9020 QUALITY ASSURANCE/QUALITY CONTROL*

9020 A. Introduction

1. General Considerations

The emphasis on microorganisms in water quality standards and enforcement activities and their continuing role in research, process control, and compliance monitoring require the establishment, documentation, and effective operation of a quality system (QS). The QS establishes an environmental testing and management operation describing both a quality assurance (QA) policy or program and quality control (QC) operational techniques and practices. These are designed to substantiate the validity of analytical data and ensure compliance with regulatory requirements, customer requirements, and applicable standards of accreditation or certification.

The laboratory practices set forth in Section 9020 are not mandatory, but represent practices that should be followed. Each laboratory must develop its own QS suitable for its needs and, in some cases, as required by regulatory agencies, standard-setting organizations, and laboratory certification or accreditation programs.

A laboratory documents its quality system's policies and objectives in a quality management plan or quality manual. The document denotes the laboratory's commitment to the QA program for integration of intra- and inter-laboratory QC activities, standardization of laboratory operating procedures, and management practices. It also clearly defines 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. Once a QA program is established, about 15% of overall laboratory time should be spent on different aspects of the program. However, additional time may be needed for more important analytical data, e.g., data for enforcement actions. When properly administered, a balanced, conscientiously applied quality system will optimize data quality, identify problems early, and increase satisfaction with the analytical results without adversely affecting laboratory productivity.

Because microbiological analyses measure constantly changing living organisms, they are inherently variable. Quality control tools available to microbiologists are different from those used by chemists because many of the measurements made by microbiologists involve discrete variables rather than continuous variables used by the analytical chemists. Discrete variables have only integer values, whereas continuous variables are not limited to particular values but only by the accuracy of the measuring tool used. Therefore, different statistics and probability distributions are used to evaluate data.

Documented quality systems will vary among laboratories as a result of differences in organizational mission, responsibilities, and objectives; laboratory size, capabilities, and facilities; and staff skills and training.

2. Guidelines for a Quality System

The laboratory must develop, document, and initiate its processes to result in controlled experimental conditions that meet its specific needs and the planned use of the data.

a. Management responsibilities: Management must evaluate the risks associated with errors, recognize and actively support the need for the QS, involve staff in development and operation of the program, commit monetary and personnel resources, and assume a leadership role. Management should meet with the laboratory supervisor and staff to develop and maintain a comprehensive program, to establish specific responsibilities for management, supervisors, and analysts, and to maintain awareness of conditions through periodic and systematic review of laboratory functions. Upper management has overall responsibility to the end customer for the QA/QC program and activities performed by the laboratory analyst. The QA officer, the laboratory supervisor, and the laboratory analyst can be delegated responsibilities to carry out a function of their individual job duties by upper management; however, upper management is ultimately responsible for the QA program and cannot avoid their managerial responsibilities by delegation to a lower authority in the organization.

b. Quality assurance officer/quality manager: In large laboratories, a QA officer has the authority and oversight responsibility for implementing the QA program. Ideally, this person has a staff position reporting directly to upper management and thus has operational independence. The QA officer must have a technical background that includes courses in microbiology, be acquainted with all aspects of laboratory work, and be aware of and familiar with the QA program and QC practices, and statistical techniques for data evaluation. The QA officer is responsible for initiating the QA program, convincing management and staff of its value, and providing necessary technical support and training. Once the QA program is functioning, the QA officer should conduct frequent (weekly to monthly) reviews with the laboratory management and staff to determine conformance to 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. In small laboratories these responsibilities will be assigned to one or more of the staff on a part-time basis or the staff may form a QA unit.

c. Staff: Laboratory and field staffs should participate with management in planning the QA program, preparing standard operating procedures, and most importantly, implementing the QA program and QC activities in their daily tasks of collecting samples, conducting analyses, performing quality control

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checks, and calculating and reporting results. Staff members are the first to identify potential problems and should work with the QA officer and management/supervisor to correct and prevent them. It is critical to the success of the QA program that staff members understand what is expected of them and actively support the QA program.

3. Quality System Objectives

The objectives of a QS 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, improving documentation and recordkeeping, developing adequate and clear reporting systems, and assuring compliance with regulations and client's requirements.

4. Elements of a Quality System Manual

Each laboratory implements a QS and develops a written management plan or manual describing the laboratory's policies and plans for ensuring the quality of their work for their clients. Updated routinely, the plan is signed by both upper management and the QA officer to indicate their approval. For a small laboratory, the owner/operator will sign the plan.

Having the upper management and the QA officer sign off on a written management plan or manual describing laboratory policies and activities makes upper management responsible. This means that personnel support, analytical instruments, and materials are ultimately the responsibility of upper management and cannot be disposed of through delegation to lesser authority, such as the QA officer.

The plan should address the following basic common aspects:

a. Quality policy statement, describing the specific objectives and commitment of the laboratory and its management to quality and data integrity. An ethics statement may be included.

b. Organization and management structure, describing the functional aspects of the laboratory and its management responsibilities with an accompanying organizational chart.

c. Personnel policies, indicating specific qualification and training requirements and job responsibilities for supervisors and analysts.

d. Equipment and instrument requirements, listing critical equipment and instruments available, noting the laboratory's requirements and frequency for calibration procedures and preventative maintenance, and ensuring acceptable functionality before equipment is put into service.

e. Specifications for supplies, noting procedures to ensure that reagents and supplies are of sufficient quality and acceptable for use.

f. Specifications for subcontracting of tests and calibrations, establishing standards for the laboratory's oversight and acceptance of products.

g. Sampling procedures (if performed by the laboratory) and sample acceptance criteria, describing procedures for collecting, handling (e.g., time and temperature), accepting, and tracking submitted samples, and procedures for chain-of-custody required if data may be subjected to litigation.

h. Analytical methods, listing the laboratory's scope for testing, and denoting the accreditation/certification status for individual methods and, for nonstandard or new methods, the laboratory's validation procedures.

i. Analytical quality control measures, stating the laboratory's requirements for measurement assurance, e.g., method verification and documentation, error prevention, and analytical checks as replicate analyses, positive and negative controls, sterility checks, and verification tests, as well as statistical methods to be used.

j. Standard operating procedures (SOPs), listing all generic laboratory processes and specific routine laboratory operations, documented and signed by management, which are available to clients upon request and readily accessible to staff.

k. Documentation control and recordkeeping requirements, identifying recordkeeping formats, e.g., hard-copy, e-notebooks, and computer files, and procedures to ensure data review, traceability, and accountability; noting procedures to ensure customer confidentiality, where applicable, and other requirements, such as control, security, storage, record retention time, and disposal of laboratory records. When confidentiality and security allow, a backup copy of the records should be stored offsite.

l. Assessments, describing the laboratory's processes to monitor and report on the effectiveness of its QA program.

1) Internal audits of the laboratory operations, performed on a routine basis, at least annually, by the QA officer and supervisor. For a small laboratory, an outside expert may be needed. These audits should involve all aspects of the laboratory including, e.g., analyses conducted, data manipulations, and reporting.

2) Onsite evaluations by outside experts to ensure that the laboratory and its personnel are following an acceptable QA program. This is a required component for laboratory certification or accreditation. For laboratories not seeking such recognition, this activity is a suggestion.

3) Proficiency test (PT) studies, in which the laboratory participates. These collaborative studies should confirm the capability of a laboratory to generate acceptable data comparable to the reference laboratory and other laboratories and to identify potential problems. PT studies are generally conducted once or twice a year.

m. Corrective and preventive activities, identifying procedures used to determine the causes of identified problems and to record, correct, and prevent their recurrence.

n. Service to the customer, describing the laboratory's commitment to and activities for responding to customer requests and complaints, and for ensuring customer confidentiality and proprietary rights.

The QC guidelines discussed in 9020B and 9020C are recommended as useful source material of elements that need to be addressed in developing policies for a QA program and QC activities. Additional information is available from several standards-setting organizations, such as the American Association for Laboratory Accreditation (A2LA), AOAC International Inc., International Organization for Standardization (ISO), National Environmental Laboratory Accreditation Conference (NELAC), Institute for National Environmental Laboratory Accreditation (INELA), and the United States Environmental Protection Agency (USEPA).

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9020 B. Intralaboratory Quality Control Guidelines

Quality control (QC) practices are designed to ensure that the laboratory's processes are in control. All laboratories have some intralaboratory QC practices that have evolved from common sense and the principles of controlled experimentation to indicate method efficiency and laboratory performance. A laboratory's QS sets in place the QA policies or program and QC activities necessary to minimize systematic and random errors resulting from variations in 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 and is discussed in 9020B.5. Additional sources of information about laboratory QC practices are available.[1-10] Laboratories should address all of the QC guidelines discussed herein, but the depth and details may differ for each laboratory. Many items mentioned here are also applicable to other laboratories such as chemical and radiological laboratories. For those microbiology laboratories testing under Good Manufacturing Practices (GMP)/Good Laboratory Practices (GLP) regulations, certain QC practices will be different from those listed here.

1. Personnel

Microbiological testing should be performed by a professional microbiologist or technician with an appropriate level of education, training, and experience in general microbiological techniques. If not, a professional microbiologist should provide close supervision to guide and train the analyst in basic microbiological laboratory procedures to perform their assigned functions. The supervisor routinely should evaluate and document the technician's skills. Sample collection (if performed by the laboratory), sample handling, media and glassware preparation, sterilization, clean room gowning and access requirements, aseptic techniques, routine analytical testing, counting, data handling, and QC techniques to identify and eliminate problems should be closely monitored. Management should assist laboratory personnel in obtaining additional training and course work to enhance their technical skills and advance their careers. An employee training record and performance score obtained by analyzing single-blind samples should be maintained. Initial demonstration of capability prior to generating data, and an ongoing demonstration of capability for each analytical method conducted should be recorded.

2. Biosafety Criteria

Biosafety is of concern to all microbiological laboratories to protect laboratory personnel and others who may be potentially exposed. There are three elements to be considered: laboratory practices, safety equipment, and facility design. The risk assessment of the work to be done with each specific biological agent will determine the appropriate combination of these elements necessary to the individual laboratory.

The Centers for Disease Control (CDC) and Prevention, U.S. Public Health Service, classifies laboratories handling potential hazardous biological agents into four biosafety levels. The four biosafety levels (BSLs 1, 2, 3, and 4) consist of a combination of laboratory practices and techniques, safety equipment, and laboratory facilities. Each combination is specifically appropriate for the operations performed, the suspected routes of transmission of the infectious agents, and the laboratory function or activity.

