



US Environmental Protection Agency Office of Pesticide Programs

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

Standard Operating Procedure for Disinfectant Sample Preparation

SOP Number: MB-22-00

Date Revised: 11-19-08

EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

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for
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1.0 SCOPE AND APPLICATION:

1.1 This standard operating procedure describes procedures for preparing the use-dilution of antimicrobial products used in AOAC Use-Dilution method (UDM), the Germicidal Spray Products as Disinfectants test (GSPT), the Tuberculocidal Activity of Disinfectants test (CTB), the Sporocidal Activity of Disinfectants test (SAT), and the Virucidal Effectiveness Test (VET). The proper preparation of antimicrobial chemicals for efficacy testing is critical to successfully conducting an efficacy test.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 NIST = National Institute of Standards and Technology

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organisms are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.

3.2 Perform work with buffer solution (Solution C) for hard water preparation in a chemical fume hood.

3.3 Disinfectants may contain a number of different active ingredients, such as quaternary ammonium compounds, halogens, phenolics, aldehydes, peroxides, and heavy metals. Latex gloves and other personal protective clothing or devices are worn during the handling of these items. A chemical fume hood or other containment equipment is employed when performing tasks with products.

4.0 CAUTIONS:

4.1 Strict adherence to the protocol is necessary for the validity of the test results.

5.0 INTERFERENCES: None

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 *Glassware.*

7.1.1 The disinfectant for UDM, SAT, and CTB testing is dispensed into autoclavable 25 × 100 mm tubes (Bellco Glass Inc., Vineland, NJ). To prepare the use-dilution of a disinfectant, use volumetric glassware (pipettes, flasks, etc.).

7.2 *Hard water solutions.*

7.2.1 *Hard Water Solution 1.* Dissolve 7.94 g MgCl₂ (or 16.94 g MgCl₂·6H₂O) and 18.50 g CaCl₂ in boiled de-ionized H₂O and dilute to 250 mL. Filter-sterilize using a 0.2 μm filter unit.

7.2.2 *Hard Water Solution 2.* Dissolve 14.01 g NaHCO₃ in boiled de-ionized H₂O and dilute to 250 mL. Filter-sterilize using a 0.2 μm filter unit.

7.2.3 *EDTA Standard Solution (Solution A).* Dissolve 4.0 g Na₂H₂EDTA·2H₂O and 0.10 g MgCl₂·6H₂O in 800 mL de-ionized H₂O and adjust by subsequent dilution so that 1 mL of solution is equivalent to 1 mg CaCO₃ when titrated against a standard CaCO₃ solution (Solution B) – Solution A may require an additional 200 mL de-ionized H₂O. Standardize Solution A every two months (refer to section 10.1).

7.2.4 *Calcium Carbonate Solution (Solution B).* Used in place of calcium standard solution described in method 960.09 section F (b) (refer to reference 15.1). 1 mL = 1 mg CaCO₃. Dilute a 10.0 mL standard HACH ampule (or equivalent) of 10,000 mg/L CaCO₃ to 100 mL with de-ionized H₂O. Mix thoroughly.

7.2.5 *Buffer Solution (Solution C).* Dilute 6.75 g NH₄Cl and 57 mL NH₄OH to 100 mL with de-ionized H₂O. Store at room temperature for up to 2 months.

7.2.6 *Inhibitor (Solution D).* Dilute 5.0 g Na₂S·9H₂O to 100 mL with de-ionized H₂O.

7.2.7 *Indicator Solution (Solution E).* Dissolve 0.5 g Chrome Black T in 100 mL (60-80%) alcohol.

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to the laboratory equipment calibration and maintenance SOPs (SOP EQ series) for details on method and frequency of calibration.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to the manufacturer's recommendations if stipulated, or at room temperature. Those disinfectants requiring activation or dilution prior to use are activated or diluted within three hours of testing unless test parameter specify otherwise.

9.2 Follow appropriate chain-of-custody (COC) guidelines during testing as stipulated in SOP COC-01, Sample Log-in and Tracking.

9.3 Hard water must be used for efficacy testing within 24 ± 2 hours of preparation. For disinfectants prepared with hard water, refer to section 10.2.

9.4 To ensure the stability of a diluted product, prepare the dilutions within three hours of the disinfectant treatment step unless specified otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 Standardization of Solution A.

10.1.1 Prior to preparation of hard water, prepare and standardize Solution A.

10.1.2 Solution A must be verified (standardized) prior to use. For Solution A to be standardized, it must accurately titrate a known concentration of CaCO_3 (see 16.2).

10.1.3 If the volume of Solution A required to titrate the standard CaCO_3 solution (Solution B) requires less volume than necessary (< 10 mL), dilute it with de-ionized water and retitrate.

10.1.4 If the volume of Solution A required to titrate Solution B requires more volume than necessary (> 10 mL), prepare a new preparation of Solution A and titrate to confirm standardization.

