



Method 1605: *Aeromonas* in Finished Water by Membrane Filtration using Ampicillin-Dextrin Agar with Vancomycin (ADA-V)

October 2001

Acknowledgments

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Disclaimer

This method has been validated by the U.S. Environmental Protection Agency through an interlaboratory validation study, and will be proposed for use in drinking water monitoring in the *Federal Register*. This method is not an EPA-approved method until it is promulgated as an approved method in the *Federal Register*.

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Introduction

Aeromonas is a common genus of bacteria indigenous to surface waters, and may be found in non-chlorinated or low-flow parts of chlorinated water distribution systems. Monitoring their presence in distribution systems is desirable because some aeromonads may be pathogenic and pose a potential human health risk. Method 1605 describes a membrane filtration technique for the detection and enumeration of *Aeromonas* species. This method uses a selective medium that partially inhibits the growth of non-target bacterial species while allowing most species of *Aeromonas* to grow. *Aeromonas* is presumptively identified by the production of acid from dextrin fermentation and the presence of yellow colonies on ampicillin-dextrin agar medium with vancomycin (ADA-V). Yellow colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test), and the ability to ferment trehalose, and produce indole.

Laboratories are not permitted to modify ADA-V media or procedures associated with filtration (Sections 10.1 through 10.10). However, the laboratory is permitted to modify method procedures related to the confirmation of colonies (Section 10.11) to improve performance or lower the costs of measurements provided that 1) presumptively identified yellow colonies submitted to confirmation are tested for the presence of cytochrome *c* (oxidase test), and the ability to ferment trehalose, and the ability produce indole, and 2) all quality control (QC) tests cited in Section 9.2.12 are performed acceptably and QC acceptance criteria are met. For example, laboratories may prefer to streak colonies that are submitted to confirmation on tryptic soy agar (TSA), instead of nutrient agar. The laboratory may not omit any quality control analyses.

This method is for use in the Environmental Protection Agency's (EPA's) data gathering and monitoring programs under the Safe Drinking Water Act.

Questions concerning this method or its application should be addressed to:

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1.0 Scope and Application

- 1.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of *Aeromonas* species in finished water samples. *Aeromonas* is a common genus of bacteria indigenous to surface waters. Its numbers are more likely to be greater during periods of warmer weather and when increased concentrations of organic nutrients are present. It is also more likely to be found in non-chlorinated water distribution systems or low-flow parts of chlorinated systems. Some *Aeromonas* species are opportunistic pathogens.
- 1.2 This method is adapted from Havelaar et al. (1987) for the enumeration of *Aeromonas* species in finished water by membrane filtration (Reference 15.1). It is a quantitative assay that uses a selective medium which partially inhibits the growth of non-target bacterial species while allowing *Aeromonas* to grow. *Aeromonas* is presumptively identified by the production of acid from dextrin fermentation producing yellow colonies. Presumptively positive colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test), and the ability to ferment trehalose, and produce indole.
- 1.3 This method is designed to meet the finished water monitoring requirements of the U.S. Environmental Protection Agency. *Aeromonas* was included on the Contaminant Candidate List (CCL) (Mar. 2, 1998, 63 FR 10274) and in the Revisions to the Unregulated Contaminant Monitoring Proposed Rule (UCMR) (September 17, 1999, 64 FR 50556). Contaminants listed in the UCMR are candidates for future regulation and may be included in a monitoring program for unregulated contaminants. Unregulated contaminant monitoring would be required for large systems and a representative sample of small and medium sized water distribution systems.
- 1.4 This method was subjected to an interlaboratory validation study involving 11 laboratories and 11 finished drinking water matrices. This method was not validated for other water types. Use of this method and appropriate validation for other water types is the responsibility of the user.

2.0 Summary of Method

- 2.1 The method provides a direct count of *Aeromonas* species in water based on the growth of yellow colonies on the surface of the membrane filter using a selective medium. A water sample is filtered through 0.45- μ m-pore-size membrane filter. The filter is placed on ampicillin-dextrin agar with vancomycin (ADA-V) and incubated at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. This medium uses ampicillin and vancomycin to inhibit non-*Aeromonas* species, while allowing most *Aeromonas* species to grow. The medium uses dextrin as a fermentable carbohydrate, and bromothymol blue as an indicator of acidity produced by the fermentation of dextrin. Presumptively identified yellow colonies are counted and confirmed by testing for the presence of cytochrome *c* (oxidase test), and the ability to ferment trehalose and produce indole.

- 2.2 The membrane filtration procedure provides a direct count of culturable *Aeromonas* in water samples that is based on the growth of bacterial colonies on the surface of the membrane filter placed on a selective medium.
- 2.3 *Aeromonas* isolates may be archived for further analysis to determine species or hybridization group by inoculating a nutrient agar slant for short term use or shipping, or nutrient broth for freezing.

3.0 Definitions

- 3.1 *Aeromonas* are bacteria that are facultative anaerobes, Gram-negative, oxidase-positive, polarly flagellated, and rod shaped. They are classified as members of the family *Aeromonadaceae*. Demarta et al. (1999) reported 15 *Aeromonas* species based on 16S rDNA sequences though not all are officially recognized. Some species have been associated with human disease. In this method, *Aeromonas* are those bacteria that grow on ampicillin-dextrin agar with vancomycin (ADA-V), produce yellow colonies, are oxidase-positive, and have the ability to ferment trehalose and produce indole.
- 3.2 Definitions for other terms are provided in the glossary at the end of the method (Section 17.3).

4.0 Interferences and Contamination

- 4.1 Water samples containing colloidal or suspended particulate material may clog the membrane filter and prevent filtration or cause spreading of bacterial colonies which could interfere with identification of target colonies.
- 4.2 Other ampicillin/vancomycin resistant bacteria that are not aeromonads may be able to grow on this medium. Some of these bacteria may also produce yellow colonies if they are able to produce acid byproducts from the fermentation of dextrin or some other media component, or if they produce a yellow pigment. *Enterococcus* are reported to produce pinpoint-size yellow colonies on ADA. Confirmation of presumptive *Aeromonas* colonies is necessary to mitigate false positives.

5.0 Safety

- 5.1 Some strains of *Aeromonas* are opportunistic pathogens. Sample containers and waste materials should be autoclaved prior to cleaning or disposal.
- 5.2 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and other materials.
- 5.3 This method does not address all safety issues associated with its use. The laboratory is responsible for maintaining a safe work environment and a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file

of material safety data sheets (MSDSs) should be available to all personnel involved in these analyses.

6.0 Equipment and Supplies

Note: Brand names, suppliers, and part numbers are for illustrative purposes only. No endorsement is implied. Equivalent performance may be achieved using apparatus and materials other than those specified here, but demonstration of equivalent performance that meets the requirements of this method is the responsibility of the laboratory.

- 6.1 Equipment for collection and transport of samples to laboratory
 - 6.1.1 Autoclavable sample container—Use sterile, non-toxic, glass or plastic containers with a leak-proof lid. Ensure that the sample container is capable of holding a 1-L sample with ample headspace to facilitate mixing of sample by shaking prior to analysis.
 - 6.1.2 Ice chest
 - 6.1.3 Ice packs
- 6.2 Autoclavable dilution bottles—125-mL marked at 99 mL or 90 mL; commercially produced dilution bottles may be used
- 6.3 Rinse water bottles
- 6.4 Sterile plastic or autoclavable glass pipettes with a 2.5% tolerance—To deliver (TD), 1- and 10-mL
- 6.5 Pipette bulbs or automatic pipetter
- 6.6 Autoclavable pipette container (if using glass pipettes)
- 6.7 Thermometer—with 0.5°C gradations checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23
- 6.8 Inoculating loop—Sterile metal, plastic, or wooden applicator sticks
- 6.9 Burner—Flame or electric incinerator for sterilizing metal inoculating loops and forceps
- 6.10 Colony counting device—Mechanical, electric or hand tally
- 6.11 Hotplate stirrer
- 6.12 Magnetic stir bar
- 6.13 Graduated cylinders—100 mL, 500 mL and 1 L, sterile, polypropylene or glass
- 6.14 Balance—Capable of weighing samples up to 200 g, with a readability of 0.1 g
- 6.15 Weigh boats
- 6.16 pH meter
- 6.17 Turbidimeter (optional)
- 6.18 Equipment for membrane filter procedure

