

Part 9000	Microbiological Examination	Part Coordinators	Margo E. Hunt and Eugene W. Rice
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The following is a list of the contents of the book. The book is divided into two main parts. The first part is the "General Microbiology" section, which covers the basic principles of microbiology. The second part is the "Specialized Microbiology" section, which covers the application of microbiology to various fields. The book is written in a clear and concise style, and it is suitable for use as a textbook or a reference work. The book is published by the American Society for Microbiology, and it is available in both print and electronic formats. The book is a valuable resource for anyone interested in microbiology.

PART 9000

MICROBIOLOGICAL EXAMINATION

Part Coordinators
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9213 Recreational Waters	Terry C. Covert
9215 Heterotrophic Plate Count	Sandra D. Canning
9218 Aerobic Endospores	Clifford H. Johnson
9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group	Mark C. Meckes, Eugene W. Rice
9222 Membrane Filter Technique for Members of the Coliform Group	Nancy H. Hall
9223 Enzyme Substrate Coliform Test	Carol Palmer
9224 Detection of Coliphages	Ronald E. Stetler, Fred P. Williams, Jr.
9225 Differentiation of the Coliform Bacteria	Eugene W. Rice
9240 Iron and Sulfur Bacteria	Richard F. Unz
9245 Nitrifying Bacteria	Nancy I. Lieu
9260 Detection of Pathogenic Bacteria	Nelson P. Moyer
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SUMMARY OF MAJOR CHANGES SINCE 1998

Material on coliphage detection from Rapid Detection Methods (9211) was expanded into a new section, Detection of Coliphages (9224). Other new sections are Aerobic Endospores (9218) and Nitrifying Bacteria (9245).

New tables for the reading and recording of Most Probable Number were added to Multiple-Tube Fermentation Technique for Members of the Coliform Group (9221). *Escherichia coli* tests using the GAD reagent and indole production also were added. New procedures for bacteria living in acidic environments were added to Iron and Sulfur Bacteria (9240) along with several new figures. The section on Pathogenic Protozoa (9711) has been updated to include an overview of methods for the detection and identification of *Giardia*, *Cryptosporidium*, and *Acanthamoeba*.

9010 INTRODUCTION*

The following sections describe procedures for making microbiological examinations of various types of water samples. The methods describe analyses for compliance monitoring and research studies of ambient water, drinking water, and wastewater. They are the best techniques currently available; however, their limitations must be thoroughly understood.

Tests for detection and enumeration of indicator organisms and of pathogens are presented. The coliform group of bacteria, as herein defined, is one of the principal indicators of the 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, in particular fecal coliforms or *Escherichia coli*, 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.

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 coliform bacteria, particularly fecal coliforms or *E. coli*, 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°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 colonization 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°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, procedures for enteric viruses are included but their routine use is not advocated. However, these methods are useful in investigations of waterborne disease and in studies designed to characterize watersheds or for determining groundwater quality.

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 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

* Approved by Standard Methods Committee, 2000.

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 in-

creased 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*

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 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 section 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.

* Approved by Standard Methods Committee, 1997.

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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, 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 off by the laboratory manager and QA officer.

Detailed descriptions of quality assurance programs are available.¹⁻⁴

The QC guidelines discussed in 9020B and 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.¹⁻³ 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.

TABLE 9020:I. KEY QUALITY CONTROL PRACTICES

Item	Action	Frequency	Further Information in Section 9020B.¶
Reagent water	Monitor quality		See Table 9020:II
Bench surface	Monitor for contamination	Weekly	2e
Air in workplace	Monitor bacterial density	Monthly	2e
Thermometers	Check accuracy	Semiannually	3a
Balances and weights	Check accuracy	Monthly	3b
Balances	Service and recalibrate	Annually	3b
pH meter	Standardize	Each use	3c
	Check against another meter	Monthly	3c
Media-dispensing apparatus	Check volume accuracy	Each use	3f
Hot-air oven	Check performance	Monthly	3g
Autoclave	Check performance	Monthly	3h, 4i2)
Refrigerator	Check temperature	Daily	3i
Freezer	Check temperature	Daily	3j
	Defrost	Semiannually	3j
Membrane filtration equipment	Check for leaks and surface scratches	Each use	3k
UV lamps	Test with UV meter	Quarterly	3l
Biohazard hood	Monitor air and UV lamps	Monthly	3m
	Inspect for airflow	Quarterly	3m
Incubator	Check temperature	Twice daily	3n and o
Microscope	Clean optics and stage	Each use	3p
Glassware	Inspect for cleanliness, chips, and etching	Each use	4a
	Check pH	Each batch	4a1)
	Conduct inhibitory residue test	Annually	4a2)
Dilution water bottles	Check pH and volume	Each use	4c
Media	Check pH and appearance	Each use	4i1)
Plate counts	Perform duplicate analyses	Weekly	8a4)
	Repeat counts	Monthly	8a2)

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 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/m²/15 min exposure (15 colonies/plate/15 min).

Plate or the swab method¹ 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 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.⁴ 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⁺.

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- μ 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 (NH₃). On storage, additional NH₃ and CO₂ 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* 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.⁵ 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 hood

* 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.

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°C (60 to 80°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 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. Sterile 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 e above. Recommended limits for reagent water quality are given in Table 9020:II. If these limits are not 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

TABLE 9020:II. QUALITY OF REAGENT WATER USED IN MICROBIOLOGY TESTING

Test	Monitoring Frequency	Maximum Acceptable Limit
Chemical tests:		
Conductivity	Continuously or with each use	>0.5 megohms resistance or <2 μ mhos/cm at 25°C
pH	With each use	5.5–7.5
Total organic carbon	Monthly	<1.0 mg/L
Heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn)	Annually*	<0.05 mg/L
Heavy metals, total	Annually*	<0.10 mg/L
Ammonia/organic nitrogen	Monthly	<0.10 mg/L
Total chlorine residual	Monthly or with each use	<0.01 mg/L
Bacteriological tests:		
Heterotrophic plate count (See Section 9215)	Monthly	< 1000 CFU/mL
Use test (see 4e)	Quarterly and for a new source	Student's $t \leq 2.78$

*Or more frequently if there is a problem.

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 Sections 9215 and 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 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 μ 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- μ 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/cm² 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 me-

dium; 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 1×10^3 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 ± 0.2 mL Endo broth.

h) Pads release less than 1 mg total acidity calculated as 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.⁶ 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.⁷

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

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 1N 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 9020B.3d 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 multi-point 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

TABLE 9020:III. TIME AND TEMPERATURE FOR AUTOCLAVE STERILIZATION

Material	Time at 121°C*
Membrane filters and pads	10 min
Carbohydrate-containing media (lauryl tryptose, BGB broth, etc.)	12–15 min
Contaminated materials and discarded cultures	30 min
Membrane filter assemblies (wrapped), sample collection bottles (empty)	15 min
Buffered dilution water, 99 mL in screw-cap bottle	15 min
Rinse water, volume > 100 mL	Adjust for volume

*Except for media, times are guidelines; check for sterility.

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.

Check effectiveness of sterilization monthly 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.†

Sterilize heat-sensitive solutions or media by filtration through a 0.22- μ 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

TABLE 9020:IV. HOLDING TIMES FOR PREPARED MEDIA

Medium	Holding Time
Membrane filter (MF) broth in screw-cap flasks at 4°C	96 h
MF agar in plates with tight-fitting covers at 4°C	2 weeks
Agar or broth in loose-cap tubes at 4°C	2 weeks
Agar or broth in tightly closed screw-cap tubes or other sealed containers	3 months
Poured agar plates with loose-fitting covers in sealed plastic bags at 4°C	2 weeks
Large volume of agar in tightly closed screw-cap flask or bottle at 4°C	3 months

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 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

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

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 multilaboratory 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.

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 9020B.11b 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.

TABLE 9020:V. CONTROL CULTURES FOR MICROBIOLOGICAL TESTS

Group	Control Culture	
	Positive	Negative
Total coliforms	<i>Escherichia coli</i> <i>Enterobacter aerogenes</i>	<i>Staphylococcus aureus</i> <i>Pseudomonas</i> sp.
Fecal coliforms	<i>E. coli</i>	<i>E. aerogenes</i> <i>Streptococcus faecalis</i>
<i>Escherichia coli</i>	<i>E. coli</i>	<i>E. aerogenes</i>
Fecal streptococci	<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i> <i>E. coli</i>
Enterococci	<i>S. faecalis</i>	<i>S. mitis/salivarius</i>

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 ($\bar{R}$) of these 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 $3.27 \bar{R}$, 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.

TABLE 9020:VI. CALCULATION OF PRECISION CRITERION

Sample No.	Duplicate Analyses		Logarithms of Counts		Range of Logarithms ($R_{\log}$) ($L_1 - L_2$)
	D_1	D_2	L_1	L_2	
1	89	71	1.9494	1.8513	0.0981
2	38	34	1.5798	1.5315	0.0483
3	58	67	1.7634	1.8261	0.0627
...	...	...	...	...	...
14	7	6	0.8451	0.7782	0.0669
15	110	121	2.0414	2.0828	0.0414

Calculations:

$$1) \sum R_{\log} = 0.0981 + 0.0483 + 0.0627 + \dots + 0.0669 + 0.0414 = 0.71889$$

$$2) \bar{R} = \frac{\sum R_{\log}}{n} = \frac{0.71889}{15} = 0.0479$$

$$3) \text{Precision criterion} = 3.27 \bar{R} = 3.27 (0.0479) = 0.1566$$

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 (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*) (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 β -D-galactopyranosidase by the *o*-nitrophenyl-

β -D-galactopyranoside (ONPG) test and indophenol by the cytochrome oxidase (CO) test. See 9225D for these tests. Coliforms are ONPG-positive and cytochrome-oxidase-negative. Verify *E. coli* using the EC MUG test (see 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:

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 9222B.5f. 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 9222B.5f. Adjust counts based on percent verification.

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

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 9221B.1 and 9221E. Adjust counts based on percent verification.

b) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in 9221B.3 and 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 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 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 9230C. Adjust counts based on percentage of verification.

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

TABLE 9020:VII. DAILY CHECKS ON PRECISION OF DUPLICATE COUNTS*

Date of Analysis	Duplicate Analyses		Logarithms of Counts		Range of Logarithms	Acceptance of Range†
	D_1	D_2	L_1	L_2		
8/29	71	65	1.8513	1.8129	0.0383	A
8/30	110	121	2.0414	2.0828	0.0414	A
8/31	73	50	1.8633	1.6990	0.1643	U

* Precision criterion = $(3.27\bar{R}) = 0.1566$.

† A = acceptable; U = unacceptable.

10. 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, 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.¹

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.

11. 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

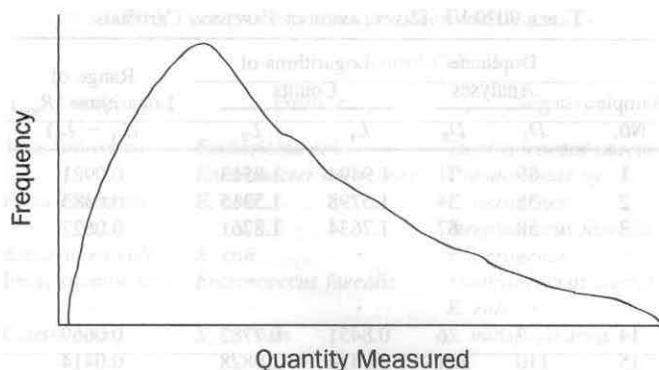


Figure 9020:1. Frequency curve (positively skewed distribution).

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.

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

TABLE 9020:VIII. COLIFORM COUNTS AND THEIR LOGARITHMS

MPN Coliform Count No./100 mL	log MPN
11	1.041
27	1.431
36	1.556
48	1.681
80	1.903
85	1.929
120	2.079
130	2.114
136	2.134
161	2.207
317	2.501
601	2.779
760	2.881
1020	3.009
3100	3.491
$\bar{x} = 442$	$\bar{x}_g = \text{antilog } 2.1825 = 152$

TABLE 9020:IX. COMPARISON OF FREQUENCY OF MPN DATA

Class Interval	Frequency (MPN)
0 to 400	11
400 to 800	2
800 to 1200	1
1200 to 1600	0
1600 to 2000	0
2000 to 2400	0
2400 to 2800	0
2800 to 3200	0

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.⁸

TABLE 9020:X. COMPARISON OF FREQUENCY OF LOG MPN DATA

Class Interval	Frequency (log MPN)
1.000 to 1.300	1
1.300 to 1.600	2
1.600 to 1.900	1
1.900 to 2.200	5
2.200 to 2.500	1
2.500 to 2.800	2
2.800 to 3.100	2
3.100 to 3.400	0
3.400 to 3.700	1

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, 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.

12. References

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4. Gas Sterilizers

Use a sterilizer equipped with automatic controls capable of carrying out a complete sterilization cycle. As a sterilizing gas

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 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:¹ 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; and 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. Reference

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*

9030 A. Introduction

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

* Approved by Standard Methods Committee, 2000.

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.

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 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.

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 $\times$ 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 $\times$ 15 mm, or tight-lid dishes, 50 $\times$ 12 mm. Sterilize glass 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* 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.

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,

* Chromel, nichrome, or equivalent.

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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9040 WASHING AND STERILIZATION*

Cleanse all glassware and plasticware thoroughly with a suitable detergent and hot water, rinse with hot water to remove all traces of residual washing compound, and finally rinse with reagent-grade water. Sterilize all contaminated laboratory ware before cleaning. If mechanical glassware washers are used, equip them with influent plumbing of stainless steel or other nontoxic material. Ensure that the spray from water jets reaches all parts of deep vessels. Do not use copper piping to distribute water. Use stainless steel or other nontoxic material for the rinse-water system.

Sterilize glassware, except when it is in metal containers, by dry heat 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. Glass or plastic sample bottles may be sterilized in an autoclave at 121°C for 15 min. Moisture present in bottles after autoclaving may be removed by placing bottles in a drying oven at 100°C for 10 to 15 min. For plastic bottles loosen caps before autoclaving to prevent distortion.

* Approved by Standard Methods Committee, 2000.

9050 PREPARATION OF CULTURE MEDIA*

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 2 weeks. 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 proceed rapidly under these conditions—resulting in marked changes in concentration of the ingredients—do not store at this temperature for more than 2 weeks. Discard tubes with growth due to contamination or 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.

* Approved by Standard Methods Committee, 2000.

Make tests to control adjustment to required pH with a pH meter. Measure pH of prepared medium as directed in Section 4500-H⁺. 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.

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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. Storage at room temperature should not exceed 1 month. Replace flexible dispensing hoses every 6 to 9 months. Aged distilled water may contain toxic volatile organic compounds absorbed from the atmosphere if stored for prolonged periods in unsealed containers. Good housekeeping practices that minimize the presence of airborne particulates usually will eliminate nutrient contamination.

See Section 9020.

2. Bibliography

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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 commercially prepared 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.

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 (KH_2PO_4) in 500 mL reagent-grade water, adjust to pH 7.2 ± 0.5 with 1N

sodium hydroxide (NaOH), and dilute to 1 L with reagent-grade water. Discard turbid stock solutions.

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 ± 2.0 mL or 9 ± 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 ± 2.0 mL or 9 ± 0.2 mL after autoclaving for 15 min.

Do not suspend sample dilutions 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*

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 Sections 9030 and 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 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 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 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 town-house 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.

* Approved by Standard Methods Committee, 1997.

Joint Task Group: 20th Edition—Eugene W. Rice (chair), Robert H. Bordner, Terry C. Covert, Charles P. Gerba, Nancy H. Hall, Walter Jakubowski, Louis A. Kaplan, Gordon A. McFeters, Nelson P. Moyer.

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 storm-water 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.

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 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,¹ 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 Geldreich² 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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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 at <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 at <10°C during transport and until time of analysis. Do not exceed 24 h holding time.

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9211 RAPID DETECTION METHODS*

9211 A. Introduction

There is a generally recognized need for methods that permit rapid estimation of the bacteriological quality of water. Applications of rapid methods may range from analysis of wastewater to potable water quality assessment. In the latter case, during emergencies involving water treatment plant failure, line breaks in a distribution network, or other disruptions to water supply

caused by disasters, there is urgent need for rapid assessment of the sanitary quality of water.

Ideally, rapid procedures would be reliable and have sensitivity levels equal to those of the standard tests routinely used. However, sensitivity of a rapid test may be compromised because the bacterial limit sought may be below the minimum bacterial concentration essential to rapid detection. Rapid tests fall into two categories, those involving modified conventional procedures and those requiring special instrumentation and materials.

* Approved by Standard Methods Committee, 2000.

9211 B. Seven-Hour Fecal Coliform Test

This method^{1,2} is similar to the fecal coliform membrane filter procedure (see Section 9222D) but uses a different medium and incubation temperature to yield results in 7 h that generally are comparable to those obtained by the standard fecal coliform method.

Heat in boiling water bath. After ingredients are dissolved, heat additional 5 min. Cool to 55 to 60°C and adjust pH to 7.3 $\pm$ 0.1 with 0.1N NaOH (0.35 mL/L usually required). Cool to about 45°C and dispense in 4- to 5-mL quantities to petri plates with tight-fitting covers. Store at 2 to 10°C. Discard after 30 d.

1. Medium

M-7 h FC agar: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract.....	3.0 g
Lactose	10.0 g
<i>d</i> -Mannitol	5.0 g
Sodium chloride, NaCl	7.5 g
Sodium lauryl sulfate	0.2 g
Sodium desoxycholate	0.1 g
Bromocresol purple	0.35 g
Phenol red	0.3 g
Agar	15.0 g
Reagent-grade water	1 L

2. Procedure

Filter an appropriate sample volume through a membrane filter, place filter on the surface of a plate containing M-7 h FC agar medium, and incubate at 41.5°C for 7 h. Fecal coliform colonies are yellow (indicative of lactose fermentation).

3. References

1. VAN DONSEL, D.J., R.M. TWEED & E.E. GELDREICH. 1969. Optimum temperature for quantitation of fecal coliforms in seven hours on the membrane filter. *Bacteriol. Proc. Abs.* No. G46, p. 25.
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9211 C. Special Techniques

Special rapid techniques are summarized in Table 9211:I. Most are not sensitive enough for potable water quality measurement or are not specific. They may be useful in monitoring wastewater effluents and natural waters but require reagents not generally available, are tedious, or require special handling or incubation schemes incompatible with most water laboratory schedules. Except for the colorimetric test, none are suitable for routine use but they may be used as research tools. The user

should refer to the literature citations for the technique listed in the table for procedural details, conditions for use, and method limitations. Only the adenosine triphosphate (ATP) procedure (the firefly bioluminescence system), the colorimetric test to estimate total microbial density, and a radiometric fecal coliform procedure that uses a ¹⁴C-labeled substrate can be recommended.

Correlate initial concentration of bacteria with ATP concentration by extracting ATP from serial dilutions of a bacterial

TABLE 9211:I. SPECIAL RAPID TECHNIQUES

Microbial Group	Rapid Method	Test Time h	Sensitivity cells/mL	Reference
Nonspecific microflora	Bioluminescence	1	100 000	1-3
	Chemiluminescence	1	500 000	3-5
	Impedance	3-12	100 000	6-9
	Colorimetric	0.02	10 000	10
	Epifluorescence/fluorometric	<1-several	—	11-13
Fecal coliforms	Radiometric	4-5	2-20	14
	Glutamate decarboxylase	10-13	0.01-500 000	15-17
	Electrochemical	1-7	1 000 000	18-20
	Impedance	6-12	200-100 000	6-9
	Gas chromatographic assay	9-12	>50	21
	Colorimetric	8-20	5-130 000	22
	Potentiometric	3.5-15	0.1->10 000 000	23
Gram-negative bacteria	Limulus assay	2	500-3000	24-27
	Fluorescent antibody	2-3	—	28-30

suspension, or for the ^{14}C radiometric method, standardize by determining the $^{14}\text{CO}_2$ released by known concentrations of fecal coliform organisms in natural samples, not pure cultures. In using any rapid procedure, determine the initial bacterial density by using an appropriate procedure such as heterotrophic plate count (Section 9215) or total (9221) or fecal (9222) coliforms, and correlate with results from the special rapid technique.

1. Bioluminescence Test (Total Viable Microbial Measurement)

The firefly luciferase test for ATP in living cells is based on the reaction between the luciferase enzyme, luciferin (enzyme substrate), magnesium ions, and ATP. Light is emitted during the reaction and can be measured quantitatively and correlated with the quantity of ATP extracted from known numbers of bacteria. When all reactants except ATP are in excess, ATP is the limiting factor. Addition of ATP drives the reactions, producing a pulse of light that is proportional to the ATP concentration.

The assay is completed in less than 1 h.¹⁻³ For monitoring microbial populations in water, the ATP assay is limited primarily by the need to concentrate bacteria from the sample to achieve the minimum ATP sensitivity level, which is 10^5 cells/mL. When combined with membrane filtration of a 1-L sample, ATP assay can provide the sensitivity level needed.

2. Radiometric Detection (Fecal Coliforms)

In this test, $^{14}\text{CO}_2$ is released from a ^{14}C -labeled substrate.¹⁴ The technique permits presumptive detection of as few as 2 to 20 fecal coliform bacteria in 4.5 h. The test uses M-FC broth, uniformly labeled ^{14}C -mannitol, and two-temperature incubation; 2 h at 35°C followed by 2.5 h at 44.5°C for fecal coliform specificity. Add labeled substrate at start of 44.5°C incubation. Use membrane filtration to concentrate organisms from sample and place membrane filter in M-FC broth in a sealable container. The $^{14}\text{CO}_2$ released is trapped by exposure to $\text{Ba}(\text{OH})_2$ -saturated filter paper disk. ^{14}C activity is assayed by liquid scintillation

spectrometry. Except for the use of the ^{14}C -mannitol substrate and liquid scintillation spectrometry to count the activity of the $^{14}\text{CO}_2$ released by the fecal coliforms, this procedure is similar to that given in Section 9222.

3. References

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9212 STRESSED ORGANISMS*

9212 A. Introduction

1. General Discussion

Indicator bacteria, including total coliforms, fecal coliforms, and fecal streptococci, may become stressed or injured in waters and wastewaters. These injured bacteria are incapable of growth and colony formation under standard conditions because of structural or metabolic damage. As a result, a substantial portion of the indicator bacteria present, i.e., 10 to greater than 90%, may not be detected.^{1,2} These false negative bacteriological findings could result in an inaccurate definition of water quality and lead to the acceptance of a potentially hazardous condition resulting from contamination by resistant pathogens³ or the penetration of undetected indicator bacteria through treatment barriers.⁴

Stressed organisms are present under ordinary circumstances in treated drinking water and wastewater effluents, saline waters, polluted natural waters, and relatively clean surface waters. High numbers of injured indicator bacteria may be associated with

partial or inadequate disinfection and the presence of metal ions or other toxic substances. These and other factors, including extremes of temperature and pH and solar radiation, may lead collectively to significant underestimations of the number of viable indicator bacteria.

Publications support the health significance of injured coliform bacteria.^{2,5-7} These reports show that enteropathogenic bacteria are less susceptible than coliforms to injury under conditions similar to those in treated drinking water and wastewater, that injured pathogens retain the potential for virulence, and that they recover after being ingested. Hence, methods allowing for the enumeration of injured coliform bacteria yield more sensitive determinations of potential health risks. This conclusion is further supported by the observation that viruses and waterborne pathogens that form cysts also are more resistant than indicator bacteria to environmental stressors.

2. Sample Handling and Collection

Certain laboratory manipulations following sample collection also may produce injury or act as a secondary stress to the

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organisms.^{2,8} These include excessive sample storage time, prolonged holding time (more than 30 min) of diluted samples before inoculation into growth media and of inoculated samples before incubation at the proper temperature, incorrect media formulations, incomplete mixing of sample with concentrated medium, and exposure to untempered liquefied agar media. Excessive numbers of nonindicator bacteria also interfere with detection of indicators by causing injury.⁹

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9212 B. Recovery Enhancement

This section describes some general procedures and considerations regarding recovery of stressed indicator organisms.

For chlorinated samples, insure that sufficient dechlorinating agent is present in the sample bottle (see Section 9060A.2).¹ Collect water samples with elevated concentrations of heavy-metal ions in a sample bottle containing a chelating agent² (see Section 9060A.2) and minimize sample storage time (see Section 9060B). Use buffered peptone dilution water rather than buffered water (see Section 9050C.1) when preparing dilutions of samples containing heavy-metal ions. After making dilutions, inoculate test media within 30 min.

Resuscitation of stressed or injured organisms is enhanced by inoculating samples and initially culturing organisms in an enriched, noninhibitory medium at a moderate temperature.

Although no simple test is available to establish the presence of injured bacteria in a given sample, bacteria in water known to contain stressors such as disinfectants or heavy metals frequently will be injured.^{1,3} When multiple-tube fermentation test results consistently are higher than those obtained from parallel membrane filter tests, or there is other indication of suboptimal

recovery, consider injury probable and use one or more of the following procedures.

1. Recovery of Injured Total Coliform Bacteria Using Membrane Filtration

a. m-T7 agar: Use m-T7 agar⁴ in the procedure described for the membrane filter test (see Section 9222B).

Proteose peptone No. 3.....	5.0 g
Yeast extract.....	3.0 g
Lactose.....	20.0 g
Tergitol 7.....	0.4 mL
Polyoxyethylene ether W1.....	5.0 g
Bromthymol blue.....	0.1 g
Bromocresol purple.....	0.1 g
Agar.....	15.0 g
Reagent-grade water.....	1 L

Adjust to pH 7.4 with 0.1N NaOH after sterilization at 121°C for 15 min. Aseptically add 1.0 µg penicillin G/mL when medium has cooled to about 45°C.

After filtering sample place filter on m-T7 agar and incubate at 35°C for 22 to 24 h. Coliform colonies are yellow. Verify not less than 10% of coliform colonies by the procedure in Section 9222B.5f. With some drinking water samples containing many non-coliform bacteria, confluent growth occurs. To obtain reliable results, carefully distinguish target yellow colonies from background growth.

b. Addition of sodium sulfite: The addition of sodium sulfite to some media (0.05 to 0.1%) can improve the detection of coliform bacteria following exposure to chloramine but not chlorine.⁵ Such modified medium is applicable to clean-water systems using chloramination or to chlorinated discharges such as wastewater effluent containing high levels of organic compounds.

2. Recovery of Injured Fecal Coliform Bacteria Using Membrane Filtration

a. Enrichment-temperature acclimation: Use two-layer agar (M-FC agar with a nonselective overlay medium that does not contain glucose, i.e., tryptic soy agar) with a 2-h incubation at 35°C followed by 22 h at 44.5°C.⁶ Prepare the M-FC agar plate in advance but do not add the overlay agar more than 1 h before use.

Alternatively, use a pre-enrichment in phenol red lactose broth incubated at 35°C for 4 h followed by M-FC agar at 44.5°C for 22 h.⁷

As a third option, prepare enrichment two-layer medium containing specific additives and incubate for 1.5 h at room temperature (22 to 26°C) followed by 35°C for 4.5 h and 44.5°C for 18 h.⁸

b. Temperature acclimation:⁹ Modify elevated temperature procedure by preincubation of M-FC cultures for 5 h at 35°C, followed by 18 ± 1 h at 44.5°C. Use a commercially available temperature-programmed incubator to make the change from 35 to 44.5°C after the 5 h preincubation period to eliminate inconvenience and provide a practical method of analysis.

c. Deletion of suppressive agent:¹⁰ Eliminate rosolic acid from M-FC medium and incubate cultures at 44.5°C ± 0.2°C for 24 h. Fecal coliform colonies are intense blue on the modified medium and are distinguished from the cream, gray, and pale-green colonies typically produced by nonfecal coliforms.

d. Alternative medium-temperature acclimation: Use m-T7 medium with an 8 h incubation at 37°C followed by 12 h at 44.5°C.¹¹

e. Verification of stressed fecal coliform bacteria: Modifications of media and procedures may decrease selectivity and differentiation of fecal coliform colonies. Therefore, if any procedural modifications are used, verify not less than 10% of the blue colonies from a variety of samples. Use lauryl tryptose broth (Section 9221B) (35°C for 48 h) with transfer of gas-

producing cultures to EC broth (Section 9221E) (44.5°C for 24 h). Gas production at 44.5°C confirms the presence of fecal coliforms.

3. Recovery of Stressed Fecal Streptococci Using Membrane Filtration

Using bile broth medium yields fecal streptococcus recoveries comparable with multiple-tube fermentation tests.¹² Preincubate membrane filters on an enrichment medium for 2 h at 35°C and follow by plating on m-Enterococcus agar (Section 9230) for 48 ± 2 h at 35°C.

Verification of stressed fecal streptococci—Verify not less than 10% of the colonies from a variety of samples using the confirmed test procedure given in Section 9230B.3.

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9213 RECREATIONAL WATERS*

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, 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 contam-

inated with fecal wastes. Enteric microorganisms that have been shown to cause gastroenteritis from recreational water contact include *Giardia*, *Cryptosporidium*, *Shigella*, *Salmonella*, *E. coli* O157: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 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.

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9213 B. Swimming Pools

1. General Discussion

a. *Characteristics*: A swimming pool is a body of water of limited size contained in a holding structure.¹ The pool water generally is potable and treated with additional disinfectant but also may come from thermal springs or salt water. Modern pools have a recirculating system for filtration and disinfection.

b. *Monitoring requirements*:

1) General—Monitor water quality in pools for changes in chemical and physical characteristics that may result in irritation to the bather's skin, eyes, and mucosal barriers or may adversely affect disinfection. Microorganisms of concern typically are those from the bather's body and its orifices and include those causing infections of the eye, ear, upper respiratory tract, skin, and intestinal or genitourinary tracts. Water quality depends on the efficacy of disinfection, sanitary conditions, the number of bathers in the pool at any one time, and the total number of bathers per day.

2) Disinfected indoor pools—Swimming pools should be disinfected continuously when in use. Test swimming pool water for residual chlorine and pH when the pool is initially opened and at least three times/d. Collect samples from at least two locations for these determinations. Evaluate clarity of the swimming pool water before opening for the day and during periods of heavy usage.² The heterotrophic plate count is the primary indicator of disinfection efficacy. Indicators of health risk include normal skin flora that are shed, such as *Pseudomonas* and *Staphylococcus*.³⁻⁶ These organisms account for a large percentage of swimming-pool-associated illnesses. In special circumstances *Mycobacterium*, *Legionella*, or *Candida albicans* may be associated with health risks related to recreational waters. Take samples for microbiological examination while the pool is in use. APHA recommends for public swimming pools that not more than 15% of the samples collected during any 30-d period shall have a heterotrophic plate count of 200/mL or show a positive confirmed total coliform test in any of five 10-mL portions of sample examined with the multiple-tube fermentation test or more than 1 total coliform/50 mL when the membrane filter test is used. Whenever swimming pool samples are examined for total staphylococci or *Staphylococcus aureus*, not more than 50 organisms/100 mL should be present.²

3) Disinfected outdoor pools—Fecal coliform bacteria and *Pseudomonas* species are the primary indicators of contamination from animal pets, rodents, stormwater runoff, and human sources. Supporting indicators include coliform bacteria, the heterotrophic plate count, and staphylococci.

4) Untreated pools—The primary indicator may be fecal coliform bacteria. Supporting indicators are those described for disinfected pools. Untreated pools are not recommended for recreational use due to increased health risks.

2. Samples

a. *Containers*: Collect samples for bacteriological examination of swimming pool water as directed in Section 9060A. Use containers with capacities of 120 to 480 mL, depending on analyses to be made. Add sufficient sodium thiosulfate,

$\text{Na}_2\text{S}_2\text{O}_3$, to the sample to provide a concentration of approximately 100 mg/L in the sample. Do this by adding 0.1 mL of 10% solution of $\text{Na}_2\text{S}_2\text{O}_3$ to a 120-mL bottle or 0.4 mL to a 480-mL bottle. After adding $\text{Na}_2\text{S}_2\text{O}_3$, stopper or cap and sterilize container.

b. *Sampling procedure*: Collect samples during periods of maximum bather load. Information on number of bathers may be helpful in subsequent interpretation of laboratory results. Use sampling frequency consistent with state and local health regulations.

Collect samples by carefully removing cap of a sterile sample bottle and holding bottle near the base at an angle of 45 deg. Fill in one slow sweep down through the water, with the mouth of the bottle always ahead of the hand. Avoid contamination of the sample by floating debris. Replace cap. Do not rinse bottle (i.e., retain sodium thiosulfate). For pools equipped with a filter, samples may be collected from sampling cocks provided in the return and discharge lines from the filter.

Most bacteria shed by bathers are in body oils, saliva, and mucus discharges that occur near the surface; collect additional samples of the surface microlayer from the area in 1-m-deep water. Collect microlayer samples by plunging a sterile glass plate (approximately 20 cm by 20 cm) vertically through the water surface and withdrawing it upward at a rate of approximately 6 cm/s. Remove surface film and water layer adhering to both sides of plate with a sterile silicone rubber scraper and collect in a sterile glass bottle. Repeat until desired volume is obtained. To minimize microbial contamination, wrap glass plate and scraper in metal foil and sterilize by autoclaving before use. Wear sterile rubber or plastic gloves during sampling or hold glass plate with forceps, clips, or tongs.

