



**US Environmental Protection Agency
Office of Pesticide Programs**

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

**Standard Operating Procedure for
Preparation of AOAC and OECD hard water and
other diluents for preparation of antimicrobial
products**

SOP Number: MB-30-01

Date Revised: 02-03-17

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Title	Preparation of AOAC and OECD hard water and other diluents for preparation of antimicrobial products
Scope	This SOP describes procedures for the preparation of diluents used to dilute test substances such as hard water, sterile de-ionized water and tap water, 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.

	Approval	Date
SOP Developer:	_____	_____
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<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 – may be prepared at various concentrations of CaCO₃ (i.e., 100 ppm-500 ppm) b. OECD hard water – prepared at a concentration of 375 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 AOAC and OECD hard water samples within 5 days of preparation. 3. Use a Media/Reagent Preparation Sheet for diluents being prepared.
<p>6. Quality Control</p>	<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 12.5.
<p>7. Interferences</p>	<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

	<p>verification.</p> <ol style="list-style-type: none"> 2. Inaccurate results will occur if the delivery tube tip is held out of the solution rather than under the surface during sample titration and/or titrator verification procedure. 3. During sample titration and titrator verification procedure, verify the delivery tube is free of air bubbles, which can interfere with titration process. 4. During titrator verification procedure: <ol style="list-style-type: none"> a. If more or less titrant was used to do the titration (based on the range of ± 4 digits), then the problem may be due to the user's technique, causing interference. b. 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. Sterilize de-ionized water and tap water according to SOP-QC-13 to prevent the presence of contamination. 7. Do not use the de-ionized water as a diluent or to prepare a diluent if it does not meet the requirements specified in SOP QC-01.
<p>8. Non-conforming Data</p>	<ol style="list-style-type: none"> 3. Errors in the preparation of the diluent, if not corrected prior to testing, will result in a repeat of the study. 4. The target hardness expressed as mg/L calcium carbonate (CaCO_3) is -10% to +5% of the target hardness. Other levels of water hardness may be used as appropriate.
<p>9. Data Management</p>	<p>Data will be archived consistent with SOP ADM-03, Records and Archives.</p>
<p>10. Cautions</p>	<p>Strict adherence to the protocol is necessary for the validity of the test results.</p>
<p>11. Special Apparatus and Materials</p>	<ol style="list-style-type: none"> 1. Glassware <ol style="list-style-type: none"> a. Glassware – such as flasks and beakers, to conduct titration assays. b. Sterile volumetric glassware (flasks, etc.) – to measure ingredients used in hard water preparation. 2. Equipment <ol style="list-style-type: none"> a. Calibrated weigh balance – to prepare hard water solutions 1, 2, A, and B. 3. AOAC hard water solutions and reagents

	<ul style="list-style-type: none">a. De-ionized waterb. 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.c. 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. <p>4. OECD hard water solutions and reagents</p> <ul style="list-style-type: none">a. <i>Note:</i> The OECD hard water (test substance diluent) is 375 ppm hard water, unless otherwise specified. Adjust the recipe for volumes other than 1 L.b. De-ionized waterc. 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.d. 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. <p>5. 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> <p>6. Apparatus</p> <ul style="list-style-type: none">a. 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).<ul style="list-style-type: none">i. Kit includes reagents, flask, digital titrator, delivery tube, and instruction sheet necessary to determine the total water hardness of the sample. Additional reagents may be purchased from manufacturer (see section 15).b. Various titrating cartridges are available depending on the water hardness range to be assayed:<ul style="list-style-type: none">i. Water hardness in the range of 10-160 ppm = titration cartridge of 0.0800 EDTA.
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	<p>ii. Water hardness in the range of 200-4,000 ppm = titration cartridge of 0.800 EDTA.</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. 2. OECD hard water preparation is conducted as described in the Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides Used on Hard Non-Porous Surfaces (dated June 21, 2013). 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 tap water, refer to section 12.4.
<p>12.1 AOAC Hard Water (as CaCO₃) – Preparation</p>	<ol style="list-style-type: none"> a. Add approximately ¾ of total de-ionized water volume to be prepared to a volumetric flask containing the appropriate amount of solution 1. Add the appropriate amount of solution 2 to the volumetric flask. Dilute with de-ionized water to volume mark. See 12.1c for example. b. Per 1 L: Add 1 mL of hard water solution 1 (for each 100 ppm hardness desired) and 4 mL of hard water solution 2 to a 1 L volumetric flask and bring to volume with sterile de-ionized H₂O. See example in 12.1c below: c. For example, to prepare 1 L of 400 ppm hard water, add 4 mL hard water solution 1 and 4 mL 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 (adjust if necessary). e. Filter-sterilize the hard water using a 0.2 µm filter unit. f. Ensure sterility of hard water prior to use in efficacy testing. g. On the day of the day of the test, measure the hardness of the water using a water hardness test kit, refer to section 12.3.
<p>12.2 OECD Hard Water (as CaCO₃) – Preparation</p>	<ol style="list-style-type: none"> a. To prepare 1 L of 375 ppm hard water, place 600-700 mL of de-ionized water in a 1,000 mL volumetric flask and add 6.0 mL of Solution A and then 8.0 mL of Solution B. b. Mix and add water to the flask to reach 1,000 mL. c. The pH of the hard water should be 7.0±0.2 at room temperature. If necessary, adjust the pH by using 1 N NaOH or 1 N HCl.

