trim any jagged edges or loose fabric.

iii. No pre-cleaning of carriers is necessary. To sterilize carriers, sterilize using a gravity cycle, 121°C for 20 minutes; ensure carriers are dry following sterilization. Test sterility of carriers prior to testing.

1. Carriers may not be entirely flat after autoclaving; however, minor distortion of carriers is acceptable for testing.

2. Prior to use in testing, document the condition of the screened and sterile carriers (e.g., digital photographs).

3) <u>Preparation of Test Culture and Carrier Inoculation</u>

a. Refer to Attachment A for preparation of the frozen stock cultures.

b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only.

c. Within 15 minutes prior to inoculation, using a calibrated pipette to aseptically add 0.1 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB.

d. Using a calibrated micropipette, add 100 µL of defrosted stock culture to 10 mL SB with dextrose, briefly vortex-mix and incubate for 24±2 h at 36±1°C.

i. Incubate without disrupting the culture.

ii. In addition, inoculate an agar plate (e.g., TSA or TSA with 5% sheep blood) from the inoculated tube and streak for isolation. Incubate plate with the test culture.

e. Following incubation, use the SB cultures to prepare a test suspension for each organism.

i. The 24±2 h culture should exhibit a titer of at least 10^8 CFU/mL.

f. For *P. aeruginosa*, inspect culture prior to harvest; visible pellicle on the surface of the culture is expected to form during incubation (record its presence). Discard the culture if pellicle has been disrupted (fragments in culture).

i. Remove visible pellicle on surface of medium and around associated interior edges of the tube by pipetting or with vacuum suction.

ii. Using a serological pipette, withdraw the remaining broth culture (at least 5 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL conical centrifuge tube.

iii. Record approximate volume harvested and transferred to 15 mL conical tube.

g. For *S. aureus*, briefly vortex-mix the 24±2 h culture and transfer to a 15 mL conical centrifuge tube.

h. Within 15 min, centrifuge the 24±2 h harvested broth cultures at 5,000g_N for 20±5 min.

i. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in 5-10 mL PBS. Record resuspension volume.

i. Prepare the final test suspension within 30 min of resuspending the culture.

ii. If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation.

j. If needed, dilute the 5-10 mL of resuspended culture in PBS to achieve a mean control carrier count level of 4.0-5.5 logs CFU/carrier for *S. aureus* and *P. aeruginosa*.

i. Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust the diluted test suspension.

k. Use the resuspended or diluted culture to prepare the final test suspension with the addition of the soil load.

l. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette:

i. 25 µL BSA stock

ii. 35 µL yeast extract stock

iii. 100 µL mucin stock

iv. Vortex soil suspension for 10s prior to adding microbial test suspension.

v. 340 µL microbial test suspension

m. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation.

i. Streak inoculate an agar plate with a loopful of the final test suspension. Incubate

plate with the treated and control carrier plates and examine for purity after incubation at 36±1°C for 72±4 h.

n. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to inoculation. Place carriers screened side up inside an empty, sterile plastic Petri dish (no more than 20 carriers/dish).

i. Privacy curtain carriers have no backing material and may be inoculated on either side.

ii. Non-PVC and vinyl carriers are layered materials comprised of a smooth, colored top surface and a white fabric bottom; only the top surface will be inoculated.

o. Vortex-mix the final test suspension for 10 s following the addition of the soil load and immediately prior to use.

p. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 5 treated) along with a few extra carriers.

q. Using a calibrated positive displacement pipette with a 10 µL tip, withdraw 10 µL of the final test suspension and deposit it at the center of each carrier (clean, screened and sterile); avoid contact of pipette tip with carrier and do not spread the final test suspension with the pipette tip.

i. For consistency, vortex-mix the inoculum frequently during inoculation of the carrier set.

ii. The same pipette tip may be used to inoculate all carriers (unless the tip is compromised).

iii. Discard any inoculated carrier where the final test suspension has run over the edge.

r. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with desiccant) and completely remove the lid of the Petri dish. Close the desiccation unit door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit.

i. Note: do not exceed 40 inoculated carriers per desiccator to ensure carriers dry within the prescribed time.

s. Maintain and monitor the vacuum level using a gauge. Achieve and maintain consistent level of vacuum (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000 Pascal) by leaving the vacuum on during the drying period with the desiccator stopcock opened or closed as necessary.

t. Hold the inoculated carriers in the evacuated desiccation unit at 21±3°C for 45 to 60 min. Visually inspect inoculated carriers to verify that they have completely dried and remove from desiccation unit. Do not use carriers that are visibly wet for testing.

i. Record the time for all timed events.

ii. Depressurize the desiccator slowly to avoid the potential for carriers to move or flip.

u. Use dried inoculated carriers for testing within 30 min following removal from desiccation unit; hold carriers in closed Petri dish at room temperature (21±3°C) until use.