Determine residual chlorine or other disinfectant at poolside at the time of sample collection (see Section 4500-Cl.G). Residual disinfectant levels, chemical, and physical quality of pool water should be consistent with local, state, or APHA standards. The permissible bathing load should adhere to local, state, or APHA-recommended regulations.

c. *Sample storage*: Analyze microbiological samples as soon as possible after collection (see Section 9060B).

d. *Sample volume*: See Section 9222B.5.

e. *Sample dilution*: If sample dilutions are required, use 0.1% peptone water or buffered dilution water as diluent to optimize recovery of stressed organisms (see Section 9222 for suggested sample volume). Because peptone water has a tendency to foam, avoid including air bubbles when pipetting to assure accurate measure.

3. Heterotrophic Plate Count

Determine the heterotrophic plate count as directed in Section 9215. Use at least two plates per dilution.

4. Tests for Total Coliforms

Determine total coliform bacteria as directed in Sections 9221, 9222, or 9223.

5. Tests for Fecal Coliforms

Test for fecal coliforms according to the multiple-tube fermentation technique (Section 9221), the membrane filter technique (Section 9222), or rapid methods (Section 9211).

6. Test for Staphylococci or *Staphylococcus aureus*

a. Baird-Parker agar base:

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Reagent-grade water	1 L

Sterilize by autoclaving. Cool to 50°C and aseptically add 50 mL commercial egg yolk tellurite enrichment/L. Mix well. Final pH should be 7.0 ± 0.2.

b. Procedure: Use membrane filter technique to prepare samples. Place membrane filter on Baird-Parker agar and incubate at 35 ± 0.5°C for 48 ± 4 h. Staphylococci typically form slate gray to jet black, smooth, entire colonies. If *S. aureus* is present egg yolk clearing may be observed if the membrane filter is raised from the medium. Verify some differentiated colonies with a commercial multi-test system or on the basis of such key characteristics as catalase reaction, coagulase production, aerobic and anaerobic acid production from certain carbohydrates, and typical microscopic morphology.

7. Test for *Staphylococcus aureus*

Use a modified multiple-tube procedure.

a. Media:

1) *M-staphylococcus* broth:

Tryptone	10.0 g
Yeast extract	2.0 g
Lactose	2.0 g
Mannitol	10.0 g
Dipotassium hydrogen phosphate, K ₂ HPO ₄	5.0 g
Sodium chloride, NaCl	75.0 g
Sodium azide, NaN ₃	0.049 g
Reagent-grade water	1 L

Sterilize by boiling for 4 min; pH should be 7.0 ± 0.2. For 10-mL inocula prepare and use double-strength medium.

2) *Lipovitellenin-salt-mannitol* agar: This medium may not be available in dehydrated form and may require preparation from the basic ingredients or by addition of egg yolk to a dehydrated base.

Beef extract	1.0 g
Polypeptone	10.0 g
Sodium chloride, NaCl	75.0 g
d-Mannitol	10.0 g
Agar	15.0 g
Phenol red	0.025 g

Egg yolk	20.0 g
Reagent-grade water	1 L

Sterilize by autoclaving; pH should be 7.4 ± 0.2.

b. Procedure: Inoculate tubes of *M-staphylococcus* broth as directed in Section 9221. Incubate at 35 ± 1°C for 24 h. Hold original enrichment sample but streak from positive (turbid) tubes on plates of lipovitellenin-salt-mannitol agar and incubate at 35 ± 1°C for 48 h. Opaque (24 h), yellow (48 h) zones around the colonies are positive evidence of lipovitellenin-lipase activity (opaque) and mannitol fermentation (yellow).

If the plate is negative, streak another plate from the original enrichment tube before discarding. Lipovitellenin-lipase activity has a 95% positive correlation with coagulase production. If necessary, confirm positive isolates as catalase-positive, coagulase-positive, fermenting mannitol, fermenting glucose anaerobically, yielding typical microscopic morphology, and gram-positive.

8. Tests for *Pseudomonas aeruginosa*

Tests for *P. aeruginosa* are presented in Sections 9213E and F and include a membrane filter procedure and a multiple-tube technique.

9. Test for Streptococci or Enterococci

Determine fecal streptococci or enterococci as described in Section 9230, and, if necessary, perform additional biochemical tests to identify species.

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9213 C. Whirlpools

1. General Discussion

a. Characteristics: A whirlpool is a shallow pool with a maximum water depth of 1.2 m; it has a closed-cycle water system, a heated water supply, and usually a hydrojet recirculation system. It may be constructed of plastic, fiberglass, redwood, or epoxy-lined surfaces. Whirlpools are designed for recreational as well as therapeutic use and may accommodate one or more bathers. These pools usually are not cleaned, drained, and refilled after each use. They are located in homes, apartments, hotels, athletic facilities, rehabilitation centers, and hospitals.

b. Monitoring requirements: Whirlpool-associated infections are common because of the inherent design and characteristics of whirlpools, which include high temperature, reduced disinfection efficacy, and increased organic material. All these factors contribute to favorable conditions for growth of microorganisms, especially *Pseudomonas aeruginosa*. Studies have shown that whirlpools can serve as a reservoir of *Legionella pneumophila*. Therefore, frequent testing for residual disinfectant levels and pH, along with scheduled maintenance, is necessary for safe whirlpool water quality.¹⁻⁵

c. Microbiological indicators: The primary indicator of disinfection efficacy is *P. aeruginosa*, with total coliforms, heterotrophic plate count, and staphylococci as supporting indicators of water quality. The standard index of water quality, i.e., total coliforms, may be insufficient to judge the microbiological quality of whirlpool water. *Pseudomonas aeruginosa* is frequently

isolated from whirlpool water that is coliform-negative.⁶ In the event of a whirlpool-associated outbreak, collect samples as close as possible to the time of the outbreak. Analyze for the suspected pathogen and *P. aeruginosa*. Methods for *P. aeruginosa* are described in Sections 9213E and 9213F.

d. Sample preservation: Examine samples as soon as possible after collection. See Section 9060B.

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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.^{1,2} 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.³⁻⁹

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.¹⁰ While the primary indicators of water quality are *E. coli* and enterococci, the enumeration of *P. aeruginosa*, *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:*

Proteose peptone	5.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Sodium chloride, NaCl	7.5 g
Dipotassium phosphate, K_2HPO_4	3.3 g
Monopotassium phosphate, KH_2PO_4	1.0 g
Sodium lauryl sulfate	0.2 g
Sodium desoxycholate	0.1 g
Bromocresol 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 ± 0.2 . Pour 4 to 5 mL liquefied agar into culture dishes (50×10 mm). Store in refrigerator.

2) Urea substrate:*

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(2)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 Sections 9213E and F. 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.

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* Difco or equivalent.

9213 E. Membrane Filter Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus

See Section 9222B.1.

2. Culture Media

a. M-PA agar: This agar may not be available in dehydrated form and may require preparation from the basic ingredients.

L-lysine HCl.....	5.0 g
Sodium chloride, NaCl.....	5.0 g
Yeast extract.....	2.0 g
Xylose.....	2.5 g
Sucrose.....	1.25 g
Lactose.....	1.25 g
Phenol red.....	0.08 g
Ferric ammonium citrate.....	0.8 g
Sodium thiosulfate, Na ₂ S ₂ O ₃	6.8 g
Agar.....	15.0 g
Reagent-grade water.....	1 L

Adjust to pH 6.5 ± 0.1 and sterilize by autoclaving. Cool to 55°C ; readjust to pH 7.1 ± 0.2 and add the following dry antibiotics per liter of agar base: sulfapyridine,* 176 mg; kanamycin,* 8.5 mg; nalidixic acid,* 37.0 mg; and cycloheximide,* 150 mg. After mixing dispense in 3-mL quantities in 50- × 12-mm petri plates. Store poured plates at 2 to 8°C . Discard unused medium after 1 month.

b. Modified M-PA agar.†

c. Milk agar (Brown and Scott Foster Modification):

Mixture A:

Instant nonfat milk‡.....	100 g
Reagent-grade water.....	500 mL

Mixture B:

Nutrient broth.....	12.5 g
Sodium chloride, NaCl.....	2.5 g
Agar.....	15.0 g
Reagent-grade water.....	500 mL

* Sigma Chemical Co., St. Louis, MO, or equivalent.

† Commercially available as M-PA-C agar. Contains magnesium, sulfate, kanamycin, and nalidixic acid.

‡ Carnation or equivalent.

Separately prepare and sterilize Mixtures A and B; cool rapidly to 55°C ; aseptically combine mixtures and dispense 20 to 25 mL per petri dish.

3. Procedure

a. Presumptive tests: Filter 200 mL or less of natural waters or up to 500 mL of swimming pool waters through sterile membrane filters. Place each membrane on a poured plate of modified M-PA agar so that there is no air space between the membrane and the agar surface. Invert plates and incubate at $41.5 \pm 0.5^\circ\text{C}$ for 72 h.

Typically, *P. aeruginosa* colonies are 0.8 to 2.2 mm in diameter and flat in appearance with light outer rims and brownish to greenish-black centers. Count typical colonies, preferably from filters containing 20 to 80 colonies. Use a 10- to 15-power magnifier as an aid in colony counting.

b. Confirmation tests: Use milk agar to confirm a number of typical and atypical colonies. Make a single streak (2 to 4 cm long) from an isolated colony on a milk agar plate and incubate at $35 \pm 1.0^\circ\text{C}$ for 24 h. *P. aeruginosa* hydrolyzes casein and produces a yellowish to green diffusible pigment.

4. Interpretation and Calculation of Density

Confirmation is not routinely required. In the absence of confirmation, report results as the number of presumptive *P. aeruginosa*/100 mL.

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9213 F. Multiple-Tube Technique for *Pseudomonas aeruginosa*

1. Laboratory Apparatus

See Section 9221.

2. Culture Media

a. Asparagine broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Asparagine, DL.....	3.0 g
Anhydrous dipotassium hydrogen phosphate, K ₂ HPO ₄	1.0 g
Magnesium sulfate, MgSO ₄ ·7H ₂ O.....	0.5 g
Reagent-grade water.....	1 L

Adjust pH to 6.9 to 7.2 before sterilization.

b. Acetamide broth: This medium may not be available in dehydrated form and may require preparation from the basic ingredients.

Acetamide	10.0 g
Sodium chloride, NaCl	5.0 g
Anhydrous dipotassium hydrogen phosphate, K_2HPO_4	1.39 g
Anhydrous potassium dihydrogen phosphate, KH_2PO_4	0.73 g
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$	0.5 g

Dissolve 1.2 g phenol red in 100 mL 0.01N NaOH and add 1 mL/L of acetamide broth. Use phenol red stock solution within 1 year. Adjust pH to 7.1 to 7.3 before sterilization. Final pH should be 7.0 ± 0.2 . Prepare acetamide broth as described above. If agar slants are preferred, prepare as described above but add 15 g agar/L, heat to dissolve agar, and dispense 8-mL quantities in 16-mm tubes. After autoclaving, incline tubes while cooling to provide a large slant surface.

3. Procedure

a. Presumptive test: Perform a five-tube multiple-tube test. Use 10 mL single-strength asparagine broth for inocula of 1 mL

or less and 10 mL double-strength asparagine broth for 10-mL inocula. For swimming pools, higher dilutions may be necessary. Incubate inoculated tubes at 35 to 37°C. After 24 h and again after 48 h of incubation, examine tubes under long-wave ultraviolet light (black light) in a darkened room. Production of a green fluorescent pigment constitutes a positive presumptive test.

b. Confirmed test: Confirm positive tubes by inoculating 0.1 mL of culture into acetamide broth or onto the surface of acetamide agar slants. Development of purple color (alkaline pH) within 24 to 36 h of incubation at 35 to 37°C is a positive confirmed test for *Pseudomonas aeruginosa*.

c. Computing and reporting results: Refer to Table 9221:IV and to Section 9221D.

9215 HETEROTROPHIC PLATE COUNT*

9215 A. Introduction

1. Applications

The heterotrophic plate count (HPC), formerly known as the standard plate count, is a procedure for estimating the number of live heterotrophic bacteria in water and measuring changes during water treatment and distribution or in swimming pools. Colonies may arise from pairs, chains, clusters, or single cells, all of which are included in the term "colony-forming units" (CFU). The final count also depends on interaction among the developing colonies; choose that combination of procedure and medium that produces the greatest number of colonies within the designated incubation time. To compare data, use the same procedure and medium. Three different methods and four different media are described.

2. Selection of Method

a. Pour plate method: The pour plate method (9215B) is simple to perform and can accommodate volumes of sample or diluted sample ranging from 0.1 to 2.0 mL. The colonies produced are relatively small and compact, showing less tendency to encroach on each other than those produced by surface growth. On the other hand, submerged colonies often are slower growing and are difficult to transfer. A thermostatically controlled water bath is essential for tempering the agar. Heat shock to bacteria

from the transient exposure of the sample to 45 to 46°C agar may occur.

b. Spread plate method: The spread plate method (9215C) causes no heat shock and all colonies are on the agar surface where they can be distinguished readily from particles and bubbles. Colonies can be transferred quickly, and colony morphology easily can be discerned and compared to published descriptions. However, this method is limited by the small volume of sample or diluted sample that can be absorbed by the agar: 0.1 to 0.5 mL, depending on the degree to which the prepoored plates have been dried. To use this procedure, maintain a supply of suitable predried, absorbent agar plates.

c. Membrane filter method: The membrane filter method (9215D) permits testing large volumes of low-turbidity water and is the method of choice for low-count waters (< 1 to 10 CFU/mL). This method produces no heat shock but adds the expense of the membrane filter. Further disadvantages include the smaller display area, the need to detect colonies by reflected light against a white background if colored filters or contrast stains are not used, possible damage to cells by excessive filtration pressures, and possible variations in membrane filter quality (see Section 9020B.4h).

3. Work Area

Provide a level table or bench top with ample area in a clean, draft-free, well-lighted room or within a horizontal-flow laminar hood. Use table and bench tops having nonporous surfaces and disinfect before any analysis is made.

* Approved by Standard Methods Committee, 2000.
Joint Task Group: 20th Edition—Sandra D. Canning (chair), Kathryn M. Craig, Ellen P. Flanagan, Timothy I. Ladd, Mark W. LeChevallier, Ansar A. Qureshi, Donald J. Reasoner, Peggy A. Roefer, Theodore J. Williams, Roy L. Wolfe.

4. Samples

Collect water as directed in Section 9060A. Initiate analysis as soon as possible after collection to minimize changes in bacterial population. The recommended maximum elapsed time between collection and analysis of samples is 8 h (maximum transit time 6 h, maximum processing time 2 h). When analysis cannot begin within 8 h, maintain sample at a temperature below 4°C but do not freeze. Maximum elapsed time between collection and analysis must not exceed 24 h.

5. Sample Preparation

Mark each plate with sample number, dilution, date, and any other necessary information before examination. Prepare at least duplicate plates for each volume of sample or dilution examined. For the pour or spread plate methods use sterile glass (65 cm²) or presterilized disposable plastic (57 cm²) petri dishes.

Thoroughly mix all samples or dilutions by rapidly making about 25 complete up-and-down (or back-and-forth) movements. Optionally, use a mechanical-vortex shaker to shake samples or dilutions for 15 s.

6. Media

Compare new lots of media with current lot in use according to Section 9020B.4i.

a. Plate count agar (tryptone glucose yeast agar): Use for pour and spread plate methods. This high-nutrient agar, widely used in the past, gives lower counts than R2A or NWRI agar. It is included for laboratories wishing to make comparisons of media or to extend the continuity of old data.

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Reagent-grade water	1 L

pH should be 7.0 ± 0.2 after autoclaving at 121°C for 15 min.

b. m-HPC agar:† Use this high-nutrient medium only for the membrane filter method.

Peptone	20.0 g
Gelatin	25.0 g
Glycerol	10.0 mL
Agar	15.0 g
Reagent-grade water	1 L

Mix all ingredients except glycerol. Adjust pH to 7.1, if necessary, with 1N NaOH, heat slowly to boiling to dissolve thoroughly, add glycerol, and autoclave at 121°C for 5 min.‡

c. R2A agar: Use for pour, spread plate, and membrane filter methods. This low-nutrient agar gives higher counts than high-nutrient formulations.

Yeast extract	0.5 g
Proteose peptone No. 3 or polypeptone	0.5 g

† Formerly called m-SPC agar.

‡ This medium may not be sterile; use with care to avoid contamination.

Casamino acids	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Dipotassium hydrogen phosphate, K ₂ HPO ₄	0.3 g
Magnesium sulfate heptahydrate, MgSO ₄ ·7H ₂ O	0.05 g
Sodium pyruvate	0.3 g
Agar	15.0 g
Reagent-grade water	1 L

Adjust pH to 7.2 with solid K₂HPO₄ or KH₂PO₄ before adding agar. Heat to dissolve agar and sterilize at 121°C for 15 min.

d. NWRI agar (HPCA): Use for pour, spread plate, and membrane filter methods. This low-nutrient medium is likely to produce higher colony counts than high-nutrient media. It is not currently available in dehydrated form and requires preparation from the basic ingredients; this makes its usage less desirable.

Peptone	3.0 g
Soluble casein	0.5 g
K ₂ HPO ₄	0.2 g
MgSO ₄	0.05 g
FeCl ₃	0.001 g
Agar	15.0 g
Reagent-grade water	1 L

Adjust pH to 7.2 before autoclaving for 15 min at 121°C.

7. Incubation

For compliance monitoring purposes under U.S. EPA's Surface Water Treatment Rule (40 CFR 141.74), provision on heterotrophic bacteria, incubate pour plates at 35°C for 48 h. Otherwise, select from among recommended times and temperatures for monitoring changes in water quality. The highest counts typically will be obtained from 5- to 7-d incubation at a temperature of 20 to 28°C.

During incubation maintain humidity within the incubator so that plates will have no moisture weight loss greater than 15%. This is especially important if prolonged incubation is used. A pan of water placed at the bottom of the incubator may be sufficient but note that to prevent rusting or oxidation the inside walls and shelving should be of high-grade stainless steel or anodized aluminum. For long incubation in nonhumidified incubators, seal plates in plastic bags.

8. Counting and Recording

a. Pour and spread plates: Count all colonies on selected plates promptly after incubation. If counting must be delayed temporarily, store plates at 5 to 10°C for no more than 24 h, but avoid this as routine practice. Record results of sterility controls on the report for each lot of samples.

Use an approved counting aid, such as the Quebec colony counter, for manual counting. If such equipment is not available, count with any other counter provided that it gives equivalent magnification and illumination. Automatic plate counting instruments are available. These generally use a television scanner coupled to a magnifying lens and an electronics package. Their use is acceptable if evaluation in parallel with manual counting gives comparable results.

In preparing plates, pipet sample volumes that will yield from 30 to 300 colonies/plate. The aim is to have at least one dilution giving colony counts between these limits, except as provided below.

Ordinarily, do not pipet more than 2.0 mL of sample; however, when the total number of colonies developing from 2.0 mL is less than 30, disregard the rule above and record result observed. With this exception, consider only plates having 30 to 300 colonies in determining the plate count. Compute bacterial count per milliliter by the following equation:

$$\text{CFU/mL} = \frac{\text{colonies counted}}{\text{actual volume of sample in dish, mL}}$$

If there is no plate with 30 to 300 colonies, and one or more plates have more than 300 colonies, use the plate(s) having a count nearest 300 colonies. Compute the count as above and report as estimated CFU per milliliter.

If plates from all dilutions of any sample have no colonies, report the count as less than one (< 1) divided by the corresponding largest sample volume used. For example, if no colonies develop from the 0.01-mL sample volume, report the count as less than 100 (< 100) estimated CFU/mL.

If the number of colonies per plate far exceeds 300, do not report result as "too numerous to count" (TNTC). If there are fewer than 10 colonies/cm², count colonies in 13 squares (of the colony counter) having representative colony distribution. If possible, select seven consecutive squares horizontally across the plate and six consecutive squares vertically, being careful not to count a square more than once. Multiply sum of the number of colonies in 13 representative square centimeters by 5 to compute estimated colonies per plate when the plate area is 65 cm². When there are more than 10 colonies/cm², count four representative squares, take average count per square centimeter, and multiply by the appropriate factor to estimate colonies per plate. The factor is 57 for disposable plastic plates and 65 for glass plates. When bacterial counts on crowded plates are greater than 100 colonies/cm², report result as greater than ($>$) 6500 divided by the smallest sample volume plated for glass plates or greater than ($>$) 5700 divided by the smallest sample volume plated for plastic plates. Report as estimated colony-forming units per milliliter.

If spreading colonies (spreaders) are encountered on the plate(s) selected, count colonies on representative portions only when colonies are well distributed in spreader-free areas and the area covered by the spreader(s) does not exceed one-half the plate area.

When spreading colonies must be counted, count each of the following types as one: a chain of colonies that appears to be caused by disintegration of a bacterial clump as agar and sample were mixed; a spreader that develops as a film of growth between the agar and bottom of petri dish; and a colony that forms in a film of water at the edge or over the agar surface. The last two types largely develop because of an accumulation of moisture at the point from which the spreader originates. They frequently cover more than half the plate and interfere with obtaining a reliable plate count.

Count as individual colonies similar-appearing colonies growing in close proximity but not touching, provided that the dis-

tance between them is at least equal to the diameter of the smallest colony. Count impinging colonies that differ in appearance, such as morphology or color, as individual colonies.

If plates have excessive spreader growth, report as "spreaders" (Spr). When plates are uncountable because of missed dilution, accidental dropping, and contamination, or the control plates indicate that the medium or other material or labware was contaminated, report as "laboratory accident" (LA).

b. Membrane filter method: Count colonies on membrane filters using a stereoscopic microscope at 10 to 15 $\times$ magnification. Preferably slant petri dish at 45° angle on microscope stage and adjust light source vertical to the colonies. Optimal colony density per filter is 20 to 200. If colonies are small and there is no crowding, a higher limit is acceptable.

Count all colonies on the membrane when there are ≤ 2 colonies per square. For 3 to 10 colonies per square count 10 squares and obtain average count per square. For 10 to 20 colonies per square count 5 squares and obtain average count per square. Multiply average count per square by 100 and divide by the sample volume to give colonies per milliliter. If there are more than 20 colonies per square, record count as > 2000 divided by the sample volume. Report averaged counts as estimated colony-forming units. Make estimated counts only when there are discrete, separated colonies without spreaders.

9. Computing and Reporting Counts

The term "colony-forming units" (CFU) is descriptive of the methods used; therefore, report all counts as colony-forming units. Include in the report the method used, the incubation temperature and time, and the medium. For example: CFU/mL, pour plate method, 35°C/48 h, plate count agar.

To compute the heterotrophic plate count, CFU/mL, divide total number of colonies or average number (if duplicate plates of the same dilution) per plate by the sample volume. Record sample volumes used and number of colonies on each plate counted or estimated.

When colonies on duplicate plates and/or consecutive dilutions are counted and results are averaged before being recorded, round off counts to two significant figures only when converting to colony-forming units.

Avoid creating fictitious precision and accuracy when computing colony-forming units by recording only the first two left-hand digits. Raise the second digit to the next higher number when the third digit from the left is 5, 6, 7, 8, or 9; use zeros for each successive digit toward the right from the second digit. For example, report a count of 142 as 140 and a count of 155 as 160, but report a count of 35 as 35.

10. Analytical Bias

Avoid inaccuracies in counting due to carelessness, damaged or dirty optics that impair vision, or failure to recognize colonies. Laboratory workers who cannot duplicate their own counts on the same plate within 5% and the counts of other analysts within 10% should discover the cause and correct such disagreements.

9215 B. Pour Plate Method

1. Samples and Sample Preparation

See 9215A.4 and 9215A.5.

2. Sample Dilution

Prepare water used for dilution blanks as directed in Section 9050C.

a. Selecting dilutions: Select the dilution(s) so that the total number of colonies on a plate will be between 30 and 300 (Figure 9215:1). For example, where a heterotrophic plate count as high as 3000 is suspected, prepare plates with 10^{-2} dilution.

For most potable water samples, plates suitable for counting will be obtained by plating 1 mL and 0.1 mL undiluted sample and 1 mL of the 10^{-2} dilution.

b. Measuring sample portions: Use a sterile pipet for initial and subsequent transfers from each container. If pipet becomes contaminated before transfers are completed, replace with a sterile pipet. Use a separate sterile pipet for transfers from each different dilution. Do not prepare dilutions and pour plates in direct sunlight. Use caution when removing sterile pipets from the container; to avoid contamination, do not drag pipet tip across exposed ends of pipets in the pipet container or across lips and necks of dilution bottles. When removing sample, do not insert pipets more than 2 to 3 cm below the surface of sample or dilution.

c. Measuring dilutions: When discharging sample portions, hold pipet at an angle of about 45° with tip touching bottom of petri dish or inside neck of dilution bottle. Lift cover of petri dish just high enough to insert pipet. Allow 2 to 4 s for liquid to drain from 1-mL graduation mark to tip of pipet. If pipet is not a blow-out type, touch tip of pipet *once* against a dry spot on petri dish bottom. Less preferably, use a cotton-plugged blow-out-type pipet and gently blow out remaining volume of sample

dilution. When 0.1-mL quantities are measured, let diluted sample drain from chosen reference graduation until 0.1 mL has been delivered. Remove pipet without retouching it to dish. Pipet 1 mL, 0.1 mL, or other suitable volume into sterile petri dish before adding melted culture medium. Use decimal dilutions in preparing sample volumes of less than 0.1 mL; in examining sewage or turbid water, do not measure a 0.1-mL inoculum of original sample, but prepare an appropriate dilution. Prepare at least two replicate plates for each sample dilution used. After depositing test portions for each series of plates, pour culture medium and mix carefully. Do not let more than 20 min elapse between starting pipetting and pouring plates.

3. Plating

a. Melting medium: Melt sterile solid agar medium in boiling water or by exposure to flowing steam in a partially closed container, but avoid prolonged exposure to unnecessarily high temperatures during and after melting. Do not resterilize plating medium. If the medium is melted in two or more batches, use all of each batch in order of melting, provided that the contents remain fully melted. Discard melted agar that contains precipitate.

Maintain melted medium in a water bath between 44 and 46°C until used, preferably no longer than 3 h. In a separate container place a thermometer in water or medium that has been exposed to the same heating and cooling as the plating medium. Do not depend on the sense of touch to indicate proper medium temperature when pouring agar.

Use plate count agar, R2A agar, or NWRI agar as specified in Section 9215A.6. Before using a new lot of medium test its suitability.

b. Pouring plates: Limit the number of samples to be plated in any one series so that no more than 20 min (preferably 10 min)

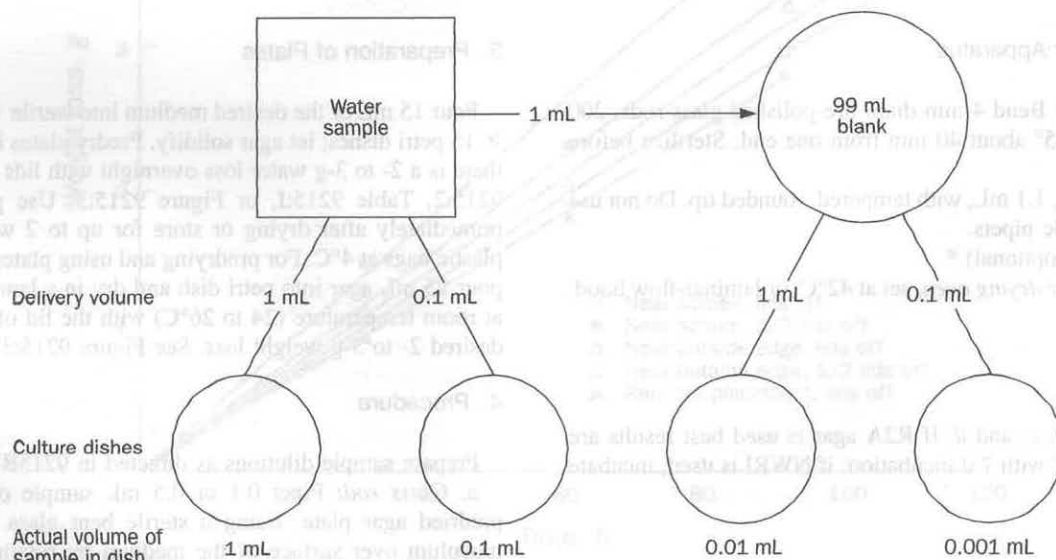


Figure 9215:1. Preparation of dilutions.

elapse between dilution of the first sample and pouring of the last plate in the series. Pour at least 10 to 12 mL liquefied medium maintained at 44 to 46°C into each dish by gently lifting cover just high enough to pour. Carefully avoid spilling medium on outside of container or on inside of dish lid when pouring. When pouring agar from flasks or tubes that have been held in a water bath, wipe with clean paper towel and flame the neck before pouring. As each plate is poured mix melted medium thoroughly with test portions in petri dish, taking care not to splash mixture over the edge, by rotating the dish first in one direction and then in the opposite direction, or by rotating and tilting. Let plates solidify (within 10 min) on a level surface. After medium solidifies, invert plates and place in incubator.

c. Sterility controls: Check sterility of medium and dilution water blanks by pouring control plates for each series of samples. Prepare additional controls to determine contamination of plates, pipets, and room air.

4. Incubation

See Section 9215A.7.

5. Counting, Recording, Computing, and Reporting

See Sections 9215A.8 and 9215A.9.

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9215 C. Spread Plate Method

1. Laboratory Apparatus

a. Glass rods: Bend 4-mm-diam fire-polished glass rods, 200 mm in length, 45° about 40 mm from one end. Sterilize before using.

b. Pipet, glass, 1.1 mL, with tempered, rounded tip. Do not use disposable plastic pipets.

*c. Turntable (optional).**

d. Incubator or drying oven, set at 42°C, or laminar-flow hood.

2. Media

See 9215A.6a, c, and d. If R2A agar is used best results are obtained at 28°C with 7 d incubation; if NWRI is used, incubate at 20°C for 7 d.

3. Preparation of Plates

Pour 15 mL of the desired medium into sterile 100 × 15 or 90 × 15 petri dishes; let agar solidify. Predry plates inverted so that there is a 2- to 3-g water loss overnight with lids on. See Figure 9215:2, Table 9215:1, or Figure 9215:3. Use predried plates immediately after drying or store for up to 2 weeks in sealed plastic bags at 4°C. For predrying and using plates the same day, pour 25 mL agar into petri dish and dry in a laminar-flow hood at room temperature (24 to 26°C) with the lid off to obtain the desired 2- to 3-g weight loss. See Figure 9215:3.

4. Procedure

Prepare sample dilutions as directed in 9215B.2.

a. Glass rod: Pipet 0.1 or 0.5 mL sample onto surface of predried agar plate. Using a sterile bent glass rod, distribute inoculum over surface of the medium by rotating the dish by hand or on a turntable. Let inoculum be absorbed completely into the medium before incubating.

* Fisher Scientific, hand operated, No. 08-758 or Lab-Line, motor driven, No. 1580, or equivalent.

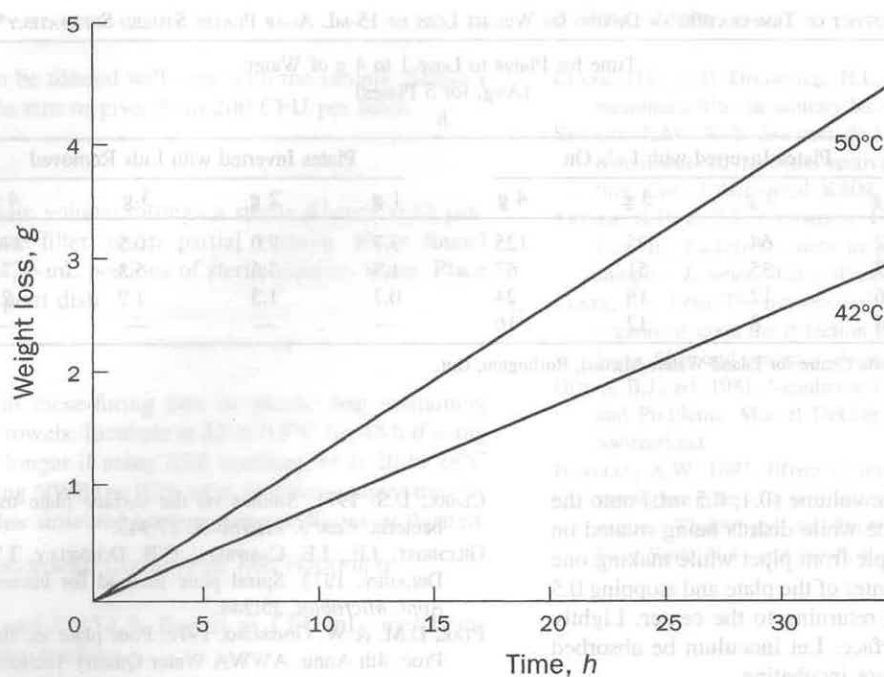


Figure 9215.2. Drying weight loss of 15-mL agar plates stored separately, inverted with lids on. Source: Unpublished data. Water Purification Lab., Chicago Dep. Water.

