9020 QUALITY ASSURANCE/QUALITY CONTROL*

9020 A. Introduction

1. General Considerations

Due to the emphasis on microorganisms in water quality standards and enforcement activities and their continuing role in research, process control, and compliance monitoring, laboratories need to implement, document, and effectively operate a quality management system (QS) for microbiological analyses. 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 processes and data and ensure compliance with regulatory requirements, customer and project quality objectives and requirements, and applicable standards of accreditation or certification.

The laboratory practices set forth in Section 9020 represent best practices to ensure high-quality data, so use of these procedures is highly recommended for both stand-alone and mobile laboratories. These practices may be required by regulatory agencies (e.g., under the U.S. Safe Drinking Water Act, standard-setting organizations, and laboratory certification or accreditation programs).

Each laboratory develops its own QS suitable for its needs. A laboratory documents its QS'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 the type, quality, and quantity required.

The program should be practical. Staff should spend about 15% of overall laboratory time on the various aspects of an established QA program. That said, more time may be needed for crucial analytical data (e.g., data for enforcement actions). When properly administered, a balanced, conscientiously applied QS will optimize data quality, identify problems early, and increase satisfaction with analytical results without affecting laboratory productivity.

Microbiological analyses are inherently variable because they measure dynamic living organisms. Several of the QC tools available to microbiologists are different from those routinely used by chemists because many of the microbiologists' measurements involve discrete variables rather than continuous ones. Discrete variables have only integer values; continuous variables are not limited to particular values but rather the accuracy of the measuring tool used. Therefore, different statistics and probability distributions are also used to evaluate chemical and microbiological data.

Documented QSs 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.

In 2012, the U.S. Environmental Protection Agency (EPA) published 12 essential QC elements for chemical pollutants analyzed under the Clean Water Act that need to be incorporated when the analytical method lacks QA/QC procedures.[1] AOAC International has developed a document[2] to address, using the EPA terminology, the 10 essential QC elements for microbiology laboratories:

- demonstration of capability (DOC),
- method blanks/sterility checks,
- laboratory QC samples/fortified blanks,
- matrix spike/matrix spike duplicates,
- calibration,
- control charts,
- corrective action,
- QC acceptance criteria,
- batch/test runs, and
- minimum frequency QC checks.

These are discussed throughout 9020 and in Part 9000 (e.g., laboratory QC samples may be considered positive and negative culture controls and matrix spikes are employed during detection of matrix effects on an analytical method and during protozoan testing).

2. Guidelines for a Quality System

The laboratory must develop, document, and implement 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 the need for and actively support the QS, involve staff in QS development and operations, 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, laboratory supervisors, and analysts; and to maintain awareness of conditions through periodic and systematic review of laboratory functions. Management has overall responsibility to the end-user or customer for the QA/QC program and activities performed by laboratory analysts. While management delegates responsibilities to the QA officer, laboratory supervisor, and laboratory analyst so they may effectively carry out their individual job duties, management is ultimately responsible for the QA/QC program.

b. Quality assurance officer/quality manager responsibilities: In large laboratories, a QA officer is responsible for overseeing the QA program. Ideally, this is a staff position reporting directly to upper management, so this person has the authority and operational independence necessary to succeed. The QA officer

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should have coursework, job experience, or specialized training in microbiological testing; be acquainted with all aspects of laboratory work; be aware of and familiar with the laboratory's QA program and QC practices; and be familiar with statistical techniques for evaluating data. The QA officer is responsible for implementing the QA program and providing necessary technical support and training where needed. Once the QA program is functioning, the QA officer should sign off on all standard operating procedures (SOPs), ensure that documents are updated routinely, and conduct frequent (weekly to monthly) reviews with laboratory management and staff to ensure that the program is being followed correctly and resolve any problems that may arise. The QA officer also reports periodically to management to secure backing for any actions needed to correct problems that threaten data quality. In small laboratories, these responsibilities may be assigned to one or more staff on a part-time basis, or staff may form a QA unit. Unavoidable conflicts of interest (e.g., reviewing and signing off on one's own data) must be clarified in advance and documented.

