US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
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Method for Testing Antimicrobial Products against Planktonic *Legionella pneumophila* in Simulated Cooling Tower Water (LSCTW) (v. 080724)

SOP Number: MB-42-00

08-28-2024

I. Overview

A. This document describes methodology to determine the efficacy of antimicrobial products against planktonic *Legionella pneumophila (L. pneumophila)* in simulated cooling tower water. The methodology is based on the AOAC method 960.09 – Germicidal and Detergent Sanitizing Action of Disinfectants (refer to section VIII.A); however, several technical attributes were derived from the European Standard (EN) Standard 13623, refer to section VIII.B.

II. Data Requirements and Special Conditions

A. Ensure the sterility of all media and reagents.

B. Confirm the effectiveness of the neutralizer.

C. Meet the target control counts: 6.0-7.0 $\log_{10}$ density of colony forming units per mL (CFU/mL).

D. Consult the Safety Data Sheet (SDS) for specific hazards associated with the test substance or other potentially hazardous materials.

III. Definitions:

A. Testing Solution = buffered ferrous hard water (BFHW) plus interferents.

B. Antimicrobial Product Stock Solution = product diluted to 100x or other required concentration using the appropriate diluent (e.g., hard water). Added to the Testing Solution during the efficacy assay.

C. Antimicrobial product = product tested at appropriate concentration, e.g., add 1 mL of 100x antimicrobial product to the volume in the test flask to achieve the target 1X concentration.

D. Final test culture = harvested and centrifuged culture of planktonic *L. pneumophila* adjusted to achieve the target control counts (6.0-7.0 $\log_{10}$ density of CFU/mL). This culture is added to the Testing Solution during the efficacy assay.

1. To conduct the neutralization confirmation assay, dilute the final test culture to achieve the appropriate concentration per Appendix 5.

E. LR = $\log_{10}$ reduction of CFU/mL.

F. CFU = colony forming unit.

G. Interferent = substance added to the testing solution to simulate the composition of cooling tower water.

IV. Special Apparatus and Materials

A. Test microbe. *Legionella pneumophila* subsp*. pneumophila* (ATCC 33152), obtained from a reputable supplier.

1. *L. pneumophila* is categorized as a biosafety level 2 (BSL-2) organism.

Appropriate safety procedures should always be used when working with test systems which include this organism. Laboratory safety is discussed in the current edition of "Biosafety in Microbiological and Biomedical Laboratories (BMBL)" from the U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Institutes for Health, refer to section VIII.C. The BMBL states that BSL-2 practices, containment equipment, and facilities are recommended for all activities involving the use or manipulation of *L. pneumophila*, including minimizing the potential for dissemination of the organism from cultures of organisms known to cause disease. For activities likely to produce extensive aerosols and when large quantities of the pathogenic organism are manipulated, such as in this method, BSL-2 with BSL-3 practices are recommended. All culture manipulations should be done in a biosafety cabinet.

B. Media

1. *Buffered Charcoal Yeast Extract Agar (BCYE).* For recovery and enumeration. Prepare according to manufacturer's instructions, use pre-poured plates, or prepare using the following recipe:
   - *L-cysteine hydrochloride solution.* Add 0.4 g to a tube with 10 mL of deionized water. Vortex-mix until in solution. Filter sterilize the solution. Prepare and use on the day of media preparation. *Iron (III) pyrophosphate solution* (CAS #: 10058-44-3). Add 0.25 g to a tube with 10 mL of deionized water. Vortex-mix and filter sterilize the solution. Prepare and use on the day of media preparation.
   - *N-2-acetamido-2 aminoethanesulfonic acid (ACES) buffer.* Add 10 g of ACES granules to 500 mL of deionized water and dissolve by standing in a water bath at 45°C to 50°C. Add 2.8 g of potassium hydroxide pellets to a separate container with 480 mL of deionized water and dissolve with gentle shaking. Mix the two solutions and use on day of media preparation.
   - *Prepare the BCYE Agar.* Add sequentially to 980 mL of ACES buffer: 2 g activated charcoal, 10 g yeast extract, and 1 g alpha-ketoglutarate, monopotassium salt. Add 0.1 M solution of potassium hydroxide (KOH) or 0.1 M sulfuric acid to the solution to bring the pH to 6.9±0.2 at 18-25°C. Add 12 g of agar, mix, and autoclave at 121°C for 15 minutes. After autoclaving, temper in a 47±2°C water bath. Add 10 mL of sterile L-cysteine solution and 10 mL of sterile iron (III) pyrophosphate solutions aseptically, mix well between additions. Aliquot approximately 20-25 mL volumes into Petri dishes. Allow excess moisture on the plates to dry and store at 2-8°C in closed containers in the dark for up to 4 weeks.

2. *Buffered Yeast Extract Broth (BYE).* For generating test cultures. Prepare by using the same method for BCYE agar except omit agar and charcoal. Store in the dark for up to 3 months at 2-8°C.

