



US Environmental Protection Agency Office of Pesticide Programs

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

**Preparation of hard water and other diluents for
preparation of antimicrobial products**

SOP Number: MB-30-03

Date Revised: 11-14-22

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Title	Preparation of Hard Water and Other Diluents for Preparation of Antimicrobial Products
Revisions Made	<ul style="list-style-type: none">• OECD Hard Water now referred to EN Hard Water and reference updated.• Created new sheets for preparation of 0.800 M and 0.08 M EDTA Hach cartridges.• Minor editorial changes for clarification purposes.

SOP Number	MB-30-03
Title	Preparation of hard water and other diluents for preparation of antimicrobial products
Scope	This SOP describes procedures for the preparation of diluents such as hard water, sterile de-ionized water, and tap water used to prepare test substances, and the accuracy verification for the digital titrator.
Application	Procedures are applicable to various types of diluents used in the preparation of liquid and spray product formulations.

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TABLE OF CONTENTS

<u>Contents</u>	<u>Page Number</u>
1. DEFINITIONS	3
2. HEALTH AND SAFETY	3
3. PERSONNEL QUALIFICATIONS AND TRAINING	3
4. INSTRUMENT CALIBRATION	3
5. SAMPLE HANDLING AND STORAGE	3
6. QUALITY CONTROL	4
7. INTERFERENCES	4
8. NON-CONFORMING DATA	4
9. DATA MANAGEMENT	4
10. CAUTIONS	4
11. SPECIAL APPARATUS AND MATERIALS	4
12. PROCEDURE AND ANALYSIS	6
13. DATA ANALYSIS/CALCULATIONS	9
14. FORMS AND DATA SHEETS	9
15. REFERENCES	10

<p>1. Definitions</p>	<ol style="list-style-type: none"> 1. Test substance = an antimicrobial formulation used in testing. 2. Ready-to-use test substance = test substance that requires no activation or dilution. 3. Concentrated liquid test substance = liquid or solid test substance that requires dilution prior to use. 4. Activation = the combination of a base and an activator to prepare the final test substance. 5. Spray test substance = trigger, aerosol, or pump-based test substance. 6. Test substance diluent = sterile liquid used to make test substance dilutions (e.g. tap water, de-ionized water, or hard water). 7. Hard water = water which contains a standardized concentration of calcium and magnesium ions. <ol style="list-style-type: none"> a. AOAC hard water (see section 15.1)– may be prepared at various concentrations of CaCO₃ (i.e., 100 ppm-500 ppm) b. EN hard water (see section 15.2) – prepared at a concentration of 375 ppm based on CaCO₃ concentration, unless otherwise specified. <ol style="list-style-type: none"> i. Previously referred to as <i>OECD Hard Water</i> [Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides Used on Hard Non-Porous Surfaces (June 21, 2013)]. c. Hard tap water – prepared at a concentration of 200 ppm based on CaCO₃ concentration, unless otherwise specified.
<p>2. Health and Safety</p>	<p>Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for hazards associated with specific products.</p>
<p>3. Personnel Qualifications and Training</p>	<p>Refer to SOP ADM-04, OPP Microbiology Laboratory Training.</p>
<p>4. Instrument Calibration</p>	<p>Refer to SOP EQ-03 (weigh balance), QC-01 (QC of water), and QC-19 (pipettes) for details on method and frequency of calibration.</p>
<p>5. Sample Handling and Storage</p>	<ol style="list-style-type: none"> 1. Refer to SOP MB-22: Preparation and Sampling Procedures for Antimicrobial Test Substances and SOP COC-01: Chain of Custody Procedures for Antimicrobial Samples for procedures on sample handling and storage. 2. Use hard water samples within 5 days of preparation. 3. Use a Media/Reagent Preparation Sheet for preparation of diluents.