The following is a brief discussion of the four different biosafety levels. Indigenous, dangerous, or exotic agents that may cause serious or potentially lethal disease are not described in *Standard Methods*; therefore, detailed information

TABLE 9020:I. KEY QUALITY CONTROL PRACTICES

Item	Action	Frequency	Further Information in Section 9020B, ¶
Reagent water	Monitor quality	See Table 9020:II	
Air in workplace	Monitor bacterial density	Monthly	3*e*
Temperature devices:			4*a*
Working units	Check accuracy	Annually	
Reference units	Recertify	Every 5 years	
Balances	Check zero	Each use	4*b*
	Check accuracy	Monthly/each use preferably	
	Service and recalibrate	Annually	
Weights:			4*b*
Working	Check with reference weights	Annually	
Reference	Recertify	Every 5 years	
pH meter	Standardize	Each use	4*c*
	Determine slope	Monthly	
Media-dispensing apparatus	Check volume dispense accuracy	Each use	4*f*
Hot-air sterilizing oven	Check performance	Monthly	4*g*
Autoclave	Check temperature with max-registering thermometer	Weekly	4*h*
	Check performance with bioindicator	Monthly	
Timer:			
Autoclave	Check timing with stopwatch	Quarterly	4*h*
Stopwatch	Check against National Time Signal	Annually	
Refrigerator	Check temperature	Daily	4*i*
Freezer	Check temperature	Daily	4*j*
	Defrost	Annually	
Membrane filtration equipment	Check for leaks and surface scratches	Each use	4*k*
	Check sterility	Pre- and post-test	
	100-mL volume check	Initially	
UV lamps (shortwave)	Test with UV meter or perform plate count check	Quarterly	4*l*
Biosafety cabinet	Inspect for airflow	Each use	4*m*
	Have certified	Annually	
Incubator	Check temperature	Twice daily	4*n* and *o*
Microscope	Clean optics and stage, check alignment	Each use	4*p*
Conductivity meter	Calibrate	Monthly	4*q*
Micropipettors	Check dispense accuracy and precision	Quarterly or more frequently if heavily used	4*s*
	Calibrate	Annually	
Glassware	Inspect for cleanliness, chips, and etching	Each use	5*a*
	Check pH with bromthymol blue	Each wash batch	
	Conduct inhibitory residue test	Initial use and new washing procedure (also may be annual)	
	Check for autofluorescence if used for testing	Each batch or lot	
Dilution water bottles	Check sterility, pH, and volume	Each batch or lot	5*c* and 9050C.1*a*
Sample bottles	Check sterility	Each batch or lot	5*d*
	Check dechlorination agent efficacy	Each batch or lot	
	Check 100 mL line	Each lot	
	Check for autofluorescence if also used for testing	Each lot	
Multi-well sealer	Check performance	Monthly	5*e*
Membrane filters	Check sterility and properties	Each new lot	5*i*
Media	Check sterility, pH and appearance	Each batch or lot	5*j*
	Check recovery of new vs. old media	Before first use	
	Check performance with + and − culture controls	Each batch or lot	
Plate counts	Perform duplicate analyses	Monthly	9*a*
	Repeat counts	Monthly	

on special practices, containment, and facilities for BSLs 3 and 4 are not included here. For further information on all BSLs, review CDC's protocols.[11]

a. Biosafety level 1 (BSL 1): As noted by CDC, BSL 1 is suitable for work involving well-characterized agents not known to consistently cause disease in healthy adults and of minimal potential hazard to laboratory personnel and the environment. Work is generally conducted on open bench tops using standard microbiological practices. The agents listed in *Standard Methods* that should be handled under BSL 1 practices are total and

thermotolerant (fecal) coliform bacteria, *E. coli*, enterococci, iron and sulfur bacteria, actinomycetes, and other nonpathogenic microorganisms. It is under the discretion of the laboratory director what biosafety practices are to be adhered to depending on the practices involved. The standard practices and safety equipment for this level are as follows:

1) Access to the laboratory is limited or restricted at the discretion of the laboratory director by posting a sign, e.g., "Restricted Area—Biohazards Laboratory Personnel Only" when experiments or work with samples are in progress. Ensure that doorways and windows are closed when aseptic work is progressing.

2) Personnel wash hands thoroughly with soap and water after they handle viable materials, after removing gloves, and before leaving the laboratory.

3) Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human use are not permitted in work areas.

4) Mouth pipetting is prohibited.

5) Policies for the safe handling of sharp items are instituted.

6) Work surfaces are decontaminated before and after each use and after any spill of viable material.

7) All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method, such as autoclaving, and that information is recorded.

8) An insect and rodent control program is in effect.

It is recommended that laboratory coats, gown, or uniforms be worn to prevent contamination or soiling of street clothes. Gloves should be worn if skin on the hands is broken or if a rash is present. All procedures should be performed so that no aerosols or splashes occur.

b. Biosafety level 2 (BSL 2): BSL 2 builds upon BSL 1 practices and involves work with agents of moderate potential hazard to personnel and the environment. The agents listed in *Standard Methods* that require BSL 2 practices are the pathogenic microorganisms described in Sections 9260, 9510, 9610, and 9711. This level differs from BSL 1 in that: laboratory personnel have specific training in handling pathogenic agents; access to the laboratory is limited when work is in progress; extreme precautions are taken with contaminated sharp items; and certain procedures in which infectious aerosols may be created are conducted in biological safety cabinets (BSC). Appropriate immunizations should be given if available.

The standard practices for this level include all those listed for BSL 1 and additional special practices, including the following:

1) A high degree of precaution is always taken with any contaminated sharp items, including needles and syringes, slides, pipets, capillary tubes, and scalpels.

2) Work surfaces are decontaminated on completion of work or at the end of the day and after any spill or splash of viable material, by using disinfectants that are effective against the agents of concern.

3) Cultures or potentially infectious wastes are placed in a container labeled "Biohazardous Waste" with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.

4) Biological safety cabinets, preferably Class II, or other appropriate personal protective equipment. are used whenever procedures with a potential for creating infectious aerosols or splashes are conducted and high concentrations or large volumes of infectious agents are used.

5) Face protection is used for anticipated splashes or sprays of infectious materials to the face whenever the microorganism must be manipulated outside the BSC.

6) Protective laboratory coats, gowns, or uniforms, and safety glasses designated for laboratory use are worn while in the laboratory and removed and left in the laboratory before leaving for nonlaboratory areas.

7) Gloves are worn when hands may contact potentially infectious materials, contaminated surfaces, or equipment.

c. Biosafety levels 3 and 4: BSLs 3 and 4 involve working with indigenous, dangerous, or exotic agents that may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Because agents in these categories are not described in *Standard Methods*, special practices, containment, and facilities for these levels are described only briefly here.

Personnel must be trained in handling infectious materials. All preceding safety practices must be followed. Access must be limited and areas secured. Work must be conducted within biological safety cabinets by personnel wearing appropriate protective clothing and devices. No one with open lesions should enter the laboratory. A passage area where personnel can change into protective clothing should be available between the entrances from the outside hallway and the inner laboratory. Prevent both doors from opening at the same time. All potentially contaminated material such as gloves, laboratory coats, etc., must be decontaminated before disposal or reuse.

BSL 4, as noted above for BSL 3, involves biological agents, often exotic, that are extremely hazardous both to personnel and/or the environment. All preceding safety practices must be followed. Access to the laboratory must be strictly controlled and situated in an area clearly marked and removed from normal operations or in a separate building. Personnel must completely disrobe and put on laboratory clothing prior to entering the test areas and must be decontaminated before leaving.

3. Facilities

Develop an environmental control policy to ensure that environmental conditions do not invalidate results, affect the required quality of the measurements, nor adversely affect personnel.[12] Factors to be considered and monitoring to be performed are described below. Much of this information applies to any laboratory facility.

a. Ventilation: Plan well-ventilated laboratories that can be maintained free of dust, drafts, and extreme temperature changes. Install air conditioning and temperature- and humidity-control systems to reduce contamination, permit more stable operation of incubators, and decrease moisture problems with media and instrumentation. Adjust air system vents so air flow does not blow directly on the working surface areas. Where feasible, air flow should be negative into the laboratory (so airflow is always into, rather than out of, the laboratory) to avoid risk of contamination of the exterior.

b. Space utilization: To ensure test and sample integrity and minimize potential contamination, design and operate the laboratory to minimize through traffic and visitors. Do not obstruct access or egress points.

Ensure that there is sufficient work space available for the volume of work to be performed. For example, maintain separate work areas for sample receipt; preparation and sterilization; decontamination of media, glassware, and equipment; testing and culturing; and data handling and storage. Maintain heat-generating equipment, such as autoclaves, in a room separate from incubators. Use of a hood or biological safety cabinet for dispensing and preparing sterile media, transferring microbial cultures, or working with pathogenic materials is recommended. In smaller laboratories it may be necessary, although undesirable, to carry out these activities in the same room. However, do not perform these activities near open doorways or open windows. Have sufficient storage space available within the laboratory so materials can be stored appropriately.

c. Laboratory bench areas: Provide at least 2 m of linear bench space per analyst and additional areas for preparation and support activities. Bench height should be reasonable and comfortable for the technicians. For stand-up work, typical bench dimensions may range from 90 to 97 cm high and 70 to 76 cm deep and for sit-down activities, such as microscopy and plate counting, benches may range from 75 to 80 cm high. Specify bench tops of stainless steel, epoxy plastic, or other smooth, impervious surfaces that are inert and corrosion-resistant with a minimum number of seams and free of cracks and crevices. Install even, glare-free lighting with about 1000 lux (100 ft-c) intensity at the working surface. Test using a photometer.

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. Specify ceiling surfaces that are smooth, nonfibrous, and with recessed lights.

e. Work area: Maintain high standards of cleanliness in work areas. Disinfect surfaces before and after testing. Institute a regular preventive maintenance policy for work areas and equipment, such as incubators and refrigerators. Sterilize contaminated supplies and media promptly after use. Avoid buildup of water in pan underneath refrigerator and clean all vent filters.

Develop an environmental monitoring program to monitor air quality routinely, at least monthly or more frequently if area is heavily used or biocontamination risk analysis indicates the need for more frequent monitoring. Use air density settling plates where aseptic work is conducted. This is a passive sampling process wherein particles can settle on the agar surface. Use active air samplers if risk assessment indicates possible aerosol conditions.[4] RODAC (replicate organism detection and counting) contact plates or the swab method[1] can be used weekly or more frequently to monitor bench surface contamination.

Average results obtained from tests over a period of time to set normal limits, i.e., set a baseline for that location. Although uniform limits for bacterial density have not been set, each laboratory can use these tests to establish a baseline for specific work areas, evaluate trends, establish alert and action levels, and take appropriate action when necessary. The number of colonies on the air density plate should not exceed 160/cm^2/15 min exposure (15 colonies/plate/15 min). In addition to this surveillance system, the laboratory may wish to identify contaminants recovered with commercially available automated identification systems.

Prevent any adverse sound and vibration levels within the laboratory. Install easy-to-clean sun shades on large glass windows to prevent heat buildup.

f. Laboratory cleanliness: Regularly clean laboratory rooms and wash benches, shelves, floors, windows, overhead lights, and exposed pipe surfaces. Wet-mop floors and treat with a disinfectant solution weekly; do not sweep or dry-mop. Wipe bench tops and treat with a disinfectant at least daily, or more frequently depending on the biosafety level required for the work being done (see 9020B.2). Do not permit laboratory to become cluttered. Store supplies and paperwork away from bench tops. Eliminate or cover any overhead pipes that cannot be cleaned routinely. Have liquid hand soap in a gravity-fed dispenser and paper towels available at laboratory sinks. Do not allow smoking or consumption of food or drink in the laboratory.

g. Electricity: Ensure a stable electric source, a sufficient number of outlets, circuit breaker (GFCI) protected where needed, and the placement of surge protectors. An emergency power backup and alarm system may be necessary where the work is critical.

4. Laboratory Equipment and Instrumentation

Have procedures in place to verify that each identified item of equipment is installed properly and is operating in a consistent and satisfactory manner.[13] Verify by constant monitoring, routine maintenance, and a regular calibration schedule that each piece of equipment or instrument meets the user's needs for precision and minimization of bias. Provide written procedures on the use, operation, calibration, and maintenance of relevant equipment and instruments (see 9020B.6) and keep manufacturers' manuals available for easy retrieval. Perform equipment calibration using reference standards and equipment maintenance on a regular basis as recommended by the manufacturer or obtain preventive maintenance contracts on autoclaves, balances, microscopes, and other critical equipment. Directly record all quality control checks in permanent log books and maintain documentation. Develop a system for "flagging" problems and actions needed for correction.