10.2 Preparation of synthetic hard water (as CaCO_3).

10.2.1 Per liter: Dilute 1 mL of Hard Water Solution 1 (for each 100 ppm

hardness desired) and 4 mL of Hard Water Solution 2 in a 1 L volumetric flask with sterile de-ionized H₂O.

For example, to prepare 1 L of 400 ppm synthetic hard water, dilute 4 mL Hard Water Solution 1 and 4 mL Hard Water Solution 2 to 1 L with sterile de-ionized H₂O.

- 10.2.2 Synthetic hard water should have a pH between 7.6 and 8.0 (adjust if necessary).
- 10.2.3 Filter-sterilize using a 0.2 µm filter unit. Store at room temperature for up to 24 hours.
- 10.2.4 Verify hardness via buret titration (refer to section 16.1). If preparing for use the following day, confirm hardness by buret titration on the day of preparation and reconfirm using a Hach water hardness test kit the following day prior to use.
- 10.2.5 Hardness may vary by no more than -10% or +5% from the stated value.
- 10.2.6 Synthetic hard water must be used within 24 hours of preparation.
- 10.2.7 Hardness of the water (as mg CaCO₃/L) is calculated as follows:

$$\text{CaCO}_3 \text{ (mg/L)} = \frac{\text{volume of Solution A used for titration (mL)} \times 1000}{\text{volume of hard water sample (mL)}}$$

- 10.3 Disinfectant preparation for UDM and CTB testing.
 - 10.3.1 Prior to opening the container of a liquid product, gently shake the container and thoroughly clean the area around the cap and spout with 70% ethanol. Allow the surface to dry. Remove the cap. Do not touch the inside surface of the cap. If present, carefully remove the seal attached to the lip of the spout with sterile instruments (i.e., razor blade, forceps).
 - 10.3.2 Pour an appropriate aliquot of the sample into a sterile beaker. Do not place a pipette or any other instrument inside the product container. Place cap on the product container and secure tightly.
 - 10.3.3 From the beaker, dispense ready-to-use products directly into sterile 25 × 100 mm tubes or initiate dilutions for concentrated products.

- 10.3.4 Aseptically prepare disinfectant samples as directed by the test parameters. Prepare all dilutions with sterile standardized volumetric glassware.
- 10.3.5 If a product requires a 1:10 dilution, 1 part product is added to 9 parts diluent.
- 10.3.6 Commonly used use-dilutions:
 - 10.3.6.1 $\frac{1}{2}$ ounce per gallon of diluent = 1:256 dilution
 - 10.3.6.2 1 ounce per gallon of diluent = 1:128 dilution
 - 10.3.6.3 $\frac{3}{4}$ cup (6 oz.) per gallon of diluent = 6:128 dilution
- 10.3.7 For diluted products, use ≥ 1.0 mL or 1.0 g of sample disinfectant to prepare the use-dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to 2 decimal places toward a stronger product.
- 10.3.8 For use-dilution and tuberculocidal efficacy testing, dispense 10 mL aliquots of the diluted disinfectant or ready-to-use product into 25×100 mm test tubes, one tube per carrier. Place the tubes in an equilibrated water bath for approximately 10 minutes to allow the test solution to come to the specified temperature.
- 10.4 Disinfectant preparation for GSPT testing.
 - 10.4.1 For spray products which require preparation (e.g., preparing a use-dilution, use of a pump or trigger based sprayer) proceed as described in 10.3.1 through 10.3.7.
 - 10.4.2 For aerosol spray products, shake the can 25 times prior to use, unless otherwise specified by the manufacturer. The cans are immobilized in the Spray Disinfectant Apparatus (see MB-06) or other suitable apparatus, and the distance from the nozzle to the inoculated carrier is measured to ensure the correct distance.
 - 10.4.3 Prior to testing, the spray nozzle is wiped with 70% ethanol and allowed to dry.
 - 10.4.4 Spray the product for 10-15 seconds prior to commencement of the

test.

10.5 Record disinfectant preparation on the Media/Reagent Preparation Sheet (refer to SOP QC-15, Media Prep and Sterilization Run Numbers).

10.6 Volume required.

10.6.1 For one UDM test or GSPT for *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *Salmonella enterica*, prepare approximately 1 L of the disinfectant.

10.6.2 For one CTB test or GSPT for *Mycobacterium bovis* (BCG), prepare approximately 250 mL of the disinfectant.

10.6.3 For one SAT, prepare approximately 250 mL of the disinfectant.

10.6.4 For one VET, prepare approximately 100 mL of the disinfectant.

11.0 DATA ANALYSIS/CALCULATIONS: None.

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms (see 16.0). Completed forms are archived in notebooks kept in secured file cabinets in room D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 For quality control purposes, the required information is documented on the appropriate forms (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the standard protocol must be recorded on the form and an explanation for the deviation given.

15.0 REFERENCES:

15.1 AOAC Official Methods of Analysis, 18th edition (1990). Germicidal and

Detergent Sanitizing Action of Disinfectants (Method 960.09, Parts E and F).
AOAC International, Gaithersburg, MD.