6. Quality Control	<ol style="list-style-type: none"> 1. For quality control purposes, the required information is documented on the appropriate record form(s), see section 14. 2. Perform accuracy verification of the digital titrator on a semi-annual basis. Details provided in section 12.6.
7. Interferences	<ol style="list-style-type: none"> 1. Inaccurate water hardness values may occur if the digital titrator is not properly used and/or does not meet the requirements for accuracy verification. 2. Inaccurate results will occur if the delivery tube tip is held above the solution rather than in the solution (e.g., under the surface) during sample titration and/or titrator verification procedure. 3. During the sample titration and titrator verification procedures, verify that the delivery tube is free of air bubbles and is not misshapen, which can interfere with the titration process. 4. During the sample titration and titrator verification procedures, conduct the verification procedure accurately and without urgency to ensure titrant is added consistently. 5. Use clean glass containers for titration samples; do not use plastic containers. 6. To prevent contamination, sterilize de-ionized water and tap water according to SOP QC-13. 7. Do not use de-ionized water as a diluent or to prepare a diluent if it does not meet the requirements specified in SOP QC-01.
8. Non-conforming Data	<ol style="list-style-type: none"> 1. Errors in the preparation of a diluent will result in an invalid study. 2. The acceptable range for hard water, expressed as mg/L (ppm) calcium carbonate (CaCO₃), is -10% to +5% of the target hardness. Other ranges of water hardness may be used as appropriate.
9. Data Management	Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	Strict adherence to the protocol is necessary for the validity of the test results.
11. Special Apparatus and Materials	<ol style="list-style-type: none"> 1. Reagents. <ol style="list-style-type: none"> a. Sterile water. Use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite quality can be met. See Standard Methods for the Examination of Water and Wastewater and SOP QC-01, Quality Assurance of Purified Water for details on reagent-grade water.

	<ul style="list-style-type: none">b. AOAC hard water solutions<ul style="list-style-type: none">i. Hard Water Solution 1. Dissolve 7.94 g MgCl_2 (anhydrous) (or 16.94 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) and 18.50 g CaCl_2 in boiled de-ionized H_2O, bring to a volume of 250 mL volumetrically. Sterilize by membrane filtration. Used for the preparation of hard water at various concentrations.ii. Hard Water Solution 2. Dissolve 14.01 g NaHCO_3 in boiled de-ionized H_2O; bring to a volume of 250 mL volumetrically. Sterilize by membrane filtration. Used for the preparation of hard water at various concentrations.c. EN hard water solutions<ul style="list-style-type: none">i. Solution A. Dissolve 19.84 g anhydrous magnesium chloride (or 42.36 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) and 46.24 g anhydrous calcium chloride (CaCl_2) in de-ionized water and dilute to 1,000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator and use for up to one month. Adjust the recipe for volumes other than 1 L.ii. Solution B. Dissolve 35.02 g sodium bicarbonate (NaHCO_3) in water and dilute to 1,000 mL. Sterilize by membrane filtration. Store the solution in the refrigerator and use for up to one month. Adjust the recipe for volumes other than 1 L.d. Hardness Voluette Ampule Standard Solution, 10,000-mg/L as CaCO_3 (Hach Company, Catalog No. 218-710). Used in the accuracy verification of the digital titrator. <p>2. Apparatus</p> <ul style="list-style-type: none">a. Glassware (e.g., flasks) to conduct titration assays; sterile volumetric glassware to prepare hard water.b. Calibrated weigh balance. To prepare hard water stock solutions.c. Digital Titrator Kit (Hach Company, Catalog No. 20636-00). For the titration of the hard water sample and to determine hardness as CaCO_3 in parts per million (ppm).d. Titration cartridges. Cartridge concentration is dependent on the water hardness range (ppm CaCO_3) to be assayed<ul style="list-style-type: none">i. For 10-160 ppm CaCO_3 use 0.0800 M EDTA titration cartridgeii. For 200-4,000 ppm CaCO_3 use 0.800 M EDTA titration cartridge
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	<p>iii. For in-house preparation of the 0.0800 M or 0.800 M EDTA solutions, see section 14.</p>
<p>12. Procedure and Analysis</p>	<ol style="list-style-type: none"> 1. AOAC hard water preparation is conducted as described in Method I of AOAC standard 960.09 (see section 15.1). 2. EN hard water preparation is conducted as described in EN 13727: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area – Test method and requirements (see section 15.2). 3. Titration is conducted as described in Method II of AOAC standard 960.09 (see section 15.1). 4. Per the study sponsor’s request, prepare the appropriate diluent for the test substance. If hard water is prescribed as the diluent, prepare and verify the water’s hardness according to sections 12.1-12.3. For products requiring the use of sterile de-ionized water or sterile tap water, refer to section 12.5. 5. Verify sterility of all diluents (e.g., hard water, tap water, etc.) prior to testing.
<p>12.1 Preparation of AOAC Hard Water</p>	<ol style="list-style-type: none"> a. Per 1 L: Add 1 mL of AOAC Hard Water Solution 1 (for each 100 ppm hardness desired) and 4 mL of AOAC Hard Water Solution 2 to a 1 L volumetric flask and bring to volume with sterile de-ionized water. b. Add approximately $\frac{3}{4}$ of total de-ionized water volume to be prepared to a volumetric flask containing the appropriate amount of AOAC Hard Water Solution 1. Add the appropriate amount of AOAC Hard Water Solution 2 to the volumetric flask. Dilute with de-ionized water to volume mark. c. Example: to prepare 1 L of 400 ppm AOAC hard water, add 4 mL AOAC Hard Water Solution 1 and 4 mL AOAC Hard Water Solution 2 to a 1 L flask and bring to volume with sterile de-ionized water. d. Determine the pH of the hard water sample. The pH should be between 7.6 and 8.0 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl. e. Filter-sterilize the hard water using a 0.2 μm filter unit. f. Measure the hardness of the water using a water hardness test kit, refer to section 12.4.
<p>12.2 Preparation of EN Hard Water</p>	<ol style="list-style-type: none"> a. To prepare 1 L of 375 ppm EN Hard water, place 600-700 mL of de-ionized water in a 1,000 mL volumetric flask and add 6.0 mL