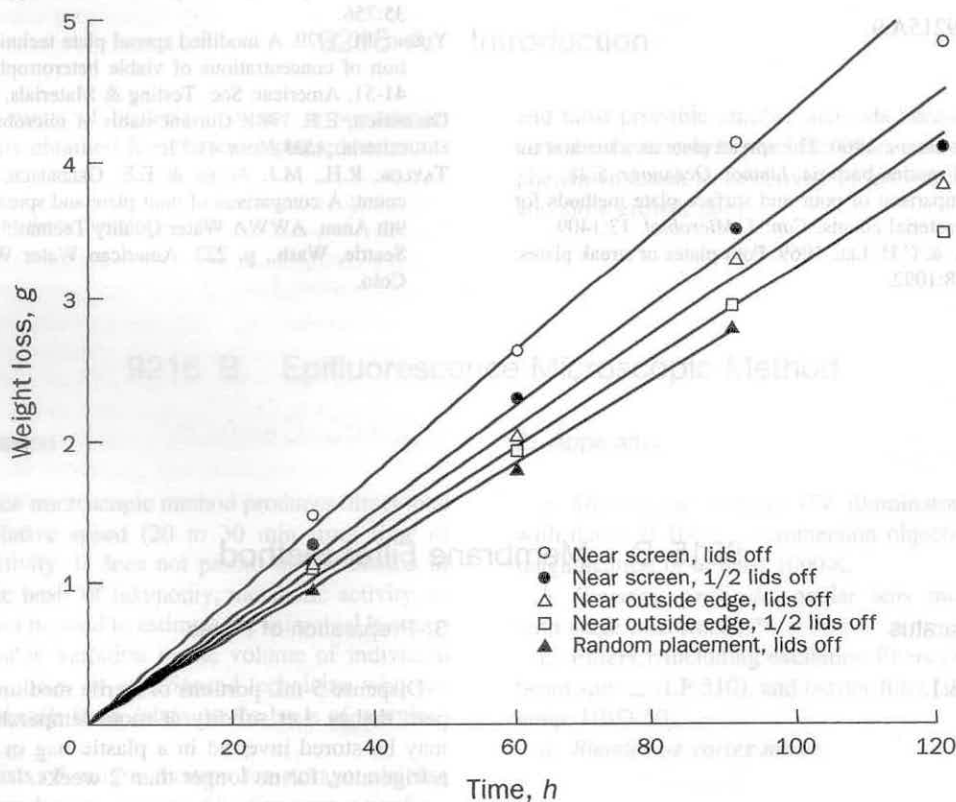


Figure 9215.3. Weight loss of 25-mL agar plates (100 x 15 mm) dried separately in a laminar-flow hood at room temperature (24 to 26°C), relative humidity (30 to 33%), and air velocity 0.6 m/s. Source: Unpublished data. Alberta Environmental Centre, Vegreville, Alta.

TABLE 9215:I. EFFECT OF TEMPERATURE OF DRYING ON WEIGHT LOSS OF 15-ML AGAR PLATES STORED SEPARATELY*

Temp. °C	Time for Plates to Lose 1 to 4 g of Water (Avg. for 5 Plates) h							
	Plates Inverted with Lids On				Plates Inverted with Lids Removed			
	1 g	2 g	3 g	4 g	1 g	2 g	3 g	4 g
24	32	64	95	125	3.7	7.0	10.5	14.0
37	17	35	51	67	1.7	3.5	5.3	7.0
50	6	12	18	24	0.7	1.3	1.9	2.7
60	4	8	12	16	—	—	—	—

* Referenced in Canada Centre for Inland Waters Manual, Burlington, Ont.

b. *Pipet*: Pipet desired sample volume (0.1, 0.5 mL) onto the surface of the predried agar plate while dish is being rotated on a turntable. Slowly release sample from pipet while making one to-and-fro motion, starting at center of the plate and stopping 0.5 cm from the plate edge before returning to the center. Lightly touch the pipet to the plate surface. Let inoculum be absorbed completely by the medium before incubating.

5. Incubation

See 9215A.7.

6. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9215A.9.

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9215 D. Membrane Filter Method

1. Laboratory Apparatus

See Section 9222B.1.

2. Media

See 9215A.6. Use m-HPC agar, or alternatively R2A or NWRI agar.

3. Preparation of Plates

Dispense 5-mL portions of sterile medium* into 50- × 9-mm petri dishes. Let solidify at room temperature. Prepared plates may be stored inverted in a plastic bag or tight container in a refrigerator, for no longer than 2 weeks.

* m-HPC agar may not be sterile.

4. Sample Size

The volume to be filtered will vary with the sample. Select a maximum sample size to give 20 to 200 CFU per filter.

5. Procedure

Filter appropriate volume through a sterile 47-mm, 0.45- μ m, gridded membrane filter, under partial vacuum. Rinse funnel with three 20- to 30-mL portions of sterile dilution water. Place filter on agar in petri dish.

6. Incubation

Place dishes in close-fitting box or plastic bag containing moistened paper towels. Incubate at $35 \pm 0.5^\circ\text{C}$ for 48 h if using m-HPC agar, or longer if using R2A medium, or at 20 to 28°C for 5 to 7 d if using NWRI or R2A agar. Duplicate plates may be incubated for other time and temperature conditions as desired.

7. Counting, Recording, Computing, and Reporting

See 9215A.8 and 9215A.9. Report as CFU/mL, membrane filter method, time, medium.

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9216 DIRECT TOTAL MICROBIAL COUNT*

9216 A. Introduction

Direct total cell counts of bacteria in water or wastewater usually exceed counts obtained from heterotrophic plate counts

and most probable number methods because, unlike those procedures, direct counts preclude errors caused by viability-related phenomena such as selectivity of growth media, cell clumping, and slow growth rates.

* Approved by Standard Methods Committee, 2000.

9216 B. Epifluorescence Microscopic Method

1. General Discussion

The epifluorescence microscopic method produces direct total cell counts with relative speed (20 to 30 min from time of sampling) and sensitivity. It does not permit differentiation of bacterial cells on the basis of taxonomy, metabolic activity, or viability, and it cannot be used to estimate the microbial biomass because of considerable variation in the volume of individual cells. The method requires an experienced technician who can distinguish microbial cells from debris on the basis of morphology.

The method consists of sample fixation for storage, staining with a chemical fluorochrome, vacuum filtration onto a nonfluorescing polycarbonate membrane, and enumeration by counting with an epifluorescence microscope.

2. Apparatus

- Microscope*, vertical UV illuminator for epifluorescence with flat field 100 $\times$ oil immersion objective lens, to give total magnification of at least 1000 $\times$.
- Counting graticule*, ocular lens micrometer* calibrated with stage micrometer.*
- Filters*,† including excitation filters (KP 490 and LP 455), beam splitter (LP 510), and barrier filter (LP 520 using mercury lamp, HBO 50).
- Blender or vortex mixer*.

* Bausch & Lomb No. 31-16-13.

† K. Zeiss or equivalent.

e. *Filtration unit*, suitable for use with 25-mm-diam membrane filters.

f. *Membrane filters*, polycarbonate,‡ 25-mm-diam, 0.2- μ m pore size (purchase nonfluorescent or prepare by soaking membrane in Irgalan black [2 g/L in 2% acetic acid] for 24 h, then rinse in water and air dry); cellulosic§ 25-mm-diam, 5- μ m pore size.

g. *Syringes*, 3-mL, disposable, with disposable syringe filters, 0.2- μ m pore size.

h. *Test tubes*, glass, screw-capped, 13- × 125-mm.

3. Reagents

a. *Phosphate buffer*: Dissolve 13.6 g KH_2PO_4 in water and dilute to 1 L. Adjust to pH 7.2 if necessary; filter through 0.2- μ m membrane filter.

b. *Fixative*, 5.0% (w/v) glutaraldehyde in phosphate buffer. Prepare fresh daily.

c. *Fluorochrome*, 0.1% (w/v) acridine orange|| in phosphate buffer.

d. *Immersion oil*, low fluorescing.#

4. Procedure

Collect water samples as directed in Section 9060. Add 9.0 mL sample to test tube containing 1.0 mL fixative. Fixed samples can be stored at 4°C for up to 3 weeks without significant decrease in cell numbers.

Disperse and dilute samples from mesotrophic or eutrophic sources to obtain reproducible results. Mix sample using blender or vortex mixer, then make tenfold dilutions in phosphate buffer as necessary. Clean water samples may not require dilution but larger sample volumes (>100 mL) may be required to obtain reliable counts.

Place 1 mL sample or dilution on a nonfluorescent polycarbonate filter supported by a cellulosic membrane filter in filter

holder. Using disposable sterile syringe filters, add 1 mL fluorochrome and wait 2 min, then add about 3 mL filtered phosphate buffer to promote more even cell distribution. Alternatively, combine fluorochrome with sample in a small clean vial, let react, and add mixture to filter holder. Filter with vacuum (about 13 kPa). Wash with 2 mL phosphate buffer and filter. Remove polycarbonate filter with forceps and air dry for 1 to 2 min. The filter can be cut into quarter sections and saved if needed. Place dried filter on a drop of immersion oil on a clean glass microscope slide. Add a small drop of immersion oil to filter surface. Gently cover filter with a clean glass cover slip. Samples can be stored in the dark for several months without significant loss of fluorescence.

Examine at least 10 randomly selected fields on the filter using the 100× oil immersion lens to establish that distribution of microbial cells is uniform and that individual cells can be enumerated (if not, dilute sample and repeat). Preferably count 10 to 50 cells per field. Count number of cells in at least 20 squares using the calibrated counting graticule.

5. Calculations

Calculate the average number of cells per filter. Obtain effective filter area from specifications of filtration unit. Extrapolate to determine number of cells per milliliter of sample:

$$\text{Total cells/mL} = (\text{avg cells/square}) \times (\text{squares/filter}) \times (\text{dilution factor}) / \text{sample volume, mL}$$

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‡ Nuclepore Corp. or equivalent.

§ Millipore Corp. or equivalent.

|| Sigma Chemical Co., biological grade, or equivalent.

Cargille Laboratories, Inc., Type B, or equivalent.

9217 ASSIMILABLE ORGANIC CARBON*

9217 A. Introduction

1. Significance

Growth of bacteria in drinking water distribution and storage systems can lead to the deterioration of water quality, violation of water quality standards, and increased operating costs. Growth or regrowth results from viable bacteria surviving the

disinfection process and utilizing nutrients in the water and biofilm to sustain growth.¹ Factors other than nutrients that influence regrowth include temperature,² residence time in mains and storage units,³ and the efficacy of disinfection.⁴ Tests to determine the potential for bacterial regrowth focus on the concentration of nutrients.⁵⁻⁷

Not all organic compounds are equally susceptible to microbial decomposition; the fraction that provides energy and carbon

* Approved by Standard Methods Committee, 1997.

for bacterial growth has been called labile dissolved organic carbon,^{8,9} biodegradable organic carbon (BDOC),⁷ or assimilable organic carbon (AOC).⁵ Easily measured chemical surrogates for AOC are not available now.^{10,11} As alternatives to chemical methods, bioassays have been proposed.^{5-7,12-14}

In a bioassay, the growth of a bacterial inoculum to maximum density can be used to estimate the concentrations of limiting nutrients; the underlying assumptions of the AOC bioassay are that nitrogen and phosphorus are present in excess, i.e., that organic carbon is limiting, and that the bioassay organism(s) represent the physiological capabilities of the distribution system microflora. Various bioassay procedures use an inoculum of one to four species of bacteria^{5,12,13,15,16} growing in log phase or present in late stationary phase, or may use undefined bacteria attached to a sand substratum,⁷ suspended in the sample,⁶ or filtered from the sample and then resuspended.¹⁴ Incubation vessels vary as to material,¹⁷ size,^{18,19} closure,¹⁸ and cleaning procedure.^{5,18,19} Water to be tested for nutrient concentrations has been variously prepared.^{5,7,14} The AOC bioassay is an indirect or surrogate method, wherein nutrient concentrations are not measured directly, but colony-forming units (CFU) of the bioassay organism(s) are the test variable. Nutrient concentrations have been estimated directly from changes in dissolved organic carbon concentrations within the test vessel⁷ or indirectly from epifluorescence microscopic counts of the maximum number of bacterial cells grown,^{13,14} turbidity,¹⁴ or incorporation of tritiated thymidine into bacterial DNA.^{6,20} CFU densities, total cell densities, or bacterial production are converted to nutrient concentration by the growth yield of bacteria, defined as either the ratio between CFU or cells produced and organic carbon used, or biomass produced and organic carbon used.^{5,6}

2. Selection of Method

The method described below is a two-species bioassay using *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX (van der Kooij)¹⁰ that has been modified to reduce problems of bacterial and carbon contamination.^{18,19} It uses a defined inoculum and miniaturized incubation vessels, requires no specialized equipment, and has been related to the presence of coliforms in a drinking water distribution system.²² The two-species inoculum probably underestimates the total quantity of AOC, is consistently lower than BDOC estimates, and does not provide an estimate of refractory organic carbon.²³ Critical aspects of the proposed method, including the preparation of the incubation vessel, test water, and inoculum, and enumeration of the test organisms, are transferable to alternate AOC assays that use a different defined inoculum.

With an undefined bacterial inoculum, enumeration by the spread plate technique is not applicable; alternate response variables, such as changes in dissolved organic carbon (DOC) concentration, turbidity, epifluorescence microscopic counts, bacterial mortality, or bacterial growth, have been used.^{6,7,14}

3. Sampling and Storage

Follow precautions outlined in Section 9060A and B for collecting and storing samples. Pasteurized and dechlorinated water samples probably can be held for several days without

deterioration if properly sealed. Initiate the AOC assay as quickly as possible after pasteurization (see ¶ B.4c).

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9217 B. *Pseudomonas fluorescens* Strain P-17, *Spirillum* Strain NOX Method

1. General Discussion

a. Principle: The AOC bioassay using *Pseudomonas fluorescens* strain P-17 and *Spirillum* strain NOX involves growth to a maximum density of a small inoculum in a batch culture of pasteurized test water. Pasteurization inactivates native microflora. The test organisms are enumerated by the spread plate method for heterotrophic plate counts (Section 9215C) and the density of viable cells is converted to AOC concentrations by an empirically derived yield factor for the growth of P-17 on acetate-carbon and NOX on oxalate-carbon as standards. The number of organisms at stationary phase is assumed to be the maximum number of organisms that can be supported by the nutrients in the sample and the yields on acetate carbon and oxalate carbon are assumed to equal the yield on naturally occurring AOC.^{1,2}

b. Interferences: Untreated surface waters, especially those with high concentrations of suspended solids or high turbidity, can contain large numbers of spore-forming bacteria that may survive pasteurization, grow, and interfere with the enumeration of P-17 and NOX on spread plates. Such waters generally have high AOC

concentrations and can be diluted with organic-free water amended with mineral salts or prefiltered through carbon-free filters. Potable waters that have been disinfected and carry a disinfectant residual will inhibit growth of the test organism unless the disinfectant is neutralized. Surface waters from reservoirs treated with copper sulfate also may be inhibitory unless a chelating agent is added to the sample,³ and lime-softened waters with elevated pH values may require pH adjustment. Any amendment to a sample requires a control for AOC contamination.

c. Minimum detectable concentration: In theory, concentrations of less than 1 µg C/L can be detected. In practice, organic carbon contamination during glassware preparation and sample handling imposes a limit of detection of approximately 5 to 10 µg AOC/L.

2. Apparatus

a. Incubation vessels: Organic-carbon-free borosilicate glass vials (45 mL capacity) with TFE-lined silicone septa.

b. Incubator, set at 15 ± 0.5°C.

c. Hot water bath capable of achieving and holding 70°C.

d. Continuously adjustable pipet* capable of delivering between 10 and 100 μ L.

e. Erlenmeyer flask, 125-mL, with ground-glass stopper.

f. Apparatus for preparing dilution water and making heterotrophic plate counts: See Sections 9050C and 9215C.

3. Reagents

a. Sodium acetate stock solution, 400 mg acetate-C/L: Dissolve 2.267 g $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 1 L organic-carbon-free, deionized water. Transfer to 45-mL vials, fill to shoulder, cap tightly, and autoclave. Although standard autoclave practice is to loosen caps, keep vials with septa capped tightly for autoclaving. Store at 5°C in tightly capped vials. Solution may be held for up to 6 months.

b. Sodium thiosulfate solution: Dissolve 30 g $\text{Na}_2\text{S}_2\text{O}_3$ in 1 L deionized water. Transfer to 45-mL vials and autoclave as directed in ¶ 3a.

c. Buffered water: See Section 9050C.

d. R2A agar: See Section 9215A.6c.

e. Sodium persulfate solution, 10% (w/v): Dissolve 100 g $\text{Na}_2\text{S}_2\text{O}_8$ in 1 L deionized water.

f. Organic-free water: See Section 5710B.3e. Alternatively, use HPLC-grade bottled water.

g. Mineral salts solution: Dissolve 171 mg K_2HPO_4 , 767 mg NH_4Cl , and 1.444 g KNO_3 in 1 L carbon-free water. Transfer to 45-mL vials and autoclave as directed in ¶ 3a.

h. Cultures of strains P-17 (ATCC 49642) and NOX (ATCC 49643).†

4. Procedure

a. Preparation of incubation vessels: Wash 45-mL vials with detergent, rinse with hot water, 0.1N HCl two times, and deionized water three times, dry, cap with foil, and heat to 550°C for 6 h. Soak TFE-lined silicone septa in a 10% sodium persulfate solution for 1 h at 60°C; rinse three times with carbon-free deionized water. Alternatively, use pre-cleaned water sampling vials‡ or an equivalent AOC-free vial.‡ Use same cleaning procedure for all glassware.

b. Preparation of stock inoculum: Prepare individual turbid suspensions of P-17 and NOX by transferring growth from a slant culture on R2A agar into 2 to 3 mL filtered (0.2 μ m), autoclaved sample. Use slant not older than 6 months. The autoclaved sample can be any water that supports growth of P-17 and NOX and is organic-carbon-limited. Neutralize chlorinated samples with sodium thiosulfate (42 μ L/50 mL). Transfer 100 μ L of suspension to 50 mL filtered, autoclaved sample in a sterile 125-mL ground-glass-stoppered erlenmeyer flask. Add 125 μ L sodium acetate solution (suspension contains 1 mg acetate-C/L). Incubate at room temperature ($\leq 25^\circ\text{C}$) until the viable cell count reaches the stationary phase. Organic-carbon limitation will insure complete utilization of acetate-C so that no AOC is transferred with the inoculum. The stationary phase is reached when the viable cell count, as measured by spread plates, reaches maximum value. Store stock cultures for

not more than 6 months at 5°C. Before inoculating a bioassay vessel, make a viable count of the culture (spread plate) to determine the appropriate volume of inoculum to be added to each bioassay vessel.

c. Preparation of incubation water: Collect samples directly into 10 45-mL vials. Use 9 vials for AOC measurement and 1 for growth control. Fill each vial to the neck (40 mL) within as short a time as possible. Place septa on the vials, TFE side down, and secure with open-topped screw caps. Alternatively, collect 500 mL sample in an organic-carbon-free vessel and pour into each vial. Neutralize samples containing disinfectant residuals with 33 μ L sodium thiosulfate solution added to each vial or 0.5 mL per 500-mL sample. Preferably, collect an extra vial to check for residual chlorine after neutralization. In the laboratory, cap vials tightly and pasteurize in 70°C water bath for 30 min.

d. Inoculation and incubation: Cool, inoculate with 500 colony-forming units (CFU)/mL each of P-17 and NOX, either by injecting through the septum or by removing cap and using a carbon-free pipet. Plastic, sterile tips for continuously adjustable pipets are suitable. Use the following equation to calculate volume of inoculum:

$$\text{Volume of inoculum} = \frac{(500 \text{ CFU/mL}) \times (40 \text{ mL/vial})}{\text{CFU/mL stock inoculum}}$$

Hold vials at 15°C in the dark for 1 week. If a 15°C incubator is unavailable, incubate at room temperature not to exceed 25°C. Because incubation temperature influences growth yield, record and report temperature. Determine yields as directed below if an alternative temperature is used.

e. Enumeration of test bacterium: On incubation days 7, 8, and 9 remove three vials from the incubator. Sample an individual vial on only 1 d. Shake vials vigorously for 1 min, remove 1 mL with a sterile pipet, and prepare a dilution series (see Section 9215B). Plate three dilutions (10^{-2} , 10^{-3} , and 10^{-4}) in duplicate. Incubate plates at 25°C for 3 to 5 d and score the number of colonies of each strain. P-17 colonies appear on plates first; they are 3 to 4 mm in diameter with diffuse yellow pigmentation. NOX colonies are small (1- to 2-mm diam) white dots. It may be necessary to count P-17 and NOX colonies at different dilutions. Sample vials on three separate days to check whether maximum density has been reached. Day-to-day variations of between 11 and 16% of the mean for batch cultures of P-17 in stationary phase are typical.¹ A consistent increase in cell densities of 20% or more over the 3-d period indicates that the cultures are not in stationary phase; repeat assay with longer incubation period. Alternatively, collect more samples (three for each additional sampling day) and prepare as in ¶ c above so that extended incubation can be used. A sharp population decrease of approximately 0.5 log over the 3 d is unusual, but may occur. If this happens repeat the assay.

f. Determination of yield of P-17 and NOX: The yields of P-17 and NOX on model carbon compounds should be constant if organic carbon is limiting and the incubation temperature is kept constant. It is acceptable to use the previously derived empirical yield values of 4.1×10^6 CFU P-17/ μ g acetate-C, 1.2×10^7 CFU-NOX/ μ g acetate-C, and 2.9×10^6 CFU-NOX/ μ g oxalate-C at 15°C.⁵ However, the determination of a yield control provides an important check on both the bioassay (see also 6, Quality Control, below) and carbon limitation in the sample.

* Eppendorf or equivalent.

† Available from the American Type Culture Collection.

‡ Pierce Vari-Clean.

5. Calculation

a. *AOC concentration*: Average viable count results for the 3 d and calculate concentration of AOC as the product of the mean of the viable counts and the inverse of the yield:

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(1/\text{yield}) + (\text{mean NOX CFU/mL})(1/\text{yield})](1000 \text{ mL/L})$$

When the empirical yield factors⁵ are used, the equation becomes:

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(\mu\text{g acetate-C}/4.1 \times 10^6 \text{ CFU}) + (\text{mean NOX CFU/mL})(\mu\text{g oxalate-C}/2.9 \times 10^6 \text{ CFU})](1000 \text{ mL/L})$$

or

$$\mu\text{g AOC/L} = [(\text{mean P-17 CFU/mL})(2.44 \times 10^{-7} \mu\text{g acetate-C/CFU}) + (\text{mean NOX CFU/mL})(3.45 \times 10^{-7} \mu\text{g oxalate-C/CFU})](1000 \text{ mL/L})$$

In practice, the densities of organisms vary during the stationary phase. Using average density over 3-d period provides a more accurate estimate of the real maximum density.

Reporting AOC as $\mu\text{g C/L}$ assumes that the yields on acetate and oxalate are equal to the yields on naturally occurring AOC. To permit data comparisons report incubation temperature, contribution of each species to AOC, and yield factors used.

6. Quality Control

See Section 9020B for general quality control procedures. Quality control specific to the AOC bioassay includes testing the inoculum for purity and viability by plating a portion on R2A agar, testing the incubation vessel, inoculum, thiosulfate solution, and any supplemental procedure such as filtration or dilution for organic carbon contamination, testing the P-17 and NOX inocula for yield, and testing the sample for carbon limitation or inhibition of assay organisms. Test all deviations in procedure (see ¶ 6f).

To make these tests, use separate controls for blank, yield, and growth. The controls outlined below use a single vial and are meant as a trouble-shooting guide. Definitive determination, for example, that the yield is different from a published value or that a sample is inhibitory, requires replication and statistical analysis.

a. *Blank control*: Dilute mineral salts solution 10:1 with carbon-free water. Follow procedures outlined above: Fill a vial to the shoulder with organic-carbon-free water, add 100 μL mineral salts and 100 μL sodium thiosulfate, pasteurize, inoculate with P-17/NOX, incubate, and enumerate growth.

b. *Yield control*: Dilute sodium acetate or sodium oxalate solution 10:1 with carbon-free water, preparing 40 mg C/L working concentrations. Follow procedures outlined above: Fill a vial to the shoulder with carbon-free water, add 100 μL mineral salts, 100 μL sodium thiosulfate, and 100 μL sodium

acetate or sodium oxalate working solution, pasteurize, inoculate with P-17/NOX, incubate, and enumerate growth. P-17, unlike NOX, will not grow with oxalate as sole carbon source (oxalate is considered a major by-product of ozonation). NOX growth in HPLC-grade water presumed to be organic carbon-free is to be expected. The yield control is a quality control measurement and is not intended to provide a conversion factor for the calculation of AOC.

c. *Growth control*: Use additional sample of test water collected with the nine AOC vials, (¶ 4c above) but amend with 100 μL diluted mineral salts and 100 μL of diluted acetate or oxalate solution per vial before pasteurization. As with other controls, inoculate with P-17/NOX, incubate, and enumerate growth.

d. *Yield calculations*: If previously derived empirical yield values (see ¶ 4f above) are not used, a conversion factor can be derived empirically by using pure cultures of P-17 and NOX. Mixed cultures of the organisms cannot be used and a separate blank control for each species is required. Convert density units to CFU/L by multiplying CFU/mL by 1000, and divide by 100 μg acetate or oxalate-C/L. Express yield as CFU P-17 or NOX/ μg acetate-C or oxalate-C. For P-17 and acetate-C, the equation is:

$$\text{Yield} = \frac{[(\text{P-17 CFU/mL:yield control}) - (\text{P-17 CFU/mL:blank control})] \times (1000 \text{ mL/L})}{100 \mu\text{g acetate-C/L}}$$

$$= \text{P-17 CFU}/\mu\text{g acetate-C}$$

e. *Interpretation of growth control*: Subtract densities of P-17 and NOX that grew in the sample amended with only thiosulfate from the densities of P-17 and NOX that grew in the growth control. Compare difference to the difference between yield and blank controls.

If: (growth control – sample) = (yield control – blank control)

Then: sample is carbon-limited and not inhibitory

If: (growth control – sample) < (yield control – blank control)

Then: sample is inhibitory to bioassay organism

If: (growth control – sample) > (yield control – blank control)

Then: sample is not carbon-limited

f. *Supplemental procedure check*: When using supplemental procedures such as filtration, dilution, or chemical amendment check for carbon contribution to the AOC values. To test a procedure, use carbon-free water and blank control as a base line. Perform the supplemental procedure on additional carbon-free water and compare to densities of P-17 and NOX that grow in the blank control.

7. Precision and Bias

The P-17 bioassay performed in a single laboratory using 45-mL vials had a precision of $\pm 17.5\%$ based on a total of 58 assays with 14 different samples.⁶

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9218 AEROBIC ENDOSPORES*

9218 A. Introduction

1. Description

Endospores are dormant, environmentally resistant structures that are formed by certain genera of bacteria. The majority of aerobic-endospore-forming bacteria are harmless, saprophytic organisms found in soil and water. The process of sporulation is initiated by vegetative cells of aerobic-endospore-forming bacteria in response to adverse environmental conditions. The bacteria can persist in the endospore form for an extended period of time. Under favorable conditions, such as a readily available nutrient supply, the endospores convert back to the vegetative bacterial cell stage in a process known as germination. Unlike vegetative cells, endospores are very resistant to environmental pressures such as heat, desiccation, and irradiation.

2. Detection and Applications

Aerobic endospores are ubiquitous in the environment. Detection of indigenously occurring aerobic endospores in water is accomplished by exposing the sample to a heat treatment to inactivate any vegetative cells; the heat treatment does not inactivate the endospores. The sample is then plated onto a nonselective nutrient medium and incubated aerobically at 35°C. The

endospores germinate and form bacterial colonies. The vast majority of these organisms will be species of *Bacillus*.

Analysis for aerobic endospores can be used to evaluate a variety of water treatment unit processes including physical removal processes (coagulation, clarification) and disinfection.^{1,2} Most surface water sources, with the exception of some lakes and reservoirs, contain sufficient levels of endospores to determine order of magnitude removal efficiencies. Since endospores are highly resistant structures, they are also used for determining the efficacy of processes such as halogen, ozone, and ultraviolet inactivation. Analyzing for endospores in groundwater is a means of evaluating whether a given groundwater source is under the influence of surface water.³ The procedure also has been used to evaluate the efficacy of river-bank infiltration. The presence of endospores may be used to determine the physical integrity of drinking water distribution systems that may have been compromised by pipeline breaks or maintenance procedures.

3. References

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* Approved by Standard Methods Committee, 2001.

Joint Task Group: Clifford H. Johnson (chair), Kathleen E. Atterbury.

9218 B. Membrane Filter Method

1. Apparatus

a. *Bottles or erlenmeyer flasks with closures*, suitable for sample size chosen and capable of withstanding hot and cold temperatures.

b. *Culture dishes*: See Section 9222B.1e.

c. *Filtration units*: See Section 9222B.1f.

d. *Water bath*, preferably shaking style, capable of heating samples to 80°C.

e. *Thermometer*.

2. Materials and Culture Media

a. Nutrient agar plus trypan blue:

Peptone	5.0	g
Beef extract	3.0	g
Trypan blue	0.015	g
Agar	15.0	g
Reagent-grade water	1.0	L

Heat to near boiling to dissolve agar. Sterilize by autoclaving for 15 min at 121°C. After autoclaving, cool to 50°C and dispense aseptically into 50- × 9-mm plastic culture plates with loose-fitting lids. The pH should be 6.8 ± 0.2 . Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. *Sample size*: See Section 9222B.5a.

b. *Heat treatment of sample*: Distribute samples into bottles or erlenmeyer flasks (§ 2a). Prepare a pilot control flask equipped

with a closure that includes a thermometer, containing the same volume as that of the water sample in the test flasks. Place control flask into the water bath along with the samples. When using a water bath with shaker, agitate samples at 60 to 80 rpm. If a shaking bath is not available, periodically shake samples by manual swirling. Monitor temperature in pilot control flask. When pilot flask reaches desired temperature, begin timing and hold sample for the specified time. At a temperature of 75°C heat samples for 15 min; at a temperature of 80°C heat samples for 10 min.^{1,2} At the end of heat treatment, cool samples immediately in a bath containing a slurry of wet ice. Let samples cool to approximately room temperature.

c. *Filtration of samples and counting*: Filter samples as described in Section 9222B.5c. Place membrane filter on agar surface and incubate for 24 ± 2 h at $35 \pm 0.5^\circ\text{C}$. Count colonies with a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device. Consider any bacterial colonies growing on the membrane as aerobic spore-forming bacteria.

d. Interpretation:

For colony counting procedure see Section 9215A.8b.

4. References

1. RICE, E.W., K.R. FOX, R.J. MILTNER, D.A. LYTLE & C.H. JOHNSON. 1996. Evaluating plant performance with endospores. *J. Amer. Water Works Assoc.* 88:122.
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9221 MULTIPLE-TUBE FERMENTATION TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9221 A. Introduction

The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae. The historical definition of this group has been based on the method used for detection (lactose fermentation) rather than on the tenets of systematic bacteriology. Accordingly, when the fermentation technique is used, this group is defined as all facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria

that ferment lactose with gas and acid formation within 48 h at 35°C.

The standard test for the coliform group may be carried out either by the multiple-tube fermentation technique or presence-absence procedure (through the presumptive-confirmed phases or completed test) described herein, by the membrane filter (MF) technique (Section 9222) or by the enzymatic substrate coliform test (Section 9223). Each technique is applicable within the limitations specified and with due consideration of the purpose of the examination. Production of valid results requires strict adherence to quality control procedures. Quality control guidelines are outlined in Section 9020.

When multiple tubes are used in the fermentation technique, results of the examination of replicate tubes and dilutions are

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reported in terms of the Most Probable Number (MPN) of organisms present. This number, based on certain probability formulas, is an estimate of the mean density of coliforms in the sample. Coliform density, together with other information obtained by engineering or sanitary surveys, provides the best assessment of water treatment effectiveness and the sanitary quality of source water.

The precision of each test depends on the number of tubes used. The most satisfactory information will be obtained when the largest sample inoculum examined shows gas in some or all of the tubes and the smallest sample inoculum shows no gas in all or a majority of the tubes. Bacterial density can be estimated by the formula given or from the table using the number of positive tubes in the multiple dilutions (9221C.2). The number of sample portions selected will be governed by the desired precision of the result. MPN tables are based on the assumption of a Poisson distribution (random dispersion). However, if the sample is not adequately shaken before the portions are removed or if clumping of bacterial cells occurs, the MPN value will be an underestimate of the actual bacterial density.

1. Water of Drinking Water Quality

When drinking water is analyzed to determine if the quality meets the standards of the U.S. Environmental Protection Agency (EPA), use the fermentation technique with 10 replicate tubes each containing 10 mL, 5 replicate tubes each containing 20 mL, or a single bottle containing a 100-mL sample portion. When examining drinking water by the fermentation technique, process all tubes or bottles demonstrating growth with or without a positive acid or gas reaction to the confirmed phase (9221B.2). Apply the completed test (9221B.3) to not less than 10% of all coliform-positive samples per quarter. Obtain at least one positive sample per quarter. A positive EC broth (9221E) or a positive EC MUG broth (9221F) test result is considered an alternative to the positive completed test phase.

For the routine examination of public water supplies the object of the total coliform test is to determine the efficiency of treatment plant operation and the integrity of the distribution system. It is also used as a screen for the presence of fecal contamination.