	<ul style="list-style-type: none"> d. Ensure sterility of hard water prior to use in efficacy testing. e. On the day of the test, measure the hardness of the water using a water hardness test kit, refer to section 12.3.
<p>12.3 Hard water titration procedure (AOAC and OECD hard water samples)</p>	<ul style="list-style-type: none"> a. Using the digital titrator with the appropriate cartridge, verify hardness of the sterile hard water, in the form of CaCO₃. b. Water hardness should be within -10% to +5% of the target hardness for both AOAC and OECD hard water samples. For example, an acceptable range of AOAC hard water hardness per 400 ppm is 360 to 420 ppm, and an acceptable range of hardness for 375 ppm OECD hard water is 338-394 ppm. c. When using the digital titrator, follow the digital titrator instructions for calculating the water hardness (refer to section 15). d. Record water hardness results in Media/Reagent Preparation Sheet. e. Verify the sterility of the hard water according to SOP MB-10.
<p>12.4 Other Diluents</p>	<ul style="list-style-type: none"> a. Sterile de-ionized water. For products requiring dilution with de-ionized water, an appropriate volume of de-ionized water is taken from the DI water faucets available in each laboratory and sterilized. b. Sterile tap water. For products requiring dilution with tap water, determine the water hardness of the tap water sample on the day of use. Collect the tap water sample from room B206 in a clean container prior to sterilization. Use a digital titrator (see sections 12.3 and 15) to determine the water hardness. Record water hardness results on the Media/Reagent Preparation Sheet. c. Appropriate sterilization procedures are available in SOP-QC-13: Performance Verification of Autoclaves. d. Verify the sterility of the de-ionized water or the tap water according to SOP MB-10, Media and Reagents Preparation and Quality Evaluation.
<p>12.5 Accuracy verification (standard additions method) using CaCO₃ hard water samples for Hach Kit</p>	<ul style="list-style-type: none"> a. On a semi-annual basis, determine the accuracy of the digital titrator using the method found in the Hach kit user's manual for accuracy verification using CaCO₃ hard water samples. Note: The accuracy verification of the digital titrator may be checked by either the CaCO₃ hard water method – described in this SOP, section 12.5.b, or by the total chlorine method – described in SOP EQ-12: Hach's Digital Titrator for Total Chlorine Titration, section 12.4. b. Accuracy verification procedure:

	<ol style="list-style-type: none"> i. Prepare a hard water sample (AOAC or OECD) and determine the water hardness as described in section 12.3. Any hard water sample may be used. ii. Use sample from step 12.5b, 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 12.5b, 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 12.5b, iv and 12.5b, 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 (11 digits of 0.714 M or 56 digits of 0.1428 M titrant). 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 12.5b, 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 ensure titration procedures are accurate. 						
12.6 Media/Reagent Preparation Sheet	<ol style="list-style-type: none"> a. Record diluent preparation and hard water titration (if applicable) on the Media/Reagent Preparation Sheet (refer to SOP QC-15, Media Prep and Sterilization Run Numbers). 						
13. Data Analysis/ Calculations	None.						
14. Forms and Data Sheets	<ol style="list-style-type: none"> 1. Media/Reagent Preparation Sheets. Sheets are stored separately from the SOP under the following file names: <table border="0" style="margin-left: 40px;"> <tr> <td>Media/Reagent Preparation Sheet for AOAC Hard Water Solution 1</td> <td style="text-align: right;">MB-30-01_F1.xlsx</td> </tr> <tr> <td>Media/Reagent Preparation Sheet for AOAC Hard Water Solution 2</td> <td style="text-align: right;">MB-30-01_F2.xlsx</td> </tr> <tr> <td>Media/Reagent Preparation Sheet for OECD Hard Water Solution A</td> <td style="text-align: right;">MB-30-01_F3.xlsx</td> </tr> </table> 	Media/Reagent Preparation Sheet for AOAC Hard Water Solution 1	MB-30-01_F1.xlsx	Media/Reagent Preparation Sheet for AOAC Hard Water Solution 2	MB-30-01_F2.xlsx	Media/Reagent Preparation Sheet for OECD Hard Water Solution A	MB-30-01_F3.xlsx
Media/Reagent Preparation Sheet for AOAC Hard Water Solution 1	MB-30-01_F1.xlsx						
Media/Reagent Preparation Sheet for AOAC Hard Water Solution 2	MB-30-01_F2.xlsx						
Media/Reagent Preparation Sheet for OECD Hard Water Solution A	MB-30-01_F3.xlsx						

	<p>Media/Reagent Preparation Sheet for OECD Hard Water Solution B MB-30-01_F4.xlsx</p> <p>Example of Media/Reagent Preparation Sheet for AOAC 400 ppm Hard Water MB-30-01_F5.xlsx</p> <p>Accuracy Verification Semi-Annual Log Sheet MB-30-01_F6.xlsx</p>
15. References	<ol style="list-style-type: none">1. AOAC Official Method 960.09: Germicidal and Detergent Sanitizing Action of Disinfectants, Revised First Action 2013.2. Guidance Document on Quantitative Methods for Evaluating the Activity of Microbicides Used on Hard Non-Porous Surfaces (June 21, 2013).3. Hach Digital Titrator Model 16900-01 manual, pages 91-98.4. Online Method– Hardness, Total. Method 8213. Hach Company. 16900-08. 25th Ed. 03/2013.