	<p>of EN Hard Water Solution A and then 8.0 mL of EN Hard Water Solution B.</p> <ol style="list-style-type: none"> b. Mix and add water to the flask to reach 1,000 mL. c. Determine the pH of the hard water sample. The pH should be 7.0 ± 0.2 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl. d. Filter sterilize the hard water using a 0.2 μm filter unit. e. Measure the hardness of the water using a water hardness test kit, refer to section 12.4.
<p>12.3 Preparation of Hard Tap Water</p>	<ol style="list-style-type: none"> a. Collect municipal tap water sample using pre-cleaned glassware free of detergents and other potentially interfering substances. Allow the tap to run 30-60 seconds prior to sample collection. Record the location and date of sample collection. b. To ensure removal of residual chlorine, sterilize tap water by autoclaving; refer to QC-13. c. Determine baseline water hardness using a water hardness test kit (refer to section 12.4) and document this hardness on the media/reagent preparation sheet. d. If the baseline hardness is below 180 ppm, increase the water hardness to 200 ppm using the following steps: <ol style="list-style-type: none"> i. Calculate the required volume of sterile AOAC Hard Water Solution 1 according to the equation below. One mL of AOAC Hard Water Solution 1 should provide approximately 100 ppm hardness per liter of water: Required vol. AOAC Hard Water Solution 1 (mL) = $[\text{desired increase in ppm} \times \text{total volume of tap water (in L)}] \div 100 \text{ ppm}$ ii. Add required volume of AOAC Hard Water Solution 1 to a volumetric flask. iii. Add approximately $\frac{3}{4}$ of total tap water volume to be prepared (e.g., approximately 750 mL of sterilized tap water if total volume of tap water is 1 L) to the volumetric flask containing the AOAC Hard Water Solution 1. iv. Add AOAC Hard Water Solution 2 to the volumetric flask based on the total volume of tap water being prepared (4 mL Solution 2 per 1 L of total volume of tap water). v. Dilute with sterilized tap water to reach the desired total volume of tap water.

	<ul style="list-style-type: none"> vi. Example: to prepare 1 L of 200 ppm hard tap water when starting with a baseline tap water hardness of 100 ppm, add 1 mL AOAC Hard Water Solution 1 and 4 mL AOAC Hard Water Solution 2 to a 1 L flask and bring to volume with sterilized tap water. vii. Determine the pH of the hard water sample. The pH should be between 7.6 and 8.0 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl. e. Filter sterilize the hard tap water using a 0.2 µm filter unit. f. Measure the hardness of the water using a water hardness test kit, refer to section 12.4.
<p>12.4 Hard water titration procedure</p>	<ul style="list-style-type: none"> a. Measure water hardness on the day the hard water is prepared and each day it is used. b. Using the digital titrator with the appropriate cartridge, verify water hardness as ppm CaCO₃. c. Water hardness should be within -10% to +5% of the target hardness for all hard water samples. For example: <ul style="list-style-type: none"> i. An acceptable range of hard water hardness for 400 ppm is 360 to 420 ppm. ii. An acceptable range of hard water hardness for 375 ppm is 338-394 ppm. iii. An acceptable range of hard tap water hardness for 200 ppm is 180-210 ppm. d. When using the digital titrator, follow the digital titrator instructions for calculating the water hardness (refer to section 15). e. Record water hardness results in Hard Water Titration Log Book (see section 14).
<p>12.5 Other Diluents</p>	<ul style="list-style-type: none"> a. Sterile de-ionized water. For products requiring dilution with de-ionized water, sterilize an appropriate volume of de-ionized water collected from a DI water faucet (available in each laboratory). b. Sterile tap water. <ul style="list-style-type: none"> i. For products requiring dilution with tap water, collect the tap water sample from room B206 in a clean container and sterilize. ii. After sterilizing, measure the hardness of the water using a water hardness test kit; refer to section 12.4.