4) Performance Assessment – Efficacy

a. Evaluate 3 control carriers and 5 treated carriers for each test substance tested (one test organism and contact time /carrier type combination) unless specified otherwise.

   i. One set of control carriers per carrier type may be used for evaluating multiple test substances against one organism on one test day (assuming the carrier material, neutralizer, and soil load are the same).

b. Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred.

c. Prepare the antimicrobial test substance. Use antimicrobial test substance within 3 hours of preparation or as specified by the manufacturer.

d. In a timed fashion with appropriate intervals, sequentially deposit 50 µL of the test substance (equilibrated to 21±3°C) with a calibrated micropipette over the dried inoculum on each test carrier, ensuring complete coverage.

   i. Note: Gently apply the antimicrobial test substance at a perpendicular angle to the inoculated carrier; do not forcefully deposit the disinfectant.

e. Use a new tip for each carrier; do not touch the carrier surface with a pipette tip during the application of the test substance or the control substance; replace with new carrier(s) and vial(s) if this occurs. Do not cap the vials.

   i. For non-foaming aerosols and pump/trigger spray products, obtain the test substance by dispensing the product into a sterile vessel for collection. Cap the vessel and use dispensed product within 30 min.

   ii. For foaming spray formulations, allow the foam to break down for at least 5-10 minutes for the generation of a 1-2 mL liquid sample. Cap the vessel and use dispensed product within 30 min.

f. Do not process carriers where the test substance runs off the carrier or does not completely cover the inoculum spot; replace with new carrier(s) and vial(s) if this occurs.

g. Conduct the test at room temperature (21±3°C) for the selected contact time. Use a certified timer to ensure that each carrier receives the required contact time.

h. Process control carriers last. Each control carrier receives 50 µL PBS, equilibrated to 21±3°C, instead of the test substance. Hold the control carriers for the same contact time as used for the test substance.

i. Within ±5 s of the end of the contact period, add 10 mL of neutralizer equilibrated to 21±3°C to each vial in the specified order according to the predetermined schedule. Briefly vortex-mix (2-3 s) each vial following the addition of the neutralizer.

   i. For calculation purposes, the solution in the neutralized vial with carrier is considered to be 10^0 dilution.

   ii. The neutralizer for the control carriers is the same as that for the treated carriers.

j. Immediately following the addition of the neutralizer and briefly (2-3 s) vortex, allow

289 carriers to sit in the vials for 5 minutes undisturbed then proceed as follows:

i. Vortex-mix vials at high speed for 30 s (vortex-mix #1).

ii. Allow carriers to sit undisturbed in the vials for 5 minutes.

iii. Vortex-mix vials at high speed for 30 s (vortex-mix #2).

iv. Allow carriers to sit undisturbed in the vials for 5 minutes.

v. Vortex-mix vials at high speed for 30 s (vortex-mix #3).

k. Initiate dilutions within 30 min after neutralization and vortex-mixing. Initiate filtration within 30 min of preparing the dilutions.

l. Dilute and filter samples from the treated and control carriers; process treated carriers first.

m. Serially dilute the eluate from the 10^0 dilution prior to filtration by transferring 1 mL into 9 mL PBS in a dilution tube.

n. Turn on vacuum and leave on for the duration of the filtration process.

o. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS.

p. Use separate membrane filters for each eluate (neutralized solution); however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.

q. Filter each sample through a separate 0.2 µm PES membrane filter.

r. For eluates from treated carriers remaining in the vial (10^0 dilution), vortex-mix the vial for ~5 s, carefully pour the eluate into the filter unit.

i. If a carrier falls onto the filter membrane, aseptically remove it using sterile forceps.

s. Rinse the treated vial with ~20 mL PBS, vortex-mix for ~5 s, pour the wash into the same filter unit. For dilution tubes, rinse tube once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit.

t. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

u. Rinse the inside surface of the funnel unit with at least 20 mL PBS and filter the contents.

v. Aseptically remove the membrane filter and place on the appropriate recovery medium. Avoid trapping any air bubbles between the filter and the agar surface.

w. Sterility controls.

i. On the day of the test, filter ~20 mL of neutralizer and ~20 mL of the PBS used in the test using two separate membrane filters and place on TSA.

ii. Incubate these filters along with a plate of recovery medium (e.g., TSA) for 72±4 h at 36±1°C, record sterility results.