15.2 Official Methods of Analysis. 2006. 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, (Testing of Disinfectants against *Staphylococcus aureus* (Methods 955.15), Testing of Disinfectants against *Pseudomonas aeruginosa* (Method 964.02), Testing of Disinfectants against *Salmonella choleraesuis* (Method 955.14), Sporicidal Activity of Disinfectants Test (Method 966.04), Germicidal Spray Products as Disinfectants (Method 961.02)).

15.3 Confirmatory Virucidal Effectiveness Test Using Feline Calicivirus as a Surrogate for Norovirus. Antimicrobials Division, US EPA.

16.0 FORMS AND DATA SHEETS:

16.1 Standardization of Solution A (EDTA)

16.2 Hard Water Titration

16.1

MEDIA/REAGENT PREPARATION SHEET
OPP MICROBIOLOGY LABORATORY

Media/Reagent Name: Standardization of Solution A (EDTA) Prep #:

Amount Prepared: N/A Preparation Date/Initials:

pH Meter #:	Dispenser: (circle one)	Water Bath #(s):	BSC #(s):	Sterilization #:
N/A	Hamilton Oxford Hand NA	N/A	N/A	N/A

Media/Chemical Ingredients:	Control No:	Amount Required:	Amount Measured:
Standard CaCO ₃ solution (1000 mg/L)		10 mL	
De-ionized water		40 mL	
Solution C		1 mL	
Solution D		1 mL	
Solution E		2 drops	
Solution A		As needed for titration.	

Preparation/Modifications/Notes:

- Add 10 mL of standard CaCO₃ solution and 40 mL de-ionized water to an Erlenmeyer flask.
- Add 1 mL Solution C, 1 mL Solution D, and 2 drops of Solution E.
- Titrate the sample using Solution A with constant stirring until the last tinge of red disappears.
- Hardness of the water as mg CaCO₃/L:

$$\text{CaCO}_3 \text{ (mg/L)} = (\text{volume of Solution A used for titration (mL)} \times 1000) \div \text{volume of CaCO}_3 \text{ solution (mL)}$$
 NOTE: For Solution A to be standardized, mg CaCO₃/L from the above equation must equal 1000.
- Check the appropriate box: **STANDARDIZED** **NOT STANDARDIZED**

Required pH:	pH Before Autoclaving with Medium Cooled to Room Temperature:	Final pH After Autoclaving with Medium Cooled to Room Temperature:
N/A	pH: N/A Temperature: N/A	pH: N/A Temperature: N/A
Volume of Acid/Base added to obtain final pH:		N/A

Sterility/Viability Test Results	Sterility			Viability		
	Pass	N/A	Fail	Pass	N/A	Fail

Storage of Reagent/Media: N/A

Media Requested by: _____ Media Prepared for: ATP R&D PIP

16.2

MEDIA/REAGENT PREPARATION SHEET
OPP MICROBIOLOGY LABORATORY

Media/Reagent Name: Prep #:

Amount Prepared: Preparation Date/Initials:

pH Meter #:	Dispenser: (circle one)	Water Bath #(s):	BSC #(s):	Sterilization #:
N/A	Hamilton Oxford Hand NA	N/A	N/A	N/A

Media/Chemical Ingredients:	Control No:	Amount Required:	Amount Measured:
Sample of hard water		20 mL	
De-ionized water		30 mL	
Solution C		1 mL	
Solution D		1 mL	
Solution E		2 drops	
Solution A		As needed for titration.	

Preparation/Modifications/Notes:

- Add 20 mL of hard water and 30 mL de-ionized water to an Erlenmeyer flask.
- Add 1 mL Solution C, 1 mL Solution D, and 2 drops of Solution E.
- Titrate the sample using Solution A with constant stirring until the last tinge of red disappears.
- Hardness of the water as mg CaCO₃/L:
 $\text{mg/L CaCO}_3 = (\text{volume of Solution A used for titration (mL)} \times 1000) \div \text{volume of hard water sample (mL)}$
 $\text{mg/L CaCO}_3 = \underline{\hspace{2cm}} \text{ mL Solution A} \times 1000 \div \underline{\hspace{2cm}} \text{ mL hard water}$
 $= \underline{\hspace{2cm}} \text{ mg/L CaCO}_3$

Hardness may vary by no more than -10% or +5% from the stated value.

Required pH:	pH Before Autoclaving with Medium Cooled to Room Temperature:	Final pH After Autoclaving with Medium Cooled to Room Temperature:
N/A	pH: N/A Temperature: N/A	pH: N/A Temperature: N/A
Volume of Acid/Base added to obtain final pH:	N/A	

Sterility/Viability Test Results	Sterility			Viability		
	Pass	N/A	Fail	Pass	N/A	Fail

Storage of Reagent/Media: N/A

Media Requested by: _____ Media Prepared for: ATP R&D PIP