A high proportion of coliform occurrences in a distribution system may be attributed not to treatment failure at the plant or the well source, but to bacterial regrowth in the mains. Because it is difficult to distinguish between coliform regrowth and new contamination, assume all coliform occurrences to be new contamination unless otherwise demonstrated.

2. Water of Other than Drinking Water Quality

In the examination of nonpotable waters inoculate a series of tubes with appropriate decimal dilutions of the water (multiples and submultiples of 10 mL), based on the probable coliform density. Use the presumptive-confirmed phase of the multiple-tube procedure. Use the more labor-intensive completed test (9221B.3) as a quality control measure on at least 10% of coliform-positive nonpotable water samples on a seasonal basis. The object of the examination of nonpotable water generally is to estimate the density of bacterial contamination, determine a source of pollution, enforce water quality standards, or trace the survival of microorganisms. The multiple-tube fermentation technique may be used to obtain statistically valid MPN estimates of coliform density. Examine a sufficient number of samples to yield representative results for the sampling station. Generally, the geometric mean or median value of the results of a number of samples will yield a value in which the effect of sample-to-sample variation is minimized.

3. Other Samples

The multiple-tube fermentation technique is applicable to the analysis of salt or brackish waters as well as muds, sediments, and sludges. Follow the precautions given above on portion sizes and numbers of tubes per dilution.

To prepare solid or semisolid samples weigh the sample and add diluent to make a 10^{-1} dilution. For example, place 50 g sample in sterile blender jar, add 450 mL sterile phosphate buffer or 0.1% peptone dilution water, and blend for 1 to 2 min at low speed (8000 rpm). Prepare the appropriate decimal dilutions of the homogenized slurry as quickly as possible to minimize settling.

9221 B. Standard Total Coliform Fermentation Technique

1. Presumptive Phase

Use lauryl tryptose broth in the presumptive portion of the multiple-tube test. If the medium has been refrigerated after sterilization, incubate overnight at room temperature (20°C) before use. Discard tubes showing growth and/or bubbles.

a. Reagents and culture medium:

1) Lauryl tryptose broth:

Tryptose.....	20.0 g
Lactose.....	5.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	2.75 g
Potassium dihydrogen phosphate, KH_2PO_4	2.75 g

Sodium chloride, NaCl.....	5.0 g
Sodium lauryl sulfate.....	0.1 g
Reagent-grade water.....	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense sufficient medium, in fermentation tubes with an inverted vial, to cover inverted vial at least one-half to two-thirds after sterilization. Alternatively, omit inverted vial and add 0.01 g/L bromocresol purple to presumptive medium to determine acid production, the indicator of a positive result in this part of the coliform test. Close tubes with metal or heat-resistant plastic caps.

TABLE 9221:I. PREPARATION OF LAURYL TRYPTOSE BROTH

Inoculum mL	Amount of Medium in Tube mL	Volume of Medium + Inoculum mL	Dehydrated Lauryl Tryptose Broth Required g/L
1	10 or more	11 or more	35.6
10	10	20	71.2
10	20	30	53.4
20	10	30	106.8
100	50	150	106.8
100	35	135	137.1
100	20	120	213.6

Make lauryl tryptose broth of such strength that adding 100-mL, 20-mL, or 10-mL portions of sample to medium will not reduce ingredient concentrations below those of the standard medium. Prepare in accordance with Table 9221:I.

b. Procedure:

1) Arrange fermentation tubes in rows of five or ten tubes each in a test tube rack. The number of rows and the sample volumes selected depend upon the quality and character of the water to be examined. For potable water use five 20-mL portions, ten 10-mL portions, or a single bottle of 100 mL portion; for nonpotable water use five tubes per dilution (of 10, 1, 0.1 mL, etc.).

In making dilutions and measuring diluted sample volumes, follow the precautions given in Section 9215B.2. Use Figure 9215:1 as a guide to preparing dilutions. Shake sample and dilutions vigorously about 25 times. Inoculate each tube in a set of five with replicate sample volumes (in increasing decimal dilutions, if decimal quantities of the sample are used). Mix test portions in the medium by gentle agitation.

2) Incubate inoculated tubes or bottles at $35 \pm 0.5^\circ\text{C}$. After 24 ± 2 h swirl each tube or bottle gently and examine it for growth, gas, and acidic reaction (shades of yellow color) and, if no gas or acidic reaction is evident, reincubate and reexamine at the end of 48 ± 3 h. Record presence or absence of growth, gas, and acid production. If the inner vial is omitted, growth with acidity signifies a positive presumptive reaction.

c. Interpretation: Production of an acidic reaction or gas in the tubes or bottles within 48 ± 3 h constitutes a positive presumptive reaction. Submit tubes with a positive presumptive reaction to the confirmed phase (9221B.2).

The absence of acidic reaction or gas formation at the end of 48 ± 3 h of incubation constitutes a negative test. Submit drinking water samples demonstrating growth without a positive gas or acid reaction to the confirmed phase (9221B.2). An arbitrary 48-h limit for observation doubtless excludes occasional members of the coliform group that grow very slowly (see Section 9212).

2. Confirmed Phase

a. Culture medium: Use brilliant green lactose bile broth fermentation tubes for the confirmed phase.

Brilliant green lactose bile broth:

Peptone.....	10.0	g
Lactose.....	10.0	g

Oxgall.....	20.0	g
Brilliant green.....	0.0133	g
Reagent-grade water.....	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 7.2 ± 0.2 after sterilization. Before sterilization, dispense, in fermentation tubes with an inverted vial, sufficient medium to cover inverted vial at least one-half to two-thirds after sterilization. Close tubes with metal or heat-resistant plastic caps.

b. Procedure: Submit all presumptive tubes or bottles showing growth, any amount of gas, or acidic reaction within 24 ± 2 h of incubation to the confirmed phase. If active fermentation or acidic reaction appears in the presumptive tube earlier than 24 ± 2 h, transfer to the confirmatory medium; preferably examine tubes at 18 ± 1 h. If additional presumptive tubes or bottles show active fermentation or acidic reaction at the end of a 48 ± 3 h incubation period, submit these to the confirmed phase.

Gently shake or rotate presumptive tubes or bottles showing gas or acidic growth to resuspend the organisms. With a sterile loop 3.0 to 3.5 mm in diameter, transfer one or more loopfuls of culture to a fermentation tube containing brilliant green lactose bile broth or insert a sterile wooden applicator at least 2.5 cm into the culture, promptly remove, and plunge applicator to bottom of fermentation tube containing brilliant green lactose bile broth. Remove and discard applicator. Repeat for all other positive presumptive tubes.

Incubate the inoculated brilliant green lactose bile broth tube at $35 \pm 0.5^\circ\text{C}$. Formation of gas in any amount in the inverted vial of the brilliant green lactose bile broth fermentation tube at any time (e.g., 6 ± 1 h, 24 ± 2 h) within 48 ± 3 h constitutes a positive confirmed phase. Calculate the MPN value from the number of positive brilliant green lactose bile tubes as described in Section 9221C.

c. Alternative procedure: Use this alternative only for polluted water or wastewater known to produce positive results consistently.

If all presumptive tubes are positive in two or more consecutive dilutions within 24 h, submit to the confirmed phase only the tubes of the highest dilution (smallest sample inoculum) in which all tubes are positive and any positive tubes in still higher dilutions. Submit to the confirmed phase all tubes in which gas or acidic growth is produced only after 48 h.

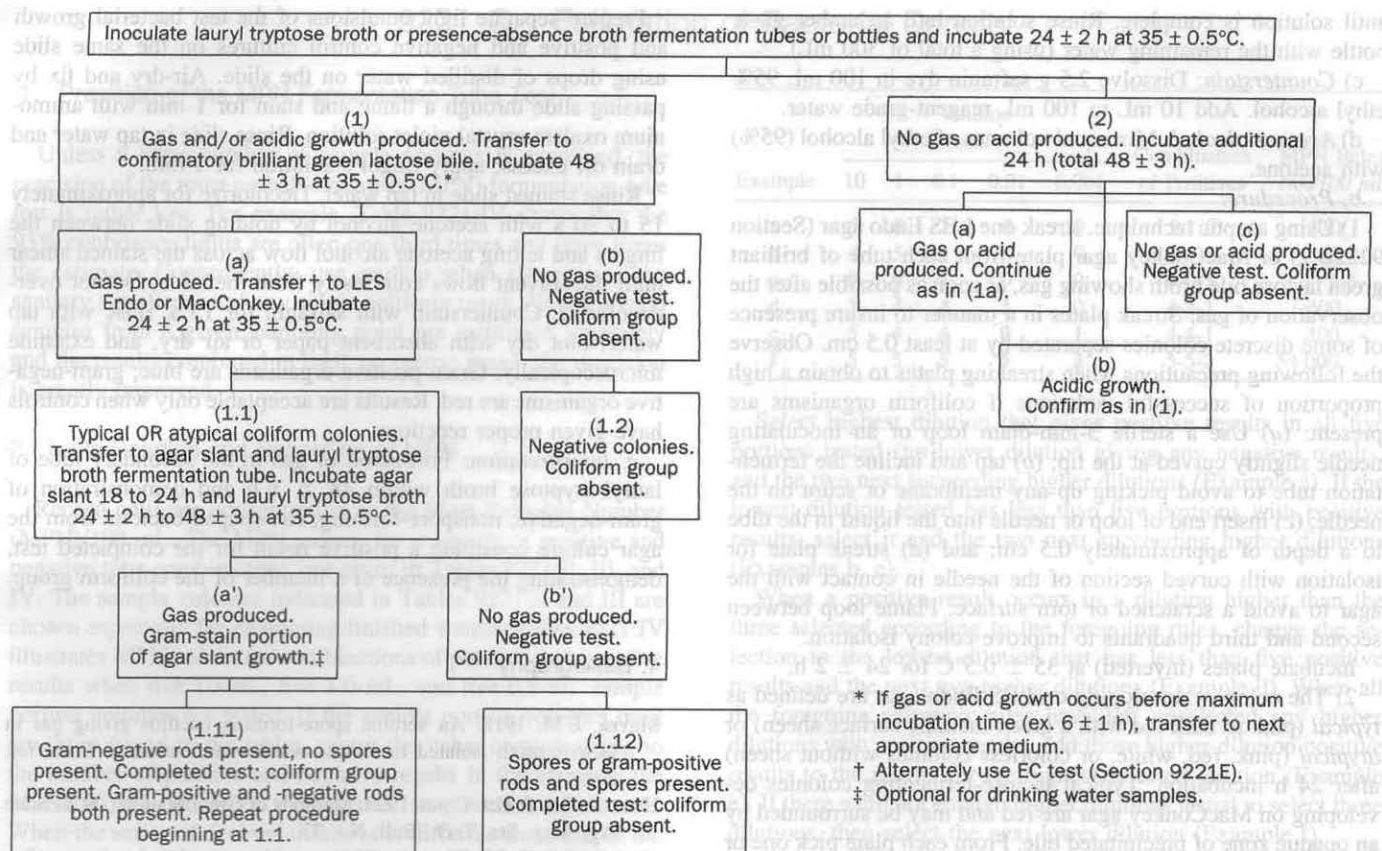


Figure 9221:1. Schematic outline of presumptive, confirmed, and completed phases for total coliform detection.

3. Completed Phase

To establish the presence of coliform bacteria and to provide quality control data, use the completed test on at least 10% of positive confirmed tubes (see Figure 9221:1). Simultaneous inoculation into brilliant green lactose bile broth for total coliforms and EC broth for fecal coliforms (see Section 9221E below) or EC-MUG broth for *Escherichia coli* may be used. Consider positive EC and EC-MUG broths elevated temperature (44.5°C) results as a positive completed test response. Parallel positive brilliant green lactose bile broth cultures with negative EC or EC-MUG broth cultures indicate the presence of nonfecal coliforms.

a. Culture media and reagents:

1) *LES Endo agar*: See Section 9222B. Use 100- × 15-mm petri plates.

2) *MacConkey agar*:

Peptone.....	17	g
Proteose peptone.....	3	g
Lactose.....	10	g
Bile salts.....	1.5	g
Sodium chloride, NaCl.....	5	g
Agar.....	13.5	g
Neutral red.....	0.03	g
Crystal violet.....	0.001	g
Reagent-grade water.....	1	L

Add ingredients to water, mix thoroughly, and heat to boiling to dissolve. Sterilize by autoclaving for 15 min at 121°C . Temper agar after sterilization and pour into petri plates (100 × 15 mm). pH should be 7.1 ± 0.2 after sterilization.

3) Nutrient agar:

Peptone.....	5.0	g
Beef extract.....	3.0	g
Agar.....	15.0	g
Reagent-grade water.....	1	L

Add ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.8 ± 0.2 after sterilization. Before sterilization, dispense in screw-capped tubes. After sterilization, immediately place tubes in an inclined position so that the agar will solidify with a sloped surface. Tighten screw caps after cooling and store in a protected, cool storage area.

4) Gram-stain reagents:

a) *Ammonium oxalate-crystal violet (Hucker's)*: Dissolve 2 g crystal violet (90% dye content) in 20 mL 95% ethyl alcohol; dissolve 0.8 g $(\text{NH}_4)_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ in 80 mL reagent-grade water; mix the two solutions and age for 24 h before use; filter through paper into a staining bottle.

b) *Lugol's solution, Gram's modification*: Grind 1 g iodine crystals and 2 g KI in a mortar. Add reagent-grade water, a few milliliters at a time, and grind thoroughly after each addition

until solution is complete. Rinse solution into an amber glass bottle with the remaining water (using a total of 300 mL).

c) *Counterstain*: Dissolve 2.5 g safranin dye in 100 mL 95% ethyl alcohol. Add 10 mL to 100 mL reagent-grade water.

d) *Acetone alcohol*: Mix equal volumes of ethyl alcohol (95%) with acetone.

b. Procedure:

1) Using aseptic technique, streak one LES Endo agar (Section 9222B.2) or MacConkey agar plate from each tube of brilliant green lactose bile broth showing gas, as soon as possible after the observation of gas. Streak plates in a manner to insure presence of some discrete colonies separated by at least 0.5 cm. Observe the following precautions when streaking plates to obtain a high proportion of successful isolations if coliform organisms are present: (a) Use a sterile 3-mm-diam loop or an inoculating needle slightly curved at the tip; (b) tap and incline the fermentation tube to avoid picking up any membrane or scum on the needle; (c) insert end of loop or needle into the liquid in the tube to a depth of approximately 0.5 cm; and (d) streak plate for isolation with curved section of the needle in contact with the agar to avoid a scratched or torn surface. Flame loop between second and third quadrants to improve colony isolation.

Incubate plates (inverted) at $35 \pm 0.5^\circ\text{C}$ for 24 ± 2 h.

2) The colonies developing on LES Endo agar are defined as *typical* (pink to dark red with a green metallic surface sheen) or *atypical* (pink, red, white, or colorless colonies without sheen) after 24 h incubation. Typical lactose-fermenting colonies developing on MacConkey agar are red and may be surrounded by an opaque zone of precipitated bile. From each plate pick one or more typical, well-isolated coliform colonies or, if no typical colonies are present, pick two or more colonies considered most likely to consist of organisms of the coliform group, and transfer growth from each isolate to a single-strength lauryl tryptose broth fermentation tube and onto a nutrient agar slant. (The latter is unnecessary for drinking water samples.)

If needed, use a colony magnifying device to provide optimum magnification when colonies are picked from the LES Endo or MacConkey agar plates. When transferring colonies, choose well-isolated ones and barely touch the surface of the colony with a flame-sterilized, air-cooled transfer needle to minimize the danger of transferring a mixed culture.

Incubate secondary broth tubes (lauryl tryptose broth with inverted fermentation vials inserted) at $35 \pm 0.5^\circ\text{C}$ for 24 ± 2 h; if gas is not produced within 24 ± 2 h reincubate and examine again at 48 ± 3 h. Microscopically examine Gram-stained preparations from those 24-h nutrient agar slant cultures corresponding to the secondary tubes that show gas.

3) Gram stain technique—The Gram stain may be omitted from the completed test for potable water samples only because the occurrences of gram-positive bacteria and spore-forming organisms surviving this selective screening procedure are infrequent in drinking water.

Various modifications of the Gram stain technique exist. Use the following modification by Hucker for staining smears of pure culture; include a gram-positive and a gram-negative culture as controls.

Prepare separate light emulsions of the test bacterial growth and positive and negative control cultures on the same slide using drops of distilled water on the slide. Air-dry and fix by passing slide through a flame and stain for 1 min with ammonium oxalate-crystal violet solution. Rinse slide in tap water and drain off excess; apply Lugol's solution for 1 min.

Rinse stained slide in tap water. Decolorize for approximately 15 to 30 s with acetone alcohol by holding slide between the fingers and letting acetone alcohol flow across the stained smear until the solvent flows colorlessly from the slide. Do not over-decolorize. Counterstain with safranin for 15 s, rinse with tap water, blot dry with absorbent paper or air dry, and examine microscopically. Gram-positive organisms are blue; gram-negative organisms are red. Results are acceptable only when controls have given proper reactions.

c. *Interpretation*: Formation of gas in the secondary tube of lauryl tryptose broth within 48 ± 3 h and demonstration of gram-negative, nonspore-forming, rod-shaped bacteria from the agar culture constitute a positive result for the completed test, demonstrating the presence of a member of the coliform group.

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9221 C. Estimation of Bacterial Density

1. Precision of the MPN Fermentation Tube Test

Unless a large number of sample portions is examined, the precision of the most probable number (MPN) fermentation tube test is rather low. For example, Table 9221:IV shows that the 95% confidence limits are often one-third times and three times the estimate. Consequently, use caution when interpreting the sanitary significance of any single coliform result. When several samples from a given sampling point are estimated separately and the results combined in their geometric mean, the precision is greatly improved.

2. Table Reading and Recording of MPN

Record coliform concentration as the Most Probable Number (MPN)/100 mL. The MPN values, for a variety of positive and negative tube combinations, are given in Tables 9221:II, III, and IV. The sample volumes indicated in Tables 9221:II and III are chosen especially for examining finished waters. Table 9221:IV illustrates MPN values for combinations of positive and negative results when five 10-mL, five 1.0-mL, and five 0.1-mL sample portion volumes are tested. If the sample portion volumes used are those found in the tables, report the value corresponding to the number of positive and negative results in the series as the MPN/100 mL, or, when appropriate, as presence or absence. When the series of decimal dilutions is different from that in the table, select the MPN value from Table 9221:IV for the combination of positive results and calculate according to the following formulas:

$$\text{MPN}/100 \text{ mL} = (\text{Table MPN}/100 \text{ mL}) \times 10/V$$

where:

V = volume of one sample portion at the lowest selected dilution.

When more than three dilutions are used in a decimal series¹ of dilutions, select the three most appropriate dilutions and refer to Table 9221:IV. Several examples (a through f) illustrate correct selection.

TABLE 9221:II. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 20-ML PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 5 (20 mL Each)	MPN Index/100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	—	3.5
1	1.1	0.051	5.4
2	2.6	0.40	8.4
3	4.6	1.0	13
4	8.0	2.1	23
5	>8.0	3.4	—

Example	Volume mL					Combination of Positives	MPN Index No./100 mL
	10	1	0.1	0.01	0.001		
a	5	5	1	0	0	5-1-0	330
b	4	5	1	0	0	4-5-1	48
c	0	0	1	0	0	0-0-1	1.8
d	5	4	4	1	0	4-4-1	400
e	5	4	4	0	1	4-4-1	400
f	5	5	5	5	2	5-5-2	54 000

Select highest dilution that gives positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions (Example a). If the lowest dilution tested has less than five portions with positive results, select it and the two next succeeding higher dilutions (Examples b, c).

When a positive result occurs in a dilution higher than the three selected according to the foregoing rules, change the selection to the lowest dilution that has less than five positive results and the next two higher dilutions (Example d). When all the foregoing selection rules have left unselected any higher dilutions with positive results, add those higher-dilution positive results to the results for the highest selected dilution (Example e). If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilution (Example f).

When it is desired to summarize with a single MPN value the results from several samples, use the geometric mean or the median. The geometric mean is computed by averaging the logarithmic values; e.g., the geometric mean of A, B, and C is 10^L where

$$L = (\log_{10} A + \log_{10} B + \log_{10} C) / 3$$

Mean values are reported as the antilog of L.

Table 9221:IV shows all but the very improbable positive tube combinations for a three-dilution series. In testing 10 different samples, there is a 99% chance of finding all the results among

TABLE 9221:III. MPN INDEX AND 95% CONFIDENCE LIMITS FOR ALL COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN TEN 10-ML PORTIONS ARE USED

No. of Tubes Giving Positive Reaction Out of 10 (10 mL Each)	MPN Index/100 mL	95% Confidence Limits (Exact)	
		Lower	Upper
0	<1.1	—	3.4
1	1.1	0.051	5.9
2	2.2	0.37	8.2
3	3.6	0.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.8	34
9	23	8.1	53
10	>23	13	—

TABLE 9221:IV. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS WHEN FIVE TUBES ARE USED PER DILUTION (10 mL, 1.0 mL, 0.1 mL)*

Combination of Positives	MPN Index/ 100 mL	Confidence Limits		Combination of Positives	MPN Index/ 100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	10	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

* Results to two significant figures.

these 95 outcomes. If untabulated combinations occur with a frequency greater than 0.1%, it indicates that the technique is faulty or that the statistical assumptions underlying the MPN estimate are not being fulfilled (e.g., growth inhibition at low dilutions).

The MPN for combinations not appearing in the table, or for other combinations of tubes or dilutions, may be estimated as

follows: First, select the lowest dilution that does not have all positive results. Second, select the highest dilution with at least one positive result. Finally, select all the dilutions between them. For example, from (5/5, 10/10, 4/10, 1/10, 0/5) use only (—, —, 4/10, 1/10, —), 4/10 @ 0.1 mL sample/tube and 1/10 @ 0.01 mL sample/tube; from (5/5, 10/10, 10/10, 0/10, 0/5) select only (—, —, 10/10, 0/10, —), 10/10 @ 0.1 mL sample/tube and 0/10 @ 0.01

mL sample/tube. Use only the selected dilutions in the following formula of Thomas¹:

$$\text{MPN/100 mL (approx.)} = 100 \times P / (N \times T)^{1/2}$$

where:

P = number of positive results,

N = volume of sample in all the negative portions combined, mL, and

T = total volume of sample in the selected dilutions, mL.

In the first example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 5 / (0.69 \times 1.1)^{1/2} =$$

$$500/0.87 = 570/100 \text{ mL}$$

In the second example above,

$$\text{MPN/100 mL (approx.)} = 100 \times 10 / (0.1 \times 1.1)^{1/2} =$$

$$1000/0.332 = 3000/100 \text{ mL}$$

The two examples compare well with the true MPNs, 590/100 mL and 2400/100 mL, respectively. The second example is a special case for which an exact solution can be calculated directly for the two selected dilutions.

Although MPN tables and calculations are described for use in the coliform test, they also determine the MPN of any other organisms provided that suitable test media are available.

When all the results at the lower dilutions are positive and all the results at higher dilutions are negative, it is possible to calculate an exact MPN for two selected dilutions as follows: When V is the volume of each individual sample portion at the highest dilution with all positive portions,

$$\text{MPN/100 mL} = (1/V) [230.3 \log_{10} (T/N)]$$

where T and N are defined as for Thomas's formula. The last

example discussed above was (5/5, 10/10, 10/10, 0/10, 0/5), with portions 10, 1, 0.1, 0.01, and 0.001. The third dilution is the highest with positive portions, so $V = 0.1$. The MPN for the third and fourth dilution would be exactly

$$\text{MPN/100 mL} = (1/0.1) \times [230.3 \log_{10} (1.1/0.1)] = 2400/100 \text{ mL}$$

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9221 D. Presence-Absence (P-A) Coliform Test

The presence-absence (P-A) test for the coliform group is a simple modification of the multiple-tube procedure. Simplification, by use of one large test portion (100 mL) in a single culture bottle to obtain qualitative information on the presence or absence of coliforms, is justified on the theory that no coliforms should be present in 100 mL of a drinking water sample. The P-A test also provides the optional opportunity for further screening of the culture to isolate other indicators (fecal coliform, *Aeromonas*, *Staphylococcus*, *Pseudomonas*, fecal streptococcus, and *Clostridium*) on the same qualitative basis. Additional advantages include the possibility of examining a larger number of samples per unit of time. Comparative studies with the membrane filter procedure indicate that the P-A test may maximize coliform detection in samples containing many organisms that could overgrow coliform colonies and cause problems in detection.

The P-A test is intended for use on routine samples collected from distribution systems or water treatment plants. When sample locations produce a positive P-A result for coliforms, it may be advisable to determine coliform densities in repeat samples. Quantitative information may indicate the magnitude of a contaminating event.

1. Presumptive Phase

a. Culture media:

1) *P-A broth*: This medium is commercially available in dehydrated and in sterile concentrated form.

Beef extract	3.0	g
Peptone	5.0	g
Lactose	7.46	g
Tryptose	9.83	g

Dipotassium hydrogen phosphate, K_2HPO_4	1.35	g
Potassium dihydrogen phosphate, KH_2PO_4	1.35	g
Sodium chloride, NaCl	2.46	g
Sodium lauryl sulfate	0.05	g
Bromocresol purple	0.0085	g
Reagent-grade water	1	L

Make this formulation triple (3 $\times$) strength when examining 100-mL samples. Dissolve the P-A broth medium in water without heating, using a stirring device. Dispense 50 mL prepared medium into a screw-cap 250-mL milk dilution bottle. A fermentation tube insert is not necessary. Autoclave for 12 min at 121°C with the total time in the autoclave limited to 30 min or less. pH should be 6.8 ± 0.2 after sterilization. When the PA medium is sterilized by filtration a 6 $\times$ strength medium may be used. Aseptically dispense 20 mL of the 6 $\times$ medium into a sterile 250-mL dilution bottle or equivalent container.

2) *Lauryl tryptose broth*: See Section 9221B.1.

b. Procedure: Shake sample vigorously for 5 s (approximately 25 times) and inoculate 100 mL into a P-A culture bottle. Mix thoroughly by inverting bottle once or twice to achieve even distribution of the triple-strength medium throughout the sample. Incubate at $35 \pm 0.5^\circ\text{C}$ and inspect after 24 and 48 h for acid reactions.

c. Interpretation: A distinct yellow color forms in the medium when acid conditions exist following lactose fermentation. If gas also is being produced, gently shaking the bottle will result in a foaming reaction. Any amount of gas and/or acid constitutes a positive presumptive test requiring confirmation.

2. Confirmed Phase

The confirmed phase is outlined in Figure 9221:1.

a. Culture medium: Use brilliant green lactose bile fermentation tubes (see 9221B.2).

b. Procedure: Transfer all cultures that show acid reaction or acid and gas reaction to brilliant green lactose bile (BGLB) broth for incubation at $35 \pm 0.5^\circ\text{C}$ (see Section 9221B.2).

c. Interpretation: Gas production in the BGLB broth culture within 48 ± 3 h confirms the presence of coliform bacteria. Report result as presence-absence test positive or negative for total coliforms in 100 mL of sample.

3. Completed Phase

The completed phase is outlined in Section 9221B.3 and Figure 9221:1.

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9221 E. Fecal Coliform Procedure

Elevated-temperature tests for distinguishing organisms of the total coliform group that also belong to the fecal coliform group are described herein. Modifications in technical procedures, standardization of methods, and detailed studies of the fecal coliform group have established the value of this procedure. The test can be performed by one of the multiple-tube procedures described here or by membrane filter methods as described in Section 9222. The procedure using A-1 broth is a single-step method.

The fecal coliform test (using EC medium) is applicable to investigations of drinking water, stream pollution, raw water sources, wastewater treatment systems, bathing waters, seawaters, and general water-quality monitoring. Prior enrichment in presumptive media is required for optimum recovery of fecal coliforms when using EC medium. The test using A-1 medium is applicable to source water, seawater, and treated wastewater.

1. Fecal Coliform Test (EC Medium)

The fecal coliform test is used to distinguish those total coliform organisms that are fecal coliforms. Use EC medium

or, for a more rapid test of the quality of shellfish waters, treated wastewaters, or source waters, use A-1 medium in a direct test.

a. EC medium:

Tryptose or trypticase	20.0	g
Lactose	5.0	g
Bile salts mixture or bile salts No. 3	1.5	g
Dipotassium hydrogen phosphate, K_2HPO_4	4.0	g
Potassium dihydrogen phosphate, KH_2PO_4	1.5	g
Sodium chloride, NaCl	5.0	g
Reagent-grade water	1	L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.9 ± 0.2 after sterilization. Before sterilization, dispense in fermentation tubes, each with an inverted vial, sufficient medium to cover the inverted vial at least partially after sterilization. Close tubes with metal or heat-resistant plastic caps.

b. Procedure: Submit all presumptive fermentation tubes or bottles showing any amount of gas, growth, or acidity within 48 h of incubation to the fecal coliform test.

1) Gently shake or rotate presumptive fermentation tubes or bottles showing gas, growth, or acidity. Using a sterile 3- or 3.5-mm-diam loop or sterile wooden applicator stick, transfer growth from each presumptive fermentation tube or bottle to EC broth (see Section 9221B.2).

2) Incubate inoculated EC broth tubes in a water bath at $44.5 \pm 0.2^\circ\text{C}$ for 24 ± 2 h.

Place all EC tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse tubes to upper level of the medium.

c. Interpretation: Gas production with growth in an EC broth culture within 24 ± 2 h or less is considered a positive fecal coliform reaction. Failure to produce gas (with little or no growth) constitutes a negative reaction. If multiple tubes are used, calculate MPN from the number of positive EC broth tubes as described in Section 9221C. When using only one tube for subculturing from a single presumptive bottle, report as presence or absence of fecal coliforms.

2. Fecal Coliform Direct Test (A-1 Medium)

a. A-1 broth: This medium may be used for the direct isolation of fecal coliforms from water. Prior enrichment in a presumptive medium is not required.

Lactose.....	5.0 g
Tryptone.....	20.0 g
Sodium chloride, NaCl.....	5.0 g
Salicin.....	0.5 g
Polyethylene glycol <i>p</i> -isooctylphenyl ether*	1.0 mL
Reagent-grade water.....	1 L

Heat to dissolve solid ingredients, add polyethylene glycol *p*-isooctylphenyl ether, and adjust to pH 6.9 ± 0.1 . Before sterilization dispense in fermentation tubes with an inverted vial

* Triton X-100, Rohm and Haas Co., or equivalent.

sufficient medium to cover the inverted vial at least partially after sterilization. Close with metal or heat-resistant plastic caps. Sterilize by autoclaving at 121°C for 10 min. Store in dark at room temperature for not longer than 7 d. Ignore formation of precipitate.

Make A-1 broth of such strength that adding 10-mL sample portions to medium will not reduce ingredient concentrations below those of the standard medium. For 10-mL samples prepare double-strength medium.

b. Procedure: Inoculate tubes of A-1 broth as directed in Section 9221B.1b1). Incubate for 3 h at $35 \pm 0.5^\circ\text{C}$. Transfer tubes to a water bath at $44.5 \pm 0.2^\circ\text{C}$ and incubate for an additional 21 ± 2 h.

c. Interpretation: Gas production in any A-1 broth culture within 24 h or less is a positive reaction indicating the presence of fecal coliforms. Calculate MPN from the number of positive A-1 broth tubes as described in Section 9221C.

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9221 F. *Escherichia coli* Procedure (PROPOSED)

Escherichia coli is a member of the fecal coliform group of bacteria. This organism in water indicates fecal contamination. Enzymatic and biochemical assays have been developed that allow for the identification of this organism. Assays for β -glucuronidase, glutamate decarboxylase, or indole may be used to determine the presence of *E. coli*. For the *E. coli* test with EC-MUG medium, ¶ 1 below, *E. coli* is defined as the species of coliform bacteria possessing the enzyme β -glucuronidase and capable of cleaving the fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide (MUG) with the corresponding release of the fluorogen when grown in EC-MUG medium at 44.5°C

within 24 ± 2 h or less. For the *E. coli* test with GAD reagent, ¶ 2 below, the *E. coli* species is defined as coliform bacteria possessing the enzyme glutamate decarboxylase (GAD) and capable of producing an alkaline reaction within 4 h in a reagent containing a lytic agent and glutamic acid. For the *E. coli* test involving indole production, ¶ 3 below, the *E. coli* species is defined as coliform bacteria that can produce indole within 24 ± 2 h or less when grown in tryptone water at 44.5°C . These procedures are used to confirm the presence of *E. coli* after prior enrichment in a presumptive medium for total coliform bacteria. These tests are performed by the tube procedure as described

here or by the membrane filter method as described in Section 9222. The chromogenic substrate procedure (Section 9223) can be used for direct detection of *E. coli*.

Tests for *E. coli* are applicable to the analysis of drinking water, surface water, groundwater, and wastewater. *E. coli* is a member of the indigenous fecal flora of warm-blooded animals. The occurrence of *E. coli* is considered a specific indicator of fecal contamination and the possible presence of enteric pathogens.