Ensure that the laboratory has all equipment and supplies required for the performance of environmental tests and calibration. Have available sufficient equipment and supplies where needed so they are not routinely moved from one laboratory area to another. Where equipment is available only off-site, document how the laboratory will ensure that the quality will be satisfactory. For molecular testing, the laboratory's equipment and supplies need to be dedicated to specific rooms.[9] Maintain all documentation showing determination of acceptability for equipment, instruments, and supplies, as well as all analytical analyses. Keep the records in a permanent record format, such as a bound notebook, e-notebook, or computer file.

Use the following quality control procedures for the basic applied as well as the research laboratory (equipment needed for specialized testing may not be listed here):

a. Temperature-sensing and recording devices: Annually or, preferably, semiannually check accuracy of all working temperature-sensing devices, such as liquid-in-glass thermometers, thermocouples, and temperature-recording instruments at the use temperature against a certified National Institute of Standards and Technology (NIST) thermometer or one traceable to NIST

and conforming to NIST specifications. Record calibration results, along with the date and the technician's signature, in a quality control logbook. Mark the necessary calibration correction factor on each temperature measuring device so that only calibrated-corrected temperature values are recorded. Verify accuracy of the reference certified thermometer as specified on the certificate of calibration or at least every 5 years. Some accreditation organizations or federal or state agencies may require more frequent calibration.

For general purposes use thermometers graduated in increments of 0.5°C or less. Maintain bulb in water or glycerol for air incubators and refrigerators. For example, for a 44.5 ± 0.2°C water bath, use a total immersion thermometer, e.g., short range and length, graduated to 0.1°C. For air convection incubators, use thermometers, e.g., short range and length, with bulbs immersed in glycerol sealed in a flask or test tube having equivalent volume to containers being used in incubators. Record the calibration-corrected temperature reading in a quality control log. Where possible, equip incubators and water baths with temperature-recording instruments that provide a continuous record of operating temperature.

Abstain, where possible, from using mercury-filled thermometers to avoid potential release of mercury into the environment when the thermometer is broken.

b. Balances: Locate balances in areas without rapid air movement and level balances on firm, even surfaces to prevent vibrations. Relevel balance each time it is moved to a new location. Follow manufacturer's instructions for operation and routine maintenance of analytical and top-loading balances. Service balances annually or more often as conditions change or problems occur.

Before each use wipe balance with a soft brush and make sure it is at zero weight when empty. If it is necessary to zero display, press tare button. Use weigh paper or boats and tare weight before adding reagents. Place item to be weighed on the pan and read the weight after the stability indicator symbol (if available) appears in display. Clean balance pans after use and wipe spills up immediately with a laboratory tissue. Replace weights if corroded or dropped. Use only a plastic-tip forceps to handle weights. Check balance routinely, preferably daily before use, with at least two working weights that bracket the normal usage range. Check working weights monthly against a set of reference weights of known tolerance[14] (e.g., ANSI/ASTM Class 1 or NIST Class S accompanied by appropriate certificate) for accuracy, precision, and linearity. Record results along with date and technician's initials. Recertify reference weights as specified in the certificate of calibration or at least every 5 years.[15,16]

Note that some regulatory agencies or accreditation organizations may require more frequent recertification of reference weights.

c. pH meter: Use a meter, graduated in 0.1 pH units or less, that includes temperature compensation, because the electrode pH response is temperature-dependent. Use digital meters, commercial buffer solutions, and electrodes suitable for a wide temperature range. A flat-head electrode can be used to measure solid agar media. Calibrate pH meter with at least two certified pH buffers that bracket the pH of sample being measured. The most desired temperature range for determining pH is 25° ± 5°C. Take pH measurement of test solution close to the temperature used to calibrate the meter.

Record calibration results, date, and technician's initials. Date buffer solutions on bottle and in logbook when opened and check monthly against another pH meter, if possible. Immediately after use, discard buffer solutions or single-use/ready-to-use pH solution packets used to calibrate meter. After 1 d, discard all buffer solutions made from packets. Replace pH buffer supply containers by the expiration date. Store electrode immersed in solution recommended by manufacturer. Do not allow electrode to dry out.

Measure and record pH meter slope after calibration at least once a month, and preferably after each use, to see if meter is malfunctioning. If the pH meter does not have a feature that automatically calculates the slope, but can provide the pH in millivolts (mV), use the following formula to calculate the slope: Slope, as % = (mV at pH 7 − mV at pH 4) × 100/177. If the slope is below 95% or above 105%, the electrode or meter may need maintenance.

For full details of pH meter use and maintenance, see Section 4500-H^+or follow manufacturer's instructions.

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 reagent-grade water. 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 that automatically regenerate the ion exchange resins are now available. Do not store reagent water unless a commercial UV irradiation device is installed and is confirmed to maintain sterility.

Monitor reagent water continuously or every day of use with a calibrated conductivity meter and analyze at least annually for trace metals. Monthly determination of heterotrophic bacteria may indicate potential problems before other test parameters. Increasing numbers of bacteria in the system can affect bacterial tests as they represent nutrient sources for bacteria being isolated. The water quality test should be performed annually and when there is a repair or change in water supply system. This bacteriological quality test is not needed for Type II water or better as defined in *Standard Methods* (18th and 19th Editions), Section 1080C, or medium-quality water or better, as defined in *Standard Methods* (20th, 21st, and Online Editions), Section 1080C, or as defined by other widely accepted standards.[17] Most systems used today meet or exceed these standards.

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, and a UV irradiation device is not available, include aseptic final filtration with a 0.2-μm-pore membrane filter and collect in a sterile container. Monitor treated water for contamination and replace 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 combined chlorine and ammonia (NH_3). On storage, additional NH_3 and CO_2 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. Mechanical media dispensing apparatus: Check volume by dispensing into a graduated cylinder at start of each volume change and periodically throughout extended runs; record results. Flush with a small volume of medium before dispensing and pump hot reagent-grade 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-grade water, and dry. Lubricate parts according to manufacturer's instructions or at least once per month.

g. Hot-air sterilizing oven: Test performance monthly with commercially available biological spore strips of a spore-forming microorganism such as *Bacillus atrophaeus,* preferably having a minimum spore density of 1×10^6 and placed in glassware similar to items being sterilized. Use a thermometer, bulb placed in sand, accurate in the 160 to 180°C range to measure temperature, or a thermocouple-type probe, or a continuous-read temperature recorder. Record results and contents when in use. Use heat-indicating tape to identify supplies and materials that have been exposed to sterilization temperatures.

h. Autoclave: Record items sterilized and sterilization temperature along with total run-time (exposure to heat), actual time period at sterilization temperature, set and actual pressure readings, and initials of responsible person for each run cycle.[18] New units may print out most of this information on tape automatically. For older units use of a recording thermometer chart is highly recommended.

For new autoclaves an initial temperature profile can be conducted to determine differences in the various locations within the autoclave. For routine use, verify the autoclave temperature weekly by using a maximum registering thermometer (MRT) to confirm that 121°C has been reached.

Test monthly for sterilization efficacy, using normal sterilization time and temperature for media, with a biological such as commercially available *Geobacillus stearothermophilus* in spore strips, suspensions, or capsules, preferably at a 1×10^6 concentration and placed into glassware containing a liquid to simulate actual autoclave sterilization performance on media.[19] With changing standards, some biological indicators may require a longer time period at sterilization temperature than is used for most carbohydrate media. If a problem is noted, use biological indicators for autoclave runs that exceed 20 min, e.g., dilution water and contaminated materials.

The additional use of a chemical steam indicator for each cycle is a practical and quick method to show if minimum exposure conditions were met. Use heat-indicating tape to identify supplies and materials that have been sterilized. Check timing quarterly by using a calibrated timer or by national time signal. Keep autoclave clean and free of debris by checking both trap and seals.

i. Refrigerator: Maintain temperature at 2 to 8°C with thermometer bulb in distilled water or glycerol solution. An initial temperature profile is suggested. Check and record calibration-corrected temperature daily when in use and clean annually or more frequently if needed. Identify and date materials stored. Defrost as required and discard outdated materials monthly. Frost-free units may result in faster dehydration of stored media. Refrigerators and freezers should be explosion-proof if they are used for storing flammable materials.

j. Freezer: Freezer temperature range will be determined by analytical need, e.g., the standard laboratory freezer may range from -10 to -20 ± 5°C to an ultra-cold freezer, which may range from -70 to -90°C. Check and record temperature daily. A recording thermometer and alarm system are highly desirable. Identify and date materials stored. Defrost and clean at least annually (semiannually if needed); discard outdated materials.

k. Membrane filtration equipment: Before initial 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. When volumetric graduation marks are used to measure sample volumes, check accuracy of graduation marks initially using a Class A graduated cylinder or volumetric pipet. Record results. For presterilized single-use funnels check one per lot or use a set percentage, e.g., 1 to 4%, for accuracy of volumetric graduation mark.

l. Ultraviolet lamps: When used, disconnect lamps monthly and clean bulbs with a soft cloth moistened with ethanol, 70% methanol/30% reagent-grade water, or use spectroscopic grade 2-propanol where baked-on material may be collecting. Test lamps quarterly with an appropriate (short-wave) UV light meter* and replace bulbs if output is less than 70% of the original. Alternatively, expose plate count agar spread plates containing 200 to 300 colony forming units (CFU) /mL of a selected bacterial suspension, for 2 min. Incubate plates at 35°C for 48 h and count colonies. Replace bulb if colony 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, used to detect fluorescence), both types of UV light can damage eyes and skin and potentially are carcinogenic.[20] Protect eyes and skin from exposure to UV light. Consider a lockout mechanism so laboratory lights cannot be turned on without turning off overhead UV lights if used. (See Section 1090B.)

m. Biohazard safety cabinet (BSC): Properly maintained Class I and II BSCs, when used in conjunction with good microbiological techniques, provide an effective containment system for safe manipulation of moderate and high-risk microorganisms (Biosafety Level 2 and 3 agents). Both Class I and II BSCs have inward face velocities (75 to 100 linear ft/min) that provide comparable levels of containment to protect laboratory workers and the immediate environment from infectious aerosols generated within the cabinet. Class II BSCs also protect the material itself through high-efficiency particulate air filtration (HEPA filtration) of the air flow down across the work surface (vertical laminar flow). Standard operating procedures are as follows:

1) Before use and after use, purge air for 10 to 15 min and wipe down unit with disinfectant. Ensure inward air flow with a piece of tissue.

2) Enter straight into cabinet and perform work in a slow methodical manner. Place material well within cabinet—not on front grill—and do not disrupt or block laminar air flow. Place discard pan within cabinet.

* 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.

3) Decontaminate interior of BSC after completion of work and before removal. Allow cabinet to run for 10 to 15 min and then shut off.[21]

Provide for testing and certification of Class I and II BSCs *in situ* at the time of installation, at any time the BSC is moved, and at least annually thereafter. Maintain cabinets as directed by the manufacturer.

n. Water bath incubator: Verify that water bath incubators maintain the set temperature, such as 35 ± 0.5°C or 44.5 ± 0.2°C. Use a total immersion thermometer (¶ *a* above) having the appropriate increments required for test incubation temperatures. When incubator is in use, monitor and record calibration-corrected temperature twice daily.

