



**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 hard water and other diluents for antimicrobial products**

SOP Number: MB-30-00

Date Revised: 12-12-13

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Title	Preparation of hard water and other diluents for antimicrobial products
Scope	This SOP describes procedures for the preparation of diluents used to dilute disinfectant products 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:	_____	
	Print Name: _____	
SOP Reviewer	_____	
	Print Name: _____	
Quality Assurance Unit	_____	
	Print Name: _____	
Branch Chief	_____	
	Print Name: _____	

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1. Definitions	<ol style="list-style-type: none"> 1. Concentrated disinfectant product = Product that requires a dilution prior to use. 2. Product diluent = Sterile liquid used to make product dilutions (e.g. tap water, de-ionized water or hard water). 3. Hard water = Water which contains a standardized concentration of calcium and magnesium ions.
2. Health and Safety	<ol style="list-style-type: none"> 1. Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Material Safety Data Sheet for hazards associated with specific products.
3. Personnel Qualifications and Training	<p>Refer to SOP ADM-04, OPP Microbiology Laboratory Training.</p>
4. Instrument Calibration	<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>
5. Sample Handling and Storage	<ol style="list-style-type: none"> 1. Refer to SOP MB-22: Disinfectant Product Preparation and Sampling Procedures and SOP COC-01: Sample Log-in and Tracking of Disinfectants for procedures on sample handling and storage. 2. Use hard water within 24 hours of preparation. 3. Use a Media/Reagent Preparation Sheet for diluents being prepared.
6. Quality Control	<p>For quality control purposes, the required information is documented on the appropriate record form(s). See section 14.</p>
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. Sterilization of de-ionized water and tap water must be conducted according to SOP-QC-13 to prevent the presence of contamination. 3. If the de-ionized water does not meet the requirements specified in SOP QC-01 then the water should not be used as a diluent or to prepare a diluent.
8. Non-conforming Data	<p>Errors in the preparation of the diluent, if not corrected prior to testing, will result in a repeat of the study.</p>
9. Data Management	<p>Data will be archived consistent with SOP ADM-03, Records and Archives.</p>
10. Cautions	<p>Strict adherence to the protocol is necessary for the validity of the test results.</p>
11. Special Apparatus and	

Materials	
11.1 Glassware	<ul style="list-style-type: none"> a. Sterile glassware – such as flasks and beakers, to conduct titration assays. b. Sterile volumetric glassware (pipettes, flasks, etc.) – to measure ingredients used in hard water preparation.
11.2 Equipment	<ul style="list-style-type: none"> a. Weigh Balance (weighs up to 5100 g) – to weigh sample containers prior to and after sample removal. b. Serological and micropipettes with tips.
11.3 Hard water solutions and reagents	<ul style="list-style-type: none"> a. De-ionized water b. Hard Water Solution 1. Dissolve 7.94 g MgCl₂ (anhydrous) (or 16.94 g MgCl₂·6H₂O) and 18.50 g CaCl₂ in boiled de-ionized H₂O, bring to a volume of 250 mL volumetrically. Filter-sterilize using a 0.2 μm filter unit. Used for the preparation of hard water at various concentrations. c. Hard Water Solution 2. Dissolve 14.01 g NaHCO₃ in boiled de-ionized H₂O; bring to a volume of 250 mL volumetrically. Filter-sterilize using a 0.2 μm filter unit. Used for the preparation of hard water at various concentrations. d. Hardness Voluette Ampule Standard Solution, 10,000-mg/L as CaCO₃ (Hach Company, Catalog No. 218-710). Used in the accuracy verification of the digital titrator.
11.4 Apparatus	<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 CaCO₃ (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 for details and users manual information). 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. ii. Water hardness in the range of 200 – 4,000 ppm = titration

