

# Standard Methods for the Examination of Water and Wastewater

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## Part 9000

## MICROBIOLOGICAL EXAMINATION

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### 9010 INTRODUCTION\*#(1)

The following sections describe procedures for making microbiological examinations of water samples to determine sanitary quality. The methods are intended to indicate the degree of contamination with wastes. They are the best techniques currently available; however, their limitations must be understood thoroughly.

Tests for detection and enumeration of indicator organisms, rather than of pathogens, are used. The coliform group of bacteria, as herein defined, is the principal indicator of suitability of a water for domestic, industrial, or other uses. The cultural reactions and characteristics of this group of bacteria have been studied extensively.

Experience has established the significance of coliform group density as a criterion of the degree of pollution and thus of sanitary quality. The significance of the tests and the interpretation of results are well authenticated and have been used as a basis for standards of bacteriological quality of water supplies.

The membrane filter technique, which involves a direct plating for detection and estimation of coliform densities, is as effective as the multiple-tube fermentation test for detecting bacteria of the coliform group. Modification of procedural details, particularly of the culture medium, has made the results comparable with those given by the multiple-tube fermentation procedure. Although there are limitations in the application of the membrane filter technique, it is equivalent when used with strict adherence to these limitations and to the specified technical details. Thus, two standard methods are presented for the detection and enumeration of bacteria of the coliform group.

It is customary to report results of the coliform test by the multiple-tube fermentation procedure as a Most Probable Number (MPN) index. This is an index of the number of coliform bacteria that, more probably than any other number, would give the results shown by the laboratory examination; it is not an actual enumeration. By contrast, direct plating methods such as the membrane filter procedure permit a direct count of coliform colonies. In both procedures coliform density is reported conventionally as the MPN or membrane filter count per 100 mL. Use of either procedure permits appraising the sanitary quality of water and the effectiveness of treatment processes. Because it is not necessary to provide a quantitative assessment of coliform bacteria for all samples, a qualitative, presence-absence test is included.

Fecal streptococci and enterococci also are indicators of fecal pollution and methods for their detection and enumeration are given. A multiple-tube dilution and a membrane filter procedure are included.

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Methods for the differentiation of the coliform group are included. Such differentiation generally is considered of limited value in assessing drinking water quality because the presence of any coliform bacteria renders the water potentially unsatisfactory and unsafe. Speciation may provide information on colonization of a distribution system and further confirm the validity of coliform results.

Coliform group bacteria present in the gut and feces of warm-blooded animals generally include organisms capable of producing gas from lactose in a suitable culture medium at  $44.5 \pm 0.2^\circ\text{C}$ . Inasmuch as coliform organisms from other sources often cannot produce gas under these conditions, this criterion is used to define the fecal component of the coliform group. Both the multiple-tube dilution technique and the membrane filter procedure have been modified to incorporate incubation in confirmatory tests at  $44.5^\circ\text{C}$  to provide estimates of the density of fecal organisms, as defined. Procedures for fecal coliforms and *Escherichia coli* include a 24-h multiple-tube test using A-1 medium, a 7-h rapid method, and chromogenic substrate coliform tests. This differentiation yields valuable information concerning the possible source of pollution in water, and especially its remoteness, because the *nonfecal* members of the coliform group may be expected to survive longer than the *fecal* members in the unfavorable environment provided by the water.

The heterotrophic plate count may be determined by pour plate, spread plate, or membrane filter method. It provides an approximate enumeration of total numbers of viable bacteria that may yield useful information about water quality and may provide supporting data on the significance of coliform test results. The heterotrophic plate count is useful in judging the efficiency of various treatment processes and may have significant application as an in-plant control test. It also is valuable for checking quality of finished water in a distribution system as an indicator of microbial regrowth and sediment buildup in slow-flow sections and dead ends.

Experience in the shipment of un-iced samples by mail indicates that noticeable changes may occur in type or numbers of bacteria during such shipment for even limited periods of time. Therefore, refrigeration during transportation is recommended to minimize changes, particularly when ambient air temperature exceeds  $13^\circ\text{C}$ .

Procedures for the isolation of certain pathogenic bacteria and protozoa are presented. These procedures are tedious and complicated and are not recommended for routine use. Likewise, tentative procedures for enteric viruses are included but their routine use is not advocated.

Examination of routine bacteriological samples cannot be regarded as providing complete information concerning water quality. Always consider bacteriological results in the light of information available concerning the sanitary conditions surrounding the sample source. For a water supply, precise evaluation of quality can be made only when the results of laboratory examinations are interpreted in the light of sanitary survey data. Consider inadequate the results of the examination of a single sample from a given source. When possible, base evaluation of water quality on the examination of a series of samples collected over a known and protracted period of time.

Pollution problems of tidal estuaries and other bodies of saline water have focused attention

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on necessary modification of existing bacteriological techniques so that they may be used effectively. In the following sections, applications of specific techniques to saline water are not discussed because the methods used for fresh waters generally can be used satisfactorily with saline waters.

Methods for examination of the waters of swimming pools and other bathing places are included. The standard procedures for the plate count, fecal coliforms, and fecal streptococci are identical with those used for other waters. Procedures for *Staphylococcus* and *Pseudomonas aeruginosa*, organisms commonly associated with the upper respiratory tract or the skin, are included.

Procedures for aquatic fungi and actinomycetes are included.

Sections on rapid methods for coliform testing and on the recovery of stressed organisms are included. Because of increased interest and concern with analytical quality control, this section continues to be expanded.

The bacteriological methods in Part 9000, developed primarily to permit prompt and rapid examination of water samples, have been considered frequently to apply only to routine examinations. However, these same methods are basic to, and equally valuable in, research investigations in sanitary bacteriology and water treatment. Similarly, all techniques should be the subject of investigations to establish their specificity, improve their procedural details, and expand their application to the measurement of the sanitary quality of water supplies or polluted waters.

### 9020 QUALITY ASSURANCE/QUALITY CONTROL\*(2)

#### 9020 A. Introduction

##### 1. General Considerations

The growing emphasis on microorganisms in water quality standards and enforcement activities and their continuing role in research, process control, and compliance monitoring require the establishment and effective operation of a quality assurance (QA) program to substantiate the validity of analytical data.

A laboratory quality assurance program is the integration of intralaboratory and interlaboratory quality control (QC), standardization, and management practices into a formal, documented program with clearly defined responsibilities and duties to ensure that the data are of the type, quality, and quantity required.

The program must be practical and require only a reasonable amount of time or it will be bypassed. Generally, about 15% of overall laboratory time should be spent on different aspects of a quality assurance program. However, more time may be needed for more important analytical data, e.g., data for enforcement actions. When properly administered, a balanced, conscientiously

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applied QA program will optimize data quality without adversely affecting laboratory productivity.

Because microbiological analyses measure constantly changing living organisms, they are inherently variable. Some quality control tools used by chemists, such as reference standards, instrument calibration, and quality control charts, may not be available to the microbiologist.

Because QA programs vary among laboratories as a result of differences in organizational mission, responsibilities, and objectives; laboratory size, capabilities, and facilities; and staff skills and training, this provides only general guidance. Each laboratory should determine the appropriate QA level for its purpose.

### 2. Guidelines for a Quality Assurance Program

Develop a QA program to meet the laboratory's specific needs and the planned use of the data. Emphasis on the use of data is particularly important where significant and costly decisions depend on analytical results. An effective QA program will confirm the quality of results and increase confidence in the data.

*a. Management responsibilities:* Management must recognize the need for quality assurance, commit monetary and personnel resources, assume a leadership role, and involve staff in development and operation of the QA program. Management should meet with the laboratory supervisor and staff to develop and maintain a comprehensive program and establish specific responsibility for management, supervisors, and analysts.

*b. Quality assurance officer:* In large laboratories, a QA officer has the authority and responsibility for application of the QA program. Ideally, this person should have a staff position reporting directly to upper management, not a line position. The QA officer should have a technical education, be acquainted with all aspects of laboratory work, and be familiar with statistical techniques for data evaluation. The QA officer is responsible for initiating the program, convincing staff of its value, and providing necessary information and training to the staff. Once the QA program is functioning, the coordinator conducts frequent (weekly to monthly) reviews with the laboratory supervisor and staff to determine the current status and accomplishments of the program and to identify and resolve problems. The QA officer also reports periodically to management to secure backing in actions necessary to correct problems that threaten data quality.

*c. Staff:* Laboratory and field staffs participate with management in planning the QA program, preparing standard operating procedures, and most importantly, implementing the QC program in their daily tasks of collecting samples, conducting analyses, performing quality control checks, and calculating and reporting results. Because the staffs are the first to see potential problems, they should identify them and work with the supervisor to correct and avoid them. It is critical to the success of the QA program that staff understand and actively support it.

### 3. Quality Assurance Program Objectives

The objectives of a QA program include providing data of known quality, ensuring a high quality of laboratory performance, maintaining continuing assessment of laboratory operations,

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identifying weaknesses in laboratory operations, detecting training needs, and improving documentation and recordkeeping.

### 4. Elements of a Quality Assurance Program

Each laboratory should develop and implement a written QA plan describing the QA program and QC activities of the laboratory. The plan should address the following basic common aspects:

- a. *Statement of objectives*, describing the specific goals of the laboratory.
- b. *Sampling procedures*, including selection of representative sites and specified holding time and temperature conditions. If data may be subjected to litigation, use chain-of-custody procedures.
- c. *Personnel policies*, describing specific qualification and training requirements for supervisors and analysts.
- d. *Equipment and instrument requirements*, providing calibration procedures and frequency and maintenance requirements.
- e. *Specifications for supplies*, to ensure that reagents and supplies are of high quality and are tested for acceptability.
- f. *Analytical methods*, i.e., standardized methods established by a standards-setting organization and validated. Ideally, these laboratory methods have documented precision, bias, sensitivity, selectivity, and specificity.
- g. *Analytical quality control measures*, including such analytical checks as duplicate analyses, positive and negative controls, sterility checks, and verification tests.
- h. *Standard operating procedures (SOPs)*, i.e., written statement and documentation of all routine laboratory operations.
- i. *Documentation requirements*, concerning data acquisition, recordkeeping, traceability, and accountability.
- j. *Assessment requirements*:
  - 1) Internal audits of the laboratory operations, performed by the QA officer and supervisor.
  - 2) On-site evaluations by outside experts to ensure that the laboratory and its personnel are following an acceptable QA program.
  - 3) Performance evaluation studies, in which the QA officer works with the supervisor to incorporate unknown challenge samples into routine analytical runs and laboratories are encouraged to participate in state and national proficiency testing and accreditation programs. The collaborative studies confirm the abilities of a laboratory to generate acceptable data comparable to those of other laboratories and identify potential problems.
- k. *Corrective actions*: When problems are identified by the staff, supervisor, and/or QA coordinator, use standard stepwise procedures to determine the causes and correct them. Nonconformances identified by external laboratory evaluation are corrected, recorded, and signed

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off by the laboratory manager and QA officer.

Detailed descriptions of quality assurance programs are available.<sup>1-4</sup>

The QC guidelines discussed in Section 9020B and Section 9020C are recommended as useful source material, but all elements need to be addressed in developing a QA program.

## 5. References

1. GASKIN, J.E. 1992. Quality Assurance in Water Quality Monitoring. Inland Water Directorate, Conservation & Protection, Ottawa, Ont., Canada.
2. RATLIFF, T.A., JR. 1990. The Laboratory Quality Assurance System. A Manual of Quality Procedures with Related Forms. Van Nostrand Reinhold, New York, N.Y.
3. GARFIELD, F.M. 1984. Quality Assurance Principles of Analytical Laboratories. Assoc. Official Analytical Chemists, Arlington, Va.
4. DUX, J.P. 1983. Quality assurance in the analytical laboratory. *Amer. Lab.* 26:54.

## 9020 B. Intralaboratory Quality Control Guidelines

All laboratories have some intralaboratory QC practices that have evolved from common sense and the principles of controlled experimentation. A QC program applies practices necessary to minimize systematic and random errors resulting from personnel, instrumentation, equipment, reagents, supplies, sampling and analytical methods, data handling, and data reporting. It is especially important that laboratories performing only a limited amount of microbiological testing exercise strict QC. A listing of key QC practices is given in Table 9020:I. Other sources of QC practices are available.<sup>1-3</sup> These practices and guidelines will assist laboratories in establishing and improving QC programs. Laboratories should address all of the QC guidelines discussed herein, but the depth and details may differ for each laboratory.