1. *Escherichia coli* Test (EC-MUG Medium)

Use EC-MUG medium for the confirmation of *E. coli*.

a. EC-MUG medium:

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3	1.5 g
Dipotassium hydrogen phosphate, K_2HPO_4	4.0 g
Potassium dihydrogen phosphate, KH_2PO_4	1.5 g
Sodium chloride, NaCl	5.0 g
4-methylumbelliferyl- β -D-glucuronide (MUG)	0.05 g
Reagent-grade water	1 L

Add dehydrated ingredients to water, mix thoroughly, and heat to dissolve. pH should be 6.9 ± 0.2 after sterilization for 15 min at 121°C . Before sterilization, dispense in tubes that do not fluoresce under long-wavelength (366 nm) ultraviolet (UV) light. An inverted tube is not necessary. Close tubes with metal or heat-resistant plastic caps.

b. *Procedure*: Submit all presumptive fermentation tubes or bottles (9221B, 9221D) showing growth, gas, or acidity within 48 ± 3 h of incubation to the *E. coli* test.

1) Gently shake or rotate presumptive fermentation tubes or bottles showing growth, gas, or acidity. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from presumptive fermentation tube or bottle to EC-MUG broth.

2) Incubate inoculated EC-MUG tubes in a water bath maintained at $44.5 \pm 0.2^\circ\text{C}$ for 24 ± 2 h. Place all EC-MUG tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in the water-bath incubator to immerse tubes to upper level of medium.

c. *Interpretation*: Examine all tubes exhibiting growth for fluorescence using a long-wavelength UV lamp (preferably 6 W). The presence of bright blue fluorescence is considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret the results and to avoid confusion of weak autofluorescence of the medium as a positive response. If multiple tubes are used, calculate MPN from the number of positive EC-MUG broth tubes as described in Section 9221C. When using only one tube or subculturing from a single presumptive bottle, report as presence or absence of *E. coli*.

2. *Escherichia coli* Test (GAD Procedure)

Use GAD reagent for the confirmation of *E. coli*.

a. GAD reagent:

L-glutamic acid	1.0 g
Sodium chloride, NaCl	90.0 g
Bromocresol green	0.05 g
Polyethylene glycol octylphenyl ether*	3.0 mL
Reagent-grade water	1 L

Add ingredients to water and mix thoroughly until all ingredients are dissolved. pH should be 3.4 ± 0.2 . The reagent is stable for 2 months when stored at 5°C . It can be filter-sterilized (0.2- μm filter) and treated as a sterile solution.

b. *Procedure*: Submit all presumptive fermentation tubes or bottles (9221B, 9221D) showing growth, gas, or acidity within 48 ± 3 h of incubation to the *E. coli* test.

1) Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a graduated pipet, transfer 5 mL broth from the fermentation tube or bottle to 15-mL centrifuge tube.

2) Concentrate the bacterial cells from the broth by centrifugation at 2500 to $3000 \times g$ for 10 min. Discard supernatant and resuspend cells in 5 mL phosphate buffer. Reconcentrate cells by centrifugation (2500 to $3000 \times g$, 10 min). Discard supernatant and add 1.0 mL GAD reagent. Vigorously swirl the tube to resuspend cells in GAD reagent.

3) Incubate tubes at 35°C and observe after 1 h. Tubes may be incubated for a maximum of 4 h.

c. *Interpretation*: Examine all tubes for a distinct color change from yellow to blue. The presence of a blue color is considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* (GAD-positive) culture, a negative control consisting of a known total coliform organism, e.g., *Enterobacter cloacae* (GAD-negative), and an uninoculated GAD reagent control may be incorporated in the assay to assist in interpretation of results. If multiple tubes are used, calculate MPN from the number of positive GAD tubes as described in Section 9221C. When using only one tube or a single presumptive bottle, report as presence or absence of *E. coli*.

3. *Escherichia coli* Test (Indole Production)

Use tryptone water and Kovacs' reagent for confirmation of *E. coli*.

a. Reagents:

1) Tryptone water:

Tryptone	20 g
Sodium chloride, NaCl	5 g
Reagent-grade water	1 L

Add ingredients to water and mix thoroughly until dissolved. Adjust pH to 7.5. Dispense 5-mL portions into tubes, cap, and sterilize for 10 min at 115°C .

2) Kovacs' reagent:

p-Dimethylaminobenzaldehyde	5 g
Amyl alcohol (analytical grade)	75 mL
Hydrochloric acid, conc	25 mL

* Triton X-100, Union Carbide Co., or equivalent.

Dissolve aldehyde in alcohol. Cautiously add acid to aldehyde-alcohol mixture and swirl to mix. Store in the dark at 4°C. This reagent should be pale yellow to light brown in color. Use of low-quality amyl alcohol may produce a dark-colored reagent; do not use such a reagent.

b. Procedure: Submit all presumptive fermentation tubes or bottles (9221B) showing growth, gas, or acidity within 48 ± 3 h of incubation to the *E. coli* test.

Gently shake or rotate presumptive tubes or bottles showing growth, gas, or acidity. Using a sterile 3- or 3.5-mm-diam metal loop or sterile wooden applicator stick, transfer growth from presumptive fermentation tube or bottle to a tube containing 5 mL tryptone water. Incubate inoculated tryptone water tubes in a water bath or incubator maintained at 44.5°C for 24 ± 2 h. After incubation, add 0.2 to 0.3 mL Kovacs' reagent to each tube of tryptone water.

c. Interpretation: Examine all tubes for appearance of a deep red color in the upper layer. The presence of a red color is considered a positive response for *E. coli*. A positive control consisting of a known *E. coli* (indole-positive) culture, a negative control consisting of a known total coliform organism, e.g., *Enterobacter cloacae* (indole-negative), and an uninoculated reagent control may be incorporated in the assay to assist in interpretation of results. If multiple tubes are used, calculate MPN from the number of indole-positive tubes as described in Section 9221C. When using only one tube or a single presumptive bottle, report as presence or absence of *E. coli*.

9222 MEMBRANE FILTER TECHNIQUE FOR MEMBERS OF THE COLIFORM GROUP*

9222 A. Introduction

The membrane filter (MF) technique is highly reproducible, can be used to test relatively large sample volumes, and usually yields numerical results more rapidly than the multiple-tube fermentation procedure. The MF technique is extremely useful in monitoring drinking water and a variety of natural waters. However, the MF technique has limitations, particularly when testing waters with high turbidity or large numbers of noncoliform (background) bacteria. When the MF technique has not been used previously, it is desirable to conduct parallel tests with the method the laboratory is using currently to demonstrate applicability and comparability.

1. Definition

As related to the MF technique, the coliform group is defined as those facultative anaerobic, gram-negative, non-spore-forming, rod-shaped bacteria that develop red colonies with a metallic

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(golden) sheen within 24 h at 35°C on an Endo-type medium containing lactose. Some members of the total coliform group may produce dark red, mucoid, or nucleated colonies without a metallic sheen. When verified these are classified as atypical coliform colonies. When purified cultures of coliform bacteria are tested, they produce negative cytochrome oxidase and positive β -galactosidase test reactions.† Generally, pink (non-mucoid), blue, white, or colorless colonies lacking sheen are considered noncoliforms by this technique.

2. Applications

Turbidity caused by the presence of algae, particulates, or other interfering material may not permit testing of a sample volume sufficient to yield significant results. Low coliform estimates may be caused by the presence of high numbers of noncoliforms or of toxic substances. The MF technique is applicable to the examination of saline waters, but not wastewaters that have received only primary treatment followed by chlorination because of turbidity in high volume samples or wastewaters

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—Nancy H. Hall (chair), Paul S. Berger, Linda R. Blish, Robert H. Bordner, John K. Brokaw, Ellen P. Flanagan, Edwin E. Geldreich, Paul J. Hickey, Mark W. LeChevallier, Shundar Lin, Gordon A. McFeters, Brian A. Nelsen, Peggy A. Ryker, Lynn P. Smith, Judy A. Starcevic, Carol A. Ziel.

† ONPG is a substrate for the β -galactosidase test.

containing toxic metals or toxic organic compounds such as phenols. For the detection of stressed total coliforms in treated drinking water and chlorinated secondary or tertiary wastewater effluents, use a method designed for stressed organism recovery (see Section 9212B.1). A modified MF technique for fecal coliforms (Section 9212) in chlorinated wastewater may be used if parallel testing over a 3-month period with the multiple-tube fermentation technique shows comparability for each site-specific type of sample.

The standard volume to be filtered for drinking water samples is 100 mL. This may be distributed among multiple membranes if necessary. However, for special monitoring purposes, such as troubleshooting water quality problems or identification of coliform breakthrough in low concentrations from treatment barriers, it may be desirable to test 1-L samples. If particulates prevent filtering a 1-L sample through a single filter, divide sample into four portions of 250 mL for analysis. Total the coliform counts on each membrane to report the number of coliforms per liter. Smaller sample volumes will be necessary for source or recreational waters and wastewater effluents that have much higher coliform densities.

9222 B. Standard Total Coliform Membrane Filter Procedure

1. Laboratory Apparatus

For MF analyses use glassware and other apparatus composed of material free from agents that may affect bacterial growth.

a. *Sample bottles:* See Section 9030B.18.

b. *Dilution bottles:* See Section 9030B.13.

c. *Pipets and graduated cylinders:* See Section 9030B.9. Before sterilization, loosely cover opening of graduated cylinders with metal foil or a suitable heavy wrapping-paper substitute. Immediately after sterilization secure cover to prevent contamination.

d. *Containers for culture medium:* Use clean borosilicate glass flasks. Any size or shape of flask may be used, but erlenmeyer flasks with metal caps, metal foil covers, or screw caps provide for adequate mixing of the medium contained and are convenient for storage.

e. *Culture dishes:* Use sterile borosilicate glass or disposable, presterilized plastic petri dishes, 60 × 15 mm, 50 × 9 mm, or other appropriate size. Wrap convenient numbers of clean, glass culture dishes in metal foil if sterilized by dry heat, or suitable heavy wrapping paper when autoclaved. Incubate loose-lidded glass and disposable plastic culture dishes in tightly closed containers with wet paper or cloth to prevent moisture evaporation with resultant drying of medium and to maintain a humid environment for optimum colony development.

Presterilized disposable plastic dishes with tight-fitting lids that meet the specifications above are available commercially and are used widely. Reseal opened packages of disposable dish supplies for storage.

f. *Filtration units:* The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, or stainless steel) consists

of a seamless funnel fastened to a base by a locking device or by magnetic force. The design should permit the membrane filter to be held securely on the porous plate of the receptacle without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on inner surface or glass funnels with chipped surfaces.

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of a seamless funnel fastened to a base by a locking device or by magnetic force. The design should permit the membrane filter to be held securely on the porous plate of the receptacle without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on inner surface or glass funnels with chipped surfaces.

Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, sterilize by autoclaving, and store until use. Alternatively expose all surfaces of the previously cleaned assembly to ultraviolet radiation (2 min exposure) for the initial sanitization before use in the test procedure, or before reusing units between successive filtration series. Field units may be sanitized by dipping or spraying with alcohol and then igniting or immersing in boiling water for 2 min. After submerging unit in boiling water, cool it to room temperature before reuse. Do not ignite plastic parts. Sterile, disposable field units may be used.

For filtration, mount receptacle of filter-holding assembly on a 1-L filtering flask with a side tube or other suitable device (manifold to hold three to six filter assemblies) such that a pressure differential (34 to 51 kPa) can be exerted on the filter membrane. Connect flask to a vacuum line, an electric vacuum pump, a filter pump operating on water pressure, a hand aspirator, or other means of securing a pressure differential (138 to 207 kPa). Connect a flask of approximately the same capacity between filtering flask and vacuum source to trap carry-over water.

g. *Membrane filter:* Use membrane filters (for additional specifications, see Section 9020) with a rated pore diameter such that there is complete retention of coliform bacteria. Use only those filter membranes that have been found, through adequate quality control testing and certification by the manufacturer, to

exhibit: full retention of the organisms to be cultivated, stability in use, freedom from chemical extractables that may inhibit bacterial growth and development, a satisfactory speed of filtration (within 5 min), no significant influence on medium pH (beyond ± 0.2 units), and no increase in number of confluent colonies or spreaders compared to control membrane filters. Use membranes grid-marked in such a manner that bacterial growth is neither inhibited nor stimulated along the grid lines when the membranes with entrapped bacteria are incubated on a suitable medium. Preferably use fresh stocks of membrane filters and if necessary store them in an environment without extremes of temperature and humidity. Obtain no more than a year's supply at any one time.

Preferably use presterilized membrane filters for which the manufacturer has certified that the sterilization technique has neither induced toxicity nor altered the chemical or physical properties of the membrane. If membranes are sterilized in the laboratory, autoclave for 10 min at 121°C. At the end of the sterilization period, let the steam escape rapidly to minimize accumulation of water of condensation on filters.

h. Absorbent pads consist of disks of filter paper or other material certified for each lot by the manufacturer to be of high quality and free of sulfites or other substances of a concentration that could inhibit bacterial growth. Use pads approximately 48 mm in diameter and of sufficient thickness to absorb 1.8 to 2.2 mL of medium. Presterilized absorbent pads or pads subsequently sterilized in the laboratory should release less than 1 mg total acidity (calculated as CaCO_3) when titrated to the phenolphthalein end point, pH 8.3, using 0.02N NaOH and produce pH levels of 7 ± 0.2 . Sterilize pads simultaneously with membrane filters available in resealable kraft envelopes, or separately in other suitable containers. Dry pads so they are free of visible moisture before use. See sterilization procedure described for membrane filters above and Section 9020 for additional specifications on absorbent pads.

i. Forceps: Smooth flat forceps, without corrugations on the inner sides of the tips. Sterilize before use by dipping in 95% ethyl or absolute methyl alcohol and flaming.

j. Incubators: Use incubators to provide a temperature of $35 \pm 0.5^\circ\text{C}$ and to maintain a humid environment (60% relative humidity).

k. Microscope and light source: To determine colony counts on membrane filters, use a magnification of 10 to 15 diameters and a cool white fluorescent light source adjusted to give maximum sheen discernment. Optimally use a binocular wide-field dissecting microscope. Do not use a microscope illuminator with optical system for light concentration from an incandescent light source for discerning coliform colonies on Endo-type media.

2. Materials and Culture Media

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

Test each new medium lot against a previously acceptable lot for satisfactory performance as described in Section 9020B. With each new lot of Endo-type medium, verify a minimum 10% of coliform colonies, obtained from natural samples or samples with known additions, to establish the comparative recovery of the medium lot.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through the filter. If controls indicate contamination, reject all data from affected samples and request resample.

a. LES Endo agar:*

Yeast extract	1.2 g
Casitone or trypticase	3.7 g
Thiopeptone or thiotone	3.7 g
Tryptose	7.5 g
Lactose	9.4 g
Dipotassium hydrogen phosphate, K_2HPO_4	3.3 g
Potassium dihydrogen phosphate, KH_2PO_4	1.0 g
Sodium chloride, NaCl	3.7 g
Sodium desoxycholate	0.1 g
Sodium lauryl sulfate	0.05 g
Sodium sulfite, Na_2SO_3	1.6 g
Basic fuchsin	0.8 g
Agar	15.0 g
Reagent-grade water	1 L

Rehydrate product in 1 L water containing 20 mL 95% ethanol. Do not use denatured ethanol, which reduces background growth and coliform colony size. Bring to a near boil to dissolve agar, then promptly remove from heat and cool to 45 to 50°C. Do not sterilize by autoclaving. Final pH 7.2 ± 0.2 . Dispense in 5- to 7-mL quantities into lower section of 60-mm glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth of 4 to 5 mm. Do not expose poured plates to direct sunlight; refrigerate in the dark, preferably in sealed plastic bags or other containers to reduce moisture loss. Discard unused medium after 2 weeks or sooner if there is evidence of moisture loss, medium contamination, or medium deterioration (darkening of the medium).

b. M-Endo medium:†

Tryptose or polypeptone	10.0 g
Thiopeptone or thiotone	5.0 g
Casitone or trypticase	5.0 g
Yeast extract	1.5 g
Lactose	12.5 g
Sodium chloride, NaCl	5.0 g
Dipotassium hydrogen phosphate, K_2HPO_4	4.375 g
Potassium dihydrogen phosphate, KH_2PO_4	1.375 g
Sodium lauryl sulfate	0.05 g
Sodium desoxycholate	0.10 g
Sodium sulfite, Na_2SO_3	2.10 g
Basic fuchsin	1.05 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

* Dehydrated Difco M-Endo Agar LES (No. 0736), dehydrated BBL M-Endo Agar LES (No. 11203), or equivalent.

† Dehydrated Difco M-Endo Broth MF (No. 0749), dehydrated BBL m-Coliform Broth (No. 11119), or equivalent may be used if absorbent pads are used.

TABLE 9222:I. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER TOTAL COLIFORM TEST

Water Source	Volume (X) To Be Filtered mL						
	100	50	10	1	0.1	0.01	0.001
Drinking water	X						
Swimming pools	X						
Wells, springs	X	X	X				
Lakes, reservoirs	X	X	X				
Water supply intake			X	X	X		
Bathing beaches			X	X	X		
River water				X	X	X	X
Chlorinated sewage				X	X	X	
Raw sewage					X	X	X

1) Agar preparation—Rehydrate product in 1 L water containing 20 mL 95% ethanol. Heat to near boiling to dissolve agar, then promptly remove from heat and cool to between 45 and 50°C. Dispense 5- to 7-mL quantities into 60-mm sterile glass or plastic petri dishes. If dishes of any other size are used, adjust quantity to give an equivalent depth. Do not sterilize by autoclaving. Final pH should be 7.2 ± 0.2 . A precipitate is normal in Endo-type media.

Refrigerate finished medium in the dark and discard unused agar after 2 weeks.

2) Broth preparation—Prepare as above, omitting agar. Dispense liquid medium (at least 2.0 mL per plate) onto absorbent pads (see absorbent pad specifications, Section 9222B.1) and carefully remove excess medium by decanting the plate. The broth may have a precipitate but this does not interfere with medium performance if pads are certified free of sulfite or other toxic agents at a concentration that could inhibit bacterial growth. Refrigerated broth may be stored for up to 4 d.

c. *Buffered dilution rinse water*: See Section 9050C.1.

3. Samples

Collect samples as directed in Sections 9060A and B.

4. Coliform Definition

Bacteria that produce a red colony with a metallic (golden) sheen within 24 h incubation at 35°C on an Endo-type medium are considered members of the coliform group. The sheen may cover the entire colony or may appear only in a central area or on the periphery. The coliform group thus defined is based on the production of aldehydes from fermentation of lactose. While this biochemical characteristic is part of the metabolic pathway of gas production in the multiple-tube test, some variations in degree of metallic sheen development may be observed among coliform strains. However, this slight difference in indicator definition is not considered critical to change its public health significance, particularly if suitable studies have been conducted to establish the relationship between results obtained by the MF and those obtained by the standard multiple-tube fermentation procedure.

5. Procedures

a. *Selection of sample size*: Size of sample will be governed by expected bacterial density. In drinking water analyses, sample

size will be limited only by the degree of turbidity or by the noncoliform growth on the medium (Table 9222:I). For regulation purposes, 100 mL is the official sample size.

An ideal sample volume will yield 20 to 80 coliform colonies and not more than 200 colonies of all types on a membrane-filter surface. Analyze drinking waters by filtering 100 to 1000 mL, or by filtering replicate smaller sample volumes such as duplicate 50-mL or four replicates of 25-mL portions. Analyze other waters by filtering three different volumes (diluted or undiluted), depending on the expected bacterial density. See Section 9215B.2 for preparation of dilutions. When less than 10 mL of sample (diluted or undiluted) is to be filtered, add approximately 10 mL sterile dilution water to the funnel before filtration or pipet the sample volume into a sterile dilution bottle, then filter the entire dilution. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

b. *Sterile filtration units*: Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is considered to be interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane filter holders in use. See Section 9222B.1f for sterilization procedures and Section 9020B.3l and m for UV cleaning and safety guidelines.

c. *Filtration of sample*: Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of receptacle. Carefully place matched funnel unit over receptacle and lock it in place. Filter sample under partial vacuum. With filter still in place, rinse the interior surface of the funnel by filtering three 20- to 30-mL portions of sterile dilution water. Alternatively, rinse funnel by a flow of sterile dilution water from a squeeze bottle. This is satisfactory only if the squeeze bottle and its contents do not become contaminated during use. Rinsing between samples prevents carryover contamination. Upon completion of final rinse and the filtration process disengage vacuum, unlock and remove funnel, immediately remove membrane filter with sterile forceps, and place it on selected medium with a rolling motion to avoid entrapment of air. If the agar-based medium is used, place prepared filter directly on agar, invert dish, and incubate for 22 to 24 h at $35 \pm 0.5^\circ\text{C}$.

If liquid medium is used, place a pad in the culture dish and saturate with at least 2.0 mL M-Endo medium and carefully remove excess medium by decanting the plate. Place prepared

filter directly on pad, invert dish, and incubate for 22 to 24 h at $35 \pm 0.5^\circ\text{C}$.

Differentiation of some colonies from either agar or liquid medium substrates may be lost if cultures are incubated beyond 24 h.

Insert a sterile rinse water sample (100 mL) after filtration of a series of 10 samples to check for possible cross-contamination or contaminated rinse water. Incubate the rinse water control membrane culture under the same conditions as the sample.

For nonpotable water samples, preferably decontaminate filter unit after each sample (as described above) because of the high number of coliform bacteria present in these samples. Alternatively, use an additional buffer rinse of the filter unit after the filter is removed to prevent carryover between samples.

d. Alternative enrichment technique: Place a sterile absorbent pad in the lid of a sterile culture dish and pipet at least 2.0 mL lauryl tryptose broth, prepared as directed in 9221B.1a1), to saturate pad. Carefully remove any excess liquid from absorbent pad by decanting plate. Aseptically place filter through which the sample has been passed on pad. Incubate filter, without inverting dish, for 1.5 to 2 h at $35 \pm 0.5^\circ\text{C}$ in an atmosphere of at least 60% relative humidity.

If the agar-based Endo-type medium is used, remove enrichment culture from incubator, lift filter from enrichment pad, and roll it onto the agar surface, which has been allowed to equilibrate to room temperature. Incorrect filter placement is at once obvious, because patches of unstained membrane indicate entrapment of air. Where such patches occur, carefully reseal filter on agar surface. If the liquid medium is used, prepare final culture by removing enrichment culture from incubator and separating the dish halves. Place a fresh sterile pad in bottom half of dish and saturate with at least 2.0 mL of M-Endo medium and carefully remove excess liquid from absorbent pad by decanting plate. Transfer filter, with same precautions as above, to new pad. Discard used enrichment pad.

With either the agar or the liquid medium, invert dish and incubate for 20 to 22 h at $35 \pm 0.5^\circ\text{C}$. Proceed to *e* below.

e. Counting: To determine colony counts on membrane filters, use a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device, with a cool white fluorescent light source directed to provide optimal viewing of sheen. The typical coliform colony has a pink to dark-red color with a metallic surface sheen. Count both typical and atypical coliform colonies. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered noncoliforms. The total count of colonies (coliform and noncoliform) on Endo-type medium has no consistent relationship to the total number of bacteria present in the original sample. A high count of noncoliform colonies may interfere with the maximum development of coliforms. Refrigerating cultures (after 22 h incubation) with high densities of noncoliform colonies for 0.5 to 1 h before counting may deter spread of confluence while aiding sheen discernment.

Samples of disinfected water or wastewater effluent may include stressed organisms that grow relatively slowly and produce maximum sheen in 22 to 24 h. Organisms from undisinfected sources may produce sheen at 16 to 18 h, and the sheen subsequently may fade after 24 to 30 h.

f. Coliform verification: Occasionally, typical sheen colonies may be produced by noncoliform organisms and atypical colonies (dark red or nucleated colonies without sheen) may be coliforms. Preferably verify all typical and atypical colony types. For drinking water, verify all suspect colonies by swabbing the entire membrane or pick at least five typical colonies and five atypical colonies from a given membrane filter culture. For waters other than drinking water, at a minimum, verify at least 10 sheen colonies (and representative atypical colonies of different morphological types) from a positive water sample monthly. See Section 9020B.8. Based on need and sample type, laboratories may incorporate more stringent quality control measures (e.g., verify at least one colony from each typical or atypical colony type from a given membrane filter culture, verify 10% of the positive samples). Adjust counts on the basis of verification results. Verification tests are listed below.

1) Lactose fermentation—Transfer growth from each colony or swab the entire membrane with a sterile cotton swab (for presence-absence results in drinking water samples) and place in lauryl tryptose broth; incubate the lauryl tryptose broth at $35 \pm 0.5^\circ\text{C}$ for 48 h. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose broth (Section 9221B.2 for medium preparation) within 48 h verifies the colony as a coliform. Simultaneous inoculation of both media for gas production is acceptable. Inclusion of EC broth inoculation for $44.5 \pm 0.2^\circ\text{C}$ incubation will provide information on the presence of fecal coliforms. Use of EC-MUG with incubation at $44.5 \pm 0.2^\circ\text{C}$ for 24 h will provide information on presence of *E. coli*. See Section 9222G for MF partition procedures.

2) Alternative coliform verifications—Apply this alternative coliform verification procedure to isolated colonies on the membrane filter culture. If a mixed culture is suspected or if colony separation is less than 2 mm, streak the growth to M-Endo medium or MacConkey agar to assure culture purity or submit the mixed growth to the fermentation tube method.

a) Rapid test—A rapid verification of colonies utilizes test reactions for cytochrome oxidase (CO) and β -galactosidase. Coliform reactions are CO negative and β -galactosidase positive within 4 h incubation of tube culture or micro (spot) test procedure.

b) Commercial multi-test systems—Verify the colony by streaking it for purification, selecting a well-isolated colony, and inoculating into a multi-test identification system for Enterobacteriaceae that includes lactose fermentation and/or β -galactosidase and CO test reactions.

6. Calculation of Coliform Density

Compute the count, using membrane filters with 20 to 80 coliform colonies and not more than 200 colonies of all types per membrane, by the following equation:

$$(\text{Total}) \text{ coliforms}/100 \text{ mL} = \frac{\text{coliform colonies counted} \times 100}{\text{mL sample filtered}}$$

If no coliform colonies are observed, report the coliform colonies counted as "<1 coliform/100 mL."

For verified coliform counts, adjust the initial count based upon the positive verification percentage and report as "verified coliform count/100 mL."

Percentage verified coliforms

$$\frac{\text{number of verified colonies}}{\text{total number of coliform colonies subjected to verification}} \times 100$$

a. Water of drinking water quality: While the EPA Total Coliform Rule for public water supply samples requires only a record of coliform presence or absence in 100-mL samples, it may be advisable to determine coliform densities in repeat sampling situations. This is of particular importance when a coliform biofilm problem is suspected in the distribution system. Quantitative information may provide an indication of the magnitude of a contaminating event.

With water of good quality, the occurrence of coliforms generally will be minimal. Therefore, count all coliform colonies (disregarding the lower limit of 20 cited above) and use the formula given above to obtain coliform density.

If confluent growth occurs, covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with (or without) coliforms." If the total number of bacterial colonies, coliforms plus noncoliforms, exceeds 200 per membrane, or if the colonies are not distinct enough for accurate counting, report results as "too numerous to count" (TNTC) or "confluent," respectively. For drinking water, the presence of coliforms in such cultures showing no sheen may be confirmed by either transferring a few colonies or placing the entire membrane filter culture into a sterile tube of brilliant green lactose bile broth. As an alternative, brush the entire filter surface with a sterile loop, applicator stick, or cotton swab and inoculate this growth to the tube of brilliant green lactose bile broth. If gas is produced from the brilliant green bile broth tube within 48 h at $35 \pm 0.5^\circ\text{C}$, coliforms are present. For compliance with the EPA Total Coliform Rule, report confluent growth or TNTC with at least one detectable coliform colony (which is verified) as a total coliform positive sample. Report confluent growth or TNTC without detectable coliforms as invalid. For invalid samples, request a new sample from the same location within 24 h and select more appropriate volumes to be filtered per membrane, observing the requirement that the standard drinking water portion is 100 mL, or choose another coliform method that is less subject to heterotrophic bacterial interferences. Thus, to reduce interference from overcrowding, instead of filtering 100 mL per membrane, filter 50-mL portions through two separate membranes, 25-mL portions through each of four membranes, etc. Total the coliform counts observed on all membranes and report as number per 100 mL.

b. Water of other than drinking water quality: As with potable water samples, if no filter has a coliform count falling in the ideal range, total the coliform counts on all filters and report as number per 100 mL. For example, if duplicate 50-mL portions were examined and the two membranes had five and three coliform colonies, respectively, report the count as eight coliform colonies per 100 mL, i.e.,

$$\frac{[(5 + 3) \times 100]}{(50 + 50)} = 8 \text{ coliforms/100 mL}$$

Similarly, if 50-, 25-, and 10-mL portions were examined and the counts were 15, 6, and <1 coliform colonies, respectively, report the count as 25/100 mL, i.e.,

$$\frac{[(15 + 6 + 0) \times 100]}{(50 + 25 + 10)} = 25 \text{ coliforms/100 mL}$$

On the other hand, if 10-, 1.0-, and 0.1-mL portions were examined with counts of 40, 9, and <1 coliform colonies, respectively, select the 10-mL portion only for calculating the coliform density because this filter had a coliform count falling in the ideal range. The result is 400/100 mL, i.e.,

$$\frac{(40 \times 100)}{10} = 400 \text{ coliforms/100 mL}$$

In this last example, if the membrane with 40 coliform colonies also had a total bacterial colony count greater than 200, report the coliform count as $\geq 400/100$ mL.

Report confluent growth or membranes with colonies too numerous to count as described in *a* above. Request a new sample and select more appropriate volumes for filtration or utilize the multiple-tube fermentation technique.

c. Statistical reliability of membrane filter results: Although the precision of the MF technique is greater than that of the MPN procedure, membrane counts may underestimate the number of viable coliform bacteria. Table 9222:II illustrates some 95% confidence limits. These values are based on the assumption that bacteria are distributed randomly and follow a Poisson distribution. For results with counts, *c*, greater than 20 organisms, calculate the approximate 95% confidence limits using the following normal distribution equations:

TABLE 9222:II. CONFIDENCE LIMITS FOR MEMBRANE FILTER COLIFORM RESULTS USING 100-ML SAMPLE

Number of Coliform Colonies Counted	95% Confidence Limits	
	Lower	Upper
0	0.0	3.7
1	0.1	5.6
2	0.2	7.2
3	0.6	8.8
4	1.0	10.2
5	1.6	11.7
6	2.2	13.1
7	2.8	14.4
8	3.4	15.8
9	4.0	17.1
10	4.7	18.4
11	5.4	19.7
12	6.2	21.0
13	6.9	22.3
14	7.7	23.5
15	8.4	24.8
16	9.2	26.0
17	9.9	27.2
18	10.7	28.4
19	11.5	29.6
20	12.2	30.8

$$\text{Upper limit} = c + 2\sqrt{c} \quad \text{Lower limit} = c - 2\sqrt{c}$$

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9222 C. Delayed-Incubation Total Coliform Procedure

Modification of the standard MF technique permits membrane shipment or transport after filtration to a distant laboratory for transfer to another substrate, incubation, and completion of the test. This delayed-incubation test may be used where it is impractical to apply conventional procedures. It also may be used: (a) where it is not possible to maintain the desired sample temperature during transport; (b) when the elapsed time between sample collection and analysis would exceed the approved time limit; or (c) where the sampling location is remote from laboratory services.

Independent studies using both fresh- and salt-water samples have shown consistent results between the delayed incubation and standard direct test. Determine the applicability of the delayed-incubation test for a specific water source by comparing with results of conventional MF methods.

To conduct the delayed-incubation test, filter sample in the field immediately after collection, place filter on the transport medium, and ship to the laboratory. Complete the coliform determination in the laboratory by transferring the membrane to standard M-Endo or LES Endo medium, incubating at $35 \pm 0.5^\circ\text{C}$ for 20 to 22 h, and counting typical and atypical coliform colonies that develop. For drinking water samples collected for compliance with the EPA Total Coliform Rule, report the presence or absence of verified coliforms in 100-mL samples. Verify colonies as outlined previously in Section 9222B.5f.

Transport media are designed to keep coliform organisms viable and generally do not permit visible growth during transit time. Bacteriostatic agents in holding/preservative media suppress growth of microorganisms en route but allow normal coliform growth after transfer to a fresh medium.

The delayed-incubation test follows the methods outlined for the total coliform MF procedure, except as indicated below. Two alternative methods are given, one using the M-Endo preservative medium and the other the M-ST holding medium.

1. Apparatus

a. *Culture dishes*: Use disposable, sterile, plastic petri dishes (50×12 mm) with tight-fitting lids. Such containers are light in weight and are less likely to break in transit. In an emergency or when plastic dishes are unavailable, use sterile glass petri dishes wrapped in plastic film or similar material. See Section 9222B.1e for specifications.

b. *Field filtration units*: See Section 9222B.1f for specifications. Disinfect by adding methyl alcohol to the filtering chamber, igniting the alcohol, and covering unit to produce formaldehyde. Ultraviolet light disinfection also may be used in the field if an appropriate power source is available (115 V, 60 Hz). Glass or metal filtration units may be sterilized by immersing in boiling water for 2 min. Use a hand aspirator to obtain necessary vacuum.