Fill unit only with reagent-quality water. Maintain water level so that it is above the upper level of the medium either in tubes or flasks. For optimum operation, equip water bath with a gable cover to prevent evaporation and with a circulating pump to maintain even temperature distribution. Use only stainless steel, plastic-coated, or other corrosion-proof racks. Use screens or weights to keep materials from floating. Empty and clean bath as needed to prevent buildup of salts and microbial growth and disinfect before refilling.

o. Incubator (air, water-jacketed, or aluminum block): Measure and establish that incubators maintain appropriate and uniform spatial test temperatures. Allow sufficient space between items to permit unobstructed airflow. Do not overload nor stack petri dishes more than four plates high. Verify initially that cold sample test media are incubated at the test temperature for the required time. Note that static air incubators will take longer to reach set incubation temperature. Bring all cold samples in media to room temperature before insertion and use incubators of sufficient size to avoid overfilling incubators with cold samples. During usage periods check and record calibration-corrected temperature twice daily (morning and afternoon, separated by at least 4 h) on the shelves in use, or at least one on the top shelf and one on the bottom shelf, to ensure and record temperature consistency throughout unit. If a glass thermometer is used, submerge bulb and stem in water or glycerin to the immersion 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). Alternatively, use well-insulated walk-in incubator rooms with forced air circulation. Clean and then sanitize incubators routinely.

p. Microscopes: Check Kohler illumination each time the microscope is put to use. Clean optics and stage after each use with lens paper and cover microscope when not in use. Further information is available in Section 9030B.20 and elsewhere.[22]

Permit only trained technicians to use fluorescence microscope and light source. Monitor fluorescence lamp and replace when a significant loss in fluorescence is observed, when manufacturer recommends replacement, or when a rule or laboratory guidance document specifies maximum hour usage, whichever occurs first. Record lamp operation time/usage, efficiency, and alignment. Always realign lamp after bulb has been replaced. Use known positive fluorescence slides as controls.

q. Conductivity meter: Conductivity measurements are temperature dependent and the effect of temperature will vary with different solutions. Therefore, calibrate meter monthly using certified low-level standard at 25°C or determine cell constant using certified low-level standard at 25°C. When solutions must be measured at a different temperature, use a meter with automatic temperature compensation or take temperature of solution, record reading, and then correct reading to 25°C using the formulae in Section 2510B.5*b* (usually 2%/°C).

r. Microwave: Units vary in power and acceptable placement of material; however, microwaves have been used successfully to melt presterilized agar media. Use at minimum time and power setting position. Check unit for quality performance and compare to standardized melting procedures by performing comparison study.

s. Micropipettors:[23,24] Micropipettors are high-precision laboratory instruments for dispensing extremely small volumes. Use with precision tips supplied by manufacturer and securely fix to the nose cone to ensure a tight seal. Maintain consistency in pipetting action, such as pre-wetting, release of plunger, and tip immersion depth (between 1 and 3 mm). Operate only in a vertical position and have both sample and equipment at equivalent temperature. Avoid over-dialing the recommended range of the micropipettor to avoid mechanical damage. Follow manufacturer's instructions to perform routine maintenance, such as cleaning, seal replacement, and re-lubrication, and have each pipet operator check accuracy and precision of volume dispensed at a frequency related to its usage, e.g., quarterly or sooner if pipettor is showing overt signs that it is inaccurate or if tip manufacturer changes. Calibrate at least annually either in-house or send to the manufacturer. Note that pipettor is calibrated with water; changes in liquid viscosity can result in a change in volume dispensed. Maintain documentation.

5. Laboratory Supplies

Retain records and manufacturer certificates of analysis, purity, or tolerance level, if supplied, for all laboratory supplies.

a. Glassware: The term "glassware" refers to both borosilicate glass and heat-resistant plastic materials. Volumetric glassware, pipets, graduated cylinders, and beakers with calibration marks should be accurate to the specified volumetric tolerances. See established standards[25] for calibration of laboratory volumetric apparatus. Volumetric glassware is generally either Class A or Class B (undesignated). Class A is the more precise volumetric glassware. Determine tolerance once per lot or at a set percentage, e.g., 1 to 4%.

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 sample, analyst, and area. Inspect glassware after washing for excessive water beading, stains, and cloudiness and rewash if necessary. Replace glassware with excessive writing if markings cannot be removed. Either cover glassware or store glassware with its bottom up to prevent dust from settling inside it.

Perform the following tests for clean glassware:

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 acceptable neutral range).

To prepare 0.04% bromthymol blue indicator solution, add 16 mL 0.01*N* 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, inhibitory, or stimulatory substances that require 6 to 12 rinses to remove all traces and ensure freedom from residual bacteriostatic action. As long as the bromthymol blue test is being done on each batch of glassware, run this test before initial use of a washing compound and whenever a new washing procedure is used. If the bromthymol blue test is not done consistently also run the toxicity test on an annual basis. Record results. 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), 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 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 *Enterobacter 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.

Follow the heterotrophic plate count method (Section 9215B) for all inoculated plates 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 is present and plasticware must not be used for microbiological analyses. A new washing procedure, equipment, or detergent supply may be needed.

b. Utensils and containers for media preparation: Use utensils and containers of borosilicate glass, stainless steel, aluminum, or other corrosion-resistant material (see Section 9030B.8). Do not use copper utensils.

c. Dilution water bottles: Use bottles scribed at 99 mL and made of nonreactive borosilicate glass or plastic with screw caps equipped with inert liners. Clean before use. Bottles prefilled with dilution water available commercially are acceptable. Before use of each batch or lot conduct sterility test, check one per lot or a set percentage, e.g., 1 to 4%, for pH and volume (99 ± 2 mL), and examine dilution water bottles 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. Recheck volume at regular intervals to determine volume loss rate under holding conditions. Discard by expiration date.

d. Sample bottles: Use wide-mouth nonreactive borosilicate glass or plastic bottles with screw caps (which should contain liners) or commercially prepared sterilized plastic bags with ties of sufficient size to collect the needed sample and still have an adequate headspace to allow shaking of the sample in the container. Clean and sterilize bottles before use and, depending upon use, add sufficient dechlorination agent to neutralize residual chlorine (Section 9060A.2). Minimally test for sterility one sample bottle per batch sterilized in the laboratory or one sample bottle per lot purchased as presterilized, or at a set percentage such as 1 to 4%. Document results. Resterilize entire batch or lot if growth occurs. Check and record efficacy of dechlorination agent, one per batch or lot. Also, check accuracy of 100-mL mark (if present) and auto-fluorescence properties (if used for fluorescence testing), one per lot. Record results.

e. Multi-well trays† and sealers: When used for growth studies, check sterility of multi-well trays one per lot by aseptically adding 100 mL of tryptic soy broth or other non-selective medium, seal, and incubate at 35 ± 0.5°C for up to 48 h. No growth indicates sterility. Note that if the wells become very turbid (indicating nonsterile condition), there could be gas production and concomitant blowout between wells.

Evaluate sealing performance of heat sealer unit monthly by adding one to two drops of a food-color dye to 100 mL deionized water sample, run through sealer, and visually check each well for leakage. Perform cleaning and preventive maintenance on sealer annually or more frequently if needed.

Microtiter plates are used in a variety of analytical procedures, e.g., DNA hybridization and immunoassay studies, and may contain >96 wells. The laboratory should examine the tray wells for consistency and run appropriate controls. The laboratory may need to detoxify the plates if their use requires this.

f. Reagent-grade water: Use reagent-grade water for preparation of solutions and media and for final rinsing of glassware. The water must be proven to be free from inhibitory and bactericidal substance. The quality of water obtainable from a water purification system differs with the system used and its maintenance. See 9020B.4*d* and *e*. Recommended limits for reagent water quality for the microbiology laboratory 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.

1) Test for bacteriological quality—This test, also known as the water suitability test, is based on the growth of *Enterobacter aerogenes* in a chemically defined minimal-growth medium. The presence of a toxic agent or a growth-promoting substance will alter the 24-h population by an increase or decrease of 20% or more when compared to a control. Perform the test at least annually, when the source of reagent water is changed, and when an analytical problem occurs. This bacteriological quality test is not needed for Type II water or better, as defined in *Standard Methods* (18th and 19th Editions), Section 1080C, or medium-quality water or better, as defined in *Standard Methods* (20th,

† For example, Quanti-Tray® or Quanti-Tray®/2000, available from IDEXX Laboratories, Inc., Westbrook, ME, 04092, or equivalent.

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

Test	Monitoring Frequency	Maximum Acceptable Limit
Chemical tests:		
Conductivity	Monthly*	<2 μmhos/cm (μmsiemens/cm) at 25°C
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
Total chlorine residual	Monthly or with each use	<0.1 mg/L
Bacteriological tests:		
Heterotrophic plate count (See Section 9215)	Monthly	<500 CFU/mL
Use test [(see 9020B.5*f*2)]	For a new source	Student's $t \leq 2.78$
Water quality test [see 9020B.5*f*1)]‡	Annually	0.8–3.0 ratio

* Monthly, if meter is in-line or has a resistivity indicator light; otherwise with each new batch of reagent water.
† Or more frequently if there is a problem.
‡ This bacteriological quality test is not needed for Type II water or better, as defined in *Standard Methods* (18th and 19th Editions), Section 1080C, or medium-quality water or better, as defined in *Standard Methods* (20th, 21st, and Online Editions), Section 1080C.

21st, and Online Editions), Section 1080C. Test to ensure continued quality of this water to meet these alternative standards.

The test is complex, requires skill and experience, and is not easily done on an infrequent basis. It requires work over 4 d, an ultrapure water from an independent source as a control, high-purity reagents, and extreme cleanliness of culture flasks, petri dishes, test tubes, pipets, and other equipment.

a) Apparatus and material—Use borosilicate glassware for all steps, although presterilized plastic petri dishes may be used in plating steps. Rinse it in water freshly redistilled from a glass still and then sterilize it with dry heat; steam sterilization will recontaminate these specially cleaned items. Test sensitivity and reproducibility depend in part on cleanliness of sample containers, flask, tubes, and pipets. It often is convenient to set aside new glassware for exclusive use in this test. Use any strain of coliform with IMViC type − − + + (*E. aerogenes*) obtained from an ambient water or wastewater sample or reference culture.

b) Reagents—Use only reagents and chemicals of ACS grade. Test sensitivity is controlled in part by the reagent purity. Prepare reagents in water freshly redistilled from a glass still as follows:

• *Sodium citrate solution:* Dissolve 0.29 g sodium citrate, $Na_3C_6H_6O_7 \cdot 2H_2O$, in 500 mL water.

• *Ammonium sulfate solution:* Dissolve 0.26 g $(NH_4)_2SO_4$ in 500 mL water.

• *Salt-mixture solution:* Dissolve 0.26 g magnesium sulfate, $MgSO_4 \cdot 7H_2O$; 0.17 g calcium chloride, $CaCl_2 \cdot 2H_2O$; 0.23 g ferrous sulfate, $FeSO_4 \cdot 7H_2O$; and 2.50 g sodium chloride, NaCl, in 500 mL water.

Phosphate buffer solution/dilution water: Dilute stock phosphate buffer solution (Section 9050C.1*a*) 1:25 in water.