c. Staff responsibilities: Laboratory and field staff should help management plan the QA program, help prepare SOPs, and—most importantly—incorporate the QA program and QC activities in their daily tasks (e.g., collecting samples, conducting analyses, and calculating and reporting results). Staff members are the first informed and credible sources in identifying potential problems and should work with the QA officer and laboratory supervisor to correct and prevent them. It is critical to QA program success that staff members understand what is expected of them and actively support the QA program.

3. Quality System Objectives

A major QS objective is to implement a system to produce data of known quality and provide a standard mechanism for ensuring and evaluating data quality and project objectives. In addition, other objectives include the assurance of excellent laboratory performance, continuously assessing laboratory operations, identifying weaknesses in laboratory operations, detecting analysts' training needs, improving documentation and recordkeeping, developing adequate and clear reporting systems to ensure traceability, and ensuring compliance with both regulations and the client's requirements.

4. Elements of a Quality System Manual

This written management plan or manual, describing the laboratory's policies and plans for ensuring the quality of their work for their clients, is to be reviewed annually, updated routinely, and signed by both management and the QA officer to indicate their approval and acceptance of their responsibilities. For a small laboratory, the plan should be signed by the owner/operator.

The plan should address the following:

a. Quality policy statement, which describes the QS's specific objectives, includes an ethics statement, and notes laboratory staff's and management's commitment to quality and data integrity.

b. Organization and management structure, which includes an organizational chart and describes the functions of key laboratory staff and management.

c. Personnel policies, which indicates specific qualifications, training requirements, and job responsibilities for all analysts and supervisors.

d. Equipment and instrument requirements, which includes a list of critical equipment and instruments available (including their serial and/or laboratory-assigned identification numbers), as well as the calibration, accuracy-check, and preventive-maintenance procedures and frequency required to ensure acceptable functionality before an item is put into service.

e. Specifications for supplies, which notes procedures to identify, track, and ensure that reagents and supplies are of sufficient quality and acceptable for use.

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

g. Sampling procedures (if performed by the laboratory) and sample-acceptance criteria, which describes procedures for identifying, collecting, handling (e.g., transport conditions, transport time, and temperature maintenance), accepting, storing, and tracking submitted samples, along with required chain-of-custody procedures if data may be subjected to litigation.

h. Analytical methods, which lists the laboratory's scope for testing, its validation procedures for nonstandard or new methods, the accreditation/certification status for individual methods and analytes, and the requirements for initial and ongoing demonstrations of capability.

i. Analytical quality control measures, which states the laboratory's requirements for measurement assurance (e.g., method verification and documentation; error prevention; analytical checks, such as replicate analyses, positive and negative culture controls, blanks, sterility checks, verification tests, performance evaluations/proficiency tests; and tests for determining analyst variability) and the statistical methods to be used, where necessary.

j. Standard operating procedures (SOPs), listing all generic laboratory processes and specific routine laboratory analyses. These are documented in a manner that reflects actual methodologies in use, signed by management, as well as appropriate staff and the QA officer, include the dates they were last revised, are readily accessible to staff, and are available to clients upon request.

k. Documentation control and recordkeeping requirements, which identifies the recordkeeping format(s) (e.g., hard-copy, e-notebooks, and computer files) and procedures to ensure data review, traceability, and accountability. It describes the procedures required to ensure customer confidentiality, where applicable; to maintain original data when revision is required; to establish levels of data access for revisions; to ensure security for data stored both onsite and offsite; and to handle other issues, such as record retention time and record disposal.

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

1) Routine internal audits of laboratory operations, performed at least annually by the QA officer and supervisor. For a small laboratory, an outside expert may be needed. These audits should be comprehensive, including analyses conducted, analyst technique, data manipulations, and reporting.