2. Materials and Transport Media

a. M-Endo methods:

1) *M-Endo preservative medium*: Prepare M-Endo medium as described in Section 9222B.2b. After cooling to below 45°C , aseptically add 3.84 g sodium benzoate (USP grade)/L or 3.2 mL 12% sodium benzoate solution to 100 mL medium. Mix ingredients and dispense in 5- to 7-mL quantities to 50- $\times$ 9-mm petri plates. Refrigerate poured plates. Discard unused medium after 96 h.

2) *Sodium benzoate solution*: Dissolve 12 g $\text{NaC}_7\text{H}_5\text{O}_2$ in sufficient reagent water to make 100 mL. Sterilize by autoclaving or by filtering through a 0.22- μm pore size membrane filter. Discard after 6 months.

3) *Cycloheximide*:* Optionally add cycloheximide to M-Endo preservative medium. It may be used for samples that previously

* Actidione®, manufactured by the Upjohn Company, Kalamazoo, MI, or equivalent.

have shown overgrowth by fungi, including yeasts. Prepare by aseptically adding 50 mg cycloheximide/100 mL to M-Endo preservative medium. Store cycloheximide solution in refrigerator and discard after 6 months. Cycloheximide is a powerful skin irritant; handle with caution according to the manufacturer's directions.

b. M-ST method:

M-ST holding medium:

Sodium phosphate, monobasic, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.1 g
Dipotassium hydrogen phosphate, K_2HPO_4	3.0 g
Sulfanilamide	1.5 g
Ethanol (95%)	10 mL
Tris (hydroxymethyl) aminomethane	3.0 g
Reagent-grade water	1 L

Dissolve ingredients by rehydrating in water. Sterilize by autoclaving at 121°C for 15 min. Final pH should be 8.6 ± 0.2 . Dispense at least 2.0 mL to tight-lidded plastic culture dishes containing an absorbent pad and carefully remove excess liquid from pad by decanting plate. Store in refrigerator for use within 96 h.

3. Procedure

a. Sample preservation and shipment: Place absorbent pad in bottom of sterile petri dish and saturate with selected coliform holding medium (see Section 9222C.2 above). Remove membrane filter from filtration unit with sterile forceps and roll it, grid side up, onto surface of medium-saturated pad. Protect membrane from moisture loss by tightly closing plastic petri dish. Seal loose-fitting dishes with an appropriate sealing tape to prevent membrane dehydration during transit. Place culture dish

containing membrane in an appropriate shipping container and send to the laboratory for test completion. The sample can be held without visible growth for a maximum of 72 h on the holding/preservative medium. This usually allows use of the mail or a common carrier. Visible growth occasionally begins on the transport medium when high temperatures are encountered during transit.

b. Transfer and incubation: At the laboratory, transfer membrane from holding medium on which it was shipped to a second sterile petri dish containing M-Endo or LES Endo medium and incubate at $35 \pm 0.5^\circ\text{C}$ for 20 to 22 h.

4. Estimation of Coliform Density

Proceed as in Section 9222B.6 above. Record times of collection, filtration, and laboratory examination, and calculate the elapsed time. Report elapsed time with coliform results.

5. Bibliography

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9222 D. Fecal Coliform Membrane Filter Procedure

Fecal coliform bacterial densities may be determined either by the multiple-tube procedure or by the MF technique. See Section 9225 for differentiation of *Escherichia coli*, the predominant fecal coliform. If the MF procedure is used for chlorinated effluents, demonstrate that it gives comparable information to that obtainable by the multiple-tube test before accepting it as an alternative. The fecal coliform MF procedure uses an enriched lactose medium and incubation temperature of $44.5 \pm 0.2^\circ\text{C}$ for selectivity. Because incubation temperature is critical, submerge waterproofed (plastic bag enclosures) MF cultures in a water bath for incubation at the elevated temperature or use an appropriate solid heat sink incubator or other incubator that is documented to hold the 44.5°C temperature within 0.2°C throughout the chamber, over a 24-h period. Areas of application for the fecal coliform method in general are stated in the introduction to the multiple-tube fecal coliform procedures, Section 9221E.

1. Materials and Culture Medium

a. M-FC medium: 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. 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.

M-FC medium:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride, NaCl	5.0 g
Lactose	12.5 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar (optional)	15.0 g
Reagent-grade water	1 L

Rehydrate product in 1 L water containing 10 mL 1% rosolic acid in 0.2N NaOH.* Heat to near boiling, promptly remove

* Rosolic acid reagent will decompose if sterilized by autoclaving. Refrigerate stock solution in the dark and discard after 2 weeks or sooner if its color changes from dark red to muddy brown.

TABLE 9222:III. SUGGESTED SAMPLE VOLUMES FOR MEMBRANE FILTER FECAL COLIFORM TEST

Water Source	Volume (X) To Be Filtered mL						
	100	50	10	1	0.1	0.01	0.0001
Lakes, reservoirs	X	X					
Wells, springs	X	X					
Water supply intake		X	X	X			
Natural bathing waters		X	X	X			
Sewage treatment plant			X	X	X		
Farm ponds, rivers				X	X	X	
Stormwater runoff				X	X	X	
Raw municipal sewage					X	X	X
Feedlot runoff					X	X	X
Sewage sludge						X	X

from heat, and cool to below 50°C. Do not sterilize by autoclaving. If agar is used, dispense 5- to 7-mL quantities to 50- × 12-mm petri plates and let solidify. Final pH should be 7.4 ± 0.2. Refrigerate finished medium, preferably in sealed plastic bags or other containers to reduce moisture loss, and discard unused broth after 96 h or unused agar after 2 weeks.

Test each medium lot against a previously acceptable lot for satisfactory performance as described in Section 9020B, by making dilutions of a culture of *E. coli* (Section 9020) and filtering appropriate volumes to give 20 to 60 colonies per filter. With each new lot of medium verify 10 or more colonies obtained from several natural samples, to establish the absence of false positives. For most samples M-FC medium may be used without the 1% rosolic acid addition, provided there is no interference with background growth. Such interference may be expected in stormwater samples collected during the first runoff (initial flushing) after a long dry period.

Before use, test each batch of laboratory-prepared MF medium for performance with positive and negative culture controls. Check for coliform contamination at the beginning and end of each filtration series by filtering 20 to 30 mL of dilution or rinse water through filter. If controls indicate contamination, reject all data from affected samples and request resample.

b. Culture dishes: Tight-fitting plastic dishes are preferred because the membrane filter cultures are submerged in a water bath during incubation. Place fecal coliform cultures in plastic bags or seal individual dishes with waterproof (freezer) tape to prevent leakage during submersion. Specifications for plastic culture dishes are given in Section 9222B.1e.

c. Incubator: The specificity of the fecal coliform test is related directly to the incubation temperature. Static air incubation may be a problem in some types of incubators because of potential heat layering within the chamber, slower heat transfer from air to the medium, and the slow recovery of temperature each time the incubator is opened during daily operations. To meet the need for greater temperature control use a water bath, a heat-sink incubator, or a properly designed and constructed incubator shown to give equivalent results. A temperature tolerance of 44.5 ± 0.2°C can be obtained with most types of water baths that also are equipped with a gable top for the reduction of water and heat losses.

2. Procedure

a. Selection of sample size: Select volume of water sample to be examined in accordance with the information in Table 9222:III. Use sample volumes that will yield counts between 20 and 60 fecal coliform colonies per membrane.

When the bacterial density of the sample is unknown, filter several volumes or dilutions to achieve a countable density. Estimate volume and/or dilution expected to yield a countable membrane and select two additional quantities representing one-tenth and ten times this volume, respectively.

b. Filtration of sample: Follow the same procedure and precautions as prescribed under Section 9222B.5b above.

c. Preparation of culture dish: Place a sterile absorbent pad in each culture dish and pipet at least 2.0 mL M-FC medium, prepared as directed above, to saturate pad. Carefully remove any excess liquid from culture dish by decanting the plate. Aseptically, place prepared filter on medium-impregnated pad as described in Section 9222B above.

As a substrate substitution for the nutrient-saturated absorbent pad, add 1.5% agar to M-FC broth as described in Section 9222B above.

d. Incubation: Place prepared dishes in waterproof plastic bags or seal, invert, and submerge petri dishes in water bath, and incubate for 24 ± 2 h at 44.5 ± 0.2°C. Anchor dishes below water surface to maintain critical temperature requirements. Place all prepared cultures in the water bath within 30 min after filtration. Alternatively, use an appropriate, accurate solid heat sink or equivalent incubator.

e. Counting: Colonies produced by fecal coliform bacteria on M-FC medium are various shades of blue. Nonfecal coliform colonies are gray to cream-colored. Normally, few nonfecal coliform colonies will be observed on M-FC medium because of selective action of the elevated temperature and addition of rosolic acid salt reagent. Count colonies with a low-power (10 to 15 magnifications) binocular wide-field dissecting microscope or other optical device.

f. Verification: Verify typical blue colonies and any atypical grey to green colonies as described in Section 9020 for fecal coliform analysis. Simultaneous inoculation at both temperatures is acceptable.

3. Calculation of Fecal Coliform Density

a. General: Compute the density from the sample quantities that produced MF counts within the desired range of 20 to 60 fecal coliform colonies. This colony density range is more restrictive than the 20 to 80 total coliform range because of larger colony size on M-FC medium. Calculate fecal coliform density as directed in Section 9222B.6 above. Record densities as fecal coliforms per 100 mL.

b. Sediment and biosolid samples: For total solid (dry weight basis) see Section 2540G.

Calculate fecal coliforms per gram dry weight for biosolid analysis as follows:

Fecal coliforms per gram dry weight

$$= \frac{\text{colonies counted}}{(\text{dilution chosen}) \times (\% \text{ dry solids})}$$

where dilution and % dry solids are expressed in decimal form.

Example 1: There were 22 colonies observed on the 1:10 000 dilution plate of a biosolid with 4% dry solids.

$$\frac{22}{(0.0001)(0.04)} = 5.5 \times 10^6 \text{ fecal coliform/g dry weight}$$

If no filter has a coliform count falling in the ideal range (20 to 60), total the coliform counts on all countable filters and report as fecal coliforms per gram dry weight.

Example 2: There were 18 colonies observed on the 1:10 000 dilution plate and 2 colonies observed on the 1:100 000 dilution plate of a biosolid sample with 4% dry solids.

$$\frac{(18 + 2)}{(0.0001 + 0.00001)(0.04)} = 4.5 \times 10^6$$

9222 E. Delayed-Incubation Fecal Coliform Procedure

This delayed-incubation procedure is similar to the delayed-incubation total coliform procedure (Section 9222C). Use the delayed-incubation test only when the standard immediate fecal coliform test cannot be performed (i.e., where the appropriate field incubator is not available, or where, under certain circumstances, a specialized laboratory service is advisable to examine, confirm, or speciate the suspect colonies).

Results obtained by this delayed method have been consistent with results from the standard fecal coliform MF test under various laboratory and field use conditions. However, determine test applicability for a specific water source by comparison with the standard MF test, especially for saline waters, chlorinated wastewaters, and waters containing toxic substances.

To conduct the delayed-incubation test filter sample in the field immediately after collection, place filter on M-ST holding medium (see Section 9222C.2b), and ship to the laboratory.

To compute a geometric mean of samples, convert coliform densities of each sample to $\log_{10}$ values. Determine the geometric mean for the given number of samples (usually seven) by averaging the $\log_{10}$ values of the coliform densities and taking the antilog of that value.

4. Bibliography

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Complete fecal coliform test by transferring filter to M-FC medium, incubating at 44.5°C for 24 ± 2 h, and counting fecal coliform colonies.

The M-ST medium keeps fecal coliform organisms viable but prevents visible growth during transit. Membrane filters can be held for up to 3 d on M-ST holding medium with little effect on the fecal coliform counts.

1. Apparatus

- Culture dishes:* See Section 9222C.1a for specifications.
- Field filtration units:* See Section 9222C.1b.

2. Materials and Transport Medium

- M-ST medium:* Prepare as described in Section 9222C.2b.
- M-FC medium:* Prepare as described in Section 9222D.1a.

3. Procedure

a. Membrane filter transport: Place an absorbent pad in a tight-lid plastic petri dish and saturate with M-ST holding medium. After filtering sample remove membrane filter from filtration unit and place it on medium-saturated pad. Use only tight-lid dishes to prevent moisture loss; however, avoid having excess liquid in the dish. Place culture dish containing membrane in an appropriate shipping container and send to laboratory. Membranes can be held on the transport medium at ambient temperature for a maximum of 72 h with little effect on fecal coliform counts.

b. Transfer: At the laboratory remove membrane from holding medium and place it in another dish containing M-FC medium.

c. Incubation: After transfer of filter to M-FC medium, place tight-lid dishes in waterproof plastic bags, invert, and submerge in a water bath at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 ± 2 h or use a solid heat sink or equivalent incubator.

d. Counting: Colonies produced by fecal coliform bacteria are various shades of blue. Nonfecal coliform colonies are gray to cream-colored. Count colonies with a binocular wide-field dissecting microscope at 10 to 15 magnifications.

e. Verification: Verify typical blue colonies and any atypical (grey to green) colonies as described in Section 9020 for fecal coliform analysis.

4. Estimation of Fecal Coliform Density

Count as directed in Section 9222D.2e above and compute fecal coliform density as described in Section 9222D.3. Record time of collection, filtration, and laboratory examination, and calculate and report elapsed time.

5. Bibliography

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9222 F. *Klebsiella* Membrane Filter Procedure

Klebsiella bacteria belong to the family Enterobacteriaceae and are included in the total coliform group. The outermost layer of *Klebsiella* bacteria consists of a large polysaccharide capsule, a characteristic that distinguishes this genus from most other bacteria in this family; this capsule provides some measure of protection from disinfectants. *Klebsiella* bacteria are commonly associated with coliform regrowth in large water supply distribution systems.

Klebsiellae may be opportunistic pathogens that can give rise to bacteremia, pneumonia, urinary tract, and several other types of human infection. Approximately 60 to 80% of all *Klebsiella* from feces and from clinical specimens are positive in the fecal coliform test and are *Klebsiella pneumoniae*.

Klebsiella bacteria also are widely distributed in nature, occurring in soil, water, grain, vegetation, etc. Wood pulp, paper mills, textile finishing plants, and sugar-cane processing operations contain large numbers of *klebsiellae* in their effluents (10^4 to 10^6), and *Klebsiella* sp. are often the predominant coliform in such effluents.

Rapid quantitation may be achieved in the MF procedure by modifying M-FC agar base through substitution of inositol for lactose and adding carbenicillin or by using M-Kleb agar. These methods reduce the necessity for biochemical testing of pure strains. Preliminary verification of differentiated colonies is recommended.

1. Apparatus

a. Culture dishes: See Section 9222B.1e for specifications.

b. Filtration units: See Section 9222B.1f.

2. Materials and Culture Medium

a. Modified M-FC agar (M-FCIC agar): This medium may not be available in dehydrated form and may require preparation from the basic ingredients:

Tryptose or biosate	10.0 g
Proteose peptone No. 3 or polypeptone	5.0 g
Yeast extract	3.0 g
Sodium chloride, NaCl	5.0 g
Inositol	10.0 g
Bile salts No. 3 or bile salts mixture	1.5 g
Aniline blue	0.1 g
Agar	15.0 g
Reagent-grade water	1 L

Heat medium to boiling and add 10 mL 1% rosolic acid dissolved in 0.2N NaOH. Cool to below 45°C and add 50 mg carbenicillin.* Dispense aseptically in 5- to 7-mL quantities into 50- × 9-mm plastic petri dishes. Refrigerate until needed. Discard unused agar medium after 2 weeks. Do not sterilize by autoclaving. Final pH should be 7.4 ± 0.2 .

b. M-Kleb agar:

Phenol red agar	31.0 g
Adonitol	5.0 g
Aniline blue	0.1 g
Sodium lauryl sulfate	0.1 g
Reagent-grade water	1 L

Sterilize by autoclaving for 15 min at 121°C . After autoclaving, cool to 50°C in a water bath; add 20 mL 95% ethyl alcohol (not denatured) and 0.05 g filter sterilized carbenicillin/L. Shake thoroughly and dispense aseptically into 50- × 9-mm plastic

* Available from Geopen, Roerig-Pfizer, Inc. New York, NY.

culture plates. The final pH should be 7.4 ± 0.2 . Refrigerated medium can be held for 20 d at 4 to 8°C.

3. Procedure

a. See Section 9222B.5 for selection of sample size and filtration procedure. Select sample volumes that will yield counts between 20 and 60 *Klebsiella* colonies per membrane. Place membrane filter on agar surface; incubate for 24 ± 2 h at $35 \pm 0.5^\circ\text{C}$. *Klebsiella* colonies on M-FCIC agar are blue or bluish-gray. Most atypical colonies are brown or brownish. Occasional false positive occurrences are caused by *Enterobacter* species. *Klebsiella* colonies on M-Kleb agar are deep blue to blue gray, whereas other colonies most often are pink or occasionally pale yellow. Count colonies with a low-power (10 to 15 $\times$ magnifications) binocular wide field dissecting microscope or other optical device.

b. *Verification*: Verify *Klebsiella* colonies from the first set of samples from ambient waters and effluents and when *Klebsiella* is suspect in water supply distribution systems. Verify a minimum of five typical colonies by transferring growth from a colony or pure culture to a commercial multi-test system for gram-negative speciation. Key tests for *Klebsiella* are citrate (positive), motility (negative), lysine decarboxylase (positive), ornithine decarboxylase (negative), and urease (positive). A *Klebsiella* strain that is indole-positive, liquefies pectin, and

demonstrates a negative fecal coliform response is most likely of nonfecal origin.

4. Bibliography

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9222 G. MF Partition Procedures

1. *Escherichia coli* Partition Methods

a. *Applications*: *Escherichia coli* is a member of the fecal coliform group of bacteria; its presence is indicative of fecal contamination. Rapid quantitation and verification may be achieved with the MF procedure by transferring the membrane from a total-coliform- or fecal-coliform-positive sample to a nutrient agar substrate containing 4-methylumbelliferyl- β -D-glucuronide (MUG). In this method *E. coli* is defined as any coliform that produces the enzyme β -glucuronidase and hydrolyzes the MUG substrate to produce a blue fluorescence around the periphery of the colony.

In the examination of drinking water samples, use this method to verify the presence of *E. coli* from a total-coliform-positive MF on Endo-type media. In the examination of wastewater and other nonpotable water samples, use this procedure to verify positive filters from M-FC medium used in the fecal coliform MF procedure.

b. Apparatus:

- 1) *Culture dishes*: See Section 9222B.1e.
- 2) *Filtration units*: See Section 9222B.1f.
- 3) *Forceps*: See Section 9222B.1i.
- 4) *Incubator*: See Section 9222B.1j.
- 5) *Ultraviolet lamp*, long wave (366 nm), preferably 6 W.
- 6) *Microscope and light source*: See Section 9222B.1k.

c. Materials and culture medium:

1) Nutrient agar with MUG (NA-MUG):

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
4-methylumbelliferyl- β -D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly, and heat to dissolve. Sterilize by autoclaving for 15 min at 121°C . Dispense aseptically into 50-mm plastic culture plates. The final pH should be 6.8 ± 0.2 . Refrigerated prepared medium may be held for 2 weeks.

2) EC broth with MUG (EC-MUG):

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Bile salts mixture or bile salts No. 3	1.5 g
Dipotassium hydrogen phosphate, K_2HPO_4	4.0 g
Potassium dihydrogen phosphate, KH_2PO_4	1.4 g
Sodium chloride, NaCl	5.0 g
4-methylumbelliferyl- β -D-glucuronide	0.1 g
Reagent-grade water	1 L

Add dehydrated ingredients to reagent-grade water, mix thoroughly and heat to dissolve. pH should be 6.9 ± 0.2 after sterilization. Before sterilization, dispense into culture tubes and cap with metal or heat-resistant plastic caps.

d. *Procedure*: See Section 9222B.5 for selection of sample size and filtration procedure. For drinking water samples using

Endo-type medium, count and record the metallic golden sheen colonies. Before transfer of the membrane, transfer a small portion of each target colony to the appropriate total coliform verification medium, using a sterile needle. See Section 9222B.5 for total coliform verification procedures.

Alternatively, after transfer and incubation on NA-MUG, swab the surface growth on the filter and transfer to the appropriate total coliform verification medium. Aseptically transfer the membrane from the Endo-type medium to NA-MUG or EC-MUG medium. If differentiation of the total coliforms is desired using NA-MUG medium, mark each sheen colony with a fine-tipped marker or by puncturing a hole in the membrane adjacent to the colony with a sterile needle. Incubate NA-MUG at $35 \pm 0.5^\circ\text{C}$ for 4 h or EC-MUG at $44.5 \pm 0.2^\circ\text{C}$ for 24 ± 2 h. Observe individual colonies or tubes using a long-wave-length (366-nm) ultraviolet light source, preferably containing a 6-W bulb. The presence of a blue fluorescence in the tube, on the periphery (outer edge) of a colony, or observed from the back of the plate is considered a positive response for *E. coli*. Count and record the number of target colonies, if quantification is desired, or just record presence or absence of fluorescence.

For nonpotable water samples, use M-FC medium for initial isolation before transfer to NA-MUG or EC-MUG medium. The procedure is the same as the above, with the exception of the total coliform verification process.

For the EC-MUG method, a positive control consisting of a known *E. coli* (MUG-positive) culture, a negative control consisting of a thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret the results and to avoid confusion of weak autofluorescence of the medium as a positive response. See Section 9221F.

2. Fecal Coliform Partition Method

a. Applications: Further partitioning of total coliforms from the original MF coliform-positive culture in a presence/absence search for fecal coliform in a drinking water sample may be achieved within 24 h. This procedure provides additional information from the original sample.

b. Materials and culture medium: EC broth. See Section 9221E.1a.

c. Procedure: See Section 9222B.5 for selection of sample size and filtration procedure. For drinking water samples using Endo-type media, count and record the metallic (golden) sheen colonies. Before transfer of membrane or swabbing of plate, transfer a small portion of each target colony to the appropriate total coliform verification media using a sterile needle (see Section 9222B.5f). Use a sterile cotton swab to collect bacteria from the membrane surface, or pick discrete colonies with a 3-mm loop or sterile applicator stick, or transfer the entire membrane to inoculate a tube of EC medium. Incubate inoculated EC broth in a water bath at $44.5 \pm 0.2^\circ\text{C}$ for 24 ± 2 h. Place all EC tubes in water bath within 30 min after inoculation. Maintain a sufficient water depth in water bath incubator to immerse tubes to upper level of the medium. Gas production in an EC broth culture in 24 h or less is considered a positive response for fecal coliform bacteria.

3. Bibliography

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9223 ENZYME SUBSTRATE COLIFORM TEST*

9223 A. Introduction

The enzyme substrate test utilizes hydrolyzable substrates for the simultaneous detection of total coliform bacteria and *Escherichia coli* enzymes. When the enzyme technique is used, the total coliform group is defined as all bacteria possessing the enzyme β -D-galactosidase, which cleaves the chromogenic substrate, resulting in release of the chromogen. *Escherichia coli* are defined as bacteria giving a positive total coliform response and possessing the enzyme β -glucuronidase, which cleaves a fluorogenic substrate, resulting in the release of the fluorogen. The test can be used in either a multiple-tube, multi-well, or a presence-absence (single 100-mL sample) format.

1. Principle

a. *Total coliform bacteria*: Chromogenic substrates, such as ortho-nitrophenyl- β -D-galactopyranoside (ONPG) or chlorophenol red- β -D-galactopyranoside (CPRG), are used to detect the enzyme β -D-galactosidase, which is produced by total coliform bacteria. The β -D-galactosidase enzyme hydrolyzes the substrate and produces a color change, which indicates a positive test for total coliforms at 24 h (ONPG) or 28 h (CPRG) without additional procedures. Noncoliform bacteria, such as *Aeromonas* and *Pseudomonas* species, may produce small amounts of the enzyme β -D-galactosidase, but are suppressed and generally will not produce a positive response within the incubation time unless more than 10^4 colony-forming units (CFU)/mL (10^6 CFU/100 mL) are present.

* Approved by Standard Methods Committee, 1997.
Joint Task Group: 20th Edition—Carol Palmer (chair), Terry C. Covert, Robert E. Grant, Nancy H. Hall, Bruce M. Roll, Jon Standridge.

9223 B. Enzyme Substrate Test

1. Substrate Media

Formulations are available commercially* in disposable tubes for the multiple-tube procedure, in disposable multi-wells† for the multi-well procedure, or in containers that will hold 100-mL samples for the presence-absence approach.* Appropriate pre-weighed portions of the reagent for mixing and dispensing into multiple tubes for 10-mL test portions or other containers for 100-mL samples also are available. The need for good quality

* Colilert® and Colilert 18® for multi-tube, P/A, and tray formats, Colilert MW® for multi-tube format, and Colisure™ for multi-tube and P/A formats, available from IDEXX Laboratories, Inc., Westbrook, ME.

† Quanti-Tray® or Quanti-Tray®/2000, available from IDEXX Laboratories, Inc., Westbrook, ME.

b. *Escherichia coli*: A fluorogenic substrate, such as 4-methylumbelliferyl- β -D-glucuronide (MUG), is used to detect the enzyme β -glucuronidase, which is produced by *E. coli*. The β -glucuronidase enzyme hydrolyzes the substrate and produces a fluorescent product when viewed under long-wavelength (366-nm) ultraviolet (UV) light. The presence of fluorescence indicates a positive test for *E. coli*. Some strains of *Shigella* spp. also may produce a positive fluorescence response. Because *Shigella* spp. are overt human pathogens, this is not considered a detriment for testing the sanitary quality of water.

2. Applications

The enzyme substrate coliform test is recommended for the analysis of drinking and source water samples. Formulations also are available for the analysis of marine waters. Initially, laboratories planning to use this procedure should conduct parallel quantitative testing (including seasonal variations) with one of the standard coliform tests to assess the effectiveness of the test for the specific water type being analyzed and to determine the comparability of the two techniques. This is particularly important when testing source waters.

Water samples containing humic or other material may be colored. If there is background color, compare inoculated tubes to a control tube containing only water sample. In certain waters, high calcium salt content can cause precipitation but this should not affect the reaction.

Do not use the enzyme substrate test to verify presumptive coliform cultures or membrane filter colonies, because the substrate may be overloaded by the heavy inoculum of weak β -D-galactosidase-producing noncoliforms, causing false-positive results.

assurance and uniformity requires the use of a commercial substrate medium. Avoid prolonged exposure of the substrate to direct sunlight. Store media according to directions and use before expiration date. Discard discolored media.

2. Procedure

a. *Multiple-tube procedure*: Select the appropriate number of tubes per sample with predisposed media for the multiple-tube test and label. Follow manufacturer's instructions for preparing serial dilutions for various formulations. Aseptically add 10 mL sample to each tube, cap tightly, and mix vigorously to dissolve. The mixture remains colorless with ONPG-based tests and turns yellow with the CPRG format. Some particles may remain

TABLE 9223:I. COLOR CHANGES FOR VARIOUS MEDIA

Substrate	Total Coliform Positive	<i>E. coli</i> Positive	Negative Result
ONPG-MUG	Yellow	Blue fluorescence	Colorless/no fluorescence
CPRG-MUG	Red or magenta	Blue fluorescence	Yellow/no fluorescence

undissolved throughout the test; this will not affect test performance. Incubate at $35 \pm 0.5^\circ\text{C}$ for period specified by substrate manufacturer.

The procedure also can be performed by adding appropriate amounts of the substrate media to the sample, mixing thoroughly, and dispensing into five or ten sterile tubes. Incubate as stated for multiple-tube procedure.

b. Multi-well procedure: The multi-well procedure is performed with sterilized disposable packets. Add sample to 100-mL container with substrate, shake vigorously, and pour into tray. The tray sealer dispenses the sample into the wells and seals the package. Incubate at $35 \pm 0.5^\circ\text{C}$ for period specified by substrate manufacturer. The MPN value is obtained from the table provided by the manufacturer.

c. Presence-absence procedure (P/A): Aseptically add preweighed enzymatic medium to 100-mL sample in a sterile, transparent, nonfluorescent borosilicate glass or equivalent bottle or container. Optionally, add 100-mL sample to the enzymatic substrate in a sterile container provided by the manufacturer. Aseptically cap and mix thoroughly to dissolve. Incubate as specified in manufacturer's instructions.

3. Interpretation

a. Total coliform bacteria: After the minimum proper incubation period, examine tubes or containers for the appropriate color change (Table 9223:I). ONPG is hydrolyzed by the bacterial enzyme to yield a yellow color. CPRG is hydrolyzed by the bacterial enzyme to yield a red or magenta color. If the color response is not uniform throughout the tube, mix by inversion before reading. Read manufacturer's instructions for interpretation guidelines. Some manufacturers suggest comparing sample tubes against a color comparator available through the manufacturer. Samples are negative for total coliforms if no color is observed in ONPG tests or if the tube is yellow when CPRG is used. If a chromogenic response is questionable after 18 or 24 h for ONPG, incubate up to an additional 4 h. If response is negative after 28 h for CPRG, incubate up to an additional 20 h. If the chromogen intensifies, the sample is total-coliform positive; if it does not, the sample is negative.

b. *Escherichia coli*: Examine positive total coliform tubes or containers for fluorescence using a long-wavelength (366-nm) ultraviolet lamp (preferably 6-W bulb). Compare each tube against the reference comparator available from a commercial source of the substrate. The presence of fluorescence is a positive test for *E. coli*. If fluorescence is questionable, incubate for an additional 4 h for ONPG tests and up to an additional 20 h for CPRG tests; intensified fluorescence is a positive test result.

4. Reporting

If performing an MPN procedure, calculate the MPN value for total coliforms and *E. coli* from the number of positive tubes as described in Section 9221C. If using the presence-absence procedure, report results as total coliform and *E. coli* present or absent in 100-mL sample.

5. Quality Control

Test each lot of media purchased for performance by inoculation with three control bacteria: *Escherichia coli*, a total coliform other than *E. coli* (e.g., *Enterobacter cloacae*), and a noncoliform. Also add a sterile water control. If the sterile water control exhibits faint fluorescence or faint positive coliform result, discard and use a new batch of substrate. Avoid using a heavy inoculum. If *Pseudomonas* is used as the representative noncoliform, select a nonfluorescent species. Incubate these controls at $35 \pm 0.5^\circ\text{C}$ as indicated above. Read and record results. Other quality-control guidelines are included in Section 9020.

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9224 DETECTION OF COLIPHAGES*

9224 A. Introduction

1. General Discussion

Coliphages are bacterial viruses that infect and replicate in *Escherichia coli*. They are shed in human and animal feces. Although coliphages are not known to be hazardous to human beings, they are potentially important microorganisms for monitoring the microbial quality of water and wastewaters.¹

The detection of coliphages has been of increasing interest since it has become clear that bacterial monitoring of waters and wastewaters may not adequately indicate the presence of viruses in those waters.² The presence of pathogenic human viruses in waters is a public health concern. Waterborne outbreaks of viral illnesses such as gastroenteritis and hepatitis A occur in the United States and elsewhere.^{3,4} Detection of human enteric viruses in water and wastewaters, however, is beyond the capabilities of most water laboratories. Such detection traditionally has required the use of cell culture techniques.⁵ These techniques are expensive, require skilled personnel, and have been both time- and labor-intensive. Coliphage assays, on the other hand, are relatively inexpensive, are easier to perform with trained personnel, and yield overnight results. Coliphage assays have been proposed as an alternative to human virus assays as an indicator of the viral quality of waters.^{6,7}

Recent progress has been made in the development of specific coliphage methods for evaluating waters and wastewaters. Much of this work has focused on the detection of the group of coliphages known as the male-specific RNA coliphages (also referred to as the F-specific RNA coliphages or FRNA coliphages).⁸ These coliphages are 20 to 30 nm in size, contain a single-stranded RNA genome, and have an isometric morphology. They exclusively infect bacterial cells that possess an F pilus, an appendage used for bacterial conjugation. Their significance lies in the fact that these coliphages are structurally similar to many human RNA viruses found in fecally contami-

nated waters. In particular, they resemble viruses of the picornavirus and calicivirus families, which include poliovirus; coxsackievirus; Norwalk and other small, round, structured viruses; hepatitis A virus; and hepatitis E virus. The human viruses cannot replicate in the environment. Similarly, the male-specific RNA coliphages have only limited replication in the environment at temperatures below 30°C.⁹ Male-specific RNA coliphages also resemble many human enteric viruses in being relatively resistant to disinfection treatment practices. Because of these characteristics, male-specific RNA coliphages are promising candidate indicators of human viruses in environmental waters.

In the procedures presented here, methods have been included for the detection of the male-specific RNA coliphages using host *E. coli* Famp and for the detection of somatic coliphages using *E. coli* C.¹⁰ Somatic coliphages, unlike the male-specific coliphages, are coliphages that do not require the presence of an F pilus to infect host cells. They represent a broad assortment of coliphage types and have often been included in environmental studies. Also presented here is a procedure that uses an alternate host bacterium, *Salmonella typhimurium* WG49. That host has been used by many laboratories to detect male-specific RNA coliphages and it previously has been used in one standard method protocol.¹¹ Although a double-agar-layer plaque assay has been specified in these procedures, a single-agar-layer method also is presented and can be used as an alternate plaque assay. A membrane filter method is presented for assaying 100-mL (and larger) sample volumes.