Boil all reagent solutions 1 to 2 min to kill vegetative cells. Store solutions in sterilized glass-stoppered bottles in the dark at 5°C for up to several months provided that they are tested for sterility before each use. Because the salt-mixture solution will develop a slight turbidity within 3 to 5 d as the ferrous salt converts to the ferric state, prepare the salt-mixture solution without $FeSO_4$ for long-term storage. To use the mixture, add an appropriate amount of freshly prepared and freshly boiled iron salt. Discard solutions with a heavy turbidity and prepare a new solution. Discard if solution becomes turbid.

c) Samples—To prepare test samples collect 150 to 200 mL laboratory reagent water and control (redistilled) water in sterile borosilicate glass flasks and boil for 1 to 2 min. Avoid longer boiling to prevent chemical changes.

d) Procedure—Label five flasks or tubes, A, B, C, D, and E. Add water samples, media reagents, and redistilled water to each flask as indicated in Table 9020:III. Add a suspension of *E. aerogenes* (IMViC type − − + +) of such density that each flask will contain 30 to 80 cells/mL, prepared as directed below. Cell densities below this range result in inconsistent ratios while densities above 100 cells/mL result in decreased sensitivity to nutrients in the test water.

e) Preparation of bacterial suspension—On the day before making the distilled-water suitability test, inoculate a strain of *E. aerogenes* onto a nutrient agar slant with a slope approximately 6.3 cm long contained in a 125- × 16-mm screw-cap tube. Streak entire agar surface to develop a continuous-growth film and incubate 18 to 24 h at 35°C.

f) Harvesting of viable cells—Pipet 1 to 2 mL sterile dilution water from a 99-mL water blank onto the 18- to 24-h culture. Emulsify growth on slant by vibrating; then pipet suspension back into original 99-mL water blank.

g) Dilution of bacterial suspension—Make a 1:100 dilution of original bottle into a second water blank, a further 1:100 dilution of second bottle into a third water blank, and a 1:10 dilution of a third bottle into a fourth water blank, shaking vigorously after each transfer. Pipet 1.0 mL of the fourth dilution ($1:10^5$) into each of Flasks A, B, C, D, and E. This procedure should produce a final dilution of the organisms to a range of 30 to 80 viable cells per milliliter of test solution.

h) Verification of bacterial density—Variations among strains of the same organism, different organisms, media, and surface area of agar slopes possibly will necessitate adjustment of the dilution procedure to arrive at a specific density range between 30 to 80 viable cells. To establish the growth range numerically for a specific organism and medium, make a series of plate counts from the third dilution to determine bacterial density. Choose proper volume from this third dilution, which when diluted by the 30 mL in Flasks A, B, C, D, and E, will contain 30 to 80 viable cells/mL. If the procedures are standardized as to slant surface area and laboratory technique, it is possible to

Table 9020:III. Reagent Additions for Water Quality Test

	Control Test *mL*		Optional Tests *mL*		
Media Reagents	Control A	Test Water B	Carbon/Nitrogen Available C	Nitrogen Source D	Carbon Source E
Sodium citrate solution	2.5	2.5	—	2.5	—
Ammonium sulfate solution	2.5	2.5	—	—	2.5
Salt-mixture solution	2.5	2.5	2.5	2.5	2.5
Phosphate buffer (7.3 ± 0.1)	1.5	1.5	1.5	1.5	1.5
Unknown water	—	21.0	21.0	21.0	21.0
Redistilled water	21.0	—	5.0	2.5	2.5
Total volume	30.0	30.0	30.0	30.0	30.0

reproduce results on repeated experiments with the same strain of microorganism.

i) Procedural difficulties—Problems in this method may be due to: storage of test water sample in soft-glass containers or in glass containers without liners for metal caps; use of chemicals in reagent preparation not of analytical-reagent grade or not of recent manufacture; contamination of reagent by distilled water with a bacterial background (to avoid this, make a heterotrophic plate count on all media and reagents before starting the suitability test, as a check on stock solution contamination); failure to obtain bacterial density or incorrect choice of dilution used to obtain 24-h plate count; delay in pouring plates; and prolongation of incubation time beyond 26-h limit, resulting in desensitized growth response.

j) Calculation—For growth-inhibiting substances:

$$\text{Ratio} = \frac{\text{colony count/mL, Flask B}}{\text{colony count/mL, Flask A}}$$

A ratio of 0.8 to 1.2 (inclusive) shows no toxic substances; a ratio of less than 0.8 shows growth-inhibiting substances in the water sample. For nitrogen and carbon sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/mL, Flask C}}{\text{colony count/mL, Flask A}}$$

For nitrogen sources that promote growth:

$$\text{Ratio} = \frac{\text{colony count/mL, Flask D}}{\text{colony count/mL, Flask A}}$$

For carbon sources that promote bacterial growth:

$$\text{Ratio} = \frac{\text{colony count/mL, Flask E}}{\text{colony count/mL, Flask A}}$$

Do not calculate the last three ratios when the first ratio indicates a toxic reaction. For these ratios a value above 1.2 indicates an available source for bacterial growth.

k) Interpretation of results—The colony count from Flask A after 20 to 24 h at 35°C will depend on number of organisms initially planted in Flask A and strain of *E. aerogenes* used. For this reason, run the control, Flask A, for each individual series of tests. However, for a given strain of *E. aerogenes* under identical environmental conditions, the terminal count should be reasonably constant when the initial plant is the same. The difference in initial plant of 30 to 80 will be about threefold larger for the 80 organisms initially inoculated in Flask A, provided that the growth rate remains constant. Thus, it is essential that initial colony counts on Flasks A and B be approximately equal.

When the ratio exceeds 1.2, assume that growth-stimulating substances are present. However, this procedure is extremely sensitive and ratios up to 3.0 have little significance in actual practice. Therefore, if the ratio is between 1.2 and 3.0, do not make Tests C, D, and E, except in special circumstances.

Usually Flask C will be very low and Flasks D and E will have a ratio of less than 1.2 when the ratio of Flask B to Flask A is between 0.8 and 1.2. Limiting growth factors in Flask A are nitrogen and organic carbon. An extremely large amount of ammonia nitrogen with no organic carbon could increase the ratio in Flask D above 1.2, or absence of nitrogen with high carbon concentration could give ratios above 1.2 in Flask E, with a B:A ratio between 0.8 and 1.2.

A ratio below 0.8 suggests that the water contains toxic substances, and this ratio includes all allowable tolerances. As indicated in the preceding paragraph, the ratio could go as high as 3.0 from 1.2 without any undesirable consequences.

Specific corrective measures cannot be recommended for every instance of defective distillation apparatus. However, make a careful inspection of the distillation equipment and review production and handling of distilled water to help locate and correct the cause of difficulty.

Feedwater to a still often is passed through a deionizing column and a carbon filter. If these columns are well maintained, most inorganic and organic contaminants will be removed. If maintenance is poor, input water may be degraded to a quality lower than that of raw tap water.

The best distillation system is made of quartz or high-silica-content borosilicate glass with special thermal endurance. Tin-lined stills are not recommended. For connecting plumbing, use stainless steel, borosilicate glass, or special plastic pipes made of polyvinyl chloride (PVC). Protect storage reservoirs from dust.

l) Test sensitivity—Taking copper as one relative measurement of distilled water toxicity, maximum test sensitivity is 0.05 mg Cu/L in a distilled water sample.

2) Use test for evaluation of reagent water, media, and membranes—When a new source of reagent-grade water or a new lot

of culture medium or membrane filters is used, checking product equivalency by testing the current lot in use (reference lot) against the test lot using reference culture is recommended. It is not possible always to conduct the use test on new reagent-grade water sources, because the previous system may no longer be available.

a) Procedure—Use a single batch of control water (redistilled or distilled water polished by deionization), glassware, membrane filters, or other needed materials to control all variables except the one factor under study. Perform replicate pour or spread plate or membrane filter plate tests on reference lot and test lot, according to procedures in Sections 9215 and 9222. At a minimum, make single analyses on five different water samples positive for the target organism or culture controls of known density. 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 known high-quality water as 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.).

b) 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:

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

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

c) 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. Software packages are available for use on personal computers for these calculations.

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.

g. Reagents:[26] 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. Maintain any Material Safety Data Sheets (MSDS) provided with reagents or standards and have them available to all personnel.

Date chemicals and reagents when received and when first opened for use. Maintain records for receipt, expiration, and subsequent preparation. During preparation bring all reagents to room temperature, 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, concentration, date prepared, name of preparer, and expiration date if known. Store under proper conditions and discard by expiration date. Include positive and negative control cultures with each series of cultural or biochemical tests.

h. Dyes and stains: In microbiological analyses, organic chemicals are used as selective agents (e.g., brilliant green), indicators (e.g., phenol red), and 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 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. For fluorescent stains, test for positive and negative reactivity each day of use.

i. Membrane filters and pads: The quality and performance of membrane filters vary with the manufacturer, type, brand, and lot as a result of differences in manufacturing methods, materials, quality control, storage conditions, and application.[27]

1) Specifications—Manufacturers of membrane filters and pads for water analyses must meet standard specifications for retention, recovery, extractables, and flow-rate characteristics.[28] 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, if desired.

2) Use test—Each new lot of membrane filters should perform satisfactorily in the use test to ensure that it does not yield low recoveries, poor differentiation, or malformation of colonies due to toxicity, chemical composition, or structural defects. For procedure, see ¶ *f*2) above.

3) Standardized tests—To maintain quality control inspect each lot of membranes before use and during testing to ensure that they are round and pliable. Critically check for brittleness if lot is held for one or more years. Discard lots showing brittleness. Record lot number and date received to maintain record of length of time in laboratory. Confirm sterility by absence of growth when a membrane filter is placed on a pad saturated with tryptone glucose extract broth (or equivalent non-selective broth or agar) and incubated at 35 ± 0.5°C for 24 h or by running a sterility control for each analytical test run.

After sample incubation, colonies should be well-developed with appropriate 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. Reject membrane lot if these criteria are not met and inform manufacturer.

j. Culture media: Because cultural methods depend on properly prepared media, use the best available materials and consistent techniques in media preparation, storage, and application, and prepare the correct medium for the intended 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. For this reason, a use test is recommended to confirm that the new batch of media is equivalent to the older media. It is also the responsibility of the laboratory to ensure that the microbiological media meet growth promotion requirements by running both positive and negative culture controls having an estimated density on both the old media lot and the new media lot. Maintain any MSDS.

Order media in quantities to last no longer than 1 year, preferably no longer than 6 months after opening. Order commercially prepared media in quantities such that it is used by the manufacturer's expiration date. Use media on a first-in, first-out basis. When practical, order media in small quantities, e.g., 0.25-lb or 125-g, rather than 1-lb or 500-g bottles, to keep the supply sealed as long as possible. Record type, amount, and appearance of media received, lot number, expiration date, and dates received and opened in a logbook or computer file; also place date of expiration and date opened on container. Check inventory quarterly for reordering.

Store all media under controlled conditions to ensure quality until expiration date is reached. Store dehydrated media in a tightly closed container in a cool (15 to 25°C), dry, controlled-temperature room or desiccator away from direct sunlight. Discard media that cake, discolor, or show other signs of deterioration. Discard unused media by manufacturer's expiration date. A conservative time limit for unopened bottles is 2 years at room temperature. Use of expired media is not recommended.

Compare growth recovery of newly purchased lots of media against proven lots, using reference cultures, preferably, or recent pure-culture isolates, or natural samples [see ¶ *f*2) above], because lot-to-lot variability may occur.

Use opened bottles of media within 6 months. Dehydrated media are hygroscopic; avoid excessive humidity. Close bottles as tightly as possible, immediately after use. If caking or discoloration of media occurs, discard media. Store opened bottles in desiccator if available.

1) Preparation of media—Prepare media in clean containers that are at least twice the volume of the medium being prepared. Prepare media using reagent-grade water. Measure water volumes and media with graduates or pipets conforming to NIST and APHA standards, respectively. Do not use blow-out pipets. Use TD (to deliver) pipets. 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.