2) Onsite evaluations by third-party experts to ensure that the laboratory and its personnel are following an acceptable QA program. This is a required component of laboratory certification or accreditation and of analyst certification.

3) Proficiency test (PT) studies, in which the laboratory generally participates once or twice a year. These collaborative studies should confirm the laboratory's ability to generate acceptable data comparable to both the reference laboratory and other participants. They also should identify any potential issues to address.

m. Corrective and preventive activities, which identify procedures used to determine the causes of identified problems and to record, correct, and prevent their recurrence. They indicate continual improvement. Another name for this process is *root-cause analyses* (the systematic process of identifying the cause of a problem or issue, generally through a multi-step process, and developing corrective action plans to prevent recurrences).

n. Customer service, which denotes the laboratory's commitment to internal and external customers. It describes procedures for responding to customer requests and complaints, as well as ensuring customer confidentiality and proprietary rights.

The QC guidelines discussed in 9020B and C are recommended as useful source material of elements that need to be addressed when developing policies for a QA program and QC activities. More information is available from several standards-setting organizations, such as the American Association for Laboratory Accreditation (A2LA), Association of Official Analytical Chemists (AOAC) International Inc., International Organization for Standardization (ISO), The NELAC Institute (TNI), and the U.S. Environmental Protection Agency (EPA).

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9020 B. Intralaboratory Quality Control Guidelines

Quality control (QC) practices are designed to ensure that the laboratory's processes are under control. All laboratories have some intralaboratory QC practices that have evolved from common sense and controlled-experimentation principles 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 discussed below. 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 (e.g., chemical and radiological laboratories). However, microbiology laboratories testing under Good Manufacturing Practices (GMP)/Good Laboratory Practices (GLP) regulations should note that certain QC practices may differ 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 laboratory bench experience in general microbiological techniques that are employed at the laboratory. If such personnel are unavailable, a professional microbiologist must provide training in specific techniques and be available to review work.

TABLE 9020:I. KEY QUALITY CONTROL PRACTICES

Item	Action	Frequency	Further Information in Section 9020B, ¶
Air in workplace	Monitor bacterial density	Monthly	3*e*
Autoclave	Check temperature with max-registering device	Weekly	4*h*
	Check performance with bioindicator	Monthly	
	Check timing	Quarterly	
Balances	Check zero	Daily before use	4*b*
	Check accuracy with at least 2 weights		
	Service and recalibrate	Monthly, preferably	
Biosafety cabinet	Inspect for airflow	Each use	4*m*
	Have certified	Annually	
Conductivity meter	Calibrate	Monthly	4*q*
Dilution water bottles	Check sterility, pH, and volume	Each batch or lot	5*c* and 9050C.1*a*
Freezer	Check temperature	Daily	4*j*
	Defrost	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	
Hot-air sterilizing oven	Check temperature	Each use	4*g*
	Check performance with bioindicator	Monthly	
Incubator	Check temperature	Twice daily when in use	4*n* and *o*
Media	Check sterility, pH, and appearance	Each batch or lot	5*j*
	Check performance with + and – culture controls	Each batch or lot	
	Check recovery of new vs. old media	Before first use	
Media-dispensing apparatus	Check volume dispense accuracy	Each volume change	4*f*
Membrane filters	Check sterility and properties	Each new lot	5*i*
Membrane-filtration equipment	Check for leaks and surface scratches	Each use	4*k*
	Check sterility	Pre- and post-test	
	100-mL volume check	Initially	
Micropipetters	Check dispense accuracy and precision	Quarterly or more frequently if heavily used	4*s*
	Calibrate	Annually	
Microscope	Clean optics and stage, check alignment	Each use	4*p*
Multi-well sealer	Check performance	Monthly	5*e*
pH meter	Standardize with at least 2 buffer solutions	Each use	4*c*
	Determine slope	Daily	
Plate counts	Perform duplicate analyses