3. *Nutrient Broth plus 15% v/v glycerol*. For the preparation of frozen stock cultures. Add 8 g of nutrient broth powder and 150 mL of glycerol to 850 mL deionized water, stir until combined. Sterilize at 121°C for 15 minutes. Store at room temperature for up to 6 months.

4. *Tryptic Soy Agar (TSA)*. For assessment of the sterility, check flask. Purchase plates from a reputable source or prepare according to manufacturer's instructions. Store at 2-8°C for up to 3 months. If purchased, use the manufacturer's expiration date.

C. Reagents

1. *Neutralizer.* A liquid reagent used to inactivate and/or dilute the antimicrobial treatment to end the contact time. Confirm neutralizer toxicity and effectiveness using the procedure outlined in Appendix 5, Neutralization Confirmation Assay. Refer to EN Standard 13623 Annex B for example neutralizers.

2. *Page's Saline.* For harvesting test culture, dilution blanks, and for rinsing dilution tubes and membranes. Add 0.120 g of sodium chloride (NaCl), 0.004 g magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$), 0.004 g calcium chloride ($CaCl_2 \cdot 2\ H_2O$), 0.142 g disodium hydrogen phosphate (Na_2HPO_4), and 0.136 g potassium dihydrogen phosphate (KH_2PO_4) to 1 L of deionized water. Sterilize by autoclaving at 121°C for 15 minutes. Store at room temperature in a screw top bottle for up to 6 months.

3. *0.2 M Boric Acid Stock Solution.* For the preparation of BFHW. Dissolve 13.6 g of boric acid in deionized water and dilute to 1,000 mL, stir to dissolve. Store at room temperature in a screw top bottle for up to 6 months.

4. *0.05 M Borax Stock Solution*. For the preparation of BFHW. Dissolve 19.07 g of borax in deionized water and dilute to 1,000 mL, stir to dissolve. Store at room temperature in a screw top bottle for up to 6 months.

5. *Ferric Sulfate Stock Solution (0.003 M).* For the preparation of BFHW. Dissolve 0.0595 g of ferric sulfate into deionized water and dilute to 50 mL. Store at room temperature for up to 6 months in a screw top bottle. This reagent does not stay in solution, therefore stir before use.

6. *Buffered Ferrous Hard Water (BFHW)* (375 ppm). For preparation of the testing solution.

    i. EN Hard Water Solution A (refer to section VIII.D): Dissolve 19.84 g

magnesium chloride ($MgCl_2$) and 46.24 g calcium chloride ($CaCl_2$) in deionized water and dilute to 1 L. Filter sterilize by membrane filtration. Store the solution at 2-8°C for up to 30 days in a screw top bottle.

ii. EN Hard Water Solution B (refer to section VIII.D): Dissolve 35.02 g sodium bicarbonate ($NaHCO_3$) in deionized water and dilute to 1 L. Filter sterilize. Store the solution at 2-8°C for up to 30 days.

iii. Place 600 mL to 700 mL of deionized water in a 1 L volumetric flask and add 70 mL of recently stirred 0.2 M boric acid, 30 mL of 0.05 M recently stirred borax, and 6.0 mL of Solution A; then add 8.0 mL of Solution B and 1.0 mL of recently stirred 0.003 M ferric sulfate solution. Mix and dilute to 1 L with deionized water.

iv. The pH of BFHW should be 8.0±0.2. If necessary, adjust the pH by using a solution of 1 M sodium hydroxide or 1 M hydrochloric acid. Filter sterilize.

v. Determine hardness by titration; final ppm = 375 ppm -10% and +5% (338-394 ppm). This step may be performed before or after filter sterilization.

vi. Prepare the BFHW under aseptic conditions and use within 12 hours.

7. *Antimicrobial product*. Use the Antimicrobial Product Stock Solution prepared at 100x of the label concentration to achieve the target 1X concentration in the test flask. Adequately mix antimicrobial product with the appropriate diluent (e.g., hard water) and use within 3 hours of preparation or as otherwise instructed by the manufacturer. If product is applied to maintain a residual concentration, concentrations greater than 100x may be used to achieve the target residual concentration in the test flask.

i. At a minimum, use 1.0 mL or 1.0 g to prepare the 100x antimicrobial product.