x. Incubate plates at 36±1°C for 48±4 h for control carriers and for a minimum of 72±4 h for treated carriers.

i. Monitor filters daily to optimize counting of colonies. CFUs may be counted daily. Record controls after 48±4 h and treated carriers after 72±4 h.

y. Count colonies and record results.

i. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.

ii. For example, contamination occuring on multiple filters within one set of serial dilutions and/or across multiple carriers is considered systemic and the test is deemed invalid.

z. For colony counts on filters in excess of 200 record as Too Numerous to Count (TNTC).

aa. If no colonies are present, record as zero.

bb. Report non-conforming data (e.g., systemic contamination and atypical serial dilution results) and repeat tests as necessary.

i. Systemic contamination

ii. Atypical serial dilution results (e.g., higher CFUs at more dilute levels).

cc. Inspect the growth on the filters for purity and typical characteristics of the test microbe, see Attachment A, Table 1.

dd. If isolated colonies are present, assess one representative colony per 5-carrier set (treated) or 3-carrier set (controls) using a Gram stain.

i. If confluent growth is present, perform a streak isolation on TSA or TSA with 5% sheep blood on growth taken from at least 1 carrier incubate at 36±1°C for 24-48 h.

ee. If additional verification of the test organism is required, perform further confirmatory analyses (e.g., Vitek and biochemical analyses) and isolation streaks on selective media.

5) Data Requirements

a. Per test, use colony counts to determine log reduction.

b. For an acceptable test, each of the three control carriers must exhibit counts between 4.0-5.5 logs CFU/carrier.

c. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with two significant figures (e.g., round up to the nearest tenth).

d. Calculate the Colony Forming Units (CFU)/carrier using the following equation:

$$\text{Log}_{10}\left(\frac{\sum_{ii=1}^{ii=n}(r_{ii})}{\sum_{ii=1}(C_{ii}\times D_{ii})}\times W\right),$$

where:

Y = CFU per filter,

C = volume filtered,

V = total volume of neutralizer,

D = 10^{-k},

k = dilution,

n = number of dilutions, and

i = lower limit of summation (the fewest number of dilutions).

e. When TNTC (Too Numerous To Count) values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculations.

f. Calculate the log density of each carrier by taking the $\log_{10}$ of the density per carrier.

g. Calculate the mean $\log_{10}$ density across treated carriers.

h. Calculate the mean $\log_{10}$ density across control carriers.

i. Calculate the $\log_{10}$ reduction (LR) for treated carriers: $\log_{10}$ reduction = the mean $\log_{10}$ density for control carriers minus the mean $\log_{10}$ density for treated carriers.

j. For a set of treated carriers: when the 10^0 dilution (the contents of the vial with the carrier) is filtered either by itself or in addition to other dilutions and the data for each carrier result in zeros for each dilution filtered, report the LR as greater than or equal to the mean $\log_{10}$ density for the control carriers.

k. Log reduction data based on estimates due to the occurrence of TNTC outcomes at each dilution in a dilution series for control and treated carriers is deemed unacceptable.

Attachment A

Preparation of Frozen Stock Culture

1) Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from a reputable vender at least every 18 months.

   a. New frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source. Begin process at step 3 below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.

2) Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, 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 thoroughly. Incubate broth culture at 36±1°C for 24±2 h.

3) At the end of the incubation timeframe, streak a loopful of the broth culture onto 2 TSA plates to obtain isolated colonies. Perform a streak isolation of the broth culture onto BAP as a purity check and streak the broth culture onto the appropriate selective media. Refer to appropriate selective media in Table 1. Incubate all plates for 24±2 h at 36±1°C.

   a. Record results at the end of the incubation timeframe. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.

4) From the TSA plates, select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. For *P. aeruginosa*, select colonies from each of the two possible phenotypes present. Spread plate 0.1 mL of the suspension onto each of 6-10 TSA plates. Incubate the plates for 24±2 h at 36±1°C. If necessary, to obtain more frozen stock cultures, a larger suspension (e.g., 2 mL) may be prepared using the same ratio of TSB (1 mL) to number of colonies (3-5 colonies).

   a. Using the TSB suspension, perform a streak isolation of the suspension onto a BAP as a purity check, and streak on the appropriate selective media (refer to Table 1).

   b. Incubate all plates for 24±2 h at 36±1°C. Record results. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.

5) After the incubation period, harvest growth from TSA plates by adding approximately 5 mL sterile cryoprotectant solution (TSB with 15% (v/v) glycerol) on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL.

6) Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

7) Immediately after mixing, dispense 0.5-1.0 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.