- 6.18.1 Incubator—Hot air or water-jacketed microbiological type to maintain a temperature of $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$
- 6.18.2 Petri dishes—sterile, 50×9 mm or other appropriate size
- 6.18.3 Membrane filtration units (filter base and funnel made of glass, plastic, or stainless steel), wrapped with aluminum foil or Kraft paper, and sterilized by autoclaving.
- 6.18.4 Vacuum source
- 6.18.5 Flasks—1-L vacuum filter with appropriate tubing; a filter manifold to hold a number of units is optional
- 6.18.6 Side-arm flask to place between vacuum source and filtration devices or filter manifold
- 6.18.7 Membrane filters—Sterile, cellulose ester, white, gridded, 47-mm-diameter with $0.45\text{-}\mu\text{m}$ pore size (Gelman E04WG04700 or equivalent)
- 6.18.8 Forceps—Sterile, straight or curved, with smooth tips to handle filters without causing damage
- 6.18.9 Ethanol or other alcohol in a container to sterilize forceps
- 6.18.10 Test tubes— 125×16 mm sterile, screw-cap tube
- 6.19 Dissecting microscope—Low power (10X to 15X), binocular, illuminated
- 6.20 Autoclave—Capable of 121°C at 15 psi. Must meet requirements set forth in the *Manual for the Certification of Laboratories Analyzing Drinking Water, 4th Edition*. (Reference 15.5)
- 6.21 Membrane filters (for sterilization purposes)—Sterile with $0.22\text{-}\mu\text{m}$ pore size (Gelman Acrodisc No. 4192 or equivalent)

7.0 Reagents and Standards

- 7.1 Purity of reagents and culture media—Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, reagents and culture media shall conform to the specifications in *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141), Section 9050 (Reference 15.2). The agar used in preparation of culture media must be of microbiological grade.
- 7.2 Purity of water—Reagent-grade water conforming to specifications in *Manual for the Certification of Laboratories Analyzing Drinking Water, 4th Edition* (Reference 15.5) or *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141), Section 9020 (Reference 15.2).
- 7.3 Phosphate buffered dilution water
 - 7.3.1 Concentrated stock phosphate buffer solution—Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL reagent-grade water. Adjust the pH to 7.2 ± 0.5 with 1N sodium hydroxide (NaOH) and dilute to 1 L with reagent-grade water. Autoclave or filter sterilize through a filter with $0.22\text{-}\mu\text{m}$ -pore-size.
 - 7.3.2 Magnesium chloride solution—Dissolve 81.1 g magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) in reagent-grade water and dilute to 1 L. Autoclave or filter sterilize through a $0.22\text{-}\mu\text{m}$ -pore-size filter.
 - 7.3.3 Prepare phosphate buffered dilution water by adding 1.25 mL of concentrated stock phosphate buffer solution (Section 7.3.1) and 5.0 mL of magnesium chloride solution

(Section 7.3.2) to a 1-L graduated cylinder and adjust final volume to 1 L with reagent-grade water. Prepare a portion of buffered dilution water in 1-L bottles for rinse water. Autoclave or filter sterilize through a filter with 0.22- μ m-pore-size.

7.3.4 Stored phosphate buffered dilution water should be free from turbidity.

7.4 Ampicillin-dextrin agar with vancomycin (ADA-V)

7.4.1 Preparation of dextrin agar—EPA highly recommends the use of commercial ADA (*m-Aeromonas* Selective Agar Base [Havelaar]), Section 7.4.1.1. However, ADA may be prepared by the laboratory (Section 7.4.1.2)

7.4.1.1 Commercial dextrin agar—Tech Pac (distributor, tech@fuse.net), Cincinnati, Ohio; Biolife (www.biolifeit.com) Italiana Srl, 272 Viale Monza, Milan, Italy, Cat. No. 401019 or equivalent. Prepare 1-L of media, according to manufacturer's instructions. Cool to room temperature, and adjust pH to 8.0 using 1N NaOH or 1N HCl. Autoclave for 15 min, cool to 50°C.

7.4.1.2 Laboratory-prepared dextrin agar.

7.4.1.2.1 5.0 g tryptose—Difco cat. no. 0124-17, or equivalent

7.4.1.2.2 11.4 g dextrin—Difco cat. no. 0161-17, or equivalent

7.4.1.2.3 2.0 g yeast extract—Difco cat. no. 0127-17, or equivalent

7.4.1.2.4 3.0 g sodium chloride (NaCl)—Baker cat. no. 3624, or equivalent

7.4.1.2.5 2.0 g potassium chloride (KCl)—Fisher cat. no. P217-500, or equivalent

7.4.1.2.6 0.1 g magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)—Fisher cat. no. M63-500, or equivalent

7.4.1.2.7 0.06 g ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)—Sigma cat. no. F-2877, or equivalent

7.4.1.2.8 0.08 g bromothymol blue—Baker cat. no. D470, or equivalent

7.4.1.2.9 Sodium deoxycholate—Sigma cat. no. D-6750, or equivalent. Add 100 mg of sodium deoxycholate to 10 mL of reagent water.

7.4.1.2.10 13.0 g agar, bacteriological grade—Fisher cat. no. BP1423-500, or equivalent.

7.4.1.2.11 Add reagents in Sections 7.4.1.2.1 through 7.4.1.2.8 to 1-L of reagent-grade water, stir to dissolve and adjust pH to 8.0 using 1N NaOH or 1N HCl. After the pH has been adjusted, add sodium deoxycholate (Section 7.4.1.2.9) and agar (Section 7.4.1.2.10) and heat to dissolve. Autoclave for 15 min, cool to 50°C.

7.4.2 Ampicillin, sodium salt—Sigma cat. no. A0166, or equivalent. Add 10 mg of ampicillin, sodium salt to 10 mL reagent water. Prepare on the same day that medium is prepared and filter sterilize through a 0.22- μ m-pore-size filter. Alternatively, use Biolife cat. no. 4240012 prepared according to manufacturer's instructions, taking care to use an appropriate amount of ampicillin for the volume of media being prepared (for example, use two vials for a 1-L batch of ADA-V). Follow manufacturer's instructions

for appropriate storage temperature and shelf-life. *Wear suitable protective clothing, gloves, and eye/face protection and prepare stock solutions in a chemical fume hood.*

- 7.4.3** Vancomycin hydrochloride—Sigma cat. no. V2002, or equivalent. Add 2 mg of vancomycin hydrochloride to 10 mL of reagent water. Filter sterilize through a 0.22- μ m-pore-size filter. Follow manufacturer's instructions for appropriate storage temperature and time. *Wear suitable protective clothing, gloves, and eye/face protection and prepare stock solutions in a chemical fume hood.*
- 7.4.4** After dextrin agar (Section 7.4.1) has been autoclaved and cooled to 50°C, add the sterile ampicillin (Section 7.4.2) and sterile vancomycin hydrochloride solutions (Section 7.4.3).
- 7.4.5** Add approximately 5 mL of ADA-V per 50 × 9 mm petri dish and allow to solidify. For larger plates, adjust volume appropriately. ADA-V plates should be stored in a tight fitting container (i.e. sealed plastic bag) at a temperature of 1°C to 5°C for no longer than 14 days.
- 7.5** Pentahydrate ACS Reagent grade sodium thiosulfate—Fisher cat. no. S445-500, or equivalent. Prepare a 3% stock solution by adding 3 g sodium thiosulfate to 100 mL reagent-grade water.
- 7.6** Disodium salt of ethylenediaminetetraacetic acid (EDTA)—Sigma cat. no. E 4884, or equivalent. EDTA should only be added to samples if metals in water samples exceed 1.0 mg/L. To prepare stock solution, add 12.4 g EDTA to 80 mL of reagent-grade water. Adjust pH to 8.0 using 10N NaOH. After the pH has been adjusted, bring the volume up to 100 mL with reagent-grade water.
- 7.7** Positive control culture—*Aeromonas hydrophila* ATCC #7966; obtained from the American Type Culture Collection (ATCC, 10801 University Blvd, Manassas, VA, 20110-2209; <http://www.atcc.org>).
- 7.8** Negative culture control—Negative culture controls serve two purposes: to ensure the laboratories are familiar with the color and morphology of non-*Aeromonas* bacteria that may grow on ADA-V and to ensure that confirmation test results are appropriate. *E. coli* (ATCC #25922) is the negative culture control for oxidase, *Pseudomonas aeruginosa* (ATCC #27853) is the negative culture control for trehalose fermentation, and *Bacillus cereus* (ATCC #11778) is the negative culture control for indole.
- 7.9** Nutrient agar—Difco cat. no. 0001-17 or equivalent. Prepare according to manufacturers instructions.
- 7.10** Oxidase reagents—Dry Slide BBL cat. no. 231746 or equivalent.
- 7.11** 0.5% Trehalose confirmation reagent
- 7.11.1** Add 5 g trehalose (Sigma cat. no. T0167, or equivalent) to 100 mL water and filter sterilize solution through a filter with 0.22- μ m-pore-size.
- 7.11.2** Prepare 900 mL purple broth base (Difco cat. no. 0222-17, or equivalent) according to manufacturer's instructions and autoclave.
- 7.11.3** Aseptically add 100 mL trehalose solution to the cooled 900 mL of purple broth base.
- 7.11.4** Dispense into 6 mL or larger size tubes and fill approximately half full. Store in refrigerator.