8. *Interferents.* Added to the Testing Solution to simulate cooling tower water. Care should be taken to ensure reagents are fully dissolved.

i. *Yeast Extract* (0.05%). Add 0.05 g of yeast extract to 100 mL of deionized water. Sterilize at 121°C for 15 minutes. Store at room temperature in screw top bottle for up to 3 months.

ii. *Scale Inhibitor*. 2-Phosphononobutane-1,2,4-Tricarboxylic Acid (50% Aqueous solution) (PBTC) – CAS No. 37971-36-1. Prepare a 450±50 ppm (prepared as 450 µL/L) stock solution of PBTC in

deionized water and filter sterilize the solution. Store at room temperature in screw top bottle for up to 3 months.

iii. *Corrosion Inhibitor*. Tolytriazole (TT) sodium salt – CAS No. 29385-43-1. Prepare a 75±25 ppm (prepared as 0.075 g/L) stock solution of TT in deionized water and filter sterilize the solution. Store at room temperature in screw top bottle for up to 3 months.

iv. *Dispersant*. Polyacrylic Acid (PAA) CAS No. 9003-01-4. Prepare a 150±50 ppm (prepared as 150 µL/L) stock solution of PAA (MW <10K Daltons) in deionized water and filter sterilize the solution. Store at room temperature in screw top bottle for up to 3 months.

v. *Humic acid sodium salt stock solution* CAS No. 68131-04. Add 0.5 g to 1 L of deionized water. Mix thoroughly and autoclave for 15 minutes at 121°C. Store at room temperature in screw top bottle for up to 3 months.

9. *Testing Solution.* Prepare sufficient testing solution volume to conduct the efficacy assay and/or neutralization confirmation assay. For example, 490 mL of testing solution is required for the efficacy assay (98 mL per flask for each of five flasks); prepare as noted below. Prepare fresh testing solution on each day of testing.
   i. Dispense 465 mL of the BFHW into a sterile vessel.
   ii. Add 5 mL of each of the interferents (yeast extract, PBTC, TT, PAA, and humic acid sodium salt) and mix thoroughly.
   iii. Other volumes may be prepared as needed using the same ratio (per flask, 93 mL BFHW + 1 mL of each interferent).
10. *Deionized Water.* Deionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media.
11. *Legionella Latex Agglutination Test*. Use for *L. pneumophila* confirmatory test.

D. *Equipment and Glassware.*

1. *250 mL Disposable Erlenmeyer flasks with vented caps (sterile)*. For conducting the efficacy assay.
2. *Plastic Petri dishes.* Sterile plates, e.g., 20 mm × 100 mm in size.
3. *50 mL screw capped tubes (sterile vented closure).* Used for test culture preparation.

4. *15 mL non-vented centrifuge tubes and 50 mL non-vented centrifuge tubes with standard caps*. Used for neutralization, test culture centrifugation, and dilution blanks.

5. *Incubated Orbital Shaker*. Used for test culture agitation and during testing. Must be able to achieve 150±5 rpm and maintain 30-36±1°C.

6. *Test tube racks.* Any convenient style.

7. *Certified timer*. For timed intervals, use any certified timer that can display time in seconds.

8. *Calibrated micropipettes* (e.g., 1000 µL) with 100-1000 µL tips.

9. *Pall Metricel Black Polyethersulfone membrane filter (PES)*. For recovery of test microbe. 47 mm diameter with 0.45 µm pore size. (Pall Metricel black filters catalog # 66585).
Disclaimer: The EPA and its employees do not endorse the products, services, or enterprises of nonfederal entities. This product was chosen because it was the only filter out of the 5 filters tested that had a recovery comparable to spread plating but may not be the only product capable of achieving this result. Use of this product does not constitute an endorsement by EPA or its employees.

10. *Vortex mixer*. For preparation of the test culture, serial dilutions, etc.

11. *Digital Titrator kit*. To measure hardness of BFHW. Titration may also be performed by manual titration methods.

12. *Sterile Forceps*. Used to transfer the filters from filter units to agar plates. Straight or curved, with smooth flat tips.

13. *Vacuum source.* For filtering samples. In-house line or suitable vacuum pump (e.g., 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000 Pascal).

14. *Centrifuge.* For test culture preparation. With fixed rotor or swinging bucket rotor capable of achieving 5,000g.

15. *Water bath*. For preparing ACES buffer and for preparing BCYE agar. Any bath capable of maintaining 45-50°C.

16. *Serological Pipets*. For the preparation of the testing solution.

V. Efficacy Procedure

Refer to Appendix 1 for the Method to Prepare a Frozen Stock Culture of *Legionella pneumophila*.

A. Preparation of the Test Culture

1. Defrost a single cryovial of frozen stock culture at room temperature and briefly vortex-mix. Use for inoculation within 15 minutes. Each cryovial is single use only.

2. Using a calibrated micropipette, add 0.1 mL of the defrosted stock culture to 10 mL of BYE broth in a 50 mL screw cap tube with vented cap, and briefly vortex-mix. Record time of inoculation.

3. In addition, use the inoculated tube to streak a BCYE plate and a TSA plate. Incubate plates with test culture. After 24 hours, the BCYE purity plate should be free of contamination, but *Legionella* will not be visible yet; the TSA purity plate should have no growth: After 72 hours, BCYE plate may be observed for *Legionella* colonies; the TSA should continue to have no growth.