### 1. Personnel

Microbiological testing should be performed by a professional microbiologist or technician trained in environmental microbiology whenever possible. If not, a professional microbiologist should be available for guidance. Train and evaluate the analyst in basic laboratory procedures. The supervisor periodically should review procedures of sample collecting and handling, media and glassware preparation, sterilization, routine analytical testing, counting, data handling, and QC techniques to identify and eliminate problems. Management should assist laboratory personnel in obtaining additional training and course work to advance their skills and career.

### 2. Facilities

*a. Ventilation:* Plan well-ventilated laboratories that can be maintained free of dust, drafts, and extreme temperature changes. Whenever possible, laboratories should have air conditioning

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to reduce contamination, permit more stable operation of incubators, and decrease moisture problems with media and instrumentation.

*b. Space utilization:* Design and operate the laboratory to minimize through traffic and visitors, with a separate area for preparing and sterilizing media, glassware, and equipment. Use a vented laminar-flow hood for dispensing and preparing sterile media, transferring microbial cultures, or working with pathogenic materials. In smaller laboratories it may be necessary, although undesirable, to carry out these activities in the same room.

*c. Laboratory bench areas:* Provide at least 2 m of linear bench space per analyst and additional areas for preparation and support activities. For stand-up work, typical bench dimensions are 90 to 97 cm high and 70 to 76 cm deep. For sit-down activities such as microscopy and plate counting, benches are 75 to 80 cm high. Specify bench tops of stainless steel, epoxy plastic, or other smooth, impervious surface that is inert and corrosion-resistant, has a minimum number of seams, and has adequate sealing of any crevices. Install even, glare-free lighting with about 1000 lux (100 ft-candles) intensity at the working surface.

*d. Walls and floors:* Assure that walls are covered with a smooth finish that is easily cleaned and disinfected. Specify floors of smooth concrete, vinyl, asphalt tile, or other impervious, sealed washable surfaces.

*e. Work-area monitoring:* Maintain high standards of cleanliness in work areas. Monitor air, at least monthly, with air density plates. The number of colonies on the air density plate test should not exceed 160/m<sup>2</sup>/15 min exposure (15 colonies/plate/15 min).

Plate or the swab method<sup>1</sup> can be used weekly or more frequently to monitor bench surface contamination. Although uniform limits for bacterial density have not been set, each laboratory can use these tests to establish a base line and take action on a significant increase.

*f. Laboratory cleanliness:* Regularly clean laboratory rooms and wash benches, shelves, floors, and windows. Wet-mop floors and treat with a disinfectant solution; do not sweep or dry-mop. Wipe bench tops and treat with a disinfectant before and after use. Do not permit laboratory to become cluttered.

### 3. Laboratory Equipment and Instrumentation

Verify that each item of equipment meets the user's needs for precision and minimization of bias. Perform equipment maintenance on a regular basis as recommended by the manufacturer or obtain preventive maintenance contracts on autoclave, balances, microscopes, and other equipment. Directly record all quality control checks in a permanent log book.

Use the following quality control procedures:

*a. Thermometer/temperature-recording instruments:* Check accuracy of thermometers or temperature-recording instruments semiannually against a certified National Institute of Standards and Technology (NIST) thermometer or one traceable to NIST and conforming to NIST specifications. For general purposes use thermometers graduated in increments of 0.5°C or less. Maintain in water or glycerol for air incubators and refrigerators and glycerol for freezers

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and seal in a flask. For a 44.5°C water bath, use a submersible thermometer graduated to 0.2°C or less. Record temperature check data in a quality control log. Mark the necessary NIST calibration corrections on each thermometer and incubator, refrigerator, or freezer. When possible, equip incubators and water baths with temperature-recording instruments that provide a continuous record of operating temperature.

*b. Balances:* Follow manufacturer's instructions in operation and routine maintenance of analytical and top-loading balances. Balances should be serviced and recalibrated by a manufacturer technician annually or more often as conditions change or problems occur. In weighing 2 g or less, use an analytical balance with a sensitivity less than 1 mg at a 10-g load. For larger quantities use a pan balance with sensitivity of 0.1 g at a 150-g load.

Wipe balance before use with a soft brush. Clean balance pans after use and wipe spills up immediately with a laboratory tissue. Inspect weights with each use and replace if corroded. Use only a plastic-tip forceps to handle weights. Check balance and working weights monthly against a set of reference weights (ANSI/ASTM Class 1 or NIST Class S) for accuracy, precision, and linearity.<sup>4</sup> Record results.

*c. pH meter:* Use a meter graduated in 0.1 pH units or less, that includes temperature compensation. Preferably use digital meters and commercial buffer solutions. With each use, standardize meter with two buffers that bracket the pH of interest and record. Date buffer solutions when opened and check monthly against another pH meter. Discard solution after each use and replace buffer supply before expiration date. For full details of pH meter use and maintenance, see Section 4500-H<sup>+</sup>.

*d. Water purification system:* Commercial systems are available that include some combination of prefiltration, activated carbon, mixed-bed resins, and reverse-osmosis with final filtration to produce a reagent-grade water. The life of such systems can be extended greatly if the source water is pretreated by distillation or by reverse osmosis to remove dissolved solids. Such systems tend to produce the same quality water until resins or activated carbon are near exhaustion and quality abruptly becomes unacceptable. Some deionization components are available now that automatically regenerate the ion exchange resins. Do not store reagent water unless a commercial UV irradiation device is installed and is confirmed to maintain sterility.

Monitor reagent water continuously or daily with a calibrated conductivity meter and analyze at least annually for trace metals. Replace cartridges at intervals recommended by the manufacturer based on the estimated usage and source water quality. Do not wait for column failure. If bacteria-free water is desired, include aseptic final filtration with a 0.22- $\mu\text{m}$ -pore membrane filter and collect in a sterile container. Monitor treated water for contamination and replace the filter as necessary.

*e. Water still:* Stills produce water of a good grade that characteristically deteriorates slowly over time as corrosion, leaching, and fouling occur. These conditions can be controlled with proper maintenance and cleaning. Stills efficiently remove dissolved substances but not dissolved gases or volatile organic chemicals. Freshly distilled water may contain chlorine and ammonia

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(NH<sub>3</sub>). On storage, additional NH<sub>3</sub> and CO<sub>2</sub> are absorbed from the air. Use softened water as the source water to reduce frequency of cleaning the still. Drain and clean still and reservoir according to manufacturer's instructions and usage.

*f. Media dispensing apparatus:* Check accuracy of volumes dispensed with a graduated cylinder at start of each volume change and periodically throughout extended runs. If the unit is used more than once per day, pump a large volume of hot reagent water through the unit to rinse between runs. Correct leaks, loose connections, or malfunctions immediately. At the end of the work day, break apparatus down into parts, wash, rinse with reagent water, and dry. Lubricate parts according to manufacturer's instructions or at least once per month.

*g. Hot-air oven:* Test performance monthly with commercially available *Bacillus subtilis* spore strips or spore suspensions. Monitor temperature with a thermometer accurate in the 160 to 180°C range and record results. Use heat-indicating tape to identify supplies and materials that have been exposed to sterilization temperatures.

*h. Autoclave:* Record items sterilized, temperature, pressure, and time for each run. Optimally use a recording thermometer. Check and record operating temperature weekly with a minimum/maximum thermometer. Test performance with *Bacillus stearothermophilus* spore strips, suspensions, or capsules monthly. Use heat-indicating tape to identify supplies and materials that have been sterilized.

*i. Refrigerator:* Maintain temperature at 1 to 4°C. Check and record temperature daily and clean monthly. Identify and date materials stored. Defrost as required and discard outdated materials quarterly.

*j. Freezer:* Maintain temperature at -20°C to -30°C. Check and record temperature daily. A recording thermometer and alarm system are highly desirable. Identify and date materials stored. Defrost and clean semiannually; discard outdated materials.

*k. Membrane filtration equipment:* Before use, assemble filtration units and check for leaks. Discard units if inside surfaces are scratched. Wash and rinse filtration assemblies thoroughly after use, wrap in nontoxic paper or foil, and sterilize.

*l. Ultraviolet lamps:* Disconnect lamps monthly and clean bulbs with a soft cloth moistened with ethanol. Test lamps quarterly with an appropriate (short- or long-wave) UV light meter\*<sup>5</sup>(3) and replace bulbs if output is less than 70% of the original. For short-wave lamps used in disinfecting work areas, expose plate count agar spread plates containing 200 to 300 organisms of interest, for 2 min. Incubate plates at 35°C for 48 h and count colonies. Replace bulb if count is not reduced 99%.

CAUTION: *Although short-wave (254-nm) UV light is known to be more dangerous than long-wave UV (365-nm), both types of UV light can damage eyes and skin and potentially are carcinogenic.<sup>5</sup> Protect eyes and skin from exposure to UV light. (See Section 1090B .)*

*m. Biohazard hood:* Once per month expose plate count agar plates to air flow for 1 h. Incubate plates at 35°C for 48 h and examine for contamination. A properly operating biohazard

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hood should produce no growth on the plates. Disconnect UV lamps and clean monthly by wiping with a soft cloth moistened with ethanol. Check lamps' efficiency as specified above. Inspect cabinet for leaks and rate of air flow quarterly. Use a pressure monitoring device to measure efficiency of hood performance. Have laminar-flow safety cabinets containing HEPA filters serviced by the manufacturer. Maintain hoods as directed by the manufacturer.

*n. Water bath incubator:* Verify that incubators maintain test temperature, such as  $35 \pm 0.5^\circ\text{C}$  or  $44.5 \pm 0.2^\circ\text{C}$ . Keep an appropriate thermometer (§ 3a, above) immersed in the water bath; monitor and record temperature twice daily (morning and afternoon). For optimum operation, equip water bath with a gable cover. Use only stainless steel, plastic-coated, or other corrosion-proof racks. Clean bath as needed.

*o. Incubator (air, water jacketed, or aluminum block):* Verify that incubators maintain appropriate test temperatures. Also, verify that cold samples are incubated at the test temperature for the required time. Check and record temperature twice daily (morning and afternoon) on the shelves in use. If a glass thermometer is used, submerge bulb and stem in water or glycerine to the stem mark. For best results use a recording thermometer and alarm system. Place incubator in an area where room temperature is maintained between  $16$  and  $27^\circ\text{C}$  ( $60$  to  $80^\circ\text{F}$ ).

*p. Microscopes:* Use lens paper to clean optics and stage after each use. Cover microscope when not in use.

Permit only trained technicians to use fluorescence microscope and light source. Monitor fluorescence lamp with a light meter and replace when a significant loss in fluorescence is observed. Log lamp operation time, efficiency, and alignment. Periodically check lamp alignment, particularly when the bulb has been changed; realign if necessary. Use known positive 4 + fluorescence slides as controls.

### 4. Laboratory Supplies

*a. Glassware:* Before each use, examine glassware and discard items with chipped edges or etched inner surfaces. Particularly examine screw-capped dilution bottles and flasks for chipped edges that could leak and contaminate the analyst and the area. Inspect glassware after washing for excessive water beading and rewash if necessary. Make the following tests for clean glassware as necessary:

1) pH check—Because some cleaning solutions are difficult to remove completely, spot check batches of clean glassware for pH reaction, especially if soaked in alkali or acid. To test clean glassware for an alkaline or acid residue add a few drops of 0.04% bromthymol blue (BTB) or other pH indicator and observe the color reaction. BTB should be blue-green (in the neutral range).

To prepare 0.04% bromthymol blue indicator solution, add 16 mL 0.01N NaOH to 0.1 g BTB and dilute to 250 mL with reagent water.

2) Test for inhibitory residues on glassware and plasticware—Certain wetting agents or detergents used in washing glassware may contain bacteriostatic or inhibiting substances that

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require 6 to 12 rinsings to remove all traces and insure freedom from residual bacteriostatic action. Perform this test annually and before using a new supply of detergent. If prewashed, presterilized plasticware is used, test it for inhibitory residues. Although the following procedure describes testing of petri dishes for inhibitory residue, it is applicable to other glass or plasticware.

a) Procedure—Wash and rinse six petri dishes according to usual laboratory practice and designate as Group A.

Wash six petri dishes as above, rinse 12 times with successive portions of reagent water, and designate as Group B.

Rinse six petri dishes with detergent wash water (in use concentration), and air-dry without further rinsing, and designate as Group C.

Sterilize dishes in Groups A, B, and C by the usual procedure.