Note: Alternatively, prepare purple broth base according to manufacturers instructions, add 5 g trehalose per liter, and filter sterilize through a filter with 0.22- μ m-pore-size.

- 7.12** Tryptone broth—Oxoid cat. no. CM0087B, or equivalent. Alternatively, the laboratory may prepare tryptone broth by adding 10 g of tryptone (Difco cat. no. 0123-17 or equivalent) and 5 g of NaCl to 1 L of reagent water. Autoclave or filter sterilize through a filter with 0.22- μ m-pore-size.
- 7.13** Kovac's reagent—Biomeriuex cat. no. V7050, or equivalent

8.0 Sample Collection, Preservation, and Storage

- 8.1** Adherence to sample preservation procedures and holding time limits specified in *Standard Methods for the examination of Water and Wastewater* (Reference 15.2) is critical to the production of valid data. Sample results will be considered invalid if those conditions are not met.
- 8.2** Preparation of sample bottles and sample collection—Samples must be representative of the drinking water distribution system. Water taps used for sampling should be free of aerators, strainers, hose attachments, mixing type faucets, and purification devices. Cold water taps should be used. The service line should be cleared before sampling by maintaining a steady water flow for at least two minutes (until the water changes temperature).
- 8.2.1** Use sterile, non-toxic, glass or plastic container (Section 6.1.1) with a leak-proof lid. Ensure that the sample container is capable of holding a 1-L sample with ample headspace to facilitate mixing of sample by shaking prior to analysis. Sampling procedures are described in detail in *Standard Methods for the Examination of Water and Wastewater*, Section 9060 (Reference 15.2).
- 8.2.2** Add 1 mL of 3% sodium thiosulfate stock (Section 7.5) per L of sample to sample bottles prior to autoclave sterilization. Alternatively, if using presterilized sample bottles, sodium thiosulfate should be autoclaved for 15 minutes or filter sterilized through a filter with 0.22- μ m-pore-size before adding to the sample bottles.
- 8.2.3** If metals in the sample exceed 1.0 mg/L, add 3 mL of EDTA stock solution (Section 7.6) per L of sample to sample bottles prior to autoclave sterilization. If using presterilized sample bottles, EDTA should be autoclaved for 15 minutes or filter sterilized through a filter with 0.22- μ m-pore-size.
- 8.2.4** Collect a minimum of 1-L of sample.
- 8.3** Sample preservation and handling
- 8.3.1** Immediately following sample collection, tighten the sample container lid(s) and place the sample container(s) upright in an insulated, plastic-lined storage cooler with ice packs or in a refrigerator to chill prior to packing the cooler for shipment. Do not freeze the sample.
- 8.3.2** Use enough solidly frozen ice packs to ensure that the samples will arrive at a temperature of 1°C to 10°C. Use a minimum of two ice packs per shipment and add extra ice packs for multiple samples. Place one or more ice packs on each side of the container to stabilize samples.

- 8.3.3** Samples must be maintained at a temperature of 1°C to 10°C during shipment. Samples must not be frozen.
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Note: Sample temperature during shipment is critical. Ice packs must be frozen solid immediately prior to shipment.

- 8.4** Verify and record sample arrival temperature when received in the laboratory. Refrigerate samples at 1°C to 5°C upon receipt at the laboratory and analyze as soon as possible after collection. Samples must be analyzed within 30 hours of sample collection.

9.0 Quality Control

- 9.1** Each laboratory that uses Method 1605 is required to operate a formal quality assurance (QA) program. The minimum QA requirements consist of the initial demonstration of capability (IDC) test (Section 9.4), ongoing analysis of spiked reagent water (ODC test, Section 9.8) and spiked finished drinking water samples (MS/MSD, Section 9.7), and analysis of negative culture controls (Section 9.6), dilution/rinse water blanks (Section 9.5), and media sterility checks (Section 9.2.6) as tests of continued acceptable performance. Spiked sample results are compared to acceptance criteria for precision, which are based on data generated during the interlaboratory validation of Method 1605 involving 11 laboratories and 11 finished water matrices. The more stringent QA requirements in this method, relative to other, currently used methods for bacterial determination, are an effort to improve overall microbiological QA. The specifications contained in this method can be met if the analytical system is maintained under control.

Laboratories are not permitted to modify ADA-V media or procedures associated with filtration (Sections 10.1 through 10.10). However, the laboratory is permitted to modify method procedures related to the confirmation of colonies (Section 10.11) to improve performance or lower the costs of measurements provided that 1) presumptively identified yellow colonies submitted to confirmation are tested for the presence of cytochrome c (oxidase test), and the ability to ferment trehalose, and the ability produce indole, and 2) all quality control (QC) tests cited in Section 9.2.12 are performed acceptably and QC acceptance criteria are met. For example, laboratories may prefer to streak colonies that are submitted to confirmation on tryptic soy agar (TSA), instead of nutrient agar. The laboratory may not omit any quality control analyses.

- 9.2 General QC requirements**—Specific quality control (QC) requirements for Method 1605 are provided below. QA and QC criteria for facilities, personnel, and laboratory equipment, instrumentation, and supplies used in microbiological analyses must be followed according to *Standard Methods for the Examination of Water and Wastewater* (latest edition approved by EPA in 40 CFR Part 141, Reference 15.2) and the U.S. EPA *Manual for the Certification of Laboratories Analyzing Drinking Water, Fourth Edition* (March 1997) (Reference 15.5).

- 9.2.1 Initial demonstration of capability (IDC).** The laboratory shall demonstrate the ability to generate acceptable performance with this method by performing an IDC test before analyzing any field samples. The procedure for performing the IDC is described in Section 9.4. IDC tests must be accompanied by a dilution/rinse water blank(s) (Section 9.2.2), negative culture controls (Section 9.2.3), and media sterility checks (Section 9.2.6).

- 9.2.2 Dilution/rinse water blanks.** The laboratory shall analyze dilution/rinse water blanks to demonstrate freedom from contamination. The procedures for analysis of dilution/rinse water blanks are described in Section 9.5. At a minimum, dilution/rinse water blanks must be processed at the beginning and end of each filtration series to check for possible cross-contamination. A filtration series ends when 30 minutes or more elapse between sample filtrations. An additional dilution/rinse water blank is also required for every 20 samples, if more than 20 samples are processed during a filtration series.
- 9.2.3 Negative culture controls.** The laboratory shall analyze negative culture controls (Section 9.6) to ensure that ADA-V and the confirmation procedures are performing properly. Negative culture controls should be run whenever a new batch of media or reagents is used. On an ongoing basis, the laboratory must perform, at a minimum, one negative culture control per week during weeks the laboratory analyzes field samples.
- 9.2.4 Matrix spike/matrix spike duplicate (MS/MSD).** The laboratory shall analyze one set of MS/MSD samples when samples are first received from a finished drinking water source for which the laboratory has never before analyzed samples (Section 9.7). Subsequently, 5% of field samples from a given source must include an MS/MSD test. Additional MS/MSD tests are also recommended when drinking water treatment is adjusted or when other events take place, for example, when scrubbing or replacing lines. When possible, MS/MSD analyses should be conducted on the same day as ODC samples, using the same spiking procedure and volume.
- 9.2.4.1 Precision.** MS/MSD sample results should meet the precision criteria set forth in Section 12, Table 1.
- 9.2.4.2 Recovery.** QC acceptance criteria for *Aeromonas* recovery are not included in this method because the number of *Aeromonas* in the spike is unknown. However, each laboratory should control chart the mean number of *Aeromonas* per MS/MSD set (adjusted for background) and maintain a record of spike preparation procedures and spike volume. The laboratory should compare number of *Aeromonas* in MS/MSD samples to results of ODC samples (Section 9.2.5 and 9.8) spiked on the same day. This comparison should help the laboratory recognize when a matrix is interfering with method recovery. If the laboratory observes consistent ODC results from week to week, control charting the MS/MSD results by source may also help to recognize fluctuations in recovery from a particular source.
- 9.2.5 Ongoing demonstration of capability (ODC).** The laboratory shall demonstrate that the analytical system is in control on an ongoing basis through analysis of ODC samples (positive control/positive control duplicate, Section 9.8).
- 9.2.5.1 Frequency.** The laboratory shall analyze one set of ODC samples after every 20 field and MS samples or one set per week that samples are analyzed, whichever occurs more frequently. No more than one set of ODC samples is required per day, provided that the same equipment (i.e., incubators) are being used for all the samples.
- 9.2.5.2 Precision.** ODC sample results must meet the precision criteria set forth in Section 12, Table 1.
- 9.2.5.3 Recovery.** QC acceptance criteria for *Aeromonas* recovery are not included in this method because the initial spike dose for ODC samples is unknown.