4. Place the culture in the incubated shaker at 36±1°C mixing at 150±5 rpm for 24±2 h. Record beginning and end of incubation time.

B. Preparation of the testing solutions

1. For each flask, dispense 98 mL of the Testing Solution into a 250 mL disposable Erlenmeyer flask with vented cap. Prepare five flasks representing three treated flasks, one untreated control flask, and one sterility check flask.

2. Place the five flasks in the incubated shaker set at 150±5 rpm and 30±1°C and equilibrate for a minimum of 30 minutes; begin efficacy test between thirty minutes and two hours of putting flasks in the shaker. Record beginning and end times of equilibration.

C. Preparation of the Antimicrobial Product Stock Solution

1. Dilute product to 100x or other required dilution using the appropriate diluent (e.g., hard water). Use diluted product within three hours of preparation.

D. Preparation of the Final Test Culture

1. Vortex-mix the 24 h culture from section V.A3. Transfer the culture to a 50 mL conical tube with screw top, tighten cap, and centrifuge at 5,000g for 20±5 min.

2. Remove supernatant without disrupting the pellet. Resuspend pellet in Page's Saline (e.g., 10 mL). Record the amount of Page's Saline used to resuspend the pellet.

3. Dilute the culture as necessary with Page's Saline to achieve the target titer in the untreated control flask (6.0-7.0 logs CFU per mL); this is the

final test culture. Initiate the efficacy test within approximately one hour of preparation of the final test culture.

4. Following the inoculation of the flasks, streak inoculate a BCYE plate and a TSA plate with a loopful of the *L. pneumophila* final test culture. Streak for isolation and incubate plates with the plates generated from the test day and examine for purity at the end of the incubation period. The BCYE purity plate should be free of contamination and the TSA purity plate should have no colonies.

E. Efficacy Assay

1. Refer to Appendix 2 (Schematic of Test Procedure) and Appendix 3 (Photographs of Key Steps in Method).

2. Remove the 5 equilibrated flasks from the incubated shaker: add 1 mL of the *L. pneumophila* final test culture to each of the three treated flasks and one untreated control flask; add 1 mL Page's Saline to the sterility check flask. Return all five flasks to the shaker and allow to mix for at least 5 minutes, but no more than 15 minutes.

3. After mixing, initiate efficacy test.

4. Using timed intervals (e.g., 2 min between each flask), sequentially remove one equilibrated flask from the incubated shaker and add either 1 mL of antimicrobial product stock solution (for treated flasks) or 1 mL of Page's Saline (for the untreated control flask and sterility check flask). Return the flask to the incubated shaker set at 150±5 rpm and 30±1°C immediately after the 1 mL addition. Continue the 1 mL additions for each flask.

   i. Apply antimicrobial product stock solution and Page's Saline while gently swirling the contents of each flask to create residual motion of the liquid to prevent pooling. Avoid touching the neck or side of flask during addition of the antimicrobial product stock solution.

   ii. Contact time begins immediately after application of the antimicrobial product. Record beginning of the contact time.

5. At the end of the contact time, remove the flask from the incubated shaker.

6. For each treated flask and the untreated control flask, gently swirl and transfer 3 separate 1 mL aliquots into 3 individual 50 mL screw cap tubes containing 9 mL of the neutralizer, briefly vortex-mix after each transfer. This corresponds to the 10^{-1} dilution. Record end of the contact time (e.g., 60±5 seconds). Transfer all 3 samples within 30 seconds of the end of the contact time.

i. Note: To achieve effective neutralization, the neutralizer volume can be increased up to 19 mL.

7. For the treated and untreated control flasks, after 1 mL aliquots have been added to the neutralizer, make 10-fold serial dilutions (1 mL into 9 mL Page's Saline) within approximately 30 min for each neutralized solution as necessary to achieve target counts of 20-200 CFU per membrane. Initiate filtration within approximately 30 min of preparing the dilutions.

8. For the sterility check flask, following exposure, gently swirl and transfer 2 separate 1 mL aliquots into 2 individual 50 mL screw cap tubes containing 9 mL of neutralizer and briefly vortex-mix.

9. For filtration, pre-wet each filter with ~10 mL Page's Saline. Vortex-mix the tube for ~5 s and pour its entire contents into the filter unit with vacuum on. Rinse each tube once with ~10 mL of Page's Saline, vortex-mix for ~5 s, and pour entire contents of the tube into the same filter unit. With the vacuum on, rinse the inside surface of each filter unit with an additional ~20 mL Page's Saline.

10. Using sterile forceps, aseptically remove the membrane filter and place onto a BCYE plate (for treated samples and untreated control samples). Avoid trapping any air bubbles between the membrane filter and the agar surface.

    i. For the sterility check samples, place one filter on a BCYE plate and the other filter on a TSA plate.