<p>12.6 Accuracy verification (standard additions method) using CaCO₃ hard water samples for Hach Kit</p>	<p>a. On a semi-annual basis, determine the accuracy of the digital titrator using the accuracy verification method found in the Hach kit user’s manual for using CaCO₃ hard water samples.</p> <p>Note: The accuracy verification of the digital titrator may be checked by either the CaCO₃ hard water method – described in this SOP, section 12.6b, or by the total chlorine method – described in SOP EQ-12: Hach’s Digital Titrator for Total Chlorine Titration, section 12.4.</p> <p>b. Accuracy verification procedure:</p> <ol style="list-style-type: none"> i. Prepare a hard water sample and determine the water hardness as described in section 12.4. Any hard water sample may be used. ii. Use sample from step 12.6b,i for accuracy verification. iii. Open the standard solution ampule (10,000 mg/L as CaCO₃). iv. Add 0.1 mL of the standard to the titrated sample in step 12.6b,i. Swirl to mix. v. Titrate the spiked sample to the end point (i.e., when the titrated sample turns from pink to blue). Record the number of digits on the digital titrator display that was used to reach the end point. vi. Repeat steps 12.6b,iv and 12.6b,v twice more for a total of three 0.1 mL samples of the standard added to the titrated sample. vii. Each 0.1 mL of standard that was added will use approximately 10 digits of the 0.800 M titration cartridge or approximately 100 digits of the 0.0800 M titration cartridge to reach the endpoint. A range of ±4 digits is acceptable. viii. Record outcome of accuracy verification procedure on the log sheet (see section 14). ix. If verification procedure fails, repeat steps 12.6b,i-vii. After the repeat, if the verification procedure continues to fail, identify the titrator as unusable. Use an alternate titrator or purchase a new one to ensure titration procedures are accurate.
<p>13. Data Analysis/ Calculations</p>	<p>None.</p>
<p>14. Forms and Data</p>	<p>1. Media/Reagent Preparation Sheets. Sheets are stored separately from</p>

<p>Sheets</p>	<p>the SOP under the following file names:</p> <p>Media/Reagent Preparation Sheet for AOAC Hard Water Solution 1 MB-30-03_F1.xlsx</p> <p>Media/Reagent Preparation Sheet for AOAC Hard Water Solution 2 MB-30-03_F2.xlsx</p> <p>Media/Reagent Preparation Sheet for EN Hard Water Solution A MB-30-03_F3.xlsx</p> <p>Media/Reagent Preparation Sheet for EN Hard Water Solution B MB-30-03_F4.xlsx</p> <p>Example of Media/Reagent Preparation Sheet for AOAC 400 ppm Hard Water MB-30-03_F5.xlsx</p> <p>Example of Media/Reagent Preparation Sheet for 200 ppm Hard Tap Water MB-30-03_F6.xlsx</p> <p>Accuracy Verification Semi-Annual Log Sheet MB-30-03_F7.xlsx</p> <p>Hard Water Titration Log Book MB-30-03_F8.docx</p> <p>Media/Reagent Preparation Sheet for 0.800 M EDTA Hach cartridge MB-30-03_F9.docx</p> <p>Media/Reagent Preparation Sheet for 0.08 M EDTA Hach cartridge MB-30-03_F10.docx</p>
<p>15. References</p>	<ol style="list-style-type: none"> 1. AOAC Official Method 960.09: Germicidal and Detergent Sanitizing Action of Disinfectants, Revised First Action 2013. 2. EN 13727: Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area – Test method and requirements. 2015. 3. Hach Digital Titrator Model 16900-01 manual, pages 139-145. 4. Online Method – Hardness, Total. Method 8213. Hach Company. 16900-08. 25th Ed. 03/2013.