As a result, each laboratory should control chart the mean number of *Aeromonas* per ODC sample set and maintain a record of spike preparation procedures and ODC spike volume. Maintaining this information will enable the laboratory to recognize when problems arise. **Example:** A laboratory that prepares spiking suspensions according to Section 9.3, spikes QC samples with 5 mL of dilution D2, and typically recovers approximately 50 *Aeromonas* per sample, and maintains a control chart of these counts. If the laboratory continues to prepare spiking suspensions the same way, but the number of *Aeromonas* counted declines noticeably (e.g. 20 *Aeromonas* per sample), then there may be a problem with the media, reagents, or the spiking suspension.

- 9.2.6 Media sterility checks.** The laboratory shall test media sterility by incubating one unit (tube or plate) from each batch of medium (ADA-V, nutrient agar slant, nutrient agar, streak plate, trehalose, and tryptone) at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours and observing for growth.
- 9.2.7 Analyst colony counting variability.** If the laboratory has two or more analysts, each are required to count target colonies on the same membrane from one ODC sample per month (Section 9.9), at a minimum.
- 9.2.8 Record maintenance.** The laboratory shall maintain records to define the quality of data that are generated. The laboratory shall maintain a record of the date and results of all QC sample analyses described in Section 9.2. A record of media sterility check, dilution/rinse water blank, analyst counting variability, IDC, ODC, and MS/MSD sample results must be maintained. Laboratories shall maintain reagent and material lot numbers along with samples analyzed using each of the lots. Laboratories shall also maintain media preparation records.
- 9.2.9 Performance studies.** The laboratory should periodically analyze external QC samples, such as performance evaluation (PE) samples, when available. The laboratory should also participate in available interlaboratory performance studies conducted by local, state, and federal agencies or commercial organizations. The laboratory should review results, correct unsatisfactory performance, and record corrective actions.
- 9.2.10 Autoclave sterilization verification.** At a minimum, the laboratory shall verify autoclave sterilization according to the procedure in Section 9.10 on a monthly basis.
- 9.2.11 Culture maintenance.** The laboratory should use 24 ± 2 hour-old nutrient agar slant cultures for preparation of IDC, ODC, and MS/MSD spiking suspension dilutions. The laboratory should use 22 to 72 hour-old nutrient agar slant cultures to inoculate ADA-V streak plates for analysis of negative culture controls. With regard to the preparation of subcultures, it is recommended that a maximum of three passages be prepared to help avoid contamination. After three passages, start a new subculture from the frozen stock.
- 9.2.12 Method modification.**
- 9.2.12.1 Membrane filtration.** Because recovery criteria are not available for this method, laboratories are not permitted to modify the membrane filtration procedures (Section 10.1 through Section 10.10.) or ADA-V media.
- 9.2.12.2 Confirmation procedures.** The confirmation procedures in Section 10.11 may be modified, provided that the laboratory demonstrates the ability to generate acceptable performance by performing an IDC test (Section 9.2.1)

and the appropriate negative culture control test(s) (Section 9.2.3) before analyzing any field samples using the modified confirmation. 100% of the colonies submitted to confirmation from IDC and negative culture control samples must give the appropriate confirmation response. These tests must be accompanied by a dilution/rinse water blank(s) (Section 9.2.2) and media sterility checks (Section 9.2.6).

9.3 Preparation of *Aeromonas* spiking suspension for use in spiking IDC, ODC, and MS/MSD samples—This dilution scheme is adapted from *Standard Methods for the Examination of Water and Wastewater, 19th Edition*, Section 9020 B (Reference 15.9). This entire process should be performed quickly to avoid loss of viable organisms. See Section 16, Flowchart 1, for an example of this dilution scheme. *Please note: Provided that all QC acceptance criteria are met and the recommended target range of 20 - 60 CFU per plate are typically observed, laboratories may prepare QC spiking suspensions using commercial products or other procedures such as growing bacteria in a broth, measuring optical density, and spiking each test sample with an equivalent volume.*

- 9.3.1** Inoculate *Aeromonas hydrophila* (ATCC #7966) onto the entire surface of several nutrient agar slants with a slope approximately 6.3 cm long in a 125 × 16 mm screw-cap tube. Incubate for 24 ± 2 hours at 35°C ± 0.5°C.
- 9.3.2** From the slant that has the best growth, prepare serial dilutions using four dilution bottles with 99 mL of sterile buffered dilution water (A, B, C and D below in Sections 9.3.3 and 9.3.4) and one dilution bottle containing 90-mL of sterile buffered dilution water (D2 below in Section 9.3.5).
- 9.3.3** Pipette 1 mL of buffered dilution water from bottle “A” to one of the slants. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipette, being careful not to tear the agar. Pipette the suspension back into dilution bottle “A.” Repeat this procedure a second time to remove any remaining growth on the agar slant, without disturbing the agar.
- 9.3.4** Make serial dilutions as follows:
- 9.3.4.1** Shake bottle “A” vigorously and pipette 1 mL to bottle “B”
 - 9.3.4.2** Shake bottle “B” vigorously and pipette 1 mL to bottle “C”
 - 9.3.4.3** Shake bottle “C” vigorously and pipette 1 mL to bottle “D”
 - 9.3.4.4** Shake bottle “D” vigorously and pipette 10 mL to bottle “D2”; this should result in a final dilution of approximately 10 CFU / mL.
- 9.3.5** Filter 1- to 5-mL portions in triplicate from bottles “D” and “D2” according to the procedure in Section 10 to determine the number of CFU in the dilutions. The recommended target dilution and spike volume is one that produces 20 to 60 colonies per ADA-V plate. (It may be difficult to count plates with more than 60 colonies due to crowding.) Dilutions should be stored at 1°C to 5°C and may be used throughout the day they are prepared. However, it should be noted that the QC acceptance criteria were established using dilutions that were prepared immediately prior to spiking samples.
- 9.3.6** Analysts may practice the dilution scheme by placing filters on nutrient agar plates instead of ADA-V plates. After a growth pattern is determined and the analyst can accurately determine the target concentrations, dilutions from Section 9.3.5 may be used for spiking IDC, ODC, and MS/MSD samples. However, multiple dilutions

should be analyzed in replicate when new cultures are received from an outside source to ensure that the analyst can accurately spike target concentrations.

Note: *If it is more convenient for your laboratory, an acceptable alternative to the dilution scheme presented in Section 9.3, is to pipette 11 mL of dilution D into a dilution bottle D2, which contains 99 mL of dilution water. There should be approximately 10^{10} Aeromonas hydrophila CFU per slant. Therefore, dilution bottles “A” through “D2” should contain approximately 10^{10} , 10^8 , 10^6 , 10^4 , and 10^3 CFU per dilution bottle, respectively. Depending on the growing conditions, these numbers may vary. As a result, until experience has been gained, more dilutions may need to be filtered to determine the appropriate dilution.*

9.4 Initial demonstration of capability (IDC)—The IDC test is performed to demonstrate acceptable performance with the method prior to analysis of field samples. IDC tests must be accompanied by a dilution/rinse water blank(s) (Section 9.2.2), negative culture controls (Section 9.2.3), and media sterility checks (Section 9.2.6).

9.4.1 Prepare an *Aeromonas* QC spiking suspension according to the procedure in Section 9.3.1 through 9.3.4.

9.4.2 For each of the four IDC test samples, spike enough volume of the appropriate dilution into 500 mL of sterile reagent water to obtain the recommended target range of 20–60 CFU per filter. (It may be difficult to count plates with more than 60 colonies due to crowding.) Filter immediately after spiking.

9.4.3 Process IDC test samples according to the procedure in Section 10.1 through 10.10 and record the number of presumptive positives for each sample. Submit 2 colonies per IDC test sample to the confirmation procedures in Section 10.11.

9.4.4 Using all four IDC sample results, compute the relative standard deviation (RSD) of *Aeromonas* CFU per 100 mL. (See glossary for definition of RSD.) Compare the RSD with the corresponding limits for IDC (Section 12). If the RSD meets the acceptance criteria, the system performance is acceptable and analysis of samples may begin. If the RSD falls outside the range, system performance is unacceptable. In this event, identify and correct the problem and repeat the test.