11. Incubate all plates at 36±1°C for 7 days.

12. Count colonies and record results. Record counts over 200 as TNTC (Too Numerous to Count).

F. Confirmatory Steps for Test Microbe

1. Evaluate representative growth from one plate per antimicrobial treatment (if growth occurs) and one plate per untreated control using an appropriate diagnostic test (e.g., *Legionella* Latex Agglutination test, or molecular detection). Refer to Appendix 4 for typical growth characteristics of strains of *L. pneumophila*.

2. If growth is atypical and/or confirmatory testing determines that the organism is not *L. pneumophila*, document the result to indicate the presence of a contaminant.

VI. Data Analysis/Calculations

A. Efficacy Assay Calculations

1. Per test, use colony counts to determine $\log_{10}$ reductions.
2. 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 to the nearest tenth).
3. Calculate the $\log_{10}$ density (LD) of colony forming units (CFU)/mL for each treated sample and each untreated control sample using the following equation: $Log_{10}\left(\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i \times D_i)}\right)$

   where:

   n = number of dilutions

   i = Lower limit of summations (the fewest number of dilutions),

   Y = CFU per filter,

   C = volume filtered,

   D = 10^{-k}, and

   k = dilution.

4. 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.
5. Calculate the mean $\log_{10}$ density across the three replicate samples from one treated flask (LD_T) using the following equation: $\frac{LD_{T1}+LD_{T2}+LD_{T3}}{3}$, where LD_{T1} = the $\log_{10}$ density for treated sample 1, LD_{T2} = $\log_{10}$ density for treated sample 2, etc.
6. Calculate the mean $\log_{10}$ density across the three treated flasks (LD_{TF}) using the following equation: $\frac{LD_{TF1}+LD_{TF2}+LD_{TF3}}{3}$, where LD_{TF1} = the mean $\log_{10}$ density for treated flask 1, LD_{TF2} = mean $\log_{10}$ density for treated flask 2, and LD_{TF3} = mean $\log_{10}$ density for treated flask 3.
7. Calculate the mean $\log_{10}$ density across the three untreated control replicate samples (LD_C) using the following equation: $\frac{LD_{C1}+LD_{C2}+LD_{C3}}{3}$, where LD_{C1} = the $\log_{10}$ density for untreated control sample 1, LD_{C2} = $\log_{10}$ density for untreated control sample 2, and LD_{C3} = $\log_{10}$ density for untreated control sample 3.

8. Calculate the mean $\log_{10}$ reduction (LR) for treated samples: Mean LR (CFU/mL) = Mean LD_C (untreated control samples) – Mean LD_{TF} (mean of three treated flasks).

9. If the test results in zero recovery for each of the three treated flasks, report the mean LR as greater than or equal to the mean $\log_{10}$ density for the untreated control samples.

   i. For the purpose of calculation, if no organism is recovered from the 10^{-1} dilution tube, the log density for that treated sample is 0 provided that the entire contents of the 10^{-1} dilution tube was filtered.

   ii. If no organism is recovered from a treated sample where the 10^{-1} dilution tube is not assayed, substitute 0.5 CFU at the least dilute dilution and scale up accordingly (e.g., if the entirety of the 10^{-2}, 10^{-3}, and 10^{-4} dilution tubes were filtered and resulted in no recovery, substitute 0.5 CFU at the 10^{-2} dilution and scale up accordingly e.g., use 0.5 CFU/mL as the number of CFU recovered from the 10^{-1} and 0 CFU for the remaining dilutions filtered).

   iii. If no organism is recovered by filtering the entirety of each of the 10^{-1} dilution tubes for each of the three treated samples, the log reduction is greater than or equal to the mean untreated control log density.

VII. **Appendices**

A. Appendix 1: Method to Prepare a Frozen Stock Culture of *Legionella pneumophila*

B. Appendix 2: Schematic of Test Procedure

C. Appendix 3: Photographs of Key Steps in Method

D. Appendix 4: Typical Growth Characteristics of strains of *L. pneumophila*

E. Appendix 5: Neutralization Confirmation Assay

F. Appendix 6: EN Hard Water (375 ppm)

VIII. **References**

A. 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.

B. EN 13623 Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity against *Legionella* of chemical disinfectants for aqueous systems – Test method and requirements (phase 2, step 1). September 2010.

C. Biosafety in Microbiological and Biomedical Laboratories, 6th Edition.US Department of Health and Human Services. HHS Publication No. (CDC) 21-1112. Revised June 2020.

D. EN 13727 Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area – Test method and requirements (phase 2, step 1). November 2015.