	cartridge of 0.800 EDTA.
12. Procedure and Analysis	<ol style="list-style-type: none"> 1. Per the study sponsor's request, prepare the appropriate diluent used to dilute the product as stated in section 12.1. If hard water is prescribed as the diluent, then verify the hardness according to section 12.2. For products requiring the use of tap water see section 12.3. 2. Conduct titration of hard water as described in section 12.2. <u>NOTE:</u> Hard water preparation is conducted as described in Method I of AOAC standard 960.09., Hard water titration is conducted as described in Method II of AOAC standard 960.09 (see section 15.1).
12.1 Hard Water (as CaCO₃) – Preparation	<ol style="list-style-type: none"> a. Prepare hard water solution 1 volumetrically as in section 11.3, b. b. Prepare hard water solution 2 volumetrically as in section 11.3, c. c. Add approximately ¾ of total deionized 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 deionized water to volume mark. See 12.1, e for example. d. Per 1L: 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 (e) below: e. 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. f. Determine the pH of the hard water sample. The pH should be between 7.6 and 8.0 (adjust if necessary). g. Filter-sterilize hard water using a 0.2 µm filter unit.

<p>12.2 Hard water titration procedure</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 CaCO_3. b. Water hardness should be within -10% to +5% of the target hardness. For example, an acceptable range of hardness per 400 ppm is 360 to 420 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. Verify the sterility of the hard water according to SOP MB-10.
<p>12.3 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 deionized water is taken from the port located in room B206. To collect the deionized water, turn on the Barnstead B-pure Pressure Cartridge System and let run until the resistivity meter in the cartridge unit reads an acceptable limit, see QC-01: Quality Assurance of Purified Water for details on the use and operation of the Barnstead B-pure Pressure Cartridge System. <ul style="list-style-type: none"> i. The Barnstead B-pure Pressure Cartridge System contains cartridges which raise the quality of the deionized water. 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 12.2 and 15) to determine the water hardness. c. Record water hardness results in Media/Reagent Preparation Sheet. Verify the sterility of the tap water accordingly to SOP MB-10: Media and Reagents Preparation and Quality Evaluation.
<p>12.4 Accuracy verification (standard additions method) for Hach Kit</p>	<ul style="list-style-type: none"> a. On a quarterly basis, determine the accuracy of the digital titrator using the method found in the Hach kit user's manual. b. Accuracy verification procedure: <ul style="list-style-type: none"> 1. Prepare a hard water sample and determine the water hardness as described in section 12.1. Any hard water sample may be used, for example a 200 ppm or 400 ppm hard water sample may be used. 2. Use sample from step 1 for accuracy verification. 3. Open the standard solution ampule (10,000 mg/L as CaCO_3)

	<ol style="list-style-type: none"> 4. Add 0.1 mL of the standard to the titrated sample in step 1. Swirl to mix. 5. Titrate the spiked sample to the end point (i.e., when the titrated sample turns from pink to blue). Write down the number of digits on the digital titrator display that was used to reach the end point. 6. Repeat steps 4 and 5, twice more, for a total of three 0.1 mL samples of the standard added to the titrated sample. 7. 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. 8. If more or less titrant was used, the problem can be due to user technique, causing interference. 9. Conduct the accuracy verification on a quarterly basis. 10. Record if verification passed or failed on the log sheet (see section 14).
12.5 Media/Reagent Preparation Sheet	<ol style="list-style-type: none"> a. Record diluent preparation on the Media/Reagent Preparation Sheet (refer to SOP QC-15, Media Prep and Sterilization Run Numbers).
13. Data Analysis/ Calculations	Hardness may vary by no more than -10% or +5% from the stated value.
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: Media/Reagent Preparation Sheet for Hard Water MB-30-00_F1.xlsx Solution 1 Media/Reagent Preparation Sheet for Hard Water MB-30-00_F2.xlsx Solution 2 Example of Media/Reagent Preparation Sheet MB-30-00_F3.xlsx for 400 ppm Hard Water Accuracy Verification Quarterly Log Sheet MB-30-00_F4.xlsx

15. References	<ol style="list-style-type: none"><li data-bbox="474 336 1481 409">1. AOAC Official Method 960.09: Germicidal and Detergent Sanitizing Action of Disinfectants, Revised First Action 2013.<li data-bbox="474 409 1481 472">2. Hach Digital Titrator Model 16900-01 manual, pages 91-98.<li data-bbox="474 472 1481 569">3. Online Method– Hardness, Total. Method 8213. Hach Company. DOC316.53.01176. 7th Ed. 2012.
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