9.5 Dilution/rinse water blanks—On an ongoing basis, dilution/rinse water blanks must be processed at the beginning and end of each filtration series to check for possible cross-contamination. A filtration series ends when 30 minutes or more elapse between sample filtrations. An additional dilution/rinse water blank is also required for every 20 samples, if more than 20 samples are processed during a filtration series. For example, if a laboratory plans to run 30 samples during a filtration series, a dilution/rinse water blank should be processed at the beginning, middle, and end of the filtration series.

9.5.1 Process 100-mL dilution/rinse water blanks according to the procedures in Section 10, as appropriate.

9.5.2 No growth should appear in dilution/rinse water blanks. If growth appears, prepare new dilution/rinse water and reanalyze a 100-mL dilution/rinse water blank. If colonies are present after analyzing the new dilution/rinse water, assess laboratory technique and reagents. If growth in dilution/rinse water blank(s) is presumptively positive, all associated sample results should be discarded and sources re-sampled immediately.

- 9.6 Negative culture controls**— Negative controls should be run whenever a new batch of medium or reagents is used. On an ongoing basis, the laboratory must perform, at a minimum, one negative culture control per week during weeks the laboratory analyzes field samples. Negative culture controls serve two purposes: to ensure the laboratories are familiar with the color and morphology of non-*Aeromonas* bacteria on ADA-V and to ensure that confirmation test results are appropriate. *E. coli* is (ATCC #25922) the negative culture control for oxidase, *Pseudomonas aeruginosa* (ATCC #27853) is the negative culture control for trehalose fermentation, and *Bacillus cereus* (ATCC #11778) is the negative culture control for indole.
- 9.6.1** Using pure cultures obtained from a qualified outside source (Sections 7.7 and 7.8), inoculate negative culture controls onto nutrient agar slants and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. Alternatively, nutrient agar slants may be inoculated up to 72 hours in advance. If nutrient agar slants will be incubated for more than 24 ± 2 hours, consider incubation at room temperature to ensure that the slants do not dry out prior to use.
- 9.6.2** For each negative culture control, place a membrane filter on an ADA-V plate, streak onto the filter, taking care not to break the filter, and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. Streaking on a filter will give the laboratory a more realistic example of the appearance of these organisms in field samples. Although not recommended, laboratories may streak directly onto the ADA-V (without the filter).
- 9.6.3** For each ADA-V negative culture control plate, pick a single colony, streak the colony onto a plate of nutrient agar medium (Section 7.9), and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ overnight to obtain isolated colonies. *Please note: Bacillus cereus typically grows only at the point of inoculation on ADA-V or not at all. If Bacillus cereus did not grow on the ADA-V plate, inoculate the streak plate from the nutrient agar slant that was originally used to inoculate the ADA-V plate.*
- 9.6.4** Negative culture control confirmation procedures
- 9.6.4.1** Oxidase negative culture control—From the streak plate, submit a single *E. coli* colony to the oxidase confirmation procedure described in Section 10.11.
- 9.6.4.2** Trehalose negative culture control—From the streak plate, submit a single *Pseudomonas aeruginosa* colony to the trehalose confirmation procedure described in Section 10.11.
- 9.6.4.3** Indole negative culture control—From the streak plate, submit a single *Bacillus cereus* colony to the indole confirmation procedure described in Section 10.11.
- 9.6.5** If any of the negative culture controls result in a positive confirmation, prepare, check and/or replace the associated media, reagents, and/or respective control organism and reanalyze the appropriate negative culture control(s). All presumptively positive colonies that have been archived from field samples (10 per sample) should be confirmed using media/reagents that exhibit the appropriate negative culture control response.
- 9.7 Matrix spike/matrix spike duplicate (MS/MSD)**—The laboratory shall analyze MS/MSD samples when samples are first received from a finished drinking water source for which the laboratory has never before analyzed samples. Subsequently, 5% of field samples from a given source must include an MS/MSD test. Additional MS/MSD tests are also recommended when drinking water treatment is adjusted or when other events take place, for example, when scrubbing or replacing lines.

- 9.7.1** Prepare an *Aeromonas* QC spiking suspension according to the procedure in Sections 9.3.1 through 9.3.4.
- 9.7.2** For each of the 500-mL MS and MSD test samples, spike enough volume of the appropriate dilution to obtain the recommended target range of 20-60 CFU per filter. (It may be difficult to count plates with more than 60 colonies due to crowding.) Filter immediately after spiking.
- 9.7.3** Process MS/MSD test samples and an unspiked finished drinking water sample according to the procedure in Section 10.1 through 10.10 and record the number of presumptive positives for each sample. (If the filter clogs during filtration, follow the instructions in Section 10, making sure to filter the same volume for both the MS and MSD. The same QC acceptance criteria apply.) Submit 10 colonies per IDC test sample to the confirmation procedures in Section 10.11.

Note: *If results exceed the optimum range because of “background” target colonies (as indicated by the results of the unspiked matrix sample), the MS/MSD should be repeated and a smaller volume of sample, for example 200-mL, should be spiked.*

- 9.7.4** For the MS and MSD test samples, calculate the number of confirmed *Aeromonas* CFU per 100 mL according to Section 11 and adjust based on any background *Aeromonas* observed in the unspiked sample.
- 9.7.5** Calculate the relative percent difference (RPD) using the following equation:

$$RPD = 100 \frac{|X_{MS} - X_{MSD}|}{X_{mean}}$$

where

RPD is the relative percent difference

X_{MS} is the number of confirmed *Aeromonas* per 100 mL in the MS sample (minus the count of any background *Aeromonas* colonies observed in the unspiked finished water sample)

X_{MSD} is the number of confirmed *Aeromonas* per 100 mL in the MSD sample (minus the count of any background *Aeromonas* colonies observed in the unspiked finished water sample)

X_{mean} is the mean number of confirmed *Aeromonas* per 100 mL in the MS and MSD

- 9.7.6** Compare the RPD with the corresponding limits in Table 1 in Section 12. If the RPD meets the acceptance criteria, the system performance is acceptable and analysis of finished water samples from this source may continue. If the MS/MSD results are unacceptable and the ODC sample results associated with this batch of samples are acceptable, a matrix interference may be causing the poor results. If the MS/MSD results are unacceptable, all associated field data should be flagged.
- 9.8 Ongoing demonstration of capability (ODC)**—The laboratory shall demonstrate that the analytical system is in control on an ongoing basis through analysis of ODC samples (positive control/positive control duplicate). The laboratory shall analyze one set of ODC samples after

every 20 field and MS samples or one set per week that samples are analyzed, whichever occurs more frequently.

- 9.8.1** Prepare an *Aeromonas* QC spiking suspension according to the procedure in Section 9.3.1 through 9.3.4.
- 9.8.2** For each of the 500-mL positive control (PC) and positive control duplicate (PC/PCD) test samples, spike enough volume of the appropriate dilution into 500 mL of sterile reagent water to obtain the recommended target range of 20-60 CFU per filter. (It may be difficult to count plates with more than 60 colonies due to crowding.) Filter immediately after spiking.
- 9.8.3** Process PC/PCD test samples according to the procedure in Section 10.1 through 10.10 and record the number of presumptive positives for each sample. Submit 2 colonies per PC/PCD test sample to the confirmation procedures in Section 10.11.
- 9.8.4** Calculate the relative percent difference (RPD) using the following equation:

$$RPD = 100 \frac{|X_{PC} - X_{PCD}|}{X_{mean}}$$

where

RPD is the relative percent difference

X_{PC} is the number of confirmed *Aeromonas* per 100 mL in the PC sample

X_{PCD} is the number of confirmed *Aeromonas* per 100 mL in the PCD sample

X_{mean} is the mean number of confirmed *Aeromonas* per 100 mL in the PC and PCD samples

- 9.8.5** Compare the RPD with the corresponding limits in Table 1 in Section 12. If the RPD meets the acceptance criteria, the system performance is acceptable and analysis of samples may continue. If RPD falls outside the range, system performance is unacceptable. Identify and correct the problem and perform another ODC test before continuing with the analysis of field samples.
- 9.8.6** As part of the QA program for the laboratory, method precision for ODC samples should be charted and records retained.
- 9.9** **Analyst colony counting variability**—If the laboratory has two or more analysts, each are required to count target colonies on the same membrane from one positive field sample per month. Compare each analyst's count of the target colonies. Counts should fall within 10% between analysts. If counts fail to fall within 10% of each other, analysts should perform additional sets of counts, until the number of target colonies counted fall within 10% between analysts for at least three consecutive samples. If there are no positive samples, an MS, MSD, or ODC sample can be used for this determination (MS or MSD are preferable to ODC samples, since they may have other background growth).
- 9.10** **Autoclave sterilization verification**—Verify autoclave sterilization monthly by placing *Bacillus stearothermophilus* spore suspensions or strips inside glassware. Sterilize at 121°C for 15 minutes. Place in trypticase soy broth tubes and incubate at 55°C for 48 hours. Check for growth to verify that sterilization was adequate. If sterilization was inadequate, determine appropriate time for autoclave sterilization. Filter sterilization may be used provided that these same QC steps are instituted for the filtrate.