Appendix 1: Method to Prepare a Frozen Stock Culture of *Legionella pneumophila*

Preparation of frozen stock cultures

1. Initiate new stock cultures from a lyophilized culture or Culti-loop of *Legionella pneumophila* from ATCC (ATCC 33152) or other reputable vendor at least every 18 months. Conduct all transfers aseptically.

    i. New frozen stock culture may be initiated <u>one</u> time using an existing, unexpired frozen stock culture as the source. Inoculate 10 mL of Buffered Yeast Extract (BYE) broth in a 50 mL conical tube with vented cap with 100 µL of frozen stock culture. Mix thoroughly. Incubate broth culture at 36±1°C with mixing (150±5 rpm) for 48-72 hours in an incubated shaker. Continue with step 2.ii below.

2. *Legionella received in lyophilized form.*

    i. Open ampule of lyophilized organism per manufacturer's instructions. Using a 50 mL conical tube with vented cap containing 5-6 mL of BYE broth, aseptically transfer 0.5 to 1.0 mL broth into the lyophilized culture and gently mix to rehydrate. Aseptically transfer the entire rehydrated pellet back into the original tube of BYE broth (primary tube). Mix thoroughly. Take 500 µL from primary tube and inoculate another 50 mL conical tube with vented cap containing 5 mL of BYE broth (secondary tube). Mix thoroughly. Incubate both broth cultures at 36±1°C with mixing (150±5 rpm) for 48-72 hours in an incubated shaker.

    ii. At the end of the incubation timeframe, streak a loopful from the secondary tube (lyophilized culture) or broth culture (existing frozen stock culture) onto 2 BCYE plates to obtain isolated colonies. Perform a streak isolation of the broth culture onto a TSA plate and blood agar plate (BAP) as a purity check. Incubate all plates for 48-72 hours at 36±1°C. Continue with step 4.

        i. Record results at the end of the incubation timeframe. Refer to Appendix 4 for colony characteristics on BCYE and diagnostic characteristics of *Legionella pneumophila;* growth should not be present on TSA or BAP. Evaluate growth from one colony from BCYE using appropriate biochemical test (e.g., *Legionella* Latex Agglutination test) or molecular detection.

3. *Legionella received as a Culti-loop:*

    i. At least 10 minutes prior to inoculation, warm two BCYE plates, one TSA plate, and one BAP in a 36±1°C incubator. Remove the sheath from the Culti-loop. Lay the Culti-loop flat on the warm, moist surface of one BCYE agar plate. Hold the Culti-loop in this manner for 10-15 seconds to allow for absorption of moisture. Streak isolate the BCYE agar plate with the Culti-

loop in the usual manner. Using the same Culti-loop, streak isolate a second BCYE plate followed by the TSA plate and BAP (five total plates may be streaked with the same Culti-loop). Incubate the plates at 36±1°C for 48-72 hours. Continue with step 4.

a. Refer to Appendix 4 for colony characteristics on BCYE and diagnostic characteristics of *Legionella pneumophila;* growth should not be present on TSA or BAP. Evaluate growth from one colony from BCYE using appropriate biochemical test (e.g., *Legionella* Latex Agglutination test) or molecular detection.

4. From the BCYE plates (from 2.ii or 3.i), select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of BYE broth. Spread plate 0.1 mL of the suspension onto each of 5-10 BCYE plates. Incubate the plates for 48-72 hours 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 BYE broth (1 mL) to number of colonies (3-5 colonies).

   i. Using the BYE broth suspension, perform a streak isolation of the suspension onto a BCYE plate, TSA plate, and BAP as a purity check.

   ii. Incubate all plates for 48-72 hours at 36±1°C. Record results at the end of the incubation timeframe. Refer to Appendix 4 for colony characteristics on BCYE and diagnostic characteristics of *Legionella pneumophila;* growth should not be present on TSA or BAP. Evaluate growth from one colony from BCYE using appropriate biochemical test (e.g., *Legionella* Latex Agglutination test) or molecular detection.

5. After the incubation period, harvest growth from BCYE plates by adding approximately 5 mL sterile cryoprotectant solution (nutrient broth with 15% (v/v) glycerol) to 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.