Check and record pH of a portion of each medium after sterilization. This is the actual pH required for adequate growth. Adjustment of pH will seldom be necessary when commercially available media are used. If needed, make minor adjustments to the pH specified in the formulation with filter-sterilized 1*N* NaOH or 1*N* HCl solutions. 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. If medium is known as requiring pH adjustment, adjust pH appropriately prior to sterilization and record final pH. Incorrect pH values may be due to reagent water quality, deterioration of medium, 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.4*d* and *e*). If water is satisfactory, remake medium and check pH; if pH is again incorrect, prepare medium using a different lot or source. Certain specific isolation media prepared with organic or fatty acids will demonstrate marked changes in pH following sterilization.

Table 9020:IV. Time and Temperature for Autoclave Sterilization*

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

* Except for media, times are guidelines.
† Certain media may require different sterilization conditions.

Document preparation activities, such as name of medium, volume produced, format, final pH, date prepared, and name of preparer. 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 occurs, discard the medium.

2) Sterilization—Sterilize media at 121°C maximum for minimum time specified. 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:IV 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 heat to removal from the autoclave. Overheating of media can result in nutrient degradation. Maintain printout records.

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. Use extreme care to avoid boiling over due to superheated liquids. Do not reautoclave media.

Sterilize heat-sensitive solutions or media by filtration through a 0.2-μm-pore-diam filter in a sterile filtration and receiving apparatus. Filter and dispense medium in a laminar-flow hood or laminar-flow safety cabinet if available. Sterilize glassware (pipets, petri dishes, sample bottles) in an autoclave or in a hot-air sterilizing oven (170 ± 10°C for a minimum of 2 h). Sterilize equipment, supplies, and other solid or dry materials that are

TABLE 9020:V. HOLDING TIMES FOR PREPARED MEDIA

Medium	Holding Time
Broth in screw-cap flasks*	96 h
Poured agar in plates with tight-fitting covers*	2 weeks
Agar or broth in loose-cap tubes*	2 weeks
Agar or broth in tightly closed screw-cap tubes†	3 months
Poured agar plates with loose-fitting covers in sealed plastic bags*	2 weeks
Large volume of agar in tightly closed screw-cap flask or bottle*	3 months

* Hold under refrigerated conditions 2–8°C.
† Hold at <30°C.

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 <50°C, preferably 44 to 46°C, until used but not for 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 suitable for use in pour plates. Add heat-sensitive solutions, e.g., antibiotics, to tempered agar. Preparation of media at least 2 d before tests is recommended to allow sufficient time for sterility and positive and negative control culture testing to be performed and read. If agar medium is not poured but allowed to solidify to use later, re-melt agar media in boiling water, flowing steam, or low-wattage microwave, use, and then discard any remainder. Agar may be re-melted only once.

The volume dispensed will change relative to the size of the petri dish and its intended use. Invert plates as soon as poured medium has solidified.

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

4) Storage of media—Prepare media in amounts that will be used within holding time limits given in Table 9020:V. Fresh medium is required to ensure proper isolation of the target microorganisms, especially for bacteria stressed or injured through the disinfection process.

For prepared ready-to-use media with a manufacturer's expiration date greater than that noted in the table, have the manufacturer supply evidence of media quality for that extended period of time. Verify usability weekly by testing recoveries with known densities of culture controls that will also meet QC check requirements.

Control of moisture content is important because recovery and selectivity may be altered with prolonged storage. When media are used for research purposes, establish appropriate media expiration dates and document results. Protect laboratory-prepared and purchased-prepared media containing dyes from light; if color changes occur, discard the media. Refrigerate poured agar plates not used on the day of preparation. To prevent dehydration, seal agar plates in plastic bags or other sealed container if they will be held more than 2 d. Store plates inverted so as to prevent condensation from falling on medium. In cases where condensate has formed, consider placing plates briefly in a 35 to 37°C incubator. For media in test tubes tighten caps before storage. Weigh plates or mark liquid level in several tubes (10% of each batch) after sterilization and monitor for loss of liquid by weight or volume stored for more than 2 weeks. If loss is 10% or more, discard the batch. Discard all petri dishes with solid media that have been stored for longer than 2 weeks; discard them earlier if they are dried out, e.g., wrinkled, cracked, or pitted.

If media are refrigerated, bring to room temperature before use and reject the batch if growth or false positive responses are present. 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 ¶s 5)–7) below.

5) Use test—Subject both laboratory-prepared and purchased media to the use test. For procedure, see ¶ *f*2) above.

6) Quality control of laboratory-prepared media—Maintain in a bound book a complete record of each batch of laboratory-prepared medium with date and name of preparer, name and lot number of medium, amount of medium weighed, volume of medium prepared, sterilization time and temperature, pH adjustments needed, final pH, and preparations of labile components. Compare quantitative recoveries of new lots with previously acceptable ones [¶ 5) above] with the microorganism of concern. Include media sterility checks and positive and negative control culture checks to determine specificity on all media as described below. Culture controls can be used to detect growth promotion and medium selectivity, and to monitor analyst technique.

A good laboratory practice is to periodically challenge prepared media with low numbers of an appropriate microorganism. Growth would be affected by media quality and media preparation, sterilization, storage time, and storage conditions.

7) Quality control of purchased-prepared media—Shipment of ready-to-use media should not invalidate any of the media holding times or conditions described above. The manufacturer should supply validation information if shipment conditions are otherwise. Record dates of receipt and expiration, lot number, and then measure and record medium. Store as directed by manufacturer and discard by expiration date. Comparison of quantitative recoveries, as directed in ¶ 5) above, is recommended. Test each new lot for sterility and with positive and negative control culture checks. For purchased-prepared media which have a longer shelf-life than those prepared in the laboratory, perform these tests more frequently.

6. Standard Operating Procedures (SOPs)[29–31]

Generic and specific SOPs are the operational backbone of an analytical laboratory and are designed to prevent deviations resulting from a misinterpretation of a process or method. Each specific SOP describes in a step-by-step fashion the details of a task or procedure performed on a routine basis, tailored to the laboratory's own equipment, instrumentation, and sample types. These laboratory operations include preparation of reagents, reagent water, standards, culture media, proper use of balances, sterilization practices, dishwashing procedures, and disposal of

contaminated material, as well as methods of sampling, sample analysis, chain of custody, record-keeping, and procedures for quality control. Simple citation of a published analytical method is not a SOP, although that information can be consolidated into the laboratory's own SOP.

SOPs are unique to the laboratory and are written by the person who is doing the work and signed as approved by the supervisor, with the effective date indicated. Follow the SOPs as written and keep them current through routine reviews and accessible to all necessary personnel. When changes are needed, document them and have the SOP re-signed. Retain outdated SOPs in files for possible future reference. Consistent use of SOPs helps to ensure uniform operations. They also provide a solid training tool and a means for determining competency when conducting an assessment.

7. Sampling

The laboratory generally is not involved with actual sample collection but personnel need to be knowledgeable about the different aspects of the sample collection process.[30]

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, sampling depth, number of samples and analyses needed, workload, and supplies. For natural waters, knowledge of the probable microbial densities, and the effects of season, weather, tide and wind patterns, known sources of pollution, and other variables, are needed to formulate the most effective sampling plan. In addition, the microbiologist can indicate when replicate samples will be needed, e.g., when a new water source is being tested or a sample is being collected from a different area of the same locale. For compliance monitoring, the sampling plan must be approved by the state.

b. Methods: Sampling plans must be specific for each sampling site and based on appropriate statistical sampling designs. 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 to ensure sample integrity and representativeness. From the information in these SOPs sampling plans can be drawn up.

c. Sample acceptance: The laboratory must determine whether sample integrity, holding conditions and time, and accompanying documentation are acceptable for the intended use of the resulting data.

8. Analytical Methods

a. Method selection: Media, temperature, time at incubation temperature, and minor variations in techniques are factors that need to be applied consistently for appropriate microbial recovery for qualitative and quantitative determinations. To avoid significant changes in results, microbiological methods must be standardized so uniform data result from multiple laboratories. Select analytical methods appropriate for the sample type from *Standard Methods* or other sources of standardized methods and ensure that methods have been properly validated in a multilaboratory study with the sample types of interest. The laboratory should validate any new method or nonstandard method to be used in the laboratory and any method being used for a matrix not specified by the method.

b. Data objectives: Review available methods and determine which best produce data meeting the program's needs for precision, bias, specificity, selectivity, detection limit, and recovery efficiency under actual test conditions. Methods that are rapid, inexpensive, and less labor-intensive are desirable, but not if verification steps are time-consuming or if the data produced will not meet the program's or customer's needs.

c. Internal QC: Written analytical methods contain the required QC checks to assure data quality, such as the use of positive and negative control cultures, sterility method blanks, replicate analyses (precision), and bacterial cultures having a known density level for quantitative methods.

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

9. Analytical Quality Control Procedures for Established Methods[6-8,18,32]

General quality control procedures:

a. Analyst colony counting variability: For routine performance evaluation, repeat counts on one or more positive samples at least monthly, record results, and compare the counts with those of other analysts testing the same samples. Replicate counts for the same analyst should agree within 5% (within analyst repeatability of counting) and those between analysts should agree within 10% (between analysts reproducibility of counting). If they do not agree, initiate investigation and any necessary corrective action. See 9020B.13*b* for a statistical calculation of data precision.

b. Positive and negative control cultures: Use certified reference cultures. For each lot of medium received, each laboratory prepared batch of medium, and each lot of purchased prepared medium, verify appropriate response by testing with known positive and negative control cultures for the organism(s) under test. Record results. Obtain certified reference cultures from nationally or internationally recognized sources or reference cultures impregnated onto discs or strips from established commercial sources. From reference culture, subculture to develop one or more primary working stocks.[33] Minimize subsequent transfers, i.e., transfer to a fresh medium to promote growth, to ensure that cultures maintain phenotypic and genotypic identity and to reduce potential contamination. Test periodically to ensure viability and performance. For each lot of medium, check analytical procedures by testing with known positive and negative control cultures for the organism(s) under test. Record results. See Table 9020:VI for examples of test cultures. For a wastewater treatment laboratory without the facilities to maintain a pure culture, use single-use culture strips or submit to another laboratory for testing.

c. Duplicate analyses[34,35]: Precision of quantitative analytical results when counting plate colonies is evaluated through replicate analyses. Perform duplicate analyses at least monthly or more frequently as needed, e.g., 10% of samples when required by the analytical method or regulations, one sample per test run,

TABLE 9020:VI. SUGGESTED CONTROL CULTURES FOR MICROBIOLOGICAL TESTS*

Group	Control Culture	
	Positive	Negative
Total coliforms	*Escherichia coli* *Enterobacter aerogenes*‡ *Klebsiella pneumoniae* (ATTC 4352)	*Staphylococcus aureus*† *Proteus vulgaris*§ *Pseudomonas aeruginosa*†
Fecal coliforms	*Escherichia coli* *Klebsiella pneumoniae* (thermotolerant)‖	*Enterobacter aerogenes*
Escherichia coli	*Escherichia coli* (MUG-positive s strain)	*Enterobacter aerogenes* *Klebsiella pneumoniae* (thermotolerant)
Enterococci#	*Enterococcus faecalis* *Enterococcus faecium*	*Staphylococcus aureus*** *Escherichia coli*††

* Use appropriate ATCC strains.
† *S. aureus, P. aeruginosa*—not lactose fermenter.
‡ *E. aerogenes*—ferments lactose, but is not typically thermotolerant.
§ *P. vulgaris*—not lactose fermenter, uses hydrolyzed lactose, indicating "overcooked" medium.
‖ *K. pneumoniae*—ferments lactose, but does not hydrolyze MUG.
Do not use closely related strains from genus *Streptococcus* as a positive control.
** *S. aureus*— sensitive to sodium nalidixic acid medium.
†† *E. coli*—sensitive to sodium azide in medium.

or one sample per week for a laboratory that conducts less than 10 tests/week. A test run is defined as an uninterrupted series of analyses. Evaluate and record results. An adequate sample volume is essential. Balance frequency of replicate analyses against the time, effort, and expense incurred. When the laboratory or analyst is first initiating a method or for a method or matrix in which considerable variability in results is expected, greater effort will need to be expended in performing replicate analyses. Replicate analyses of environmental samples can result in widely different counts and can be considered estimates only.

d. Sterility checks: Test media sterility before first use. Incubate minimally one per lot or a set percentage, e.g., 1 to 4%, of laboratory-prepared and ready-to-use medium, broth, or agar, at an appropriate temperature for the amount of time the test would be performed, e.g., 48 h for coliforms, and observe for growth. For enzyme defined substrate tests, check for sterility by adding media packet to 100 mL sterile deionized water and incubating at 35°C for 18 to 24 h. Certain granulated ready-to-use enzyme-substrate media may not be sterile but only free of coliforms; use of nonselective broth could result in growth and turbidity but should not produce a positive reaction.