10.0 Procedure

- 10.1 The membrane filter (MF) procedure with ampicillin-dextrin agar with vancomycin (ADA-V) is used to enumerate *Aeromonas* in finished waters.
 - 10.2 Label each petri dish with sample identification, preparation date, and analysis start date/time.
 - 10.3 Use a sterile MF unit assembly (Section 6.18.3) at the beginning of each filtration series. The laboratory must sanitize each MF unit between filtrations by using a UV sanitizer, flowing steam, or boiling water for 2 min. A filtration series ends when 30 minutes or more elapse between sample filtrations.
 - 10.4 Sterilize forceps with alcohol. Flame off excess alcohol. Using sterile forceps, place the MF (grid side up) over the sterilized funnel. Carefully place the top half of the filtration unit over the funnel and lock it in place.
 - 10.5 Shake the sample bottle vigorously approximately 25 times to distribute the bacteria uniformly. Using aseptic technique, transfer one, 500-mL aliquot of sample to a single funnel. Use a graduated cylinder with a “to deliver” tolerance of approximately 2.5%.
-

Note: *Laboratories must filter the entire 500-mL sample volume unless the filter clogs. If the filter clogs, a minimum of 100 mL of sample must be filtered, which may require multiple filtrations. If less than 500 mL are filtered and analyzed due to filter clogging, measure the residual, unfiltered volume to determine the volume filtered, and adjust the reporting limit accordingly.*

- 10.6 Filter each sample under partial vacuum through a sterile membrane filter. Rinse the funnel after each sample filtration by filtering three, 30-mL portions of sterile buffered dilution water, being sure to thoroughly rinse the sides of the funnel.
- 10.7 Upon completion of the final rinse, disengage the vacuum and remove the funnel.
- 10.8 Using sterile forceps, immediately remove the MF and place it grid-side-up on the ADA-V medium with a rolling motion to avoid trapping air under the filter. Reseat the membrane filter if bubbles occur. Place the inverted petri dishes in the $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ incubator within 30 minutes of preparation. Sterilize forceps and sanitize the MF unit between the analysis of each sample.
- 10.9 After 24 ± 2 hours of incubation at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$, count and record yellow colonies under magnification using a dissecting microscope.
- 10.10 Isolation of a yellow colony on ampicillin-dextrin agar with vancomycin (ADA-V) should be considered presumptively positive for *Aeromonas*.
- 10.11 **Confirmation**—All presumptive colonies, up to ten per sample, must be submitted to confirmation. In this method, any presumptive colony that is positive for oxidase (Section 10.11.2), ferments trehalose (Section 10.11.3), and produces indole (Section 10.11.4) is considered to be *Aeromonas*. If the result for any confirmation procedure is negative, no further confirmation steps are necessary. Slight variations in color and morphology may be present between different *Aeromonas* species grown on ADA-V medium. The colonies selected for confirmation should be representative of all yellow (presumptively positive) colony morphology types on ADA-V plate. For example, if 30 bright yellow colonies and 20 dull yellow colonies are observed, then 6 bright yellow and 4 dull yellow colonies should be submitted to confirmation.

Note: *It is important to record the number of colonies of each presumptively positive morphological type so that the final density of Aeromonas can be reported based on percent confirmation of each morphological type. Also, the laboratory may submit more than ten presumptively positive colonies to the confirmation step.*

- 10.11.1 Nutrient agar streak plate.** To confirm as *Aeromonas*, pick a colony and streak the colony onto a plate of nutrient agar medium (Section 7.9) and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ overnight to obtain isolated colonies.
- 10.11.2 Oxidase confirmation.** Apply a very small amount of a discreet colony from the nutrient agar to the oxidase dry slide using a wooden or plastic applicator. Do not use iron or other reactive wire because it may cause false positive reactions. Also, do not transfer any medium with the culture material, as this could lead to inconsistent results. A blue/purple color reaction within 10 seconds is considered a positive oxidase test. For commercially-prepared reagent, adhere to manufacturer's expiration date. Freshly-made solutions should be used within one week. *Please note: This method was validated using nutrient agar, if the oxidase reagent is to be dropped directly on colonies, use tryptic soy agar plates because nutrient agar plates give inconsistent results. The use of tryptic soy agar plates for streaking (Section 10.11.1) has not been validated and is considered a method modification and, as a result, the laboratory must demonstrate acceptable performance for the QC analyses described in Section 9.2.12.*
-

Note: *Timing of the color reaction is critical, as some Gram-positive bacteria may give false positives after 10 seconds. Also, it is important to put just a small amount of the colony on the oxidase dry slide or saturated pad, as too much bacteria can also cause a false positive oxidase test.*

- 10.11.3 Trehalose confirmation.** If the oxidase test is positive, then test for trehalose fermentation. Trehalose fermentation is determined by inoculating a tube containing 3-10 mL (depending on the size of the tube used - fill about half full) of 0.5% trehalose in purple broth base (Section 7.11) with a colony from the nutrient agar and incubating at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. A change in color of the medium from purple to yellow is considered a positive for trehalose fermentation.
- 10.11.4 Indole confirmation.** If the oxidase and trehalose tests are positive, then test for indole production. (If the laboratory prefers, the indole confirmation procedure may be started on the same day as the trehalose confirmation.) Indole production is determined by inoculating a tube containing 3-10 mL (depending on the size of the tube used - fill about half full) of tryptone broth (Section 7.12) with a colony from the nutrient agar and incubating at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. After incubation, add 0.2 to 0.3 mL (4 to 6 drops) of Kovac's test reagent (Section 7.13) to each tube, let stand for approximately 10 minutes and observe results. A pink to red color in the surface layer constitutes a positive indole test. The original color of the Kovac's reagent indicates a negative indole test. An orange color probably indicates the presence of skatole, a breakdown product of indole, and is considered a positive result.
- 10.11.5** If a colony is oxidase, trehalose, and indole positive, report as a confirmed *Aeromonas* and archive the colony for further identification.

Note: *If samples are to be archived for further analysis to determine species or hybridization group, from the nutrient agar plate (Section 10.11.1), inoculate a nutrient agar slant for short term use or shipment to another laboratory.*

11.0 Data Analysis and Calculations

11.1 See *Standard Methods for the Examination of Water and Wastewater* (Reference 15.2) for general counting rules. The density of *Aeromonas* determined by the membrane filter (MF) procedure is calculated by direct identification and enumeration of yellow colonies by a dissecting microscope (Section 6.19) followed by oxidase, trehalose, and indole confirmation. Bacterial density is recorded as presumptive *Aeromonas* colony forming units (CFU) per 100 mL of sample and confirmed *Aeromonas* CFU per 100 mL.

11.2 Counting colonies on ADA-V

11.2.1 Record the number of presumptive *Aeromonas* CFU/100mL. If there is more than one morphological type that is considered to be presumptively positive, record the number of presumptive positives for each morphological type, as well as the total number of presumptive positives.

11.2.2 If there are more than 200 colonies, including background colonies, report results as too numerous to count (TNTC) and resample. If the filter is TNTC with more background colonies than presumptive aeromonads, split the 500 mL resample between 3 or 4 filters in order to better differentiate the colony morphology types. If the filter is TNTC with mostly aeromonads, a minimum of three dilutions (e.g. 100 mL, 10 mL and 1 mL) should be analyzed.

11.2.3 If the colonies are not discrete and appear to be growing together, report results as confluent growth (CG) and resample.

11.3 Confirmation and calculation of *Aeromonas* density

11.3.1 In this method, any presumptive colony that is positive for oxidase (Section 10.11.2), ferments trehalose (Section 10.11.3), and produces indole (Section 10.11.4) is considered to be *Aeromonas*. For the final density of confirmed *Aeromonas*, adjust the initial, presumptive count based on the positive confirmation percentage for each presumptively positive morphological type and report as confirmed CFU per 100 mL.