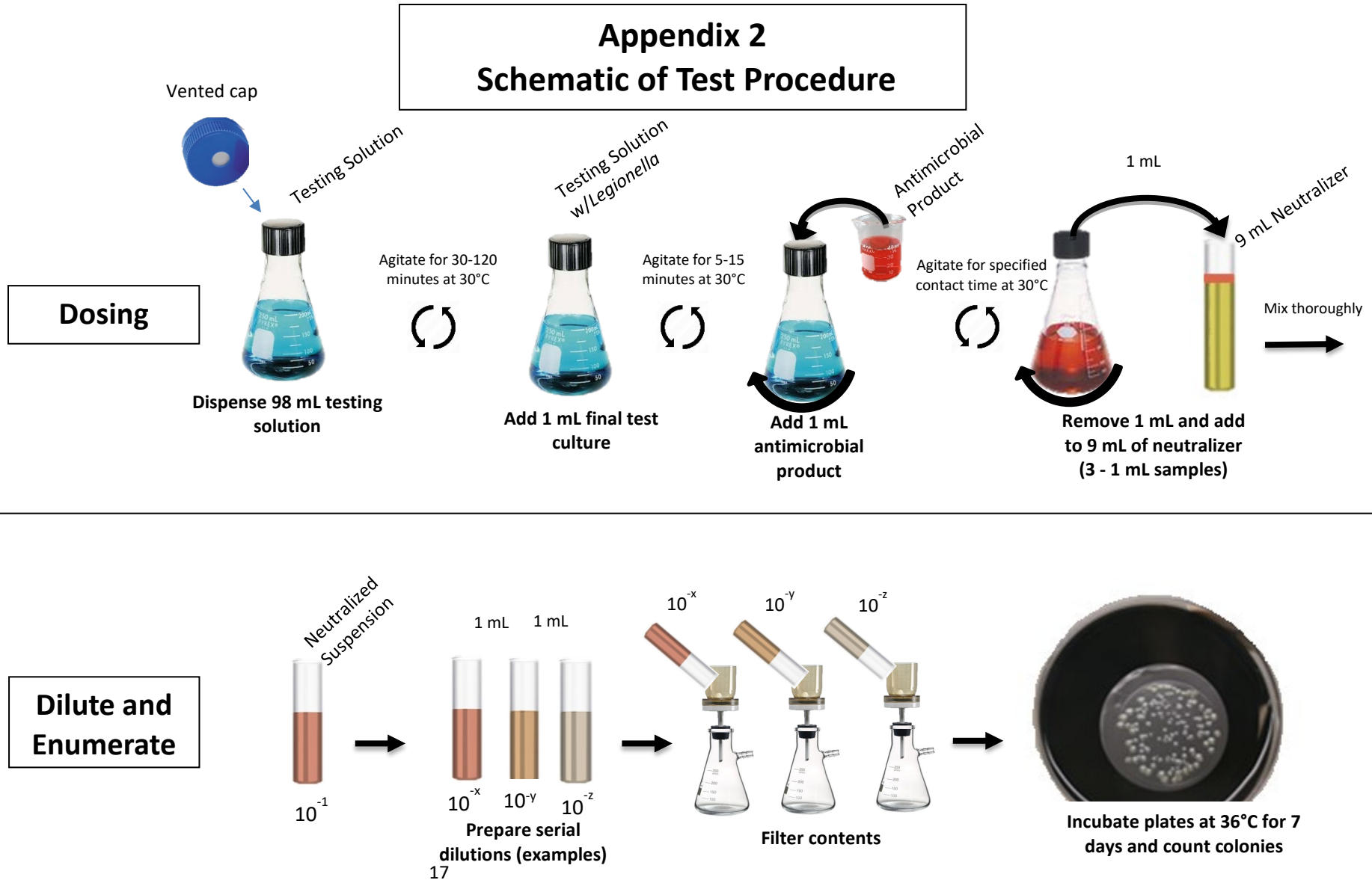
   i. For QC purposes, conduct purity check of culture by performing a streak isolation of the BYE broth suspension on a BCYE plate, a TSA plate, and a BAP. Incubate all plates for 48-72 h at 36±1°C. Record results. Refer to Appendix 4 for colony characteristics on BCYE and diagnostic characteristics

of *Legionella pneumophila;* growth should not be present on TSA or BAP. Evaluate growth from one colony from BCYE using appropriate biochemical test (e.g., *Legionella* Latex Agglutination test) or molecular detection.

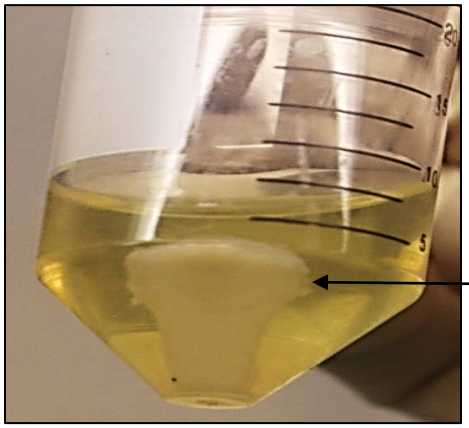
8. Store the cryovials at -70°C or lower for a maximum of 18 months; reinitiate with a new lyophilized culture, Culti-loop, or frozen stock culture vial.

9. If the characteristics of *Legionella pneumophila* are not consistent with the information in Appendix 4 at any step in the process, discard the cultures and re-initiate the process.