For presterilized plasticware, set up six plastic petri dishes and designate them as Group D.

Prepare and sterilize 200 mL plate count agar and hold in a 44 to 46°C water bath.

Prepare a culture of *E. aerogenes* known to contain 50 to 150 colony-forming units/mL. Preliminary testing may be necessary to achieve this count range. Inoculate three dishes from each test group with 0.1 mL and the other three dishes from each group with 1 mL culture.

Analyze the four sets of six plates each, following heterotrophic plate count method (Section 9215B), and incubate at 35°C for 48 h. Count plates with 30 to 300 colonies and record results as CFU/ mL.

b) Interpretation of results—Difference in averaged counts on plates in Groups A through D should be less than 15% if there are no toxic or inhibitory effects.

Differences in averaged counts of less than 15% between Groups A and B and greater than 15% between Groups A and C indicate that the cleaning detergent has inhibitory properties that are eliminated during routine washing. Differences between B and D greater than 15% indicate an inhibitory residue.

*b. Utensils and containers for media preparation:* Use utensils and containers of borosilicate glass, stainless steel, aluminum, or other corrosion-resistant material (see Section 9030). Do not use copper utensils.

*c. Dilution water bottles:* Use scribed bottles made of nonreactive borosilicate glass or plastic with screwcaps containing inert liners. Clean before use. Disposable plastic bottles prefilled with dilution water are available commercially and are acceptable. Before use of each lot, check pH and volume and examine sterile bottles of dilution water for a precipitate; discard if present. Reclean bottles with acid if necessary, and remake the dilution water. If precipitate repeats, procure a different source of bottles.

*d. Reagent-grade water quality:* The quality of water obtainable from a water purification system differs with the system used and its maintenance. See ¶ 3d and ¶ 3e above. Recommended limits for reagent water quality are given in Table 9020:II. If these limits are not

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met, investigate and correct or change water source. Although pH measurement of reagent water is characterized by drift, extreme readings are indicative of chemical contamination.

*e. Use test for evaluation of reagent water, media, and membranes:* When a new lot of culture medium, membrane filters, or a new source of reagent-grade water is to be used make comparison tests, at least quarterly, of the current lot in use (reference lot) against the new lot (test lot).

1) Procedure—Use a single batch of control water (redistilled or distilled water polished by deionization), glassware, membrane filters, or other needed materials to control all variables except the one factor under study. Make parallel pour or spread plate or membrane filter plate tests on reference lot and test lot, according to procedures in Section 9215 and Section 9222. As a minimum, make single analyses on five different water samples positive for the target organism. Replicate analyses and additional samples can be tested to increase the sensitivity of detecting differences between reference and test lots.

When conducting the use test on reagent water, perform the quantitative bacterial tests in parallel using a known high-quality water as a control water. Prepare dilution/rinse water and media with new source of reagent and control water. Test water for all uses (dilution, rinse, media preparation, etc.).

2) Counting and calculations—After incubation, compare bacterial colonies from the two lots for size and appearance. If colonies on the test lot plates are atypical or noticeably smaller than colonies on the reference lot plates, record the evidence of inhibition or other problem, regardless of count differences. Count plates and calculate the individual count per 1 mL or per 100 mL. Transform the count to logarithms and enter the log-transformed results for the two lots in parallel columns. Calculate the difference,  $d$ , between the two transformed results for each sample, including the + or – sign, the mean,  $\bar{d}$  and the standard deviation  $s_d$  of these differences (see Section 1010B).

Calculate Student's  $t$  statistic, using the number of samples as  $n$ :

$$t = \frac{\bar{d}}{s_d / \sqrt{n}}$$

These calculations may be made with various statistical software packages available for personal computers.

3) Interpretation—Use the critical  $t$  value, from a Student's  $t$  table for comparison against the calculated value. At the 0.05 significance level this value is 2.78 for five samples (four degrees of freedom). If the calculated  $t$  value does not exceed 2.78, the lots do not produce significantly different results and the test lot is acceptable. If the calculated  $t$  value exceeds 2.78, the lots produce significantly different results and the test lot is unacceptable.

If the colonies are atypical or noticeably smaller on the test lot or the Student's  $t$  exceeds

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2.78, review test conditions, repeat the test, and/or reject the test lot and obtain another one.

*f. Reagents:* Because reagents are an integral part of microbiological analyses, their quality must be assured. Use only chemicals of ACS or equivalent grade because impurities can inhibit bacterial growth, provide nutrients, or fail to produce the desired reaction. Date chemicals and reagents when received and when first opened for use. Make reagents to volume in volumetric flasks and transfer for storage to good-quality inert plastic or borosilicate glass bottles with borosilicate, polyethylene, or other plastic stoppers or caps. Label prepared reagents with name and concentration, date prepared, and initials of preparer. Include positive and negative control cultures with each series of cultural or biochemical tests.

*g. Dyes and stains:* In microbiological analyses, organic chemicals are used as selective agents (e.g., brilliant green), as indicators (e.g., phenol red), and as microbiological stains (e.g., Gram stain). Dyes from commercial suppliers vary from lot to lot in percent dye, dye complex, insolubles, and inert materials. Because dyes for microbiology must be of proper strength and stability to produce correct reactions, use only dyes certified by the Biological Stain Commission. Check bacteriological stains before use with at least one positive and one negative control culture and record results.

*h. Membrane filters and pads:* The quality and performance of membrane filters vary with the manufacturer, type, brand, and lot. These variations result from differences in manufacturing methods, materials, quality control, storage conditions, and application.

1) Membrane filters and pads for water analyses should meet the following specifications:

a) Filter diam 47 mm, mean pore diam 0.45  $\mu\text{m}$ . Alternate filter and pore sizes may be used if the manufacturer provides data verifying performance equal to or better than that of 47-mm-diam, 0.45- $\mu\text{m}$ -pore size filter. At least 70% of filter area must be pores.

b) When filters are floated on reagent water, the water diffuses uniformly through the filters in 15 s with no dry spots on the filters.

c) Flow rates are at least 55 mL/min/cm<sup>2</sup> at 25°C and a differential pressure of 93 kPa.

d) Filters are nontoxic, free of bacterial-growth-inhibiting or stimulating substances, and free of materials that directly or indirectly interfere with bacterial indicator systems in the medium; ink grid is nontoxic. The arithmetic mean of five counts on filters must be at least 90% of the arithmetic mean of the counts on five agar spread plates using the same sample volumes and agar media.

e) Filters retain the organisms from a 100-mL suspension of *Serratia marcescens* containing  $1 \times 10^3$  cells.

f) Water-extractables in filter do not exceed 2.5% after the membrane is boiled in 100 mL reagent water for 20 min, dried, cooled, and brought to constant weight.

g) Absorbent pad has diam 47 mm, thickness 0.8 mm, and is capable of absorbing  $2.0 \pm 0.2$  mL Endo broth.

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h) Pads release less than 1 mg total acidity calculated as  $\text{CaCO}_3$  when titrated to the phenolphthalein end point with 0.02N NaOH.

i) If filter and absorbent pad are not sterile, they should not be degraded by sterilization at 121°C for 10 min. Confirm sterility by absence of growth when a membrane filter is placed on a pad saturated with tryptone glucose extract broth or tryptone glucose extract agar and incubated at  $35 \pm 0.5^\circ\text{C}$  for 24 h.

j) Some lots of membrane filters yield low recoveries, poor differentiation, or malformation of colonies due to toxicity, chemical composition, or structural defects.<sup>6</sup> Perform the use test (¶ 4e) on new lots of filters.

### 2) Standardized tests:

Standardized tests are available for evaluating retention, recovery, extractables, and flow rate characteristics of membrane filters.<sup>7</sup>

Some manufacturers provide information beyond that required by specifications and certify that their membranes are satisfactory for water analysis. They report retention, pore size, flow rate, sterility, pH, percent recovery, and limits for specific inorganic and organic chemical extractables. Although the standard membrane filter evaluation tests were developed for the manufacturers, a laboratory can conduct its own tests.

To maintain quality control inspect each lot of membranes before use and during testing to insure they are round and pliable, with undistorted gridlines after autoclaving. After incubation, colonies should be well-developed with well-defined color and shape as defined by the test procedure. The gridline ink should not channel growth along the ink line nor restrict colony development. Colonies should be distributed evenly across the membrane surface.

*i. Culture media:* Because cultural methods depend on properly prepared media, use the best available materials and techniques in media preparation, storage, and application. For control of quality, use commercially prepared media whenever available but note that such media may vary in quality among manufacturers and even from lot to lot from the same manufacturer.

Order media in quantities to last no longer than 1 year. Use media on a first-in, first-out basis. When practical, order media in quarter pound (114 g) multiples rather than one pound (454 g) bottles, to keep the supply sealed as long as possible. Record kind, amount, and appearance of media received, lot number, expiration date, and dates received and opened. Check inventory quarterly for reordering.

Store dehydrated media at an even temperature in a cool dry place, away from direct sunlight. Discard media that cake, discolor, or show other signs of deterioration. If expiration date is given by manufacturer, discard unused media after that date. A conservative time limit for unopened bottles is 2 years at room temperature. Compare recovery of newly purchased lots of media against proven lots, using recent pure-culture isolates and natural samples.

Use opened bottles of media within 6 months. Dehydrated media are hygroscopic. Protect opened bottles from moisture. Close bottles as tightly as possible, immediately after use. If

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caking or discoloration of media occurs, discard media. Store opened bottles in a dessicator.

1) Preparation of media—Prepare media in containers that are at least twice the volume of the medium being prepared. Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes heated on a hot plate or gas burner. Preferably use hot plate-magnetic stirrer combinations. Label and date prepared media. Prepare media in reagent water. Measure water volumes and media with graduates or pipets conforming to NIST and APHA standards, respectively. Do not use blow-out pipets. After preparation and storage, remelt agar media in boiling water or flowing steam.

Check and record pH of a portion of each medium after sterilization and cooling. Check pH of solid medium with a surface probe. Record results. Make minor adjustments in pH (<0.5 pH units) with 1*N* NaOH or HCl solution to the pH specified in formulation. If the pH difference is larger than 0.5 units, discard the batch and check preparation instructions and pH of reagent water to resolve the problem. Incorrect pH values may be due to reagent water quality, medium deterioration, or improper preparation. Review instructions for preparation and check water pH. If water pH is unsatisfactory, prepare a new batch of medium using water from a new source (see Section 9020B.3*d* and *e*). If water is satisfactory, remake medium and check; if pH is again incorrect, prepare medium from another bottle.

Record pH problems in the media record book and inform the manufacturer if the medium is indicated as the source of error. Examine prepared media for unusual color, darkening, or precipitation and record observations. Consider variations of sterilization time and temperature as possible causes for problems. If any of the above occur, discard the medium.

2) Sterilization—Sterilize media at 121 to 124°C for the minimum time specified. A double-walled autoclave permits maintenance of full pressure and temperature in the jacket between loads and reduces chance for heat damage. Follow manufacturer's directions for sterilization of specific media. The required exposure time varies with form and type of material, type of medium, presence of carbohydrates, and volume. Table 9020:III gives guidelines for typical items. Do not expose media containing carbohydrates to the elevated temperatures for more than 45 min. Exposure time is defined as the period from initial exposure to removal from the autoclave.

Some currently available autoclave models are automatic and include features such as vertical sliding, self-sealing and opening doors, programmable sterilization cycles, and continuous multipoint monitoring of chamber temperature and pressure. These units also may incorporate solution cooling and vapor removal features. When sterilizer design includes heat exchangers and solution cooling features as part of a factory-programmed liquid cycle, strict adherence to the 45-min total elapsed time in the autoclave is not necessary provided that printout records verify normal cycle operation and chamber cooling during exhaust and vapor removal.

Remove sterilized media from autoclave as soon as chamber pressure reaches zero, or, if a fully automatic model is used, as soon as the door opens. Do not reautoclave media.

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Check effectiveness of sterilization weekly by placing *Bacillus stearothermophilus* spore suspensions or strips (commercially available) inside glassware. Sterilize at 121°C for 15 min. Place in trypticase soy broth tubes and incubate at 55°C for 48 h. If growth of the autoclaved spores occurs after incubation at 55°C, sterilization was inadequate. A small, relatively inexpensive 55°C incubator is available commercially. †#(4)

Sterilize heat-sensitive solutions or media by filtration through a 0.22- $\mu\text{m}$ -pore-diam filter in a sterile filtration and receiving apparatus. Filter and dispense medium in a safety cabinet or biohazard hood if available. Sterilize glassware (pipets, petri dishes, sample bottles) in an autoclave or an oven at 170°C for 2 h. Sterilize equipment, supplies, and other solid or dry materials that are heat-sensitive, by exposing to ethylene oxide in a gas sterilizer. Use commercially available spore strips or suspensions to check dry heat and ethylene oxide sterilization.

3) Use of agars and broths—Temper melted agars in a water bath at 44 to 46°C until used but do not hold longer than 3 h. To monitor agar temperature, expose a bottle of water or medium to the same heating and cooling conditions as the agar. Insert a thermometer in the monitoring bottle to determine when the temperature is 45 to 46°C and suitable for use in pour plates. If possible, prepare media on the day of use. After pouring agar plates for streaking, dry agar surfaces by keeping dish slightly open for at least 15 min in a bacteriological hood to avoid contamination. Discard unused liquid agar; do not let harden or remelt for later use.

Handle tubes of sterile fermentation media carefully to avoid entrapping air in inner tubes, thereby producing false positive reactions. Examine freshly prepared tubes to determine that gas bubbles are absent.

4) Storage of media—Prepare media in amounts that will be used within holding time limits given in Table 9020:IV. Protect media containing dyes from light; if color changes occur, discard the media. Refrigerate poured agar plates not used on the day of preparation. Seal agar plates with loose-fitting lids in plastic bags if held more than 2 d. Prepare broth media that will be stored for more than 2 weeks in screw-cap tubes, other tightly sealed tubes, or in loose-capped tubes placed in a sealed plastic bag or other tightly sealed container to prevent evaporation.

Mark liquid level in several tubes and monitor for loss of liquid. If loss is 10% or more, discard the batch. If media are refrigerated, incubate overnight at test temperature before use and reject the batch if false positive responses occur. Prepared sterile broths and agars available from commercial sources may offer advantages when analyses are done intermittently, when staff is not available for preparation work, or when cost can be balanced against other factors of laboratory operation. Check performance of these media as described in ¶ 5 below.

5) Quality control of prepared media—Maintain in a bound book a complete record of each prepared batch of medium with name of preparer and date, name and lot number of medium, amount of medium weighed, volume of medium prepared, sterilization time and temperature, pH measurements and adjustments, and preparations of labile components. Compare quantitative recoveries of new lots with previously acceptable ones. Include sterility and positive and negative

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control culture checks on all media as described below.

### 5. Standard Operating Procedures (SOPs)

SOPs are the operational backbone of an analytical laboratory. SOPs describe in detail all laboratory operations such as preparation of reagents, reagent water, standards, culture media, proper use of balances, sterilization practices, and dishwashing procedures, as well as methods of sampling, analysis, and quality control. The SOPs are unique to the laboratory. They describe the tasks as performed on a day-to-day basis, tailored to the laboratory's own equipment, instrumentation, and sample types. The SOPs guide routine operations by each analyst, help to assure uniform operations, and provide a solid training tool.

### 6. Sampling

*a. Planning:* Microbiologists should participate in the planning of monitoring programs that will include microbial analyses. They can provide valuable expertise on the selection of sampling sites, number of samples and analyses needed, workload, and equipment and supply needs. For natural waters, knowledge of the probable microbial densities, and the impact of season, weather, tide and wind patterns, known sources of pollution, and other variables, are needed to formulate the most effective sampling plan.

*b. Methods:* Sampling plans must be specific for each sampling site. Prior sampling guidance can be only general in nature, addressing the factors that must be considered for each site. Sampling SOPs describe sampling equipment, techniques, frequency, holding times and conditions, safety rules, etc., that will be used under different conditions for different sites. From the information in these SOPs sampling plans will be drawn up.

### 7. Analytical Methods

*a. Method selection:* Because minor variations in technique can cause significant changes in results, microbiological methods must be standardized so that uniform data result from multiple laboratories. Select analytical methods appropriate for the sample type from *Standard Methods* or other source of standardized methods and ensure that methods have been validated in a multi-laboratory study with the sample types of interest.

*b. Data objectives:* Review available methods and determine which produce data to meet the program's needs for precision, bias, specificity, selectivity, and detection limit. Ensure that the methods have been demonstrated to perform within the above specifications for the samples of interest.

*c. Internal QC:* The written analytical methods should contain required QC checks of positive and negative control cultures, sterile blank, replicate analyses (precision), and a known quantitative culture, if available.

*d. Method SOPs:* As part of the series of SOPs, provide each analyst with a copy of the analytical methods written in step-wise fashion exactly as they are to be performed and specific to the sample type, equipment, and instrumentation used in the laboratory.

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## 8. Analytical Quality Control Procedures

### a. General quality control procedures:

- 1) New methods—Conduct parallel tests with the standard procedure and a new method to determine applicability and comparability. Perform at least 100 parallel tests across seasons of the year before replacement with the new method for routine use.
- 2) Comparison of plate counts—For routine performance evaluation, repeat counts on one or more positive samples at least monthly and compare the counts with those of other analysts testing the same samples. Replicate counts for the same analyst should agree within 5% and those between analysts should agree within 10%. See Section 9020B.10b for a statistical calculation of data precision.
- 3) Control cultures—For each lot of medium check analytical procedures by testing with known positive and negative control cultures for the organism(s) under test. See Table 9020:V for examples of test cultures.
- 4) Duplicate analyses—Perform duplicate analyses on 10% of samples and on at least one sample per test run. A test run is defined as an uninterrupted series of analyses. If the laboratory conducts less than 10 tests/week, make duplicate analyses on at least one sample each week.
- 5) Sterility checks—For membrane filter tests, check sterility of media, membrane filters, buffered dilution and rinse water, pipets, flasks and dishes, and equipment as a minimum at the end of each series of samples, using sterile reagent water as the sample. If contaminated, check for the source. For multiple-tube and presence-absence procedures, check sterility of media, dilution water, and glassware. To test sterility of media, incubate a representative portion of each batch at an appropriate temperature for 24 to 48 h and observe for growth. Check each batch of buffered dilution water for sterility by adding 20 mL water to 100 mL of a nonselective broth. Alternatively, aseptically pass 100 mL or more dilution water through a membrane filter and place filter on growth medium suitable for heterotrophic bacteria. Incubate at  $35 \pm 0.5^\circ\text{C}$  for 24 h and observe for growth. If any contamination is indicated, determine the cause and reject analytical data from samples tested with these materials. Request immediate resampling and reanalyze.

b. *Precision of quantitative methods:* Calculate precision of duplicate analyses for each different type of sample examined, for example, drinking water, ambient water, wastewater, etc., according to the following procedure:

1) Perform duplicate analyses on first 15 positive samples of each type, with each set of duplicates analyzed by a single analyst. If there is more than one analyst, include all analysts regularly running the tests, with each analyst performing approximately an equal number of tests. Record duplicate analyses as  $D_1$  and  $D_2$ .

2) Calculate the logarithm of each result. If either of a set of duplicate results is  $<1$ , add 1 to both values before calculating the logarithms.

3) Calculate the range ( $R$ ) for each pair of transformed duplicates as the mean ( $\hat{t}$ ) of these

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ranges.

See sample calculation in Table 9020:VI.

4) Thereafter, analyze 10% of routine samples in duplicate. Transform the duplicates as in ¶ 2) and calculate their range. If the range is greater than  $3.27 R$ , there is greater than 99% probability that the laboratory variability is excessive. Determine if increased imprecision is acceptable; if not, discard all analytical results since the last precision check (see Table 9020:VII). Identify and resolve the analytical problem before making further analyses.

5) Update the criterion used in ¶ 4) by periodically repeating the procedures of ¶s 1) through 3) using the most recent sets of 15 duplicate results.

### 9. Verification

For the most part, the confirmation/verification procedures for drinking water differ from those for other waters because of specific regulatory requirements.

#### a. Multiple-tube fermentation (MTF) methods:

##### 1) Total coliform procedure (Section 9221B)

a) Drinking water—Carry samples through confirmed phase only. Verification is not required. For QC purposes, if normally there are no positive results, analyze at least one positive source water quarterly to confirm that the media produce appropriate responses. For samples with a history of heavy growth without gas in presumptive-phase tubes, carry the tubes through the confirmed phase to check for false negative responses for coliform bacteria. Verify any positives for fecal coliforms or *E. coli*.

b) Other water types—Verify by performing the completed MTF Test on 10% of samples positive through the confirmed phase.

##### 2) Enzyme substrate coliform test (total coliform/*E. coli*) (Section 9223B)

a) Drinking water—Verify at least 5% of total coliform positive results from enzyme substrate coliform tests by inoculating growth from a known positive sample and testing for lactose fermentation or for  $\beta$ -D-galactopyranosidase by the *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) test and indophenol by the cytochrome oxidase (CO) test. See Section 9225D for these tests. Coliforms are ONPG-positive and cytochrome-oxidase-negative. Verify *E. coli* using the EC MUG test (see Section 9221F).

b) Other water types—Verify at least 10% of total coliform positive samples as in ¶ 2a above.

3) Fecal streptococci procedure—Verify as in 9230C.5. Growth of catalase-negative, gram-positive cocci on bile esculin agar at 35°C and in brain-heart infusion broth at 45°C verifies the organisms as fecal streptococci. Growth at 45°C and in 6.5% NaCl broth indicates the streptococci are members of the enterococcus group.

4) Include known positive and negative pure cultures as a QC check.

#### b. Membrane filter methods:

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### 1) Total coliform procedures

a) Drinking water—Pick all, up to 5 typical and 5 atypical (nonsheen) colonies from positive samples on M-Endo medium and verify as in Section 9222B.5 *f*. Also verify any positives for fecal coliforms or *E. coli*. If there are no positive samples, test at least one known positive source water quarterly.

b) Other water types—Verify positives monthly by picking at least 10 sheen colonies from a positive water sample as in Section 9222B.5 *f*. Adjust counts based on percent verification.

c) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Section 9222B.5 *f*.

### 2) Fecal coliform procedure

a) Verify positives monthly by picking at least 10 blue colonies from one positive sample. Verify in lauryl tryptose broth and EC broth as in Section 9221B.3 and Section 9221E. Adjust counts based on percent verification.

b) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Section 9221B.3 and Section 9221E.

### 3) *Escherichia coli* procedure

a) Drinking water—Verify at least 5% of MUG-positive and MUG-negative results. Pick from well-isolated sheen colonies that fluoresce on nutrient agar with MUG (NA MUG), taking care not to pick up medium, which can cause a false positive response. Also verify nonsheen colonies that fluoresce. Verify by performing the citrate test and the indole test as described in Section 9225D, but incubate indole test at 44.5°C. *E. coli* are indole-positive and yield no growth on citrate.

b) Other water types—Verify one positive sample monthly as in ¶ a) above. Adjust counts based on percentage of verification.

4) Fecal streptococci procedure—Pick to verify monthly at least 10 isolated esculin-positive red colonies from m-Enterococcus agar to brain heart infusion (BHI) media. Verify as described in Section 9230C. Adjust counts based on percentage of verification.

5) *Enterococci* procedures—Pick to verify monthly at least 10 well-isolated pink to red colonies with black or reddish-brown precipitate from EIA agar. Transfer to BHI media as described in Section 9230C. Adjust counts based on percentage of verification.

6) Include known positive and negative pure cultures as a quality control check.

## 9. Documentation and Recordkeeping

a. *QA plan*: The QA program documents management's commitment to a QA policy and sets forth the requirements needed to support program objectives. The program describes overall policies, organization, objectives, and functional responsibilities for achieving the quality goals. In addition, the program should develop a project plan that specifies the QC requirements for each project. The plan specifies the QC activities required to achieve the data representativeness,

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completeness, comparability, and compatibility. Also, the QA plan should include a program implementation plan that ensures maximum coordination and integration of QC activities within the overall program (sampling, analyses, and data handling).

*b. Sampling records:* A written SOP for sample handling records sample collection, transfer, storage, analyses, and disposal. The record is most easily kept on a series of printed forms that prompt the user to provide all the necessary information. It is especially critical that this record be exact and complete if there is any chance that litigation may occur. Such record systems are called chain of custody. Because laboratories do not always know whether analytical results will be used in future litigation, some maintain chain-of-custody on all samples. Details on chain of custody are available in Section 1060B and elsewhere.<sup>1</sup>

*c. Recordkeeping:* An acceptable recordkeeping system provides needed information on sample collection and preservation, analytical methods, raw data, calculations through reported results, and a record of persons responsible for sampling and analyses. Choose a format agreeable to both the laboratory and the customer (the data user). Ensure that all data sheets are signed and dated by the analyst and the supervisor. The preferable record form is a bound and page-numbered notebook, with entries in ink and a single line drawn through any change with the correction entered next to it.