11.3.2 Calculate the number of positive confirmations for *each* presumptively positive morphological type **from all filters of a given sample** using the following equation:

$$\left(\frac{\text{Number positively confirmed}}{\text{Number submitted to confirmation}} \times \text{Number of presumptive positives} \right) \times \frac{100}{\text{mL filtered}} = \text{Confirmed } Aeromonas / 100\text{mL}$$

11.3.3 Record the number of confirmed *Aeromonas* per 100 mL for each colony morphology.

11.3.4 Sum the number of confirmed *Aeromonas* per 100 mL for all presumptively positive colony types (Section 11.3.2) and report as the density of confirmed *Aeromonas* per 100 mL.

11.3.5 Example 1: In this example, **500 mL** of sample was filtered and two different morphological types of presumptively positive colonies were observed.

Example 1

Morphological Description	Number of presumptively positive colonies per volume filtered	Number submitted to confirmation steps	Number positively confirmed	Number of confirmed <i>Aeromonas</i> per 100 mL
Type A: Bright yellow, round, opaque	30	6	6	6
Type B: Dull yellow, oval, translucent	20	4	3	3
Total number of confirmed <i>Aeromonas</i> per sample:				9 per 100 mL

$$\left(\frac{6}{6} \times 30\right) \times \frac{100}{500} = 6 \text{ Confirmed Type A } *Aeromonas* / 100\text{mL}$$

$$\left(\frac{3}{4} \times 20\right) \times \frac{100}{500} = 3 \text{ Confirmed Type B } *Aeromonas* / 100\text{mL}$$

Example 1 results in 9 confirmed *Aeromonas* / 100 mL.

11.3.6 Example 2: In this example, **200 mL** of sample was filtered and two different morphological types of presumptively positive colonies were observed.

Example 2

Morphological Description	Number of presumptively positive colonies per volume filtered	Number submitted to confirmation steps	Number positively confirmed	Number of confirmed <i>Aeromonas</i> per 100 mL
Type A: Dull yellow, round, opaque	40	5	5	20
Type B: Dull yellow, round, translucent	40	5	3	12
Total number of confirmed <i>Aeromonas</i> per sample:				32 per 100 mL

$$\left(\frac{5}{5} \times 40\right) \times \frac{100}{200} = 20 \text{ Confirmed Type A } *Aeromonas* / 100\text{mL}$$

$$\left(\frac{3}{5} \times 40\right) \times \frac{100}{200} = 12 \text{ Confirmed Type B } *Aeromonas* / 100\text{mL}$$

Example 2 results in 32 confirmed *Aeromonas* / 100 mL.

11.3.7 If there were no presumptively positive colonies or if none of the presumptive colonies are confirmed, then report the results as less than the detection limit (DL) in CFU per 100 mL based on sample volume filtered. If less than 500 mL are filtered, then adjust the reporting limit per 100 mL accordingly. The DL may be calculated as follows:

$$\text{DL per 100 mL} = 100 / \text{volume filtered CFU per 100mL}$$

11.3.7.1 Example 3: If 500 mL of sample was filtered and there were no confirmed colonies, then report as <0.2 CFU/100 mL.

11.3.7.2 Example 4: If 100 mL of sample was filtered and there were no confirmed colonies, then report as <1.0 CFU/100 mL.

12.0 Method Performance

12.1 Specificity of media

12.1.1 Please refer to Section 16, Table 2, for results of *Aeromonas* growth after 24 hours on ADA at 30°C and 35°C and ADA-V at 35°C .

12.1.2 ADA-V was able to support the growth of the *Aeromonas* species (*hydrophila*, *caviae*, and *veronii/sobria*) most often associated with human disease.

12.1.3 Efforts continue to identify colonies which give a presumptive positive on the ADA-V media but do not confirm.

12.2 The QC acceptance criteria listed in Table 1, below are based on data generated through the interlaboratory validation of Method 1605 involving 11 laboratories and 11 finished drinking water matrices. Detailed method QC procedures applicable to these criteria are discussed in Section 9.

Table 1. QC Acceptance Criteria for Method 1605

QC specification	Maximum acceptable precision
Initial demonstration of capability (IDC): This test will require the analysis of 4 spiked reagent water samples	RSD = 22%
Ongoing demonstration of capability (ODC): This test will require the analysis of 2 spiked reagent water samples	RPD = 37%
Matrix spike/matrix spike duplicate (MS/MSD) precision: This test will require the analysis of 2 spiked finished water (matrix) samples	RPD = 48%

13.0 Pollution Prevention

13.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

13.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

14.0 Waste Management

14.1 It is the laboratory’s responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

- 14.2** Samples, reference materials, and equipment known or suspected of having bacterial contamination from this work must be sterilized prior to disposal.
- 14.3** For further information on waste management, consult “The Waste Management Manual for Laboratory Personnel” and “Less is Better: Laboratory Chemical Management for Waste Reduction,” both of which are available from the American Chemical Society’s Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

15.0 References

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- 15.2** Standard Methods for the Examination of Water and Wastewater. 1998. 20th Edition. Eds. A.D. Eaton, L.S. Clesceri, and A. Greenberg. American Public Health Association, American Water Works Association, and Water Environment Federation. American Public Health Association, Washington, D.C., publisher.
- 15.3** Demarta, A., M. Tonolla, A. Caminada, N. Ruggeri, and R. Peduzzi. 1999. Signature region within the 16S rDNA sequences of *Aeromonas popoffii*. *FEMS Microbiol. Letts*. 172:239-246.
- 15.4** Annual Book of ASTM Standards, Vol. 11.01. American Society for Testing and Materials. Philadelphia, PA 19103.
- 15.5** Manual for the Certification of Laboratories Analyzing Drinking Water. 1997. 4th Edition. EPA-815-B-97-001. Office of Ground Water and Drinking Water. U.S. EPA.
- 15.6** Moyer, N. P. 1996. Isolation and enumeration of aeromonads. *In: The Genus Aeromonas*. Eds. B. Austin, M. Altwegg, P. Gosling, and S. Joseph. John Wiley and Sons publisher, Chichester, U.K.
- 15.7** Reagent Chemicals, American Chemical Society Specifications. American Chemical Society, Washington, D.C.
- 15.8** Handfield, M., P. Simard, and R. Letarte. 1996. Differential media for quantitative recovery of waterborne *Aeromonas hydrophila*. *Applied Environmental Microbiology* 62:3544-3547.
- 15.9** Standard Methods for the Examination of Water and Wastewater. 1995. 19th Edition. Eds. A.D. Eaton, L.S. Clesceri, and A. Greenberg. American Public Health Association, American Water Works Association, and Water Environment Federation. American Public Health Association, Washington, D.C., publisher.
- 15.10** Janda, J.M. and S.L. Abbott. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. *Journal of Clinical Infectious diseases*. 27:332-344.

16.0 Tables and Flowcharts

Table 2. Growth of *Aeromonas* cultures in 24 hours on ADA at 30°C and 35°C and ADA-V at 35°C

Collection #	Hybridization group	<i>Aeromonas</i> species	ADA at 30°C	ADA at 35°C	ADA-V at 35°C
ATCC 7966	Group 1	<i>hydrophila</i>	+	+	+
ATCC 35654	Group 1	<i>hydrophila</i>	+	+	+
AMC 12723-W	Group 1	<i>hydrophila</i>	+	+	+
ATCC 51108	Group 2	<i>bestiarum</i>	+	+	+
AMC 14228-V	Group 2	<i>bestiarum</i>	+	+	+
ATCC 33658 ¹	Group 3	<i>salmonicida/salmonicida</i>	-	-	NA
AMC 15228-V	Group 3	<i>salmonicida</i>	+	+	+
ATCC 15468	Group 4	<i>caviae</i>	+	+	+
MML 1685-E	Group 4	<i>caviae</i>	+	+	+
ATCC 33907	Group 5	<i>media</i>	-	-	NA
AMC Leftwich	Group 5	<i>media</i>	-	-	NA
ATCC 23309 ¹	Group 6	<i>eucrenophila</i>	+	-	NA
ATCC 35993	Group 7	<i>sobria</i>	+	+	+
Muldoon SMHC	Group 7	<i>sobria</i>	+	+	+
ATCC 9071	Group 8	<i>veronii/sobria</i>	+	+	+
AMC 1123-W	Group 8	<i>veronii/sobria</i>	+	+	+
ATCC 43700	Group 12	<i>schubertii</i>	+ ²	+ ⁵	+ ⁵
AMC 1108-W	Group 12	<i>schubertii</i>	+	-	NA
ATCC 49657 ³	unknown	<i>trota</i>	-	-	NA
NMRI 206	unknown	<i>trota</i>	-	-	NA
ATCC 51208	unknown	<i>allosaccharophila</i>	+	+	+
ATCC 49568	Group 9	<i>jandaei</i>	+	+	+
AS 14	Group 9	<i>jandaei</i>	+	+	+
ATCC 35622	Group 10	<i>veronii/veronii</i>	+	+	+
WR 4659	Group 10	<i>veronii/veronii</i>	+	+	+
CECT 4342	Group 11	<i>encheleia</i>	+	-	NA
LMG 17541 ⁴	unknown	<i>popoffii</i>	+	+	+
AMC (ATCC 35941)	unknown	ornithine positive	-	-	NA
AMC (ATCC 43946)	unknown	Group 501	+	+	+
CDC 0434-84	Group 3	Motile Group 3	+	+	+

(1) Respective *Aeromonas* cultures grew on ADA medium when streaked, but not when filtered.