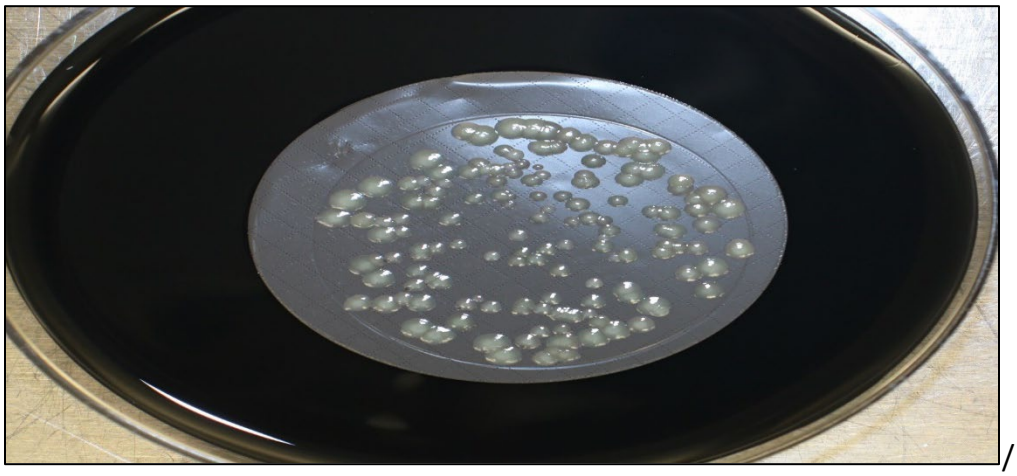
Appendix 2: Schematic of Test Procedure



Appendix 3: Example Photos of Key Steps in Method



Pellet of *L. pneumophila* after centrifugation



L. pneumophila colonies on PES filter on BCYE plate after 5 days of incubation at 36±1°C.

Appendix 4: Typical Growth Characteristics of strains of *L. pneumophila*

	Result
Latex agglutination test	(+)
Typical Growth Characteristics on Solid Media	
BCYE	Grey-white, textured cut glass
Typical Microscopic Characteristics	
Cell dimensions	0.3-0.9 in width x 2.0-20 μm in length
Cell appearance	Rod shaped or filamentous.

Appendix 5: Neutralization Confirmation Assay

1. The neutralization assay must demonstrate the recovery of a low-level of *L. pneumophila* on BCYE plates.
2. Prepare Treatments 1, 2, and 3 using testing solution that has been equilibrated at 30±1°C for approximately 30 min in 50 mL non-vented centrifuge tubes.
    i. Treatment 1: Neutralizer Effectiveness (Tubes 1-3)
    ii. Treatment 2: Neutralizer Toxicity Control (Tubes 4-6)
    iii. Treatment 3: Titer Control (Tubes 7-9)
3. Serially dilute the final test culture using Page's Saline and select the appropriate dilution(s) to deliver 20-200 CFU per 0.1 mL. Use the diluted culture within one hour.
    i. Two or more separate serial dilutions may be used to ensure at least one dilution with an average challenge of 20-200 CFU per 0.1 mL.
4. Follow Schematic of Neutralization Confirmation Assay to perform neutralization confirmation assay.
5. After the 10 min±30 seconds holding period, vortex-mix each solution and pour entire contents onto a filter pre-wetted with ~10 mL of Page's Saline, with vacuum on. Rinse each tube once with ~10 mL of Page's Saline, vortex-mix, and pour the contents of the tube into the same filter. Rinse the inside surface of each filter unit with an additional ~20 mL Page's Saline.
6. Using sterile forceps, aseptically remove the membrane filter and place on BCYE plates. Avoid trapping any air bubbles between the membrane filter and the agar surface.
7. Incubate plates at 36±1ºC for 7 days and record number of colonies.
8. Neutralization Results and Calculations
    i. Determine the average CFU of the **Titer Control,** the **Neutralizer Toxicity Control,** and **Neutralizer Effectiveness** treatment.
    ii. Determine the percent difference in CFU between the treatments using the following equations:
    $\left(\frac{Mean\ CFU_{Neutralizer\ Toxicity\ Control}}{Mean\ CFU_{Titer\ Control}}\right) \times 100$ and
    $\left(\frac{Mean\ CFU_{Neutralizer\ Effectiveness\ Treatment}}{Mean\ CFU_{Titer\ Control}}\right) \times 100.$
    iii. For determining the suitability of the neutralizer, ensure that the average CFU in the **Neutralizer Toxicity Control** is at least 50% of the **Titer Control**. A count lower than 50% indicates that the neutralizer is harmful to the test organism. Average CFU for the **Neutralizer Toxicity Control** that are higher than the **Titer Control** (counts greater than 100% of the **Titer Control)** are also deemed valid.

iv. To verify effectiveness of the neutralization, the average number of CFU in the **Neutralizer Effectiveness** treatment is at least 50% of the **Titer Control**. Average CFU for the **Neutralizer Effectiveness** treatment that are higher than the **Titer Control** (counts greater than 100% of the Titer Control) are also deemed valid.

v. If the criteria are not met, verify another neutralizer or mixture of neutralizers.

vi. If the counts fall below 20 or above 200 CFU/filter, repeat the neutralization test using a different dilution of test culture.

9.