Check each new batch (or lot, if commercially prepared) of buffered water for sterility before first use by adding 50 mL of the water to 50 mL of a double-strength broth (e.g., tryptic soy, trypticase soy, or tryptose broth). Alternatively, aseptically pass 100 mL or more dilution water through a membrane filter and place filter on nonselective medium. Incubate at 35 ± 0.5°C for 24 h and observe for growth.

Record results. If any contamination is indicated, discard medium, invalidate any data associated with that batch, and check for contamination source. Request immediate resampling.

For membrane filter tests, check the sterility of the entire process by using sterile reagent or dilution water as the sample at the beginning and end of each filtration series of samples and test for growth. With a processing interruption of more than 30 min use new sterilized funnels and repeat sterility test. Record results. If contamination is indicated, invalidate data associated with that batch and check for source. Request immediate resampling and reanalyze.

For multiple-tube and presence-absence procedures, check sterility of prepared media and dilution water as outlined above. If any contamination is indicated, determine the cause and reject analytical data from samples tested with these materials. Request immediate resampling and reanalyze.

For pour plate procedures check sterility by pouring at least one uninoculated plate per batch or lot of media and record results. If any contamination is indicated, determine the cause. Document both problem and corrective action and request resampling.

Laboratories interested in contaminant identification can use either standardized phenotypic testing systems or genotypic procedures.

e. Precision of quantitative methods[33,34]*:* Calculate precision of replicate analyses for each different type of sample examined, for example, drinking water, ambient water, or wastewater, according to the following procedure and record results:

Perform duplicate analyses on first 15 positive samples of each matrix 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 an approximately equal number of tests. Record duplicate analyses as D_1 and D_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. Calculate the range (R) for each pair of transformed duplicates and the mean ($\bar{R}$) of these ranges. See sample calculation in Table 9020:VII.

Thereafter, analyze 10% of routine samples in duplicate or one per test run. Transform the duplicates and calculate their range as above. If the range is greater than $3.27\bar{R}$, there is greater than 99% probability that the laboratory variability is excessive; in such a case, discard all analytical results since the last precision check (see Table 9020:VIII). Identify and resolve the analytical problem before making further analyses.

TABLE 9020:VII. CALCULATION OF PRECISION CRITERION

Sample No.	Duplicate Analyses		Logarithms of Counts		Range of Logarithms (R_{log})
	D_1	D_2	L_1	L_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:

$$\Sigma \text{ of } R_{log} = 0.0981 + 0.0483 + 0.0627 + \ldots + 0.0669 + 0.0414 = 0.718\ 89$$

$$\bar{R} = \frac{\Sigma R_{log}}{n} = \frac{0.71889}{15} = 0.0479$$

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

Update by periodically repeating the procedures using the most recent sets of 15 duplicate results.

10. Verification

Verification is a general process used to determine whether the microbiological analytical method is performing as expected to provide reliable data. If a laboratory finds a low percentage of verification with a certain water supply or matrix, another test method must be chosen. For the most part, the confirmation/verification procedures for drinking water differ from those for other waters because of specific regulatory requirements. The following is a brief summary; further information may be found in the appropriate discussions of the specific microorganism or microbial group.

a. Multiple-tube fermentation (MTF) methods:

1) Total coliform procedure (Section 9221B)

a) Drinking water—Carry tests through confirmed phase only. The Completed Test is not required.

For QC purposes, if normally there are no positive results within a quarter, analyze at least one positive source water sample to confirm that the media and laboratory procedures and equipment 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 thermotolerant (fecal) coliforms or *E. coli.*

b) Other water types—Verification can be achieved by performing the completed phase at a frequency established by the laboratory, such as 10% of positive samples, or one sample per test run, or a certain percentage depending on normal laboratory work load. For large laboratories analyzing a significant number of samples daily, 10% of positive samples may result in an unnecessary burden and a lower percentage value may be used.

2) Fecal streptococci procedure—Verification can be performed as outlined in Section 9230C.5 at a frequency established by the laboratory. Growth of catalase-negative, gram-positive cocci appearing as brownish-black colonies with brown halos on bile esculin agar at 35°C and in brain-heart infusion broth at 45°C verifies the organisms as fecal streptococci. Growth also in 6.5% NaCl broth and in brain-heart infusion broth at 10°C indicates that the streptococci are members of the *Enterococcus* group.

b. Membrane filter methods:

1) Total coliform procedures

a) Drinking water—Swab entire membrane or pick up five typical and five atypical (nonsheen) colonies from positive samples on M-Endo or LES-Endo agar medium and verify as in Section 9222B.4*f*. Also verify any positives for thermotolerant coliforms. If there are no positive samples, test at least one known positive source water sample quarterly.

b) Other water types—Verify positives monthly by picking at least 10 typical and atypical colonies from a positive water

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

Analyses	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	0.0384	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.

sample as in Section 9222B.4*f*. Adjust counts based on percent verification.

c) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Section 9222B.4*f*.

2) Thermotolerant (fecal) coliform procedure—Verify positives monthly by picking at least 10 blue colonies from one positive sample using lauryl tryptose broth and EC broth as in Section 9221E.1*b*. Adjust counts based on percent verification. To determine false negatives, pick representative atypical colonies of different morphological types and verify as in Presumptive Phase, Section 9221B.2.

3) *Escherichia coli* procedure

a) Drinking water—Verification is not required.

b) Other water types—Verify one positive sample monthly by picking from well-isolated colonies while taking care not to pick up medium, which can cause a false positive response. Perform the citrate test and the indole test as described in Section 9225D.4 and 7, or other equivalent identification procedures or systems. Incubate indole test at 44.5°C. *E. coli* are indole-positive and yield no growth on citrate. Adjust counts according to percentage of verification.

c) To determine false negatives, pick representative atypical colonies of different morphological types and verify as in ¶ b) above.

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

c. Enzyme defined substrate tests:

1) Total coliform test Section 9223

a) Drinking water—Verification is not required.

b) Other water types—No confirmation/verification step is required. Enzyme substrate tests use a defined substrate in which noncoliform bacterial growth is inhibited. The following is a brief description for those who desire to conduct verification testing.

For total coliform analyses aseptically transfer material from a certain percentage (e.g., 5%) of ONPG or CPRG-positive wells and ONPG or CPRG-negative wells to mEndo or Levine EMB or other suitable media. Streak for isolation. Test for lactose fermentation (a number of coliforms can be either slow lactose fermenters or may not ferment lactose at all) or for β-D galactopyranosidase by *o*-nitrophenyl-β-D galactopyranoside (ONPG) test and indophenol cytochrome oxidase (CO) test or organism identification. See Section 9225D for test descriptions or use other equivalent identification procedures or systems.

2) *E. coli*—For *E. coli* analyses verification, if desired, can be accomplished by aseptically transferring material from a certain percentage (e.g., 5%) MUG-positive and MUG-negative wells to MacConkey or Levine EMB or other suitable media. Streak for isolation. Verify by confirmation of MUG reaction using EC+MUG or NA+MUG media or *E. coli* biochemical identification as described in Section 9225D or other equivalent identification procedure or system. Adjust counts according to percentage of verification.

3) Enterococci—Verify colonies by selecting 10 typical colonies (positives) and 10 atypical colonies (negative) once per month or 1 typical and 1 atypical colony from 10% of positive samples, whichever is greater.[36]

11. Validation of New or Nonstandard Methods[37–43]

All nonstandard methods, laboratory-developed methods, and standard methods used under different test conditions, e.g., matrix, must be validated by the laboratory before gathering data with these methods. Validation involves establishing and demonstrating that the performance criteria of a method or process provide accurate and reliable data for its intended use. The term "validation" has been applied historically to the field of chemistry. Validation is now applied to microbiology, using the same terms used in chemistry. The main difference is that, where discrete variables are used, i.e., plate counts, different statistics are applied and different probability distributions are used.

For the culture-based microbiologist, validation focuses on the suitability of the test method or process to detect and/or quantify a specific microorganism or group of microorganisms having set characteristics in the matrix of concern. For the culture-independent methods, such as immunoassays and molecular genetic techniques, the same need exists to demonstrate process control and confidence in the reliability of the information. This is essentially a proof of concept.

For standard compliance methods obtain validation data from the manufacturer and/or the regulatory agency. Before a method is adopted by the laboratory, conduct parallel tests with the standard or reference procedure to determine comparability to the stated performance criteria of the standard and its suitability for use. Obtain at least 30 positive data points over the year to allow a statistical determination of equivalence to the established or standard method before replacement with the new method for routine use. This can be called a secondary or cross validation.

For methods in development, such as research methods, establish confidence in the analytical method or process by conducting full intralaboratory validation studies on a statistically significant number of samples to ensure reliability before final determination of usability. Conduct interlaboratory studies (also called collaborative studies) to validate the method for wider use. The following is a brief discussion of microbial method validation and desired quality performance criteria to be ascertained. Review the cited references for further information and for programs involved with microbial method validation.

To determine the effect of matrix on recoveries add a known concentration set at an anticipated ambient level to a field sample collected from the same site as the original. Use commercial‡ or laboratory-prepared suspensions of the target microorganism.

a. Qualitative test methods: Validation of presence or absence (growth versus no-growth) methods involve establishing method performance characteristics in the matrix of choice, such as:

1) Accuracy and precision (repeatability and reproducibility)—For qualitative tests, the number of replicates would need to be extremely large to reach a statistical evaluation of comparability. Therefore these data quality indicators generally are not determined.

‡ Bioball™, BTF Pty LTD, Australia, or equivalent.

2) Specificity/selectivity—the ability of the test method or process to preferentially select or distinguish the target organisms from the nontarget species in the matrix of choice under normal laboratory sample analysis conditions, i.e., fitness for use. For qualitative methods growth of the target organism is the indicator. It is determined by verifying all responses, e.g., by microbial identification testing.

3) Detection limit—the lowest microbial density that can be determined under the stated conditions. Determine by using dilutions of reference cultures and measurement of recovery among replicates of each dilution.

4) Robustness—the measure of how well a test method can perform under changing conditions. This test is conducted by the initial developer of the method and is determined by changing variables, such as sample holding time or conditions, incubation temperature, medium pH, and incubation time.

5) Repeatability—the degree of agreement between replicate analyses or measurements conducted under the same conditions, e.g., laboratory, technician, and equipment. Use a target microorganism or microbial group density such that at least 75% will be positive, i.e., growth, so a sufficient number of responses can be detected[44] for either a quantitative or qualitative test. This can serve as one measure of uncertainty.

b. Quantitative test methods: Validation of a method or process concerned with numerical determinations, e.g., count per unit volume, involves ascertaining the method's performance characteristics as noted above, in addition to the following:

1) Accuracy—the degree of agreement, or lack of uncertainty, between the observed and the true value. It is estimated by using known reference cultures at the anticipated range of environmental densities and comparing the test method results to that of the reference or standard method. It is usually expressed as the percentage of recovery.