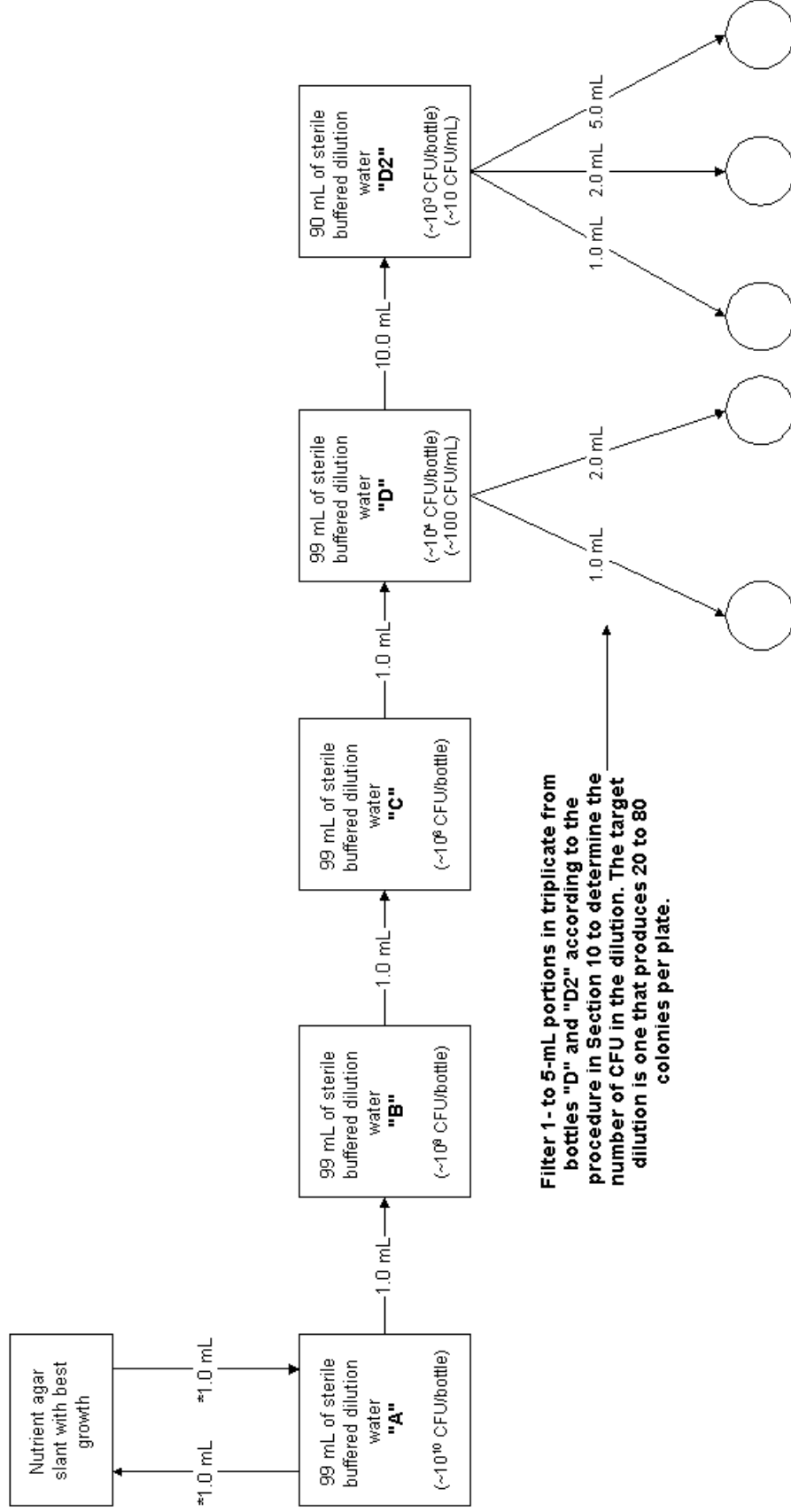
- (2) Respective *Aeromonas* cultures grew when streaked on ADA medium at 30°C, however filtration was not performed with these cultures.
- (3) Respective *Aeromonas* cultures did not grow on ADA medium when streaked.
- (4) Respective *Aeromonas* cultures grew poorly on ADA medium at both temperatures and on ADA-V at 35°C. The same pattern of poor growth was also observed on non-selective media.
- (5) Respective *Aeromonas* cultures grew poorly on ADA and ADA-V medium at 35°C. The same pattern of poor growth was also observed on non-selective media.

Results:

- NA Based on ADA results, it was assumed that the culture would not grow on ADA-V at 35°C.
- + positive growth
- No growth

ATCC = American Type Culture Collection, Manassas, VA Other cultures were obtained from Amy Carnahan, University of Maryland. Serial dilutions representing approximately 10-200 CFU were filtered and the membrane placed on ADA or ADA-V medium as described in Section 10. Additional membranes representing the same dilution for each of the respective cultures were placed on brain heart infusion agar as a control.

Flowchart 1. Example Dilution And Inoculation Scheme For Determining Organism Density (Section 9.3)



Filter 1- to 5-mL portions in triplicate from bottles "D" and "D2" according to the procedure in Section 10 to determine the number of CFU in the dilution. The target dilution is one that produces 20 to 80 colonies per plate.

***Pipet 1 mL of buffered dilution water from bottle "A" to the nutrient agar slant with the best growth. Emulsify the growth on the slant by gently rubbing the bacterial film with the pipet, being careful not to tear the agar, and pipet the solution back into bottle "A". Repeat this procedure a second time to remove any growth remaining on the agar slant.**

17.0 Glossary

17.1 Symbols

°C	degrees Celsius
μm	micrometer
±	plus or minus
<	less than
%	percent

17.2 Alphabetical characters and acronyms

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CFR	Code of Federal Regulations
CG	confluent growth
EDTA	ethylenediaminetetraacetic acid
g	gram
L	liter
mg	milligram
mL	milliliter
mm	millimeter
Na ₂ S ₂ O ₃	sodium thiosulfate
NIST	National Institute of Standards and Technology
OSHA	Occupational Safety and Health Administration
psi	pounds per square inch
RSD	relative standard deviation
QC	quality control
TNTC	too numerous to count
USEPA	United States Environmental Protection Agency
X	“times”

17.3 Definitions

Confirmed colonies—Presumptively positive colonies that test positive for oxidase, ferment trehalose, and produce indole

Dilution/rinse water blank—A 100-mL aliquot of dilution/rinse water that is treated exactly as a sample and carried through all portions of the procedure until determined to be negative or positive. The Dilution/rinse water blank is used to determine if the sample has become contaminated by the introduction of a foreign microorganism through poor technique.

Initial demonstration of capability (IDC)—The IDC test is performed to demonstrate acceptable performance with the method prior to analysis of field samples.

Must—This action, activity, or procedural step is required.

Negative culture control—A non-*Aeromonas* bacteria processed to ensure the laboratories are familiar with the color and morphology of non-*Aeromonas* bacteria on ADA-V and to ensure that confirmation test results are appropriate.

Ongoing demonstration of capability (ODC)—The laboratory shall demonstrate that the analytical system is in control on an ongoing basis through analysis of ODC samples (positive control/positive control duplicate).

Positive control—A 500-mL reagent water spiked with 20 - 80 CFU of *Aeromonas*. The positive control is analyzed exactly like a sample. Its purpose is to ensure that the results produced by the laboratory remain within the limits specified in this method for precision and recovery.

Presumptive positive colonies—Colonies that are yellow on ADA-V.

Relative Standard Deviation (RSD)—The standard deviation times 100 divided by the mean.

Selective medium—A culture medium designed to suppress the growth of unwanted microorganisms and encourage the growth of the target bacteria.

Should—This action, activity, or procedural step is suggested but not required.