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9224 B. Somatic Coliphages Assay

1. General Discussion

This plaque assay procedure for detecting somatic coliphages is a variant of the widely used double-agar-layer method;¹ it is based on the somatic coliphage assay procedure developed for regulatory purposes.^{2,3} The procedure can be used, without supplementary methods, to assay small volumes of water and wastewater samples directly. In conjunction with large-scale concentration methods used to detect enteric viruses (Section 9510), the procedure also can be used to assay samples of much larger volume (>100 L). However, the use of electronegative filters at pH 3.5 is not recommended, because substantial loss of coliphage viability may occur. It is advisable to predetermine the suitability of any large-volume method in measured coliphage recovery trials. This plaque assay procedure uses *E. coli* C, one of the most effective host bacteria for detecting somatic coliphages.⁴ Plaques produced with this host are readily distinguished, making plaque quantification relatively simple. The major advantage in detecting somatic coliphages is that they frequently are found in waters and wastewaters in greater abundance than the male-specific RNA coliphages. This can be an important consideration, especially for the assay of waters where pollution indices are expected to be low. The somatic coliphage group, however, is composed of different phage types (including both tailed and non-tailed phages) that exhibit widely varied characteristics. Somatic coliphages can replicate in the environment if a suitable host is present. Because of these factors, the somatic coliphage group may be less representative of human enteroviruses than the more uniform RNA phages of the male-specific coliphage group. The somatic coliphage group thus may not be the best group to serve as a specific indicator for human viruses in waters and wastewaters. However, the detection of somatic phages using *E. coli* C may be useful as a general indicator of water quality.⁵

2. Apparatus

- a. Cryovials, 2-mL.
- b. Erlenmeyer flasks, 125- and 250-mL, and 2-L.
- c. Graduated cylinders, 100- and 500-mL.

d. Inoculating loop.

e. Laboratory balance.

f. Pipets, 1-, 5-, and 10-mL.

g. Petri dishes, 100- × 15-mm.

h. Filters, 0.22- μ m. When passing material containing phage, always pass about 10 mL 1.5% beef extract through filter just before use to minimize phage adsorption to filter. Use pre-sterilized filters or sterilize filters before use by autoclaving at 121°C for 15 min. At the time of use, use sterile technique to place filters into sterile sample filtration apparatus.

i. Test tubes, 16- × 150-mm, screw-capped and with closures.

j. Water bath set at 44.5 ± 1°C.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

a. *Beef extract, 1.5%*: Dissolve 1.5 g beef extract powder and 0.375 g glycine (final glycine concentration = 0.05M) in 90 mL water. Adjust pH to 7.0 to 7.5, if necessary, and bring final volume to 100 mL with water. Autoclave at 121°C for 15 min and use at room temperature. Store at 4°C.

b. *Glycerol solution, 50%*: Add equal volumes of water and undiluted glycerol. Autoclave at 121°C for 15 min. Store at 4°C.

c. Tryptone agar slants:

Tryptone	1.0 g
Yeast extract	0.1 g
Glucose	0.1 g
NaCl	0.8 g
CaCl ₂	0.022 g
Agar	1.2 g
Reagent-grade water	100 mL

With gentle mixing, add ingredients to water in a 250-mL flask. Dissolve and sterilize by autoclaving at 121°C for 20 min and dispense 8-mL portions into 16- × 150-mm test tubes with tube closures. Prepare slants by letting agar solidify with the

tubes held at about a 20° angle. Slants may be stored at 4°C for up to 3 months.

d. Tryptone bottom agar: Prepare before sample analysis, using ingredients and concentrations listed for tryptone agar slants, but use 1.5 g agar. After autoclaving, pipet 15-mL portions aseptically into sterile 100- × 15-mm petri dishes and let agar harden. Store dishes in sleeve bags at 4°C for up to 2 weeks and warm to room temperature for 1 h before use.

e. Tryptone broth: Prepare as for tryptone agar slants, excluding agar. Autoclave at 121°C for 20 min, and allow to cool before use. Broth may be stored at 4°C for up to 3 months in sterile, capped containers.

f. Tryptone dilution tubes: Aseptically, dispense 9-mL portions of sterile tryptone broth into 16- × 150-mm screw-capped test tubes presterilized by autoclaving at 121°C for 15 min.

g. Tryptone top agar: Use ingredients and concentrations listed for tryptone agar slants, but use 0.7 g agar. Autoclave and place in the 44.5 ± 1°C water bath. Agar may be stored at 4°C for up to 3 months in sterile, capped containers. Melt solidified agar before use.

4. Procedure

a. Storage of E. coli C host culture: For short-term storage inoculate an *Escherichia coli* C* host culture onto tryptone agar slants with a sterile inoculating loop by spreading the inoculum evenly over entire slant surface. Incubate the culture overnight at 36.5 ± 2°C. Store at 4°C for up to 2 weeks. For long-term storage inoculate a 5- to 10-mL tube of tryptone broth with host culture. Incubate overnight at 36.5 ± 2°C. Add 1/5th volume of 50% glycerol solution. Dispense into 1-mL portions in 2-mL cryovials and store at -70°C, or lower.

b. Preparation of host: Inoculate 5 mL tryptone broth with *E. coli* C from a slant with an inoculating loop and incubate for 16 h at 36.5 ± 2°C. Transfer 1.5 mL of the 16-h culture to 30 mL tryptone broth in a 125-mL flask and incubate for 4 h at 36.5 ± 2°C with gentle shaking. The amount of inoculum and broth used can be altered proportionally according to need.

c. Preparation of coliphage ΦX174 positive control: Rehydrate a stock culture of somatic coliphage ΦX174† and store at 4°C. Prepare a 30-mL culture of *E. coli* C as described above. Incubate for 2 h at 36.5 ± 2°C with shaking. Add 1 mL rehydrated phage stock and incubate for an additional 4 h at 36.5 ± 2°C. Filter through a beef-extract-treated 0.22-μm filter (see ¶2h). Prepare 10⁻⁷, 10⁻⁸, and 10⁻⁹ dilutions of the filtrate using tryptone dilution tubes. These dilutions should be sufficient for most ΦX174 stocks. Some stocks may require higher or lower dilutions. Add 3 mL melted tryptone top agar held in the 44.5 ± 1°C water bath to fifteen 16- × 150-mm test tubes. Keep these test tubes in heated water bath to avoid premature solidifying of agar. Add 0.1 mL host culture to each test tube. Add 1 mL 10⁻⁹ dilution to each of five test tubes, Add 1 mL 10⁻⁸ dilution to five additional tubes and 1 mL 10⁻⁷ dilution to the remaining five tubes. Label tubes with appropriate dilution. For each tube, mix and immediately pour contents over bottom agar of a petri dish labeled with the dilution assayed. Rotate dish to spread suspen-

sion evenly and place it on a level surface to let agar solidify. Incubate at 36.5 ± 2°C overnight and examine for plaques the following day. Count number of plaques on each dish. To determine the titer of the stock filtrate, use the dilution with dishes exhibiting 20 to 100 plaques. Average the plaque counts on these five dishes and multiply result by the reciprocal of the dilution to obtain that titer. For use as a positive control in the coliphage assay, dilute stock filtrate to 30 to 80 PFU/mL in tryptone broth. Store original filtrate and diluted positive control preparation at 4°C. Before using the positive control preparation for the first time, assay 10 mL by adding 1-mL volumes of the preparation to ten test tubes containing agar and host culture and pouring their contents into ten petri dishes. Count plaques on all dishes and divide by 10. If result is not 30 to 80, adjust dilution of the positive control sample and re-assay.

d. Assay procedure: Add 3 mL melted tryptone top agar held at 44.5 ± 1°C to each of ten 16- × 150-mm test tubes for sample assay and to each of two additional test tubes to serve as negative and positive controls. Hold test tubes in water bath to avoid premature solidifying of agar. Add 0.1 mL of host culture to each of the 12 test tubes. Add 1 mL tryptone broth to test tube serving as negative control. Add 1 mL ΦX174 preparation (30 to 80 PFU/mL) to test tube serving as positive control. To each of the remaining 10 tubes, add 1 mL sample. For each tube, mix and immediately pour contents over bottom agar of a petri dish that has been suitably labeled. Tilt and rotate dish to spread suspension evenly and place it on a level surface to let agar solidify. Incubate at 36.5 ± 1°C overnight and examine for plaques the following day. Count total number of plaques on the ten dishes receiving the sample. Calculate the somatic coliphage concentration according to the formula:

$$C_a = (P \div 10) \times D$$

where:

C_a = somatic coliphage concentration, PFU/mL,

P = total number of plaques from the 10 dishes, and

D = reciprocal of dilution made on the inoculum before plating ($D = 1$ for undiluted samples).

If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again. In the calculation for diluted samples, D will be greater than 1 (e.g., $D = 10$, for a sample diluted 1:10 or $D = 100$, for a sample diluted 1:100).

If the sample assayed is the product of a concentration procedure, such as an eluate from a large-volume filter sampling of water or wastewater, calculate coliphage concentration of the sampled water according to the formula:

$$C_b = \frac{C_a \times V_a}{V_b}$$

where:

C_b = somatic coliphage concentration of sampled water, PFU/mL,

C_a = coliphage concentration of concentrated material, PFU/mL,

V_a = volume of that material, mL, and

V_b = volume of water processed in sampling procedure, L.

* American Type Culture Collection Product No. 13706.

† American Type Culture Collection Product No. 13706-B1.

The resultant concentration generally is reported as PFU/L or PFU/100 L. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again. Count plaques on the positive control dish. Maintain a record of the plaque count as a check on the virus sensitivity of the *E. coli* C host. Assay any water eluate samples again where the positive control counts are more than one log below their normal average. Should plaques be detected on the negative control dish, discard assay results and repeat assay.

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9224 C. Male-Specific Coliphage Assay Using *Escherichia coli* Famp

1. General Discussion

Like the somatic coliphage assay, this male-specific assay is a variant of the double-agar-layer method¹ and is based on procedures developed for regulatory purposes.² Similarly, this male-specific assay can be used, without supplementary methods, to assay small volumes of water and wastewater samples directly. In conjunction with large-scale concentration methods used to detect enteric viruses (Section 9510), the procedure also can be used to assay larger-volume samples (>100 L). However, the use of electronegative filters at pH 3.5 is not recommended because substantial loss of coliphage viability may occur. Determine the suitability of any large-volume method in measured coliphage recovery trials, before using it. This procedure uses *E. coli* Famp as the host bacterium.³ This strain is resistant to ampicillin and streptomycin antibiotics. It is one of two bacterial strains that have been developed to selectively detect male-specific coliphages. For a procedure using the other bacterial strain, *Salmonella typhimurium* WG49, see Section 9224D. It has not been universally established that one strain performs better than the other. Individual laboratories may evaluate both strains before selecting one for routine use. Male-specific plaques can be difficult to recognize in comparison to somatic plaques. They are generally less well-defined than somatic plaques. Use diligence when examining dishes for male-specific plaques. As male-specific DNA coliphages and some somatic coliphages also can produce plaques on host *E. coli* Famp, preferably confirm that plaques quantified by the assay are caused by RNA phages. An RNase procedure that permits differential quantification of the RNA coliphages also is presented below. The advantage in detecting male-specific RNA coliphages is that these coliphages more closely resemble human enteroviruses than do the varied phage types of the somatic group. These male-specific RNA coliphages appear to be useful as a model for human viruses.^{4,5} As an indicator of the presence of human viruses in waters and wastewaters, their role remains to be fully established.

2. Apparatus

See 9224B.2.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in making media and reagents.

a. *Ampicillin solution*: Dissolve 0.15 g ampicillin in 100 mL water and filter with the 0.22- μ m filter (beef extract pretreatment not necessary). Store at 4°C.

b. *Beef extract, 1.5%*: See 9224B.3a.

c. *Glycerol solution, 50%*: See 9224B.3b.

d. *Ribonuclease (RNase) solution*: Dissolve 100 mg RNase containing 50 to 100 Kunitz units/mg in 100 mL water by heating to 100°C for 10 min. Store at -20°C in 0.5-mL portions.

e. *Streptomycin solution*: Dissolve 1.5 g streptomycin sulfate in 100 mL water and filter-sterilize with 0.45- μ m filter (beef extract pretreatment not necessary). Store at 4°C.

f. *Tryptone agar slants*: Mix ingredients, dissolve, and sterilize as directed in 9224B.3c. Let autoclaved agar equilibrate in water bath set at 44.5 $\pm$ 1°C, then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution. Dispense portions, prepare, and store as directed in 9224B.3c.

g. *Tryptone bottom agar*: See 9224B.3d. After autoclaving, let agar equilibrate in water bath set at 44.5 $\pm$ 1°C, and then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution/100-mL volume of warm agar. Dispense portions and store as directed in 9224B.3d.

h. *Tryptone broth*: Use ingredients listed in 9224B.3c, excluding agar. Sterilize by autoclaving at 121°C for 20 min. Cool and add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution/100 mL broth. Store at 4°C. Use for growth medium. Store as directed in 9224B.3c.

i. *Tryptone dilution tubes*: See 9224B.3f.

j. *Tryptone top agar*: Use ingredients listed in 9224B.3c, but use 0.7 g agar. After autoclaving, let agar equilibrate in a 44.5 $\pm$

1°C water bath, then add 1.0 mL filtered ampicillin solution and 1.0 mL filtered streptomycin solution / 100 mL volume of warm agar. Store as directed in 9224B.3g.

4. Procedure

a. *Storage of E. coli Famp host culture*: Follow procedures of 9224B.4a, using *Escherichia coli* Famp.* For this strain, use media containing ampicillin and streptomycin as described above.

b. *Preparation of host*: Follow procedures of 9224B.4b. Place on ice until used for assay to prevent loss of F pili (use within 3 h).

c. *Preparation of coliphage MS2 positive control*: Rehydrate a stock culture of male-specific RNA coliphage MS2† and store at 4°C. Prepare a 30-mL culture of *E. coli* Famp as described in 9224B.4b. Incubate and proceed as directed in 9224B.4c, using the MS2 and *E. coli* Famp.

d. *Assay procedure*: Proceed as in 9224B.4d, using MS2 as positive control. Calculate C_a as directed. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again.

If the sample assayed is the product of a concentration procedure, calculate C_b as directed in 9224B.4d.

e. *Assay procedure with additional confirmation*: Proceed as in 9224B.4d, using MS2 as positive control, but assay an additional 10 mL of sample. For the additional 10 mL of sample, add RNase solution to the melted tryptone top agar to a concentration of 40 µg/mL. Before pouring tube contents to petri dishes, label dishes so that the 10 dishes containing RNase are readily distin-

guished from the 10 dishes without RNase. Calculate the male-specific RNA coliphage concentration (C_a) in PFU/mL according to the formula:

$$C_a = [(P \div 10) - (P_{RNase} \div 10)] \times D$$

where:

C_a = male-specific RNA coliphage concentration, PFU/mL,

P = total number of plaques from the 10 dishes without RNase,

P_{RNase} = total number of plaques from the 10 dishes with RNase, and

D = reciprocal of the dilution made on the inoculum before plating.

If the sample assayed is the product of a concentration procedure, calculate coliphage concentration as previously described in ¶ d.

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* American Type Culture Collection Product No. 700891.

† American Type Culture Collection Product No. 15597-B1.

9224 D. Male-Specific Coliphage Assay Using *Salmonella typhimurium* WG49

1. General Discussion

This male-specific assay uses *Salmonella typhimurium* WG49 as host bacterium. It is a variant of the double-agar-layer method¹ and has been adapted from several published procedures.^{2,3} The assay can be used with large-scale concentration methods (see 9224C.1). The use of *S. typhimurium* WG49 in this assay effectively eliminates plaque production by somatic coliphages. This is an advantage over the *E. coli* Famp-based assay. However, somatic salmonella phages do form plaques with *S. typhimurium* WG49 and these somatic salmonella phages also may be present in waters under investigation. Because it has not been established that either *S. typhimurium* WG49 or *E. coli* Famp is universally superior as host bacterium for the male-specific phages, individual laboratories may find it useful to evaluate both assays before making a selection. With either assay, take care in examining dishes for male-specific plaques and confirm that the plaques detected are indeed caused by RNA coliphages

and not other phages. An RNase procedure for this purpose is presented below. Also presented below is a treatment to neutralize the somatic salmonella phage content of samples before assay. This treatment may be necessary if assay interference by somatic salmonella phages is encountered.⁴⁻⁶ Such interference may be expected because somatic salmonella phages do outnumber male-specific phages in some waters.⁷ The advantage of the *S. typhimurium* WG49-based assay, as with the *E. coli* Famp-based assay, is that the targeted male-specific RNA phages possess a similarity to human enteroviruses not found with the somatic coliphage group. Assay of these male-specific phages can be useful in evaluating water and wastewater treatment processes but as a specific indicator of the presence of human viruses, their role remains to be clearly established.

2. Apparatus

See 9224B.2.

3. Media and Reagents

Adjust amount of media prepared proportionally to the number of samples. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

a. *Beef extract*: See 9224B.3a.

b. *Glycerol solutions, 50%*: See 9224 B.3b.

c. *Kanamycin solution*: Dissolve 1 g kanamycin monosulfate in 100 mL water and filter with the 0.22- μ m filter (beef extract pretreatment not necessary). Store at 4°C.

d. *Lipopolysaccharide solution (LPS)*: Prepare stock solution of phenol-extracted *S. typhimurium* LPS* by dissolving the lyophilized LPS in sterile phosphate-buffered saline to yield an LPS concentration of 2 mg/mL. Store at 4°C.

e. *Nalidixic acid solution*: Dissolve 1 g nalidixic acid in 100 mL water and filter with the 0.22- μ m filter (beef extract pretreatment not necessary). Store at 4°C.

f. *Phosphate-buffered saline*: Dissolve 0.8 g NaCl, 20 mg KCl, 12 mg KH_2PO_4 , and 91 mg Na_2HPO_4 in 100 mL water. Sterilize by autoclaving at 121°C for 15 min.

g. *Ribonuclease (RNase) solution*: See 9224C.3d.

h. *Tryptone agar slants*: Mix ingredients, dissolve, and filter as directed in 9224B.3c. Let autoclaved agar equilibrate in water bath at $44.5 \pm 1^\circ\text{C}$, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution. Dispense portions and store as directed in 9224B.3c.

i. *Tryptone bottom agar*: See 9224 B.3d. After autoclaving, let agar equilibrate in water bath at $44.5 \pm 1^\circ\text{C}$, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL volume of warm agar. Dispense portions and store as directed in 9224B.3d.

j. *Tryptone broth*: Use ingredients listed in 9224B.3c, excluding agar. Sterilize by autoclaving at 121°C for 20 min. Cool and add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL broth. Use for growth medium. Store as directed in 9224B.3c.

k. *Tryptone dilution tubes*: See 9224B.3f.

l. *Tryptone top agar*: Use ingredients listed in 9224B.3c, but use 0.7 g agar. After autoclaving let agar equilibrate in a $44.5 \pm 1^\circ\text{C}$ water bath, then add 1 mL filtered nalidixic acid solution and 0.2 mL filtered kanamycin solution/100 mL volume of warm agar. Store as directed in 9224B.3g.

4. Procedure

a. *Storage of *S. typhimurium* WG49 host culture*: Follow procedures of 9224B.4a, using *S. typhimurium* WG49† host culture. *S. typhimurium* WG49 is resistant to nalidixic acid and kanamycin. Accordingly, use media containing nalidixic acid and kanamycin as described above.

b. *Preparation of host*: Follow procedures of 9224B.4b. Place on ice until used in assay to prevent loss of F pili (use within 3 h).

c. *Preparation of coliphage MS2 positive control*: Rehydrate a stock culture of male-specific RNA coliphage MS2‡ and store at 4°C. Prepare a 30-mL culture of *S. typhimurium* WG49 as in 9224B.4b. Incubate and proceed as directed in 9224B.4c, using the MS2 and *S. typhimurium* WG49.

d. *Assay procedure*: Proceed as in 9224B.4d, using MS2 as positive control. Calculate C_a as directed. If the sample dishes are completely lysed or yield plaques that are too numerous to count, dilute sample and assay again.

If the sample assayed is the product of a concentration procedure, calculate C_b as directed in 9224B.4d.

e. *Assay procedure with additional confirmation*: See 9224C.4e.

f. *Sample treatment procedure to reduce somatic salmonella phages*: Results of the confirmation procedure may reveal assay interference by somatic salmonella phages present in some water or wastewater samples. To neutralize these somatic salmonella phages before assay, add enough LPS stock solution to the sample to produce a final LPS concentration of 20 $\mu\text{g/mL}$. Mix thoroughly and hold at ambient temperature for 15 to 30 min. Then assay as described above.

5. References

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* Sigma Chemical Co., St. Louis, MO, item #L6511, or equivalent.

† American Type Culture Collection Product No. 700730.

‡ American Type Culture Collection Product No. 15597-B1.

9224 E. Single-Agar-Layer Method

1. General Discussion

This method can be used as an alternative to the double-agar-layer procedures described above. It uses a single-agar-layer format and larger petri dishes. The method enables more sample material to be assayed per dish and can be used to directly assay 100-mL volumes of water and wastewater.¹ Although originally described for use with *E. coli* C, the procedure is suitable for use with *E. coli* Famp and *S. typhimurium* WG49 host bacteria. The general procedure is described below. For specific details relating to each of the three bacterial hosts, refer to Sections 9224B, C, and D.

2. Apparatus

See 9224B.2. Use 150- × 15-mm petri dishes for sample assay.

3. Media and Reagents

The amount of media prepared may be increased proportionally to the number of samples to be analyzed. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

For all host cultures:

a. *Beef extract*: See 9224B.3a.

b. *Calcium chloride solution*: Add 22 g CaCl₂ to 50 mL water and sterilize by autoclaving at 121°C for 15 min. Use at room temperature.

c. *Glycerol solution, 50%*: See 9224B.3b.

d. *Tryptone agar slants*: See 9224B.3c.

e. *Tryptone agar*:

Tryptone	2.0	g
Yeast extract	0.2	g
Glucose	0.2	g
NaCl	1.6	g
CaCl ₂	0.022	g
Agar	1.2	g
Reagent-grade water	100	mL

Sterilize by autoclaving at 121°C for 20 min. Place in the 44.5 ± 1°C water bath.

f. *Tryptone broth*: See 9224B.3d, excluding the agar.

g. *Tryptone dilution tubes*: See 9224B.3f.

For *E. coli* Famp host only:

h. *Ampicillin solution*: See 9224C.3a.

i. *Streptomycin solution*: See 9224C.3e.

For *S. typhimurium* WG49 host only:

j. *Kanamycin solution*: See 9224D.3c.

k. *Lipopolysaccharide solution (LPS)*: See 9224D.3d.

l. *Nalidixic acid solution*: See 9224D.3e.

For *E. coli* Famp and *S. typhimurium* WG49 hosts:

m. *Ribonuclease (RNase) solution*: See 9224C.3d.

4. Procedure

a. *Storage of host cultures*: Follow procedures of 9224B.4a, using appropriate host culture.

b. *Preparation of host*: Follow procedures of 9224B.4b.

c. *Preparation of coliphage positive controls*: Rehydrate a stock culture of appropriate positive control and proceed as directed in 9224B.4c.

d. *Sample filtration* (somatic coliphage assay, only): The bacteria present in samples for analysis can interfere with the plaque assay. To eliminate such interference, filter sample before assay. Use sterile 0.22-μm filter unit. To minimize phage adsorption to the filter, pass about 10 mL 1.5% beef extract through filter before passing sample through filter. NOTE: As an alternative to sample filtration, use *E. coli* strain CN-13* in place of *E. coli* C in the somatic coliphage assay. *E. coli* CN-13 is a nalidixic-acid-resistant variant of *E. coli* C; using it permits the addition of nalidixic acid to the assay media (see Section 9224D) to hinder growth of indigenous bacteria. *E. coli* CN-13 appears equivalent to *E. coli* C in somatic coliphage detection.²

e. *Assay procedure*: Place 100 mL sample in the 44.5 ± 1°C water bath for 3 min. Add 5 mL CaCl₂ solution and 5 mL appropriate host bacterium preparation to the warmed sample. Mix inoculated sample with 100 mL melted tryptone agar at 44.5 ± 1°C and distribute to eight 150- × 15-mm petri dishes. For a positive control, mix 1 mL of appropriate positive control preparation (30 to 80 PFU/mL) and 1 mL host bacterium with 12.5 mL warmed agar that has been diluted with an equal volume of warm sterile water. Pour to a single 150- × 15-mm petri dish. Repeat for a negative control but omit the 1 mL of phage preparation. Incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count total number of plaques on the eight dishes that received the sample; total is coliphage concentration/100 mL sample. However, when assaying for male-specific RNA coliphages using *E. coli* Famp and *S. typhimurium* WG49, this count could include some somatic and male-specific DNA coliphages or some somatic salmonella phages present in the sample. For appropriate procedures to address the presence of undesired phage, see ¶s 4f and g, below.

f. *Assay procedure with additional confirmation* (male-specific assays only): Assay an additional 100-mL sample portion as described above, but add RNase solution to the melted tryptone agar. For the additional material, the melted tryptone agar should contain RNase at a concentration of 60 μg/mL (before sample is added). Appropriately label dishes so that the eight dishes containing RNase are readily distinguished from the eight dishes without RNase. Calculate the male-specific coliphage concentration according to the formula:

$$C_a = P - P_{RNase}$$

where:

C_a = male-specific coliphage concentration, PFU/100 mL.

P = total number of plaques from dishes without RNase, and

P_{RNase} = total number of plaques from dishes with RNase.

g. *Sample treatment procedure to reduce somatic salmonella phages* (WG49 host assay only): To neutralize these somatic

* American Type Culture Collection Product No. 700609.

salmonella phages before assay, add enough LPS stock solution to sample to produce a final LPS concentration of 20 $\mu\text{g/mL}$. (NOTE: Add LPS after adding CaCl_2 to sample and before adding WG49 host.) Mix thoroughly and hold at ambient temperature for 15 to 30 min. Then add WG49 host and assay as described above.

5. References

1. GRABOW, W.O.K. 1986. Practical direct plaque assay for coliphages in 100-mL samples of drinking water. *Appl. Environ. Microbiol.* 52:430.

2. SOBSEY, M.D., A. AMANTI & T.R. HANDZEL. 1996. Detection and occurrence of coliphage indicator viruses in water. In Proc. Amer. Water Works Assoc. Water Quality Technol. Conf., New Orleans, La., Nov. 12-16, 1995. American Water Works Assoc., Denver, Colo.

9224 F. Membrane Filter Method

1. General Discussion

This method uses a filter-adsorption technique to recover coliphages from water samples. With this method, 100-mL and larger volume samples can be assayed effectively by a simple procedure in which the sample is first passed through a membrane filter. Coliphages present in the sample are adsorbed onto the filter and the phage-adsorbed filter is then directly plaque assayed.^{1,2} The advantage of this method is that, unlike Methods B through E, it does not require multiple assay dishes for each sample. A single assay dish is utilized for each phage-adsorbed filter. This significantly reduces the time and materials required. The disadvantage is that extraneous material, which can interfere with subsequent plaque assay, can accumulate on filters as water is passed through them. The amount of accumulation depends on the quality of the water being sampled and increases in proportion to the sample volume. Keep sample volumes as small as possible to prevent observable accumulation of discoloring material on the filter. However, with poor-quality waters, rapid accumulation may be observed with even small sample volumes. With such waters, use larger-diameter filters to increase sample volume. In the procedure below, the use of 47-mm membrane filters is described, but 90-mm membrane filters may be used if sample volumes prove inadequate with the smaller filters. Although originally described for use with *S. typhimurium* WG49, the procedure is suitable for use with *E. coli* C, and *E. coli* Famp host bacteria. The general procedure is described below. For specific details relating to each of the three bacterial hosts, refer to the preceding sections (Section 9224B, 9224C, and 9224D). Although a membrane-filter method is presented here, other approaches also may prove effective for the assay of 100-mL and larger volume samples. Methods such as the most probable number method could be adapted to larger volumes,³ and such non-filter-based methods probably would be less susceptible to poor water quality. Consider their use if water conditions are encountered that make the membrane filter method impractical.

2. Apparatus

See 9224B.2a through f and h through j, and in addition:

- a. Petri dishes, 60 $\times$ 15 mm.

- b. Sample vacuum filtration apparatus, including a 47-mm membrane filter holder and filtrate receiving vessel. Sterilize apparatus before use by autoclaving at 121°C for 15 min.

- c. Sample membrane filters, 0.45- μm pore size with a 47-mm diam and a cellulose nitrate and acetate composition. Sterilize filters before use by autoclaving at 121°C for 15 min (or use pre-sterilized filters). At the time of use, use sterile technique to place sterile filter into sterile sample filtration apparatus.

3. Media and Reagents

The amount of media prepared may be increased proportionally to the number of samples to be analyzed. Use reagent-grade water (see Table 9020:II) in preparing media and reagents.

For all host cultures:

- a. Beef extract: See 9224B.3a.

- b. Magnesium chloride solution, 2M: Dissolve 19.04 g MgCl_2 in 100 mL water and sterilize by autoclaving at 121°C for 15 min. Use at room temperature.

- c. Glycerol solution, 50%: See 9224B.3b.

- d. Phosphate-buffered saline: Dissolve 0.8 g NaCl , 20 mg KCl , 12 mg KH_2PO_4 , and 91 mg Na_2HPO_4 in 100 mL water. Sterilize by autoclaving at 121°C for 15 min.

- e. Tetrazolium violet solution: Add 3 g tetrazolium violet to 100 mL water and filter-sterilize with 0.45- μm filter. Use 1 mL tetrazolium violet solution/100 mL agar.

- f. Tryptone agar slants: See 9224B.3c.

- g. Tryptone agar: Use ingredients and concentrations listed for tryptone broth, and add 0.9 g agar and 15 mg MgSO_4 /100 mL of broth. Sterilize by autoclaving at 121°C for 15 min. Place autoclaved agar in the $44.5 \pm 1^\circ\text{C}$ water bath. After agar has cooled, add 1 mL polysorbate solution and 1 mL tetrazolium violet solution/100 mL agar. Then add 2 mL of appropriate host bacterium preparation per 100 mL agar. Dispense agar by pipet in 5-mL portions to sterile 60- $\times$ 15-mm petri dishes and let agar harden. If plates are to be used the following day, store at 4°C overnight and warm to room temperature for 1 h before use.

- h. Tryptone broth: See 9224 B.3e.

- i. Tryptone dilution tubes: See 9224B.3f.

j. *Polysorbate solution*: Add 30 g polysorbate* to 100 mL water and filter with 0.22- μ m filter. Use 1 mL/100 mL agar.

For *E. coli* Famp host only:

k. *Ampicillin solution*: See 9224C.3a.

l. *Streptomycin solution*: See 9224C.3e.

For *S. typhimurium* host only:

m. *Kanamycin solution*: See 9224D.3c.

n. *Lipopolysaccharide solution (LPS)*: See 9224D.3d.

o. *Nalidixic acid solution*: See 9224D.3e.

For *E. coli* Famp and *S. typhimurium* WG49 hosts:

p. *Ribonuclease (RNase) solution*: See 9224C.3d.

4. Procedure

a. *Storage of host cultures*: Follow procedures of 9224B.4a, using appropriate host culture.

b. *Preparation of host*: Follow procedures of 9224B.4b.

c. *Preparation of coliphage positive controls*: Rehydrate a stock culture of appropriate positive control and proceed as directed in 9224B.4c.

d. *Assay procedure*: For each 100 mL sample, use a single petri dish assay. After dishes have been prepared, add 2.5 mL $MgCl_2$ solution to sample to yield a final concentration of 0.05M. Pass magnesium-chloride-supplemented sample through sterile sample filter at a rate sufficient to clear entire 102.5 mL within 1 to 3 min. Using sterile technique, remove filter from sample filter apparatus. Taking care to prevent formation of bubbles, apply filter, face down, onto surface of one of the pre-poured agar petri dishes. Incubate at $36.5 \pm 2^\circ C$ overnight and examine for plaques the following day. Count total number of plaques. That total is the coliphage concentration/100 mL sample. How-

ever, in assays for the male-specific RNA coliphages using *E. coli* Famp and *S. typhimurium* WG49, this count could include some somatic and male-specific DNA coliphages or some somatic salmonella phages present in the sample. For appropriate procedures to address the presence of undesired phage see 4e and 4f, below.

e. *Assay procedure with additional confirmation* (male specific coliphage assay only): Assay an additional 100-mL sample portion. Proceed as described above, but add RNase solution to the melted tryptone agar to give a concentration of RNase of 40 μ g/mL. Label assay dish containing RNase so that it is readily distinguished from the corresponding assay dish without RNase. After overnight incubation at $36.5 \pm 2^\circ C$, determine male-specific RNA coliphage concentration/100 mL sample by subtracting total number of plaques counted on the RNase-containing dish from total number counted on the dish without RNase.

f. *Sample treatment procedure to reduce somatic salmonella phages* (WG49 host assay only): To neutralize somatic salmonella phages before assay, treat sample filters with an LPS solution made in PBS that contains 50 mM $CaCl_2$ and has an LPS concentration of 20 μ g/mL. After passing 100 mL sample through filter, pass 5 mL of LPS solution through filter. Handle LPS-treated filter as described in the above assay procedure with untreated filters.