2) Precision/repeatability—the degree of agreement between replicate analyses or measurements conducted under the same conditions, e.g., laboratory, technician, and equipment. Use a target microorganism or microbial group density such that at least 75% will be positive, so a sufficient number of responses can be detected[44] for either a quantitative or qualitative test. This can serve as one measure of uncertainty.

3) Precision/reproducibility—the degree of variability when the same method or process is conducted under changed conditions, e.g., more than one analyst following the method or procedure in another area or room in the laboratory and/or using different equipment. This serves as another measure of uncertainty.

4) Recovery/sensitivity—the capability of a test method to recognize or detect the target microorganism or component thereof in the matrix of choice. Determine by analyzing a sufficient number of samples using at least two added suspension levels of the target microorganism or by increasing or decreasing the sample volume or dilution analyzed, followed then by determination of statistical confidence.

5) Detection limit—the lowest microbial density that can be determined. Determine by using dilutions of reference cultures and measurement of recovery among replicates of each dilution.

6) Upper counting limit—the level at which quantitative measurements become unreliable, e.g., due to overcrowding on an agar plate. Determine as above.

7) Range—the interval between the upper and lower detection limits determined as above.

12. Documentation and Recordkeeping

a. QA Plan: The laboratory's QA Plan or Quality Manual documents management's commitment to a QA policy and sets forth the requirements needed to support program objectives. The plan describes overall policies, organization, objectives, and functional responsibilities for achieving the quality goals and specifies the QC activities required to achieve the data representativeness, completeness, comparability, and compatibility. In addition, the QA plan includes the laboratory's implementation plan to ensure maximum coordination and integration of QC activities within the overall program (sampling, analyses, and data handling) and indicates compliance with federal, state, and local regulations and accreditation requirements where applicable.

b. Sampling records: A written SOP for sample handling records the laboratory's procedures for sample collection, acceptance, transfer, storage, analyses, and disposal. The sampling record is most easily kept in a computer file or 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" and may be required by certain federal or state programs to ensure integrity of the samples. 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.2 and elsewhere.[1] A laboratory system that uniquely identifies samples within the laboratory and that is tied to the field sample number will ensure that samples cannot be confused.

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, sample acceptance, and analyses. Choose a format agreeable to both the laboratory and the customer (the data user). Use preprinted forms if available. 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, as well as the initials of the correction recorder entered next to it, or in a computer file, e.g., an e-notebook.

Keep records of microbiological analyses for at least 5 years in a secure location. Off-site storage is recommended as backup for all records. Data expected to become part of a legal action must be maintained for a longer period of time. 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 and time of sample receipt; condition and temperature of received sample; dates of sample analysis start and completion; 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.

When a laboratory information management system (LIMS) is used, verify the software input and output and arithmetic computations. Back up all laboratory data on disk or hardcopy system to meet the customer and laboratory needs for both data management and reporting. Verify

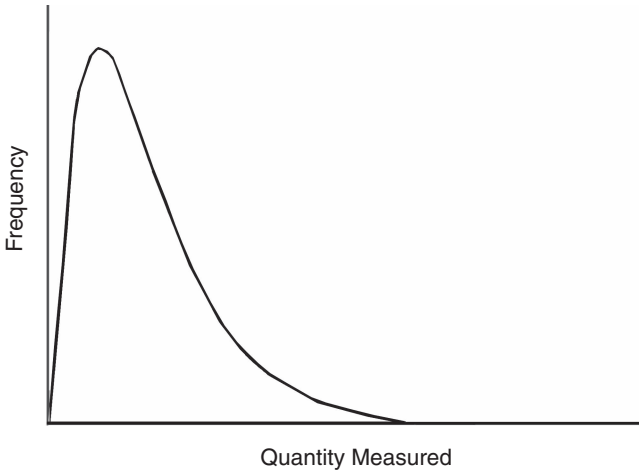

Figure 9020:1. Frequency curve (positively skewed distribution).

TABLE 9020:IX. 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	$\bar{x}_g$ = antilog 2.1825 = 152

data on the printouts. Always back up electronic data by protected tape or disk or hard copy.[44] If the system (hardware or software) is changed, transfer old data to the new system so it remains retrievable within the specified period of time. Data expected to become part of a legal action must be maintained for a longer period of time; check with the laboratory's legal counsel. Further guidance is available.[45-47]

13. Data Handling

a. Distribution of bacterial populations: In most chemical analyses the distribution of analytical results follows a normal (Gaussian) curve, which has symmetrical distribution of values about the mean (see Section 1010B). Microbial distributions are not necessarily symmetrical and rarely fit a normal distribution curve. 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 higher 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. Natural random variation in the distribution of microorganisms within a sample may be unique to the sample and matrix, and not a function of laboratory performance.[48] In addition, the microbial counts obtained represent colony-forming units (CFUs), which may have resulted from one cell or multiples thereof,[49] resulting in variation in colony count numbers in replicate plating or multiple dilutions.

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

b. Central tendency measures of skewed distribution: The best estimate of central tendency of log-normal data is the geometric mean. The term "mean" in geometric mean is misleading. What is being determined is the maximum likelihood estimate, which is based on the mode or maximum frequency of the distribution curve. The probability of observing results by multiple dilutions is expressed by a multiple Poisson distribution.[50,51] The curve generated appears skewed to the right; the characteristic is similar to that of a log-normal distribution curve. This results from the fact that colony counts under the envelope of the log-normal curve result from multiple Poisson distribution curves for numerous other organisms that are not of interest and cause the distribution curve to be further skewed. When the maximum likelihood approach[52,53] is used the maxima of these organisms are spread out under the curve because different organisms respond differently to the nutrients and media. This process is affected by temperature, pH, and time of incubation. The process of analyzing the data for the maximum frequency insures that the correct organism is selected for colony count.

When the MPN curves for 1, 2, 3, and 4 positive tubes out of 5 total tubes incubated are examined, the log-normal probability graph is close to being linear (thus indicating approximate normality) but it bows upward and could indicate possible kurtosis, a sharpness, brought about by measuring the cumulative probability on the low and high ends of the distribution curve. The error is in the extreme values of the tails of the distribution because measurement is difficult at the extreme values of the log-normal distribution curve.

The log-normal probability assumption is confirmed when the log of values is plotted against colony count MPN (maximum probable number) on log-normal−cumulative probability graph paper.

The use of the geometric mean,[54] calculated as the *n*th root of the product of all the data values, is based on the likelihood of a probability distribution. The likelihood estimate is based on both frequency of *n* observations and the count of a random sample on *n* observations.

When the likelihood ratio is observed before and after the log transformation of the variable, *x*, it can be shown that the ratios are the same.[55] By means of the log-likelihood ratio, product properties are converted into summation properties, which are easy to understand and deal with.

The likelihood approach differs from ordinary arithmetic averages in that both frequency and variable colony count are considered, rather than only the arithmetic average of colony counts.

The geometric average is the log of the inverse of the average log of likelihoods of a parameter being measured. This is quite different from the average of MPNs and will generally give a

TABLE 9020:X. COMPARISON OF FREQUENCY OF MPN DATA

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

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

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

lower possible number than an arithmetic average.[56] The geometric average of the maximum likelihood estimates is a better estimate than the arithmetic average for living organisms.

In the derivation of the Maximum Likelihood[56] for a Poisson Probability Distribution, the log of the products of MPN can be shown to be a function of the log of frequency. Thus, geometric averages are justified as the method of obtaining a maximum likelihood estimate of multiple MPN determinations.

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.[57,58]

There are valid reasons for not including "less than" values, whether modified or not. If the database is fairly large with just a few such 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 "less than" values, inclusion of modified forms of such values would exert an undue influence on the final results and could result in an artificial negative or positive bias. Including "less than" 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 such values are first noted, adjust or expand test volumes. The only exception to this caution would be regulatory testing with defined compliance limits, such as the <1/100 mL values reported for drinking water systems where the 100-mL volume is required.

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9020 C. Interlaboratory Quality Control

1. Background

Interlaboratory QC programs are a means of establishing an agreed-upon, common performance criteria system that will ensure an acceptable level of data quality and comparability among laboratories with similar interests and/or needs. A number of different publications[1–4] and organizations* are concerned with interlaboratory programs.

A certification program is one in which an independent authority issues a written assurance or certificate that the laboratory's management is compliant to that authority's standards. An accreditation program is one in which a specialized accreditation body sets standards and a certification body then determines whether the laboratory exhibits competence in following the standards. The laboratory then receives formal recognition. Often the term "accreditation" is used interchangeably with "certification."

Usually, interlaboratory QA programs have three elements: uniform criteria for laboratory operations, external review of the program, and external proficiency testing. These programs assist the laboratories in addressing continual improvement efforts.

2. Uniform Criteria

Interlaboratory quality control programs begin as a voluntary 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 certifying or accrediting organization and an external quality assessment is requested. The type of assessment and the organization performing the assessment will depend on a number of variables, such as the request for accreditation and whether the samples to be tested will be for compliance purposes. An experienced external QA professional or team then arranges an onsite visit to evaluate the QA program for acceptability and to work with the laboratory to solve any problems. Laboratories applying for review will have their laboratory documentation and procedures reviewed. 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 onsite evaluations are repeated and may be announced or unannounced.

4. External Proficiency Testing

Laboratories applying for certification or accreditation are required to participate in routine proficiency testing for those analytical, technological, or matrix-specific procedures the laboratory intends to use. Challenge samples are prepared and sent as unknowns on a set schedule for analyses and reporting of results. The proficiency test samples are to be processed as routine samples by the analyst routinely running the method being reviewed. 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. Failure to successfully evaluate the proficiency test sample can result in loss of recognition.

For those laboratories not applying for certification or accreditation, control samples or proficiency test samples can be purchased.

5. Maintenance

The laboratory needs to undergo an external evaluation and successfully pass a set number of proficiency test samples. Upon successful completion of both, the laboratory will receive formal notification. To maintain this recognition the laboratory must successfully complete annual or semiannual proficiency test samples at a rate set by the authority and to pass an onsite assessment about once every 3 years.

6. 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: Criteria are established for laboratory operations and methodology; onsite 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

* American Association for Laboratory Accreditation, www.a2la.net, and National Cooperation for Laboratory Accreditation, www.nacla.net.

the onsite inspection or performance evaluation and requires corrections within a set period of time. Individual state programs may exceed the federal criteria.

To maintain accreditation by the National Environmental Laboratory Accreditation Conference, acceptable performance on two of the last three Proficiency Tests, as well as successful on-site assessments conducted on a routine basis, are required.

Onsite 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.

7. References

1. Working Group Food of the EA Laboratory Committee & Eurachem. 2002. Accreditation for Microbiological Laboratories, 2nd rev. EA-04/10, European Co-operation for Accreditation.
2. U.S. Environmental Protection Agency. 2002. NELAC Constitution, Bylaws and Standards. EPA/600/R-03/049, U.S. Environmental Protection Agency, Washington, D.C
3. Stein, P. 2004. Why buy accredited? *Qual. Progress* 7:94.
4. U.S. Environmental Protection Agency. 2005. Manual for the Certification of Laboratories Analyzing Drinking Water, 5th ed. EPA-815-R-05-004, U.S. Environmental Protection Agency, Cincinnati, Ohio.