Keep records of microbiological analyses for at least 5 years. Actual laboratory reports may be kept, or data may be transferred to tabular summaries, provided that the following information is included: date, place, and time of sampling, name of sample collector; identification of sample; date of receipt of sample and analysis; person(s) responsible for performing analysis; analytical method used; the raw data and the calculated results of analysis. Verify that each result was entered correctly from the bench sheet and initialed by the analyst. If an information storage and retrieval system is used, double check data on the printouts.

### 10. Data Handling

*a. Distribution of bacterial populations:* In most chemical analyses the distribution of analytical results follows the Gaussian curve, which has symmetrical distribution of values about the mean (see Section 1010B). Microbial distributions are not necessarily symmetrical. Bacterial counts often are characterized as having a skewed distribution because of many low values and a few high ones. These characteristics lead to an arithmetic mean that is considerably larger than the median. The frequency curve of this distribution has a long right tail, such as that shown in Figure 9020:1, and is said to display positive skewness.

Application of the most rigorous statistical techniques requires the assumption of symmetrical distributions such as the normal curve. Therefore it usually is necessary to convert skewed data so that a symmetrical distribution resembling the normal distribution results. An approximately normal distribution can be obtained from positively skewed data by converting numbers to their logarithms, as shown in Table 9020:VIII. Comparison of the frequency tables for the original data (Table 9020:IX) and their logarithms (Table 9020:X) shows that the logarithms approximate a symmetrical distribution.

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*b. Central tendency measures of skewed distribution:* If the logarithms of numbers from a positively skewed distribution are approximately normally distributed, the original data have a log-normal distribution. The best estimate of central tendency of log-normal data is the geometric mean, defined as:

$$\bar{x}_g = \sqrt[n]{(x_1)(x_2) \cdots (x_n)}$$

and

$$\log \bar{x}_g = \frac{\sum (\log x_i)}{n}$$

that is, the geometric mean is equal to the antilog of the arithmetic mean of the logarithms. For example, the following means calculated from the data in Table 9020:VIII are drastically different.

$$\log \bar{x}_g = \frac{\sum (\log x_i)}{n} = \frac{32.737}{15} = 2.1825$$

geometric mean

$$\bar{x}_g = \text{antilog}(2.1825) = 152$$

and arithmetic mean

$$\bar{x} = \frac{\sum x_i}{n} = \frac{6632}{15} = 442$$

Therefore, although regulations or tradition may require or cause microbiological data to be reported as the arithmetic mean or median, the preferred statistic for summarizing microbiological monitoring data is the geometric mean. An exception may be in the evaluation of data for risk assessment. The arithmetic mean may be a better measure for this purpose because it may generate a higher central tendency value and possibly provide a greater safety factor.<sup>8</sup>

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c. “Less than” (<) values: There has always been uncertainty as to the proper way to include “less than” values in calculation and evaluation of microbiological data because such values cannot be treated statistically without modification. Proposed modifications involve changing such numbers to zero, choosing values halfway between zero and the “less than” value, or assigning the “less than” value itself, i.e., changing <1 values to 1,  $\frac{1}{2}$ , or 0.

There are valid reasons for not including < values, whether modified or not. If the database is fairly large with just a few < values, the influence of these uncertain values will be minimal and of no benefit. If the database is small or has a relatively large number of < values, inclusion of modified < values would exert an undue influence on the final results and could result in an artificial negative or positive bias. Including < values is particularly inappropriate if the < values are <100, <1000, or higher because the unknown true values could be anywhere from 0 to 99, 0 to 999, etc. When < values are first noted, adjust or expand test volumes. The only exception to this caution would be regulatory testing with defined compliance limits, such as the <1/100 mL values reported for drinking water systems where the 100-mL volume is required.

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### 9020 C. Interlaboratory Quality Control

#### 1. Background

Interlaboratory QC programs are a means of establishing an agreed-upon, common performance criteria system that will assure an acceptable level of data quality and comparability among laboratories with similar interests and/or needs.

These systems may be volunteer, such as that for the cities in the Ohio River Valley Water Sanitation Commission (ORSANCO), or regulatory, such as the Federal Drinking Water Laboratory Certification Program (see below). Often, the term “accreditation” is used interchangeably with certification. Usually, interlaboratory quality control programs have three elements: uniform criteria for laboratory operations, external review of the program, and external proficiency testing.

#### 2. Uniform Criteria

Interlaboratory quality control programs begin as a volunteer or mandatory means of establishing uniform laboratory standards for a specific purpose. The participants may be from one organization or a group of organizations having common interests or falling under common regulations. Often one group or person may agree to draft the criteria. If under regulation, the regulating authority may set the criteria for compliance-monitoring analyses.

Uniform sampling and analytical methods and quality control criteria for personnel, facilities, equipment, instrumentation, supplies, and data handling and reporting are proposed, discussed, reviewed, modified if necessary, and approved by the group for common use. Criteria identified as necessary for acceptable data quality should be mandatory. A formal document is prepared and provided to all participants.

The QA/QC responsibilities of management, supervisors, and technical staff are described in 9020A. In large laboratories, a QA officer is assigned as a staff position but may be the supervisor or other senior person in smaller laboratories.

After incorporation into laboratory operations and confirmation that the QA program has been adapted and is in routine use, the laboratory supervisor and the QA officer conduct an internal program review of all operations and records for acceptability, to identify possible problems and assist in their resolution. If this is done properly, there should be little concern that subsequent external reviews will find major problems.

#### 3. External Program Review

Once a laboratory has a QA program in place, management informs the organization and a qualified external QA person or team arranges an on-site visit to evaluate the QA program for

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acceptability and to work with the laboratory to solve any problems. An acceptable rating confirms that the laboratory's QA program is operating properly and that the laboratory has the capability of generating valid defensible data. Such on-site evaluations are repeated and may be announced or unannounced.

### 4. External Proficiency Testing

Whenever practical, the external organization conducts formal performance evaluation studies among all participant laboratories. Challenge samples are prepared and sent as unknowns on a set schedule for analyses and reporting of results. The reported data are coded for confidentiality and evaluated according to an agreed-upon scheme. The results are summarized for all laboratories and individual laboratory reports are sent to participants. Results of such studies indicate the quality of routine analyses of each laboratory as compared to group performance. Also, results of the group as a whole characterize the performance that can be expected for the analytical methods tested.

### 5. Example Program

In the Federal Drinking Water Laboratory Certification Program, public water supply laboratories must be certified according to minimal criteria and procedures and quality assurance described in the EPA manual on certification:<sup>1</sup> criteria are established for laboratory operations and methodology; on-site inspections are required by the certifying state agency or its surrogate to verify minimal standards; annually, laboratories are required to perform acceptably on unknown samples in formal studies, as samples are available; the responsible authority follows up on problems identified in the on-site inspection or performance evaluation and requires corrections within a set period of time. Individual state programs may exceed the federal criteria.

On-site inspections of laboratories in the present certification program show that primary causes for discrepancies in drinking water laboratories have been inadequate equipment, improperly prepared media, incorrect analytical procedures, and insufficiently trained personnel.

### 6. References

1. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1997. Manual for the Certification of Laboratories Analyzing Drinking Water, 4th ed. EPA-814B-92-002, U.S. Environmental Protection Agency, Cincinnati, Ohio.

## 9030 LABORATORY APPARATUS\*(5)

### 9030 A. Introduction

This section contains specifications for microbiological laboratory equipment. For testing and maintenance procedures related to quality control, see Section 9020.

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## 9030 B. Equipment Specifications

### 1. Incubators

Incubators must maintain a uniform and constant temperature at all times in all areas, that is, they must not vary more than  $\pm 0.5^{\circ}\text{C}$  in the areas used. Obtain such accuracy by using a water-jacketed or anhydric-type incubator with thermostatically controlled low-temperature electric heating units properly insulated and located in or adjacent to the walls or floor of the chamber and preferably equipped with mechanical means of circulating air.

Incubators equipped with high-temperature heating units are unsatisfactory, because such sources of heat, when improperly placed, frequently cause localized overheating and excessive drying of media, with consequent inhibition of bacterial growth. Incubators so heated may be operated satisfactorily by replacing high-temperature units with suitable wiring arranged to operate at a lower temperature and by installing mechanical air-circulation devices. It is desirable, where ordinary room temperatures vary excessively, to keep laboratory incubators in special rooms maintained at a few degrees below the recommended incubator temperature.

Alternatively, use special incubating rooms well insulated and equipped with properly distributed heating units, forced air circulation, and air exchange ports, provided that they conform to desired temperature limits. When such rooms are used, record the daily temperature range in areas where plates or tubes are incubated. Provide incubators with open metal wire or perforated sheet shelves so spaced as to assure temperature uniformity throughout the chamber. Leave a 2.5-cm space between walls and stacks of dishes or baskets of tubes.

Maintain an accurate thermometer, traceable to the National Institute of Standards and Technology (NIST), with the bulb immersed in liquid (glycerine, water, or mineral oil) on each shelf in use within the incubator and record daily temperature readings (preferably morning and afternoon). It is desirable, in addition, to maintain a maximum and minimum registering thermometer within the incubator on the middle shelf to record the gross temperature range over a 24-h period. At intervals, determine temperature variations within the incubator when filled to maximum capacity. Install a recording thermometer whenever possible, to maintain a continuous and permanent record of temperature.

Ordinarily, a water bath with a gabled cover to reduce water and heat loss, or a solid heat sink incubator, is required to maintain a temperature of  $44.5 \pm 0.2^{\circ}\text{C}$ . If satisfactory temperature control is not achieved, provide water recirculation. Keep water depth in the incubator sufficient to immerse tubes to upper level of media.

### 2. Hot-Air Sterilizing Ovens

Use hot-air sterilizing ovens of sufficient size to prevent internal crowding; constructed to give uniform and adequate sterilizing temperatures of  $170 \pm 10^{\circ}\text{C}$ ; and equipped with suitable thermometers. Optionally use a temperature-recording instrument.

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## 3. Autoclaves

Use autoclaves of sufficient size to prevent internal crowding; constructed to provide uniform temperatures within the chambers (up to and including the sterilizing temperature of 121°C); equipped with an accurate thermometer the bulb of which is located properly on the exhaust line so as to register minimum temperature within the sterilizing chambers (temperature-recording instrument is optional); equipped with pressure gauge and properly adjusted safety valves connected directly with saturated-steam supply lines equipped with appropriate filters to remove particulates and oil droplets or directly to a suitable special steam generator (do not use steam from a boiler treated with amines for corrosion control); and capable of reaching the desired temperature within 30 min. Confirm, by chemical or toxicity tests, that the steam supply has not been treated with amines or other corrosion-control chemicals that will impart toxicity.

Use of a vertical autoclave or pressure cooker is not recommended because of difficulty in adjusting and maintaining sterilization temperature and the potential hazard. If a pressure cooker is used in emergency or special circumstances, equip it with an efficient pressure gauge and a thermometer the bulb of which is 2.5 cm above the water level.

## 4. Gas Sterilizers

Use a sterilizer equipped with automatic controls capable of carrying out a complete sterilization cycle. As a sterilizing gas use ethylene oxide (CAUTION: *Ethylene oxide is toxic—avoid inhalation, ingestion, and contact with the skin. Also, ethylene oxide forms an explosive mixture with air at 3-80% proportion.*) diluted to 10 to 12% with an inert gas. Provide an automatic control cycle to evacuate sterilizing chamber to at least 0.06 kPa, to hold the vacuum for 30 min, to adjust humidity and temperature, to charge with the ethylene oxide mixture to a pressure dependent on mixture used, to hold such pressure for at least 4 h, to vent gas, to evacuate to 0.06 kPa, and finally, to bring to atmospheric pressure with sterile air. The humidity, temperature, pressure, and time of sterilizing cycle depend on the gas mixture used.

Store overnight sample bottles with loosened caps that were sterilized by gas, to allow last traces of gas mixture to dissipate. Incubate overnight media sterilized by gas, to insure dissipation of gas.

In general, mixtures of ethylene oxide with chlorinated hydrocarbons such as freon are harmful to plastics, although at temperatures below 55°C, gas pressure not over 35 kPa, and time of sterilization less than 6 h, the effect is minimal. If carbon dioxide is used as a diluent of ethylene oxide, increase exposure time and pressure, depending on temperature and humidity that can be used.

Determine proper cycle and gas mixture for objects to be sterilized and confirm by sterility tests.

## 5. Optical Counting Equipment

a. *Pour and spread plates:* Use Quebec-type colony counter, dark-field model preferred, or

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one providing equivalent magnification (1.5 diameters) and satisfactory visibility.

*b. Membrane filters:* Use a binocular microscope with magnification of 10 to 15 $\times$ . Provide daylight fluorescent light source at angle of 60 to 80° above the colonies; use low-angle lighting for nonpigmented colonies.

*c. Mechanical tally.*

### 6. pH Equipment

Use electrometric pH meters, accurate to at least 0.1 pH units, for determining pH values of media.

### 7. Balances

Use balances providing a sensitivity of at least 0.1 g at a load of 150 g, with appropriate weights. Use an analytical balance having a sensitivity of 1 mg under a load of 10 g for weighing small quantities (less than 2 g) of materials. Single-pan rapid-weigh balances are most convenient.

### 8. Media Preparation Utensils

Use borosilicate glass or other suitable noncorrosive equipment such as stainless steel. Use glassware that is clean and free of residues, dried agar, or other foreign materials that may contaminate media.

### 9. Pipets and Graduated Cylinders

Use pipets of any convenient size, provided that they deliver the required volume accurately and quickly. The error of calibration for a given manufacturer's lot must not exceed 2.5%. Use pipets having graduations distinctly marked and with unbroken tips. Bacteriological transfer pipets or pipets conforming to APHA standards may be used. *Do not pipet by mouth; use a pipet aid.*

Use graduated cylinders meeting ASTM Standards (D-86 and D-216) and with accuracy limits established by NIST where appropriate.

### 10. Pipet Containers

Use boxes of aluminum or stainless steel, end measurement 5 to 7.5 cm, cylindrical or rectangular, and length about 40 cm. When these are not available, paper wrappings for individual pipets may be substituted. To avoid excessive charring during sterilization, use best-quality sulfate pulp (kraft) paper. *Do not use copper or copper alloy cans or boxes as pipet containers.*

### 11. Refrigerator

Use a refrigerator maintaining a temperature of 1 to 4.4°C to store samples, media, reagents, etc. Do not store volatile solvents, food, or beverages in a refrigerator with media. Frost-free refrigerators may cause excessive media dehydration on storage longer than 1 week.

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### 12. Temperature-Monitoring Devices

Use glass or metal thermometers graduated to 0.5°C to monitor most incubators and refrigerators. Use thermometers graduated to 0.1°C for incubators operated above 40°C. Use continuous recording devices that are equally sensitive. Verify accuracy by comparison with a NIST-certified thermometer, or equivalent.

### 13. Dilution Bottles or Tubes

Use bottles or tubes of resistant glass, preferably borosilicate glass, closed with glass stoppers or screw caps equipped with liners that do not produce toxic or bacteriostatic compounds on sterilization. Do not use cotton plugs as closures. Mark graduation levels indelibly on side of dilution bottle or tube. Plastic bottles of nontoxic material and acceptable size may be substituted for glass provided that they can be sterilized properly.

### 14. Petri Dishes

For the plate count, use glass or plastic petri dishes about 100 × 15 mm. Use dishes the bottoms of which are free from bubbles and scratches and flat so that the medium will be of uniform thickness throughout the plate. For the membrane filter technique use loose-lid glass or plastic dishes, 60 × 15 mm, or tight-lid dishes, 50 × 12 mm. Sterilize petri dishes and store in metal cans (aluminum or stainless steel, but not copper), or wrap in paper—preferably best-quality sulfate pulp (kraft)—before sterilizing. Presterilized petri dishes are commercially available.

### 15. Membrane Filtration Equipment

Use filter funnel and membrane holder made of seamless stainless steel, glass, or autoclavable plastic that does not leak and is not subject to corrosion. Field laboratory kits are acceptable but standard laboratory filtration equipment and procedures are required.

### 16. Fermentation Tubes and Vials

Use fermentation tubes of any type, if their design permits conforming to medium and volume requirements for concentration of nutritive ingredients as described subsequently. Where tubes are used for a test of gas production, enclose a shell vial, inverted. Use tube and vial of such size that the vial will be filled completely with medium, at least partly submerged in the tube, and large enough to make gas bubbles easily visible.

### 17. Inoculating Equipment

Use wire loops made of 22- or 24-gauge nickel alloy\*#(6) or platinum-iridium for flame sterilization. Use loops at least 3 mm in diameter. Sterilize by dry heat or steam. Single-service hardwood or plastic applicators also may be used. Make these 0.2 to 0.3 cm in diameter and at least 2.5 cm longer than the fermentation tube; sterilize by dry heat and store in glass or other nontoxic containers.

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### 18. Sample Bottles

For bacteriological samples, use sterilizable bottles of glass or plastic of any suitable size and shape. Use bottles capable of holding a sufficient volume of sample for all required tests and an adequate air space, permitting proper washing, and maintaining samples uncontaminated until examinations are completed. Ground-glass-stoppered bottles, preferably wide-mouthed and of resistant glass, are recommended. Plastic bottles of suitable size, wide-mouthed, and made of nontoxic materials such as polypropylene that can be sterilized repeatedly are satisfactory as sample containers. Presterilized plastic bags, with or without dechlorinating agent, are available commercially and may be used. Plastic containers eliminate the possibility of breakage during shipment and reduce shipping weight.

Metal or plastic screw-cap closures with liners may be used on sample bottles provided that no toxic compounds are produced on sterilization.

Before sterilization, cover tops and necks of sample bottles having glass closures with aluminium foil or heavy kraft paper.

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Dairy Products, 16th ed. American Public Health Assoc., Washington, D.C.

## 9040 WASHING AND STERILIZATION\*(7)

Cleanse all glassware thoroughly with a suitable detergent and hot water, rinse with hot water to remove all traces of residual washing compound, and finally rinse with laboratory-pure water. If mechanical glassware washers are used, equip them with influent plumbing of stainless steel or other nontoxic material. Do not use copper piping to distribute water. Use stainless steel or other nontoxic material for the rinse water system.

Sterilize glassware, except when in metal containers, for not less than 60 min at a temperature of 170°C, unless it is known from recording thermometers that oven temperatures are uniform, under which exceptional condition use 160°C. Heat glassware in metal containers to 170°C for not less than 2 h.

Sterilize sample bottles not made of plastic as above or in an autoclave at 121°C for 15 min. For plastic bottles loosen caps before autoclaving to prevent distortion.

## 9050 PREPARATION OF CULTURE MEDIA\*(8)

### 9050 A. General Procedures

#### 1. Storage of Culture Media

Store dehydrated media (powders) in tightly closed bottles in the dark at less than 30°C in an atmosphere of low humidity. Do not use them if they discolor or become caked and lose the character of a free-flowing powder. Purchase dehydrated media in small quantities that will be used within 6 months after opening. Additionally, use stocks of dehydrated media containing selective agents such as sodium azide, bile salts or derivatives, antibiotics, sulfur-containing amino acids, etc., of relatively current lot number (within a year of purchase) so as to maintain optimum selectivity. See also Section 9020.

Prepare culture media in batches that will be used in less than 1 week. However, if the media are contained in screw-capped tubes they may be stored for up to 3 months. See Table 9020:IV for specific details. Store media out of direct sun and avoid contamination and excessive evaporation.

Liquid media in fermentation tubes, if stored at refrigeration or even moderately low temperatures, may dissolve sufficient air to produce, upon incubation at 35°C, a bubble of air in the tube. Incubate fermentation tubes that have been stored at a low temperature overnight before use and discard tubes containing air.

Fermentation tubes may be stored at approximately 25°C; but because evaporation may

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proceed rapidly under these conditions—resulting in marked changes in concentration of the ingredients—do not store at this temperature for more than 1 week. Discard tubes with an evaporation loss exceeding 1 mL.

### 2. Adjustment of Reaction

State reaction of culture media in terms of hydrogen ion concentration, expressed as pH.

The decrease in pH during sterilization will vary slightly with the individual sterilizer in use, and the initial reaction required to obtain the correct final reaction will have to be determined. The decrease in pH usually will be 0.1 to 0.2 but occasionally may be as great as 0.3 in double-strength media. When buffering salts such as phosphates are present in the media, the decrease in pH value will be negligible.

Make tests to control adjustment to required pH with a pH meter. Measure pH of prepared medium as directed in Section 4500-H<sup>+</sup>. Titrate a known volume of medium with a solution of NaOH to the desired pH. Calculate amount of NaOH solution that must be added to the bulk medium to reach this reaction. After adding and mixing thoroughly, check reaction and adjust if necessary. The required final pH is given in the directions for preparing each medium. If a specific pH is not prescribed, adjustment is unnecessary.

The pH of reconstituted dehydrated media seldom will require adjustment if made according to directions. Such factors as errors in weighing dehydrated medium or overheating reconstituted medium may produce an unacceptable final pH. Measure pH, especially of rehydrated selective media, regularly to insure quality control and media specifications.

### 3. Sterilization

After rehydrating a medium, dispense promptly to culture vessels and sterilize within 2 h. Do not store nonsterile media.

Sterilize all media, except sugar broths or broths with other specifications, in an autoclave at 121°C for 15 min after the temperature has reached 121°C. When the pressure reaches zero, remove medium from autoclave and cool quickly to avoid decomposition of sugars by prolonged exposure to heat. To permit uniform heating and rapid cooling, pack materials loosely and in small containers. Sterilize sugar broths at 121°C for 12 to 15 min. The maximum elapsed time for exposure of sugar broths to any heat (from time of closing loaded autoclave to unloading) is 45 min. Preferably use a double-walled autoclave to permit preheating before loading to reduce total needed heating time to within the 45-min limit. Presterilized media may be available commercially.

### 4. Bibliography

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Manual of Clinical Microbiology, 5th ed. American Soc. Microbiology, Washington, D.C.

## 9050 B. Water

### 1. Specifications

To prepare culture media and reagents, use only distilled or demineralized reagent-grade water that has been tested and found free from traces of dissolved metals and bactericidal or inhibitory compounds. Toxicity in distilled water may be derived from fluoridated water high in silica. Other sources of toxicity are silver, lead, and various unidentified organic complexes. Where condensate return is used as feed for a still, toxic amines or other boiler compounds may be present in distilled water. Residual chlorine or chloramines also may be found in distilled water prepared from chlorinated water supplies. If chlorine compounds are found in distilled water, neutralize them by adding an equivalent amount of sodium thiosulfate or sodium sulfite.

Distilled water also should be free of contaminating nutrients. Such contamination may be derived from flashover of organics during distillation, continued use of exhausted carbon filter beds, deionizing columns in need of recharging, solder flux residues in new piping, dust and chemical fumes, and storage of water in unclean bottles. Store distilled water out of direct sunlight to prevent growth of algae and turn supplies over as rapidly as possible. Aged distilled water may contain toxic volatile organic compounds absorbed from the atmosphere if stored for prolonged periods in unsealed containers. Good housekeeping practices usually will eliminate nutrient contamination.

See Section 9020.

### 2. Bibliography

- STRAKA, R.P. & J.L. STOKES. 1957. Rapid destruction of bacteria in commonly used diluents and its elimination. *Appl. Microbiol.* 5:21.
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## 9050 C. Media Specifications

The need for uniformity dictates the use of dehydrated media. Never prepare media from basic ingredients when suitable dehydrated media are available. Follow manufacturer's directions for rehydration and sterilization. Commercially prepared media in liquid form (sterile ampule or other) also may be used if known to give equivalent results. See Section 9020 for quality-control specifications.

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The terms used for protein source in most media, for example, peptone, tryptone, tryptose, were coined by the developers of the media and may reflect commercial products rather than clearly defined entities. It is not intended to preclude the use of alternative materials provided that they produce equivalent results.

NOTE—The term “percent solution” as used in these directions is to be understood to mean “grams of solute per 100 mL solution.”

### 1. Dilution Water

*a. Buffered water:* To prepare stock phosphate buffer solution, dissolve 34.0 g potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), in 500 mL reagent-grade water, adjust to  $\text{pH } 7.2 \pm 0.5$  with 1N sodium hydroxide (NaOH), and dilute to 1 L with reagent-grade water.