5. References

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2. SOBSEY, M.D., D.A. BATTIGELLI, T.R. HANDZEL & K.J. SCHWAB. 1995. Male-specific coliphages as indicators of viral contamination of drinking water. American Water Works Association Research Foundation, Denver, Colo.
3. KOTT, Y. 1966. Estimation of low numbers of *Escherichia coli* bacteriophage by use of the most probable number method. *Appl. Microbiol.* 14:141.

* Tween 80 or equivalent.

9225 DIFFERENTIATION OF THE COLIFORM BACTERIA*

9225 A. Introduction

Identification of bacteria that constitute the coliform group sometimes is necessary in water analysis. It is of particular importance in reference to distinguishing the presence of *Escherichia coli*. Special procedures for detection of *E. coli* are given in Sections 9221F, 9222G, and 9223. Differential tests for identification must be used with the knowledge that all strains taxonomically assigned to the coliform group do not conform necessarily to the coliform definition stated in this manual because they may not ferment lactose, or if they

do, they may not produce gas. Furthermore, gram-negative bacteria other than coliforms ferment lactose and produce sheen (e.g., *Aeromonas* spp.) and not all strains of a species will react uniformly in media. Unusual strains (such as *E. coli*, inactive, Table 9225:I), mutants, and injured organisms may not give classical responses. The traditional "IMViC" tests (i.e., indole, methyl red, Voges-Proskauer, and citrate utilization) are useful for coliform differentiation, but do not provide complete identification. Additional biochemical tests often are necessary. Commercial kits for identification are available and may serve as economical alternatives to traditional differential media. Automated systems of identifying large numbers of isolates also are available.

* Approved by Standard Methods Committee, 2000.

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The significance of various coliform organisms in water has been and is a subject of considerable study. Collectively, the coliforms are referred to as indicator organisms. The genera

Enterobacter, *Klebsiella*, *Citrobacter*, and *Escherichia* usually are represented in the majority of isolations made from raw and treated municipal water supplies.

9225 B. Culture Purification

1. Procedure

A pure culture is essential for accurate identification. Obtain a pure culture by carefully picking a well-isolated colony that gives typical responses on an appropriate solid medium or membrane filter, and streaking on a tryptic soy or nutrient agar plate. Better distribution of colonies in the subculture is obtained if a portion of the picked colony is emulsified in peptone broth or physiological saline (0.85% w/v) and then streaked. When picking a colony from a primary culture on a selective medium, be aware that viable cells, which have not formed colonies themselves, may surround the picked colony. Incubate the subculture at $35 \pm 0.5^\circ\text{C}$ for 24 h and test a single well-isolated colony by the Gram stain to confirm the sole presence of gram-negative, non-spore-forming rods (Section 9221B). Also determine that the culture is oxidase-negative (Section 9225D). Oxidase-positive, gram-negative, non-spore-forming rods are not coliform bacteria, but may be organisms such as *Aeromonas*, which is not regarded as an indicator of fecal pollution.

Variation in organisms of the coliform group occurs occasionally and mixed reactions in differential media may indicate a pure culture undergoing variation. Persistent variations of reac-

tions in differential media indicate a mixed culture caused by inadequate purification.

2. Bibliography

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9225 C. Identification

1. Definition

Coliforms are defined here as facultative anaerobic, gram-negative non-spore-forming rods that ferment lactose with gas formation within 48 h at 35°C or, as applied to the membrane filter method, produce a dark red colony with a metallic sheen within 24 h on an Endo-type medium containing lactose. However, anaerogenic (non-gas-producing) lactose-fermenting strains of *Escherichia coli* and coliforms that do not produce metallic sheen on Endo medium may be encountered. These organisms, as well as typical coliforms, can be considered indicator organisms, but they are excluded from the current definition of coliforms. More extensive testing may be required for proper identification.

2. Characteristics and Tests

Coliforms belong to the bacterial taxonomic family Enterobacteriaceae. Table 9225:I provides data on some of the

biochemical reactions used for differentiating these organisms.

Preparing differential media and reagents may not be as economical for many laboratories as using commercially prepared and prepackaged multiple-test kits, which reduce quality-control work. These commercial kits are simple to store and use, and give reproducible and generally accurate results. Periodically test reactions with known stock cultures of bacteria to assure accuracy and reproducibility of results. Make further tests if the kit provides equivocal results.

3. Bibliography

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TABLE 9225:I. BIOCHEMICAL REACTIONS OF KEY SPECIES OF THE FAMILY ENTEROBACTERIACEAE*

Percentage Isolates Positive in 1 to 2 Days†

Species	Lactose Fermentation	ONPG Hydrolysis	Indole Production	Methyl Red	Voges- Proskauer	Simmons' Citrate	Ornithine Decarboxylase	Lysine Decarboxylase	Sorbitol Fermentation	Cellobiose Fermentation	Motility, 35–37°C	Yellow Pigment
<i>Citrobacter diversus</i>	35	96	99	100	0	99	99	0	99	99	95	0
<i>Citrobacter freundii</i>	50	95	5	100	0	95	20	0	98	55	95	0
<i>Enterobacter aerogenes</i>	95	100	0	5	98	95	98	98	100	100	97	0
<i>Enterobacter agglomerans</i>	40	90	20	50	70	50	0	0	30	55	85	75
<i>Enterobacter cloacae</i>	93	99	0	5	100	100	96	0	95	99	95	0
<i>Escherichia coli</i>	95	95	98	99	0	1	65	90	94	2	95	0
<i>Escherichia coli</i> , inactive	25	45	80	95	0	1	20	40	75	2	5	0
<i>Escherichia fergusonii</i>	0	83	98	100	0	17	100	95	0	96	93	0
<i>Escherichia hermannii</i>	45	98	99	100	0	1	100	6	0	97	99	98
<i>Escherichia vulneris</i>	15	100	0	100	0	0	0	85	1	100	100	50
<i>Hafnia alvei</i>	5	90	0	40	85	10	98	100	0	15	85	0
<i>Klebsiella oxytoca</i>	100	100	99	20	95	95	0	99	99	100	0	1
<i>Klebsiella ozaenae</i>	30	80	0	98	0	30	3	40	65	92	0	0
<i>Klebsiella pneumoniae</i>	98	99	0	10	98	98	0	98	99	98	0	0
<i>Klebsiella rhinoscleromatis</i>	0	0	0	100	0	0	0	0	100	100	0	0
<i>Serratia fonticola</i>	97	100	0	100	9	91	97	100	100	6	91	0
<i>Serratia marcescens</i>	2	95	1	20	98	98	99	99	99	5	97	0

*Modified after Farmer, J.J. III, 1985. Clinical identification of new species and biogroups of Enterobacteriaceae. *J. Clin. Microbiol.* 21:46.

†Reactions that become positive after 2 d are not considered.

9225 D. Media, Reagents, and Procedures

Commercially available media and reagents can reduce work and cost; however, include negative and positive controls with known stock cultures to assure accuracy and reliability. Detailed methods are available. Expected test results are shown in Table 9225:I.

1. Lactose, Sorbitol, and Cellobiose Fermentation Tests

Suspend 16 g phenol red broth base and 5 g of the desired carbohydrate in 1 L reagent-grade water and stir to dissolve completely. Dispense in tubes to a depth of one-third tube length. To determine gas production place a small inverted vial (Durham tube) in the tubes of media at the time of preparation. Close tubes and sterilize at 121°C for 15 min. Store tubes in the dark (refrigeration preferred) and discard if evaporation exceeds 10% of the volume.

To conduct a test, inoculate with a loopful of growth from a well-isolated colony or slant and incubate for 24 to 48 h at 35 ± 0.5°C. Carbohydrate fermentation (acid production) is indicated by a decrease in pH, resulting in a change in color of the pH indicator, phenol red, from red-orange to yellow (pH <6.6). Alternatively, for lactose fermentation, lauryl tryptose broth (Section 9221B) may be used.

2. ONPG Hydrolysis

Numerous commercial test kits and disks for determining ONPG hydrolysis are available, or an ONPG-containing medium (Section 9222) can be used. Alternatively, prepare peptone water by dissolving 1 g peptone and 0.5 g NaCl in 100 mL reagent-grade water. Sterilize at 121°C for 15 min. Also prepare ONPG solution by dissolving 0.6 g *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 100 mL 0.01M Na₂HPO₄, sterilize by filtration, and store in the dark at 4 to 10°C. To prepare ONPG broth, aseptically combine 25 mL ONPG solution and 75 mL peptone water, dispense aseptically in 2.5-mL amounts in sterile 13- × 100-mm tubes, and store in the dark for up to 1 month at 4 to 10°C. Do not use the ONPG solution if it becomes yellow.

To conduct the test, inoculate 0.5 mL ONPG broth with a heavy loopful of growth from a slant and incubate at 35 ± 0.5°C for up to 24 h. A yellow color, compared with an uninoculated tube or (preferably) a tube inoculated with an ONPG-negative culture, is a positive test. Interpret tests of yellow-pigmented organisms with caution. Do not use the enzyme substrate method (Section 9223) to test ONPG hydrolysis.

3. Indole Test

Indole is a product of the metabolism of tryptophane.

a. Reagents:

1) *Medium*: Use tryptophane broth. Dissolve 10.0 g tryptone or trypticase/L reagent-grade water. Dispense in 5-mL portions in test tubes and sterilize.

2) *Test reagent*: Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 mL isoamyl (or normal amyl) alcohol, ACS grade, and add 25 mL conc HCl. The reagent should be yellow. Some brands of *p*-dimethylaminobenzaldehyde are not satisfactory and some good brands become unsatisfactory on aging.

The amyl alcohol solution should have a pH value of less than 6.0. Purchase both amyl alcohol and benzaldehyde in as small amounts as will be consistent with the volume of work to be done.

b. *Procedure*: Inoculate 5-mL portions of medium from a pure culture and incubate at $35 \pm 0.5^\circ\text{C}$ for 24 ± 2 h. Add 0.2 to 0.3 mL test reagent and gently shake. Let stand for about 10 min and observe results.

A dark red color in the amyl alcohol surface layer constitutes a positive indole test; the original color of the reagent, a negative test. An orange color probably indicates the presence of skatole, a breakdown product of indole.

4. Methyl Red Test

The methyl red test measures the ability of organisms to produce stable acid end products from glucose fermentation.

a. Reagents:

1) *Medium*: Use buffered glucose broth. Dissolve 7.0 g proteose peptone or equivalent peptone, 5.0 g glucose, and 5.0 g dipotassium hydrogen phosphate (K_2HPO_4) in 1 L reagent-grade water. Dispense in 5-mL portions in test tubes and sterilize by autoclaving at 121°C for 12 to 15 min, making sure that total time of exposure to heat is not longer than 30 min.

2) *Indicator solution*: Dissolve 0.1 g methyl red in 300 mL 95% ethyl alcohol and dilute to 500 mL with reagent-grade water.

b. *Procedure*: Inoculate 10-mL portions of medium from a pure culture. Incubate at $35 \pm 0.5^\circ\text{C}$ for 5 d. To 5 mL of the culture add 5 drops methyl red indicator solution.

Incubation for 48 h is adequate for most cultures, but do not incubate for less than 48 h. If test results are equivocal at 48 h repeat with cultures incubated for 4 or 5 d. In such cases incubate duplicate cultures at 22 to 25°C . Testing of culture portions at 2, 3, 4, and 5 d may provide positive results sooner.

Record a distinct red color as methyl-red-positive and a distinct yellow color as methyl-red-negative. Record a mixed shade as questionable and possibly indicative of incomplete culture purification.

5. Voges-Proskauer Test

The Voges-Proskauer test measures the ability of organisms to produce a neutral end product (acetoin) from glucose fermentation.

a. Reagents:

1) *Medium*: See ¶ 4a1) above.

2) *Naphthol solution*: Dissolve 5 g purified α -naphthol (melting point 92.5°C or higher) in 100 mL absolute ethyl alcohol. When stored at 5 to 10°C , this solution is stable for 2 weeks.

3) *Potassium hydroxide, 7N*: Dissolve 40 g KOH in 100 mL reagent-grade water.

b. *Procedure*: Inoculate 5 mL medium and incubate for 48 h at $35 \pm 0.5^\circ\text{C}$. To 1 mL of culture add 0.6 mL naphthol solution and 0.2 mL KOH solution. Shake well after the addition of each reagent. Development of a pink to crimson color at the surface within 5 min constitutes a positive test. Do not read after 10 min. Disregard tubes developing a copper color.

6. Simmons' Citrate Test

The citrate test measures the ability of bacteria to utilize citrate as the sole source of carbon.

a. *Medium*: Use Simmons' citrate agar. To make Simmons' citrate agar, add 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$), 1.0 g K_2HPO_4 , 2.0 g sodium citrate dihydrate, 5.0 g NaCl, 15.0 g agar, and 0.08 g bromthymol blue to 1 L reagent-grade water. Tube for long slants.

b. *Procedure*: Inoculate agar medium by the streak technique using a light inoculum.

Incubate 48 h at $35 \pm 0.5^\circ\text{C}$. Record growth on the medium with a blue color as a positive reaction; record absence of growth or color change as negative.

7. Motility Test

The motility test measures whether an organism is motile in a semi-solid medium.

a. *Medium*: Use motility test medium made by adding 3.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, and 4.0 g agar to 1 L reagent-grade water. Adjust pH to 7.4, dispense in 3-mL portions in 13- × 100-mm tubes or 8-mL portions in 16- × 125-mm tubes, and sterilize.

b. *Procedure*: Inoculate by stabbing into the center of the medium, using an inoculating needle, to a depth of 5 mm. Incubate for 1 to 2 d at 35°C . If negative, incubate an additional 5 d at 22 to 25°C .

Diffuse growth through the medium from the point of inoculation is positive. In a negative test, growth is visible only along the stab line and the surrounding medium stays clear. Alternatively, prepare the medium without agar and examine a young culture using the hanging drop slide technique for motile organisms.

8. Lysine and Ornithine Decarboxylase Tests

This procedure tests the ability of bacteria to metabolize the amino acids lysine and ornithine.

a. Reagents:

1) *Media*: Use a basal medium made according to the Moeller or Falkow methods. For the Moeller method, dissolve 5.0 g peptone (Orthana special, thiotone, or equivalent), 5.0 g beef extract, 0.625 mL bromocresol purple (1.6%), 2.5 mL cresol red (0.2%), 0.5 g glucose, and 5.0 mg pyridoxal in 1 L reagent-grade water and adjust to pH 6.0 to 6.5. For the Falkow method, dissolve 5.0 g peptone, 3.0 g yeast extract, 1.0 g glucose, and 1.0 mL bromocresol purple (1.6%) in 1 L reagent-grade water and

adjust to pH 6.7 to 6.8. For either decarboxylase test divide into three portions: make no addition to the first portion, add enough L-lysine dihydrochloride to the second portion to make a 1% solution, and add L-ornithine dihydrochloride to the third to make 1% (for the Falkow method, add only 0.5% of the L-amino acid). After adding ornithine readjust pH of the medium to 6.0 ± 0.2 . Dispense in 3- to 4-mL portions in screw-capped test tubes and sterilize by autoclaving at 121°C for 10 min. A floccular precipitate in the ornithine medium does not interfere with its use.

2) *Mineral oil*: Use mineral oil sterilized by autoclaving at 121°C for 30 to 60 min depending on the size of the container.

b. Procedure: Lightly inoculate each of the three media, add a layer of about 10 mm thickness of mineral oil, and incubate at 37°C for up to 4 d. Examine tubes daily. A color change from yellow to violet or reddish-violet constitutes a positive decarboxylase test; a change to bluish gray indicates a weak positive; no color change or a yellow color represents a negative test. See Table 9225.I.

9. Oxidase Test

The oxidase test determines the presence of oxidase enzymes. Coliform bacteria are oxidase-negative.

a. Reagents:

1) *Media*: Use either nutrient agar or tryptic soy agar plates to streak cultures and produce isolated colonies. From these obtain the inoculum for oxidase testing on impregnated filter paper. Do not use any medium that includes a carbohydrate in its formulation. Use only tryptic soy agar if reagent is dropped on colonies.

Tryptic soy agar:

Tryptone	15.0 g
Soytone	5.0 g
Sodium chloride, NaCl	5.0 g
Agar	15.0 g
Reagent-grade water	1.0 L

pH should be 7.3 ± 0.2 after sterilization.

2) *Tetramethyl p-phenylenediamine dihydrochloride*, 1% aqueous solution, freshly prepared or refrigerated for no longer than 1 week. Impregnate a filter paper strip* with this solution. Alternatively, prepare a 1% solution of dimethyl p-phenylenediamine hydrochloride. Single-use reagent ampules, commercially available, are convenient and economical, but use them with caution. When the reagent is to be dropped directly on colonies, use tryptic soy agar plates because nutrient agar plates give inconsistent results; when smearing a portion of a picked colony on reagent-impregnated filter paper, do not transfer any medium with the culture material.

b. Procedure: Remove some of a colony from agar plate with a platinum wire, a wooden or plastic applicator stick, or a glass rod and smear on the test strip. Do not use iron or other reactive wire because it will cause false positive reactions. A dark purple color that develops within 10 s is a positive oxidase test. Test positive and negative cultures concurrently. If the liquid reagent is used, drop it on colonies on the culture plate. Oxidase-positive colonies develop a pink color that successively becomes maroon, dark red, and finally, black.

10. Yellow Pigment

Observe isolated colonies on nutrient agar slants and plates or plates of tryptic soy agar incubated at $35 \pm 0.5^\circ\text{C}$ for up to 48 h. Pigmentation often intensifies as time of incubation proceeds.

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* Whatman No. 1 or equivalent.

9230 FECAL STREPTOCOCCUS AND ENTEROCOCCUS GROUPS*

9230 A. Introduction

This section is currently undergoing substantial revision; consensus on proposed changes could not be reached in time for this publication. The methods below are reprinted from the 20th Edition of *Standard Methods*.

1. Fecal Streptococcus Group

The fecal streptococcus group consists of a number of species of the genus *Streptococcus*, such as *S. faecalis*, *S. faecium*, *S.*

avium, *S. bovis*, *S. equinus*, and *S. gallinarum*. They all give a positive reaction with Lancefield's Group D antisera¹ and have been isolated from the feces of warm-blooded animals. In addition, *S. avium* sometimes reacts with Lancefield's Group Q antisera. *S. faecalis* subsp. *liquefaciens* and *S. faecalis* subsp. *zymogenes* are differentiated based on the ability of these strains to liquefy gelatin and hemolyze red cells. However, the validity of these subspecies is questionable.^{2,3}

The normal habitat of fecal streptococci is the gastrointestinal tract of warm-blooded animals. *S. faecalis* and *S. faecium* once were thought to be more human-specific than other *Streptococ-*

* Approved by Standard Methods Committee, 1993.

cus species. Other species have been observed in human feces but less frequently.⁴ Similarly, *S. bovis*, *S. equinus*, and *S. avium* are not exclusive to animals, although they usually occur at higher densities in animal feces.⁵ Certain streptococcal species predominate in some animal species and not in others, but it is not possible to differentiate the source of fecal contamination based on speciation of fecal streptococci.

The fecal streptococci have been used with fecal coliforms to differentiate human fecal contamination from that of other warm-blooded animals. Editions of *Standard Methods* previous to the 17th suggested that the ratio of fecal coliforms (FC) to fecal streptococci (FS) could provide information about the source of contamination. A ratio greater than four was considered indicative of human fecal contamination, whereas a ratio of less than 0.7 was suggestive of contamination by nonhuman sources. The value of this ratio has been questioned because of variable survival rates of fecal streptococcus group species. *S. bovis* and *S. equinus* die off rapidly, once exposed to aquatic environments, whereas *S. faecalis* and *S. faecium* tend to survive longer.⁶ Furthermore, disinfection of wastewaters appears to have a significant effect on the ratio of these indicators, which may result in misleading conclusions regarding the source of contaminants.⁷ The ratio is affected also by the methods for enumerating fecal streptococci. The KF membrane filter procedure has a false-positive rate ranging from 10 to 90% in marine and fresh waters.⁸⁻¹⁰ For these reasons, the FC/FS ratio cannot be recommended, and should not be used as a means of differentiating human and animal sources of pollution.

2. Enterococcus Group

The enterococcus group is a subgroup of the fecal streptococci that includes *S. faecalis*, *S. faecium*, *S. gallinarum*, and *S. avium*. The enterococci are differentiated from other streptococci by their ability to grow in 6.5% sodium chloride, at pH 9.6, and at 10°C and 45°C.

The enterococci portion of the fecal streptococcus group is a valuable bacterial indicator for determining the extent of fecal contamination of recreational surface waters. Studies at marine and fresh water bathing beaches indicated that swimming-associated gastroenteritis is related directly to the quality of the bathing water and that enterococci are the most efficient bacterial indicator of water quality.^{11,12} Water quality guidelines based on enterococcal density have been proposed for recreational waters.¹³ For recreational fresh waters the guideline is 33 enterococci/100 mL while for marine waters it is 35/100 mL. Each guideline is based on the geometric mean of at least five samples per 30-d period during the swimming season.

3. Selection of Method

The multiple-tube technique is applicable primarily to raw and chlorinated wastewater and sediments, and can be used for fresh and marine waters. The membrane filter technique also may be used for fresh and saline water samples, but is unsuitable for highly turbid waters.

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9230 B. Multiple-Tube Technique

1. Materials and Culture Media

a. Azide dextrose broth:¹

Beef extract	4.5	g
Tryptone or polypeptone	15.0	g
Glucose	7.5	g
Sodium chloride, NaCl	7.5	g
Sodium azide, NaN ₃	0.2	g
Reagent-grade water	1	L

pH should be 7.2 ± 0.2 at 25°C after sterilization.

b. Pfizer selective enterococcus (PSE) agar:²

Peptone C	17.0	g
Peptone B	3.0	g
Yeast extract	5.0	g
Bacteriological bile	10.0	g
Sodium chloride, NaCl	5.0	g
Sodium citrate	1.0	g
Esculin	1.0	g
Ferric ammonium citrate	0.5	g
Sodium azide, NaN ₃	0.25	g
Agar	15.0	g
Reagent-grade water	1	L

pH should be 7.1 ± 0.2 after sterilization. Hold medium for not more than 4 h at 45 to 50°C before plates are poured.

2. Presumptive Test Procedure

Inoculate a series of tubes of azide dextrose broth with appropriate graduated quantities of sample. Use sample of 10 mL portions or less. Use double-strength broth for 10-mL inocula. The portions used will vary in size and number with the sample

character. Use only decimal multiples of 1 mL (see Section 9221 for suggested sample sizes).

Incubate inoculated tubes at $35 \pm 0.5^\circ\text{C}$. Examine each tube for turbidity at the end of 24 ± 2 h. If no definite turbidity is present, reincubate, and read again at the end of 48 ± 3 h.

3. Confirmed Test Procedure

Subject all azide dextrose broth tubes showing turbidity after 24- or 48-h incubation to the confirmed test.

Streak a portion of growth from each positive azide dextrose broth tube on PSE agar. Incubate the inverted dish at $35 \pm 0.5^\circ\text{C}$ for 24 ± 2 h. Brownish-black colonies with brown halos confirm the presence of fecal streptococci.

Brownish-black colonies with brown halos may be transferred to a tube of brain-heart infusion broth containing 6.5% NaCl. Growth in 6.5% NaCl broth and at 45°C indicates that the colony belongs to the enterococcus group.

4. Computing and Recording of MPN

Estimate fecal streptococci densities from the number of tubes in each dilution series that are positive on PSE agar. Similarly, estimate enterococci densities from the number of tubes in each dilution series containing streptococci that can grow in 6.5% NaCl broth. Compute the combination of positives and record as the most probable number (MPN). Refer to Section 9221C.

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9230 C. Membrane Filter Techniques

1. Laboratory Apparatus

See Section 9222B.1.

2. Materials and Culture Media

a. mE agar for enterococci:¹

Peptone	10.0	g
Sodium chloride, NaCl	15.0	g
Yeast extract	30.0	g
Esculin	1.0	g
Actidione (cycloheximide)	0.05	g
Sodium azide, NaN ₃	0.15	g
Agar	15.0	g
Reagent-grade water	1	L

Heat to dissolve ingredients, sterilize, and cool in a water bath at 44 to 46°C . Mix 0.25 g nalidixic acid in 5 mL reagent-grade water, add a few drops of 0.1N NaOH to dissolve the antibiotic, and add to the basal medium. Add 0.15 g 2,3,5-triphenyl tetrazolium chloride and mix well to dissolve. Pour the agar into 9- × 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. The final pH should be 7.1 ± 0.2 . Store poured plates in the dark at 2 to 10°C . Discard after 30 d. (NOTE: This medium is recommended for culturing enterococci in fresh and marine recreational waters.)

b. EIA substrate:¹

Esculin	1.0	g
Ferric citrate	0.5	g
Agar	15.0	g
Reagent-grade water	1	L

The pH should be 7.1 ± 0.2 before autoclaving. Heat to dissolve ingredients, sterilize, and cool in a water bath at 44 to 46°C . Pour medium into 50-mm petri dishes to a depth of 4 to 5 mm (approximately 4 to 6 mL) and let solidify. Store poured plates in the dark at 2 to 10°C . Discard after 30 d.

*c. m Enterococcus agar for fecal streptococci:*²

Tryptose	20.0 g
Yeast extract	5.0 g
Glucose	2.0 g
Dipotassium phosphate, K_2HPO_4	4.0 g
Sodium azide, NaN_3	0.4 g
2,3,5-Triphenyl tetrazolium chloride	0.1 g
Agar	10.0 g
Reagent-grade water	1 L

Heat to dissolve ingredients. Do not autoclave. Dispense into 9- × 50-mm petri plates to a depth of 4 to 5 mm (approximately 4 to 6 mL), and let solidify. Prepare fresh medium for each set of samples. (NOTE: This medium is recommended for Group D streptococci in fresh and marine waters.)

d. Brain-heart infusion broth:

Infusion of calf brain	200 g
Infusion of beef heart	250 g
Proteose peptone	10.0 g
Glucose	2.0 g
Sodium chloride, NaCl	5.0 g
Disodium hydrogen phosphate, Na_2HPO_4	2.5 g
Reagent-grade water	1 L

The pH should be 7.4 after sterilization.

e. Brain-heart infusion agar: Add 15.0 g agar to the ingredients for brain-heart infusion broth. The pH should be 7.4 after sterilization. Tube for slants.

*f. Bile esculin agar:*³

Beef extract	3.0 g
Peptone	5.0 g
Oxgall	40.0 g
Esculin	1.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Reagent-grade water	1 L

Heat to dissolve ingredients. Dispense 8 to 10 mL into tubes for slants or an appropriate volume into a flask for subsequent pouring into plates. Autoclave at 121°C for 15 min. Do not overheat because this may cause darkening of the medium. Cool to 44 to 46°C and slant the tubes or dispense 15 mL into 15- × 100-mm petri dishes. The final pH should be 6.6 ± 0.2 after sterilization. Store at 4 to 10°C .

3. Procedures

*a. mE Method:*¹

1) Selection of sample size and filtration—Filter appropriate sample volumes through a $0.45\text{-}\mu\text{m}$, gridded, sterile membrane to give 20 to 60 colonies on the membrane surface. Transfer filter to agar medium in petri dish, avoiding air bubbles beneath the membrane.

2) Incubation—Invert culture plates and incubate at $41^\circ\text{C} \pm 0.5^\circ\text{C}$ for 48 h.

3) Substrate test—After 48 h incubation, carefully transfer filter to EIA medium. Incubate at $41^\circ\text{C} \pm 0.5^\circ\text{C}$ for 20 min.

4) Counting—Pink to red enterococci colonies develop a black or reddish-brown precipitate on the underside of the filter. Count colonies using a fluorescent lamp and a magnifying lens.

*b. m Enterococcus method:*²

1) Selection of sample size and filtration—See ¶ 3a.

2) Incubation—Let plates stand for 30 min, then invert and incubate at $35 \pm 0.5^\circ\text{C}$ for 48 h.

3) Counting—Count all light and dark red colonies as enterococci. Count colonies using a fluorescent lamp and a magnifying lens.

4. Calculation of Fecal Streptococci or Enterococci Density

Compute density from sample quantities producing membrane filter counts within the desired 20- to 60-fecal streptococcus or enterococcus colony range. Calculate as in Section 9222B.6. Record densities as fecal streptococci or enterococci per 100 mL.

5. Verification Tests

Pick selected typical colonies from a membrane and streak for isolation onto the surface of a brain-heart infusion agar plate. Incubate at $35^\circ\text{C} \pm 0.5^\circ\text{C}$ for 24 to 48 h.

Transfer a loopful of growth from a well-isolated colony on brain-heart infusion agar into a brain-heart infusion broth tube and to each of two clean glass slides. Incubate the brain-heart infusion broth at $35 \pm 0.5^\circ\text{C}$ for 24 h. Add a few drops of freshly prepared 3% hydrogen peroxide to the smear on a slide. The appearance of bubbles constitutes a positive catalase test and indicates that the colony is not a member of the fecal streptococcus group. If the catalase test is negative, i.e., no bubbles, make a Gram stain of the second slide. Fecal streptococci and enterococci are gram-positive, ovoid cells, 0.5 to $1.0\text{ }\mu\text{m}$ in diameter, mostly in pairs or short chains.

Transfer a loopful of growth from the brain-heart infusion broth to each of the following media: bile esculin agar (incubate at $35 \pm 0.5^\circ\text{C}$ for 48 h); brain-heart infusion broth (incubate at $45 \pm 0.5^\circ\text{C}$ for 48 h); brain-heart infusion broth with 6.5% NaCl (incubate at $35 \pm 0.5^\circ\text{C}$ for 48 h).

Growth of catalase-negative, gram-positive cocci on bile esculin agar and at 45°C in brain-heart infusion broth verifies that the colony is of the fecal streptococcus group. Growth at 45°C and in 6.5% NaCl broth indicates that the colony belongs to the enterococcus group.

6. Serological Verification of Group D Fecal Streptococci

An alternate verification test for Group D streptococci can be performed using the precipitin method of Lancefield.⁴ This test is highly specific for *S. faecalis*, *S. faecium*, *S. avium*, *S. gallinarum*, *S. bovis*, and *S. equinus*.

a. Antigen preparation: Pick typical single colonies from the membrane filter and streak for isolation on brain-heart infusion agar or blood agar plates. Pick a well-isolated colony and inoculate into 30 to 50 mL of Todd-Hewitt broth.⁵ Incubate at 35°C for 24 h under aerobic conditions. Concentrate bacterial suspen-

TABLE 9230:I. SELECTED KEY BIOCHEMICAL CHARACTERISTICS OF THE STREPTOCOCCUS SPECIES WITHIN THE FECAL STREPTOCOCCUS AND ENTEROCOCCUS GROUPS*

Test	Fecal Streptococcus Group					
	Enterococcus Group					
	<i>S. faecalis</i>	<i>S. faecium</i>	<i>S. avium</i>	<i>S. gallinarum</i>	<i>S. bovis</i>	<i>S. equinus</i>
Catalase	—	—	—	—	—	—
40% Bile	+	+	+	+	+	+
Esculin ⁶	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	—	—
Growth at 10°C	+	+	+	+	—	—
Pyruvate utilization ⁷	+	—	—	—	—	—
Phosphatase activity ⁸	+	—	+	+	—	—
Arginine hydrolysis ⁸	+	+	— ^d	—	—	—
L-Sorbose fermentation ⁹	—	—	+	—	—	—
Lactose fermentation ⁸	+	+	+	+	+	+
<i>n</i> -Acetyl- β -glucosaminidase activity ⁹	— ^d	—	—	+	—	—
Starch ⁶	—	—	—	—	+	—
Arabinose ⁶	—	+	+	—	—	—

* + = 90% or more of strains are positive

— = 90% or more of strains are negative

^d = reactions variable

sion by centrifuging (3000 $\times$ *g* for 5 min). Draw supernatant off and resuspend cells in 0.5 mL saline solution. Autoclave resuspended cells for 15 min at 121°C. Centrifuge the bacteria and decant clear supernatant fluid containing the group antigen.

b. Capillary precipitin test: Antisera for this test may be obtained from commercial sources.

Dip a 1.2- to 1.5-mm-OD capillary tube into antiserum and draw up about 1 cm of serum. Place a finger over upper end of tube so that no air will be drawn up and carefully wipe off excess antiserum. Dip tube into streptococcal antigen extract solution and draw up an equal volume of antigen. Carefully wipe off excess extract. Place a finger over upper end of tube and force lower end into plasticine to plug lower opening. Invert tube and place it in plasticine groove of a capillary holding rack.

A positive test for Group D antigen is characterized by a white precipitate that appears at the antigen-antiserum interface within

15 min and usually by 5 min. If no reaction has occurred by 30 min, the test is negative. Examination of the tubes is more effective if they are read in a bright light against a dark background.

Serological verification of Group D streptococci also can be done using commercially available agglutination tests.* The slide agglutination tests are simple and appear to be reliable. Group D streptococci are verified directly from isolated colonies on membrane filter plates or from broth culture tubes. To verify presumptive enterococci cultures, test also for salt tolerance (growth in 6.5% NaCl broth).

7. Identification of Individual Species within Fecal Streptococcus and Enterococcus Groups

Table 9230:I shows some of the key biochemical reactions for identifying fecal streptococci, enterococci, and species within these two groups.

8. References

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* Phadebact Strep D test, Pharmacia Diagnostics, Piscataway, NJ and Streptex Test, Burroughs-Wellcome Co., Research Triangle Park, NC.