Add 1.25 mL stock phosphate buffer solution and 5.0 mL magnesium chloride solution (81.1 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O/L}$  reagent-grade water) to 1 L reagent-grade water. Dispense in amounts that will provide  $99 \pm 2.0$  mL or  $9 \pm 0.2$  mL after autoclaving for 15 min.

*b. Peptone water:* Prepare a 10% solution of peptone in distilled water. Dilute a measured volume to provide a final 0.1% solution. Final pH should be 6.8.

Dispense in amounts to provide  $99 \pm 2.0$  mL or  $9 \pm 0.2$  mL after autoclaving for 15 min.

Do not suspend bacteria in any dilution water for more than 30 min at room temperature because death or multiplication may occur.

### 2. Culture Media

Specifications for individual media are included in subsequent sections. Details are provided where use of a medium first is described.

## 9060 SAMPLES\*#(9)

### 9060 A. Collection

#### 1. Containers

Collect samples for microbiological examination in nonreactive borosilicate glass or plastic bottles that have been cleansed and rinsed carefully, given a final rinse with deionized or distilled water, and sterilized as directed in Section 9030 and Section 9040. For some applications samples may be collected in presterilized plastic bags.

#### 2. Dechlorination

Add a reducing agent to containers intended for the collection of water having residual chlorine or other halogen unless they contain broth for direct planting of sample. Sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) is a satisfactory dechlorinating agent that neutralizes any residual halogen

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and prevents continuation of bactericidal action during sample transit. The examination then will indicate more accurately the true microbial content of the water at the time of sampling.

For sampling chlorinated wastewater effluents add sufficient  $\text{Na}_2\text{S}_2\text{O}_3$  to a clean sterile sample bottle to give a concentration of 100 mg/L in the sample. In a 120-mL bottle 0.1 mL of a 10% solution of  $\text{Na}_2\text{S}_2\text{O}_3$  will neutralize a sample containing about 15 mg/L residual chlorine. For drinking water samples, the concentration of dechlorinating agent may be reduced: 0.1 mL of a 3% solution of  $\text{Na}_2\text{S}_2\text{O}_3$  in a 120-mL bottle will neutralize up to 5 mg/L residual chlorine.

Cap bottle and sterilize by either dry or moist heat, as directed (Section 9040). Presterilized plastic bags or bottles containing  $\text{Na}_2\text{S}_2\text{O}_3$  are available commercially.

Collect water samples high in metals, including copper or zinc ( $>1.0$  mg/L), and wastewater samples high in heavy metals in sample bottles containing a chelating agent that will reduce metal toxicity. This is particularly significant when such samples are in transit for 4 h or more. Use 372 mg/L of the disodium salt of ethylenediaminetetraacetic acid (EDTA). Adjust EDTA solution to pH 6.5 before use. Add EDTA separately to sample bottle before bottle sterilization (0.3 mL 15% solution in a 120-mL bottle) or combine it with the  $\text{Na}_2\text{S}_2\text{O}_3$  solution before addition.

### 3. Sampling Procedures

When the sample is collected, leave ample air space in the bottle (at least 2.5 cm) to facilitate mixing by shaking, before examination. Collect samples that are representative of the water being tested, flush or disinfect sample ports, and use aseptic techniques to avoid sample contamination.

Keep sampling bottle closed until it is to be filled. Remove stopper and cap as a unit; do not contaminate inner surface of stopper or cap and neck of bottle. Fill container without rinsing, replace stopper or cap immediately, and if used, secure hood around neck of bottle.

*a. Potable water:* If the water sample is to be taken from a distribution-system tap without attachments, select a tap that is supplying water from a service pipe directly connected with the main, and is not, for example, served from a cistern or storage tank. Open tap fully and let water run to waste for 2 or 3 min, or for a time sufficient to permit clearing the service line. Reduce water flow to permit filling bottle without splashing. If tap cleanliness is questionable, choose another tap. If a questionable tap is required for special sampling purposes, disinfect the faucet (inside and outside) by applying a solution of sodium hypochlorite (100 mg  $\text{NaOCl/L}$ ) to faucet before sampling; let water run for additional 2 to 3 min after treatment. Do not sample from leaking taps that allow water to flow over the outside of the tap. In sampling from a mixing faucet remove faucet attachments such as screen or splash guard, run hot water for 2 min, then cold water for 2 to 3 min, and collect sample as indicated above.

If the sample is to be taken from a well fitted with a hand pump, pump water to waste for about 5 to 10 min or until water temperature has stabilized before collecting sample. If an outdoor sampling location must be used, avoid collecting samples from frost-proof hydrants. If

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there is no pumping machinery, collect a sample directly from the well by means of a sterilized bottle fitted with a weight at the base; take care to avoid contaminating samples by any surface scum. Other sterile sampling devices, such as a trip bailer, also may be used.

In drinking water evaluation, collect samples of finished water from distribution sites selected to assure systematic coverage during each month. Carefully choose distribution system sample locations to include dead-end sections to demonstrate bacteriological quality throughout the network and to ensure that localized contamination does not occur through cross-connections, breaks in the distribution lines, or reduction in positive pressure. Sample locations may be public sites (police and fire stations, government office buildings, schools, bus and train stations, airports, community parks), commercial establishments (restaurants, gas stations, office buildings, industrial plants), private residences (single residences, apartment buildings, and townhouse complexes), and special sampling stations built into the distribution network. Preferably avoid outdoor taps, fire hydrants, water treatment units, and backflow prevention devices. Establish sampling program in consultation with state and local health authorities.

*b. Raw water supply:* In collecting samples directly from a river, stream, lake, reservoir, spring, or shallow well, obtain samples representative of the water that is the source of supply to consumers. It is undesirable to take samples too near the bank or too far from the point of drawoff, or at a depth above or below the point of drawoff.

*c. Surface waters:* Stream studies may be short-term, high-intensity efforts. Select bacteriological sampling locations to include a baseline location upstream from the study area, industrial and municipal waste outfalls into the main stream study area, tributaries except those with a flow less than 10% of the main stream, intake points for municipal or industrial water facilities, downstream samples based on stream flow time, and downstream recreational areas. Dispersion of wastewaters into the receiving stream may necessitate preliminary cross-section studies to determine completeness of mixing. Where a tributary stream is involved, select the sampling point near the confluence with the main stream. Samples may be collected from a boat or from bridges near critical study points. Choose sampling frequency to be reflective of changing stream or water body conditions. For example, to evaluate waste discharges, sample every 4 to 6 h and advance the time over a 7- to 10-d period.

To monitor stream and lake water quality establish sampling locations at critical sites. Sampling frequency may be seasonal for recreational waters, daily for water supply intakes, hourly where waste treatment control is erratic and effluents are discharged into shellfish harvesting areas, or even continuous.

*d. Bathing beaches:* Sampling locations for recreational areas should reflect water quality within the entire recreational zone. Include sites from upstream peripheral areas and locations adjacent to drains or natural contours that would discharge stormwater collections or septic wastes. Collect samples in the swimming area from a uniform depth of approximately 1 m. Consider sediment sampling of the water-beach (soil) interface because of exposure of young children at the water's edge.

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To obtain baseline data on marine and estuarine bathing water quality include sampling at low, high, and ebb tides.

Relate sampling frequency directly to the peak bathing period, which generally occurs in the afternoon. Preferably, collect daily samples during the recognized bathing season; as a minimum include Friday, Saturday, Sunday, and holidays. When limiting sampling to days of peak recreational use, preferably collect a sample in the morning and the afternoon. Correlate bacteriological data with turbidity levels or rainfall over the watershed to make rapid assessment of water quality changes.

*e. Sediments and biosolids:* The bacteriology of bottom sediments is important in water supply reservoirs, in lakes, rivers, and coastal waters used for recreational purposes, and in shellfish-growing waters. Sediments may provide a stable index of the general quality of the overlying water, particularly where there is great variability in its bacteriological quality.

Sampling frequency in reservoirs and lakes may be related more to seasonal changes in water temperatures and stormwater runoff. Bottom sediment changes in river and estuarine waters may be more erratic, being influenced by stormwater runoff, increased flow velocities, and sudden changes in the quality of effluent discharges.

Microbiological examination of biosolids from water and wastewater treatment processes is desirable to determine the impact of their disposal into receiving waters, ocean dumping, land application, or burial in landfill operations.

Collect and handle biosolids with less than 7% total solids using the procedures discussed for other water samples. Biosolids with more than 7% solids and exhibiting a “plastic” consistency or “semisolid” state typical of thickened sludges require a finite shear stress to cause them to flow. This resistance to flow results in heterogeneous distribution of biosolids in tanks and lagoons. Use cross-section sampling of accumulated biosolids to determine distribution of organisms within these impoundments. Establish a length-width grid across the top of the impoundment, and sample at intercepts. A thief sampler that samples only the solids layer may be useful. Alternatively use weighted bottle samplers that can be opened up at a desired depth to collect samples at specific locations.

Processed biosolids having no free liquids are best sampled when they are being transferred. Collect grab samples across the entire width of the conveyor and combine into a composite sample. If solids are stored in piles, classification occurs. Exteriors of uncovered piles are subject to various environmental stresses such as precipitation, wind, fugitive dusts, and fecal contamination from scavengers. Consequently, surface samples may not reflect the microbiological quality of the pile. Therefore, use cross-section sampling of these piles to determine the degree of heterogeneity within the pile. Establish a length-width grid across the top of the pile, and sample intercepts. Sample augers and corers may prove to be ineffective for sampling piles of variable composition. In such cases use hand shovels to remove overburden.

*f. Nonpotable samples (manual sampling):* Take samples from a river, stream, lake, or reservoir by holding the bottle near its base in the hand and plunging it, neck downward, below

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the surface. Turn bottle until neck points slightly upward and mouth is directed toward the current. If there is no current, as in the case of a reservoir, create a current artificially by pushing bottle forward horizontally in a direction away from the hand. When sampling from a boat, obtain samples from upstream side of boat. If it is not possible to collect samples from these situations in this way, attach a weight to base of bottle and lower it into the water. In any case, take care to avoid contact with bank or stream bed; otherwise, water fouling may occur.

*g. Sampling apparatus:* Special apparatus that permits mechanical removal of bottle stopper below water surface is required to collect samples from depths of a lake or reservoir. Various types of deep sampling devices are available. The most common is the ZoBell J-Z sampler,<sup>1</sup> which uses a sterile 350-mL bottle and a rubber stopper through which a piece of glass tubing has been passed. This tubing is connected to another piece of glass tubing by a rubber connecting hose. The unit is mounted on a metal frame containing a cable and a messenger. When the messenger is released, it strikes the glass tubing at a point that has been slightly weakened by a file mark. The glass tube is broken by the messenger and the tension set up by the rubber connecting hose is released and the tubing swings to the side. Water is sucked into the bottle as a consequence of the partial vacuum created by sealing the unit at time of autoclaving. Commercial adaptations of this sampler and others are available.

Bottom sediment sampling also requires special apparatus. The sampler described by Van Donsel and Geldreich<sup>2</sup> has been found effective for a variety of bottom materials for remote (deep water) or hand (shallow water) sampling. Construct this sampler preferably of stainless steel and fit with a sterile plastic bag. A nylon cord closes the bag after the sampler penetrates the sediment. A slide bar keeps the bag closed during descent and is opened, thereby opening the bag, during sediment sampling.

For sampling wastewaters or effluents the techniques described above generally are adequate; in addition see Section 1060.

### 4. Size of Sample

The volume of sample should be sufficient to carry out all tests required, preferably not less than 100 mL.

### 5. Identifying Data

Accompany samples by complete and accurate identifying and descriptive data. Do not accept for examination inadequately identified samples.

### 6. References

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## 7. Bibliography

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- U.S. ENVIRONMENTAL PROTECTION AGENCY. 1992. Environmental Regulations and Technology Control of Pathogens and Vector Attraction in Sewage Sludge. EPA-625/R-92-013. Washington, D.C.

## 9060 B. Preservation and Storage

### 1. Holding Time and Temperature

*a. General:* Start microbiological analysis of water samples as soon as possible after collection to avoid unpredictable changes in the microbial population. For most accurate results, ice samples during transport to the laboratory, if they cannot be processed within 1 h after collection. If the results may be used in legal action, employ special means (rapid transport, express mail, courier service, etc.) to deliver the samples to the laboratory within the specified time limits and maintain chain of custody. Follow the guidelines and requirements given below for specific water types.

*b. Drinking water for compliance purposes:* Preferably hold samples at <10°C during transit to the laboratory. Analyze samples on day of receipt whenever possible and refrigerate overnight if arrival is too late for processing on same day. Do not exceed 30 h holding time from collection to analysis for coliform bacteria. Do not exceed 8 h holding time for heterotrophic plate counts.

*c. Nonpotable water for compliance purposes:* Hold source water, stream pollution, recreational water, and wastewater samples below 10°C during a maximum transport time of 6 h. Refrigerate these samples upon receipt in the laboratory and process within 2 h. When transport conditions necessitate delays in delivery of samples longer than 6 h, consider using either field laboratory facilities located at the site of collection or delayed incubation procedures.

*d. Other water types for noncompliance purposes:* Hold samples below 10°C during transport and until time of analysis. Do not exceed 24 h holding time.

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- PUBLIC HEALTH LABORATORY SERVICE WATER SUB-COMMITTEE. 1952. The effect of storage on the coliform and *Bacterium coli* counts of water samples. Overnight storage at room and refrigerator temperatures. *J. Hyg.* 50:107.
- PUBLIC HEALTH LABORATORY SERVICE WATER SUB-COMMITTEE. 1953. The effect of storage on the coliform and *Bacterium coli* counts of water samples. Storage for six hours at room and refrigerator temperatures. *J. Hyg.* 51:559.
- MCCARTHY, J.A. 1957. Storage of water sample for bacteriological examinations. *Amer. J. Pub. Health* 47:971.
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## 9213 RECREATIONAL WATERS\*#(10)

### 9213 A. Introduction

#### 1. Microbiological Indicators

Recreational waters include freshwater swimming pools, whirlpools, and naturally occurring fresh and marine waters. Many local and state health departments require microbiological monitoring of recreational waters. Historically, the most common microbiological tests to assess sanitary quality have been heterotrophic counts and total and fecal coliform tests. Total coliform tests and heterotrophic counts usually are performed on treated waters and fecal coliform tests performed on untreated waters. Although detection of coliform bacteria in water indicates that it may be unsafe to drink, other bacteria have been isolated from recreational waters that may suggest health risks through body contact, ingestion, or inhalation. Other bacteria suggested as indicators of recreational water quality include *Pseudomonas aeruginosa*, fecal streptococci,

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enterococci, and staphylococci. Ideally, recreational water quality indicators are microorganisms for which densities in the water can be related quantitatively to potential health hazards resulting from recreational use, particularly where upper body orifices are exposed to water. The ideal indicator is the one with the best correlation between density and the health hazards associated with a given type of pollution. The most common potential sources of infectious agents in recreational waters include untreated or poorly treated municipal and industrial effluents or sludge, sanitary wastes from seaside residences, fecal wastes from pleasure craft, drainage from sanitary landfills, stormwater runoff, and excretions from animals. In addition, the source of infectious agents may be the aquatic environment itself. The potential health hazards from each of these sources are not equal. Exposure to untreated or inadequately treated human fecal wastes is considered the greatest health hazard. The presence of microbiological indicators in treated swimming pools or whirlpools indicate possible insufficient water exchange, disinfection, and maintenance. Bather density is a major factor in determining the probability of swimmer-associated illnesses with swimming pools, particularly when there is insufficient disinfection and water circulation. The bathers themselves may be the source of pollution by shedding organisms associated with the mouth, nose, and skin.

### 2. Infectious Diseases from Water Exposure

In general, infections or disease associated with recreational water contact fall into two categories. The first group is gastroenteritis resulting from unintentional ingestion of water contaminated with fecal wastes. Enteric microorganisms that have been shown to cause gastroenteritis from recreational water contact include *Giardia*, *Cryptosporidium*, *Shigella*, *Salmonella*, *E. coli* 0157:H7, Hepatitis A, Coxsackie A and B, and Norwalk virus. Leptospirosis is not an enteric infection but also is transmitted through contact with waters contaminated with human or animal wastes. The second group or category of infections or disease is associated mainly with microorganisms that are indigenous to the environment, which include the following: *Pseudomonas aeruginosa*, *Staphylococcus* sp., *Legionella* sp., *Naegleria fowleri*, *Mycobacterium* sp., and *Vibrio* sp. The illnesses or waterborne diseases caused by these organisms include dermatitis or folliculitis, otitis externa, Pontiac fever, granulomas, primary amebic meningoencephalitis (PAM), and conjunctivitis. Commonly occurring illnesses or infections associated with recreational water contact are dermatitis caused by *Pseudomonas aeruginosa* and otitis externa, “swimmer’s ear,” frequently caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

### 3. Microbiological Monitoring Limitations

Routine examination for pathogenic microorganisms is not recommended except for investigations of water-related illness and special studies; in such cases, focus microbiological analyses on the known or suspected pathogen. Methods for several of these pathogens are given in Section 9260, Detection of Pathogenic Bacteria, Section 9510, Detection of Enteric Viruses, and Section 9711, Pathogenic Protozoa. Because some pathogenic organisms such as *Giardia*, *Cryptosporidium*, *Mycobacterium*, and *Naegleria* are more resistant to changes in environmental

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conditions than indicator bacteria, routine monitoring may not always reflect the risk of infection from these organisms. Described below are recommended methods for microbial indicators of recreational water quality. Consider the type(s) of water examined in selecting the microbiological method(s) or indicator(s) to be used. No single procedure is adequate to isolate all microorganisms from contaminated water. While bacterial indicators may not adequately reflect risk of viral, fungal, or parasitic infection from recreational waters, available technology limits monitoring for such organisms in routine laboratory operations.

### 4. Bibliography

- CABELLI, V.J. 1977. Indicators of recreational water quality. *In* Bacterial Indicators/Health Hazards Associated with Waters. STP 635, American Soc. Testing & Materials, Philadelphia, Pa.
- DUFOUR, A.P. 1986. Diseases caused by water contact. *In* Waterborne Diseases in the United States. CRC Press Inc., Boca Raton, Fla.
- MOE, C.L. 1996. Waterborne transmission of infectious agents. *In* Manual of Environmental Microbiology. American Soc. Microbiology, ASM Press, Washington, D.C.

## 9213 D. Natural Bathing Beaches

### 1. General Discussion

*a. Characteristics:* A natural bathing beach is any area of a stream, lake, ocean, impoundment, or hot spring that is used for recreation. A wide variety of pathogenic microorganisms can be transmitted to humans through use of natural fresh and marine recreational waters contaminated by wastewater.<sup>1,2</sup> These include enteric pathogens such as *Salmonella*, *Shigella*, enteroviruses, protozoa, multicellular parasites, and “opportunists” such as *P. aeruginosa*, *Klebsiella* sp., *Vibrio* sp., and *Aeromonas hydrophila*, which can multiply in recreational waters with sufficient nutrients. Other organisms of concern are those associated with the skin, mouth, or nose of bathers, such as *Staphylococcus aureus* and other organisms, e.g., nontuberculous mycobacteria and leptospira, and *Naegleria* sp..<sup>3-9</sup>

*b. Monitoring requirements:* Historically, fecal coliforms have been recommended as the indicator of choice for evaluating the microbiological quality of recreational waters. Many states have adopted use of this indicator in their water quality standards. Recent studies have demonstrated that *E. coli* and enterococci showed a stronger correlation with swimming-associated gastroenteritis than do fecal coliforms, and that both indicators were equally acceptable for monitoring fresh-water quality. For marine water, enterococci showed the strongest relationship of density to gastroenteritis. The recommended densities of these indicator organisms were calculated to approximate the degree of protection previously accepted for fecal coliforms. EPA-recommended water quality criteria are based on these findings.<sup>10</sup> While the primary indicators of water quality are *E. coli* and enterococci, the enumeration of *P. aeruginosa*,

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*Aeromonas hydrophila*, and *Klebsiella* sp. in recreational waters may be useful in cases of discharge of pulp and paper wastes and effluents from textile finishing plants into receiving waters.

### 2. Samples

a. *Containers*: Collect samples as directed in Section 9060A. The size of the container varies with the number and variety of tests to be performed. Adding  $\text{Na}_2\text{S}_2\text{O}_3$  to the bottle is unnecessary.

b. *Sampling procedure*: Collect samples 0.3 m below the water surface in the areas of greatest bather load. Take samples over the range of environmental and climatic conditions, especially during times when maximal pollution can be expected, i.e., periods of tidal, current, and wind influences, stormwater runoff, wastewater treatment bypasses. See Section 9213B.2b for methods of sample collection and Section 9222 for suggested sample volumes.

c. *Sample storage*: See Section 9060B.

### 3. Tests for *Escherichia coli*

a. *Media*:

1) mTEC agar:\*(11)

Proteose peptone	5.0	g
Yeast extract	3.0	g
Lactose	10.0	g
Sodium chloride, NaCl	7.5	g
Dipotassium phosphate, $\text{K}_2\text{HPO}_4$	3.3	g
Monopotassium phosphate $\text{KH}_2\text{PO}_4$	1.0	g
Sodium lauryl sulfate	0.2	g
Sodium desoxycholate	0.1	g
Bromcresol purple	0.08	g
Bromphenol red	0.08	g
Agar	15.0	g
Reagent-grade water	1	L

Sterilize by autoclaving; pH should be  $7.3 \pm 0.2$ . Pour 4 to 5 mL liquefied agar into culture dishes ( $50 \times 10$  mm). Store in refrigerator.

2) Urea substrate:\* #(12)

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Urea	2.0 g
Phenol red	10 mg
Reagent-grade water	100 mL

Adjust pH to between 3 and 4. Store at 2 to 8°C. Use within 1 week.

*b. Procedure:* Filter sample through a membrane filter (see Section 9222), place membrane on mTEC agar, incubate at  $35 \pm 0.5^\circ\text{C}$  for 2 h to rejuvenate injured or stressed bacteria, and then incubate at  $44.5 \pm 0.2^\circ\text{C}$  for 22 h. Transfer filter to a filter pad saturated with urea substrate. After 15 min, count yellow or yellow-brown colonies, using a fluorescent lamp and a magnifying lens. *E. coli* produces yellow or yellow-brown colonies. Verify a portion of these differentiated colonies with a commercial multi-test system [see Section 9222B.5 f2)b)].

#### 4. Tests for Enterococci

Perform tests for enterococci by the multiple-tube technique (Section 9230B) or membrane filter technique (Section 9230C).

#### 5. Tests for *Pseudomonas aeruginosa*

Perform tests for *P. aeruginosa* as directed in Section 9213E and Section 9213F. Use the multiple-tube test with samples but note that the procedures may not be applicable to marine samples.

#### 6. Tests for *Salmonella/Shigella*

See Section 9260.

#### 7. References

1. CABELLI, V.J. 1980. Health Effects Criteria for Marine Recreational Waters. EPA-600/1-80-031, U.S. Environmental Protection Agency, Research Triangle Park, N.C.
2. DUFOUR, A.P. 1984. Health Effects Criteria for Fresh Recreational Waters. EPA-600/1-84-004, U.S. Environmental Protection Agency, Research Triangle Park, N.C.
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5. CABELLI, V.J., H. KENNEDY & M.A. LEVIN. 1976. *Pseudomonas aeruginosa* and fresh recreational waters. *J. Water Pollut. Control Fed.* 48: 367.

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10. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1986. Ambient Water Quality Criteria for Bacteria—1986. EPA-440/5-84-002, U.S. Environmental Protection Agency, Washington, D.C.

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- RICE, E.W., T.C. COVERT, D.K. WILD, D. BERMAN, S.A. JOHNSON & C.H. JOHNSON. 1993. Comparative resistance of *Escherichia coli* and *Enterococci* to chlorination. *J. Environ. Health.* A28:89.

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## Endnotes

### **1 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1993.

### **2 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1997.

### **3 (Popup - Footnote)**

\* Fisher Scientific, short wave meter (Cat. No. 11-924-54) and long wave meter (Cat. No. 11-984-53), Pittsburgh, PA 15219-4785, or equivalent.

### **4 (Popup - Footnote)**

† 3M Health Care, St. Paul, MN 55144, or equivalent.

### **5 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1993.

### **6 (Popup - Footnote)**

\* Chromel, nichrome, or equivalent.

### **7 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1993.

### **8 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1993.

### **9 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1997.

### **10 (Popup - Footnote)**

\* APPROVED BY STANDARD METHODS COMMITTEE, 1997.

### **11 (Popup - Footnote)**

\* Difco or equivalent.

### **12 (Popup - Footnote)**

\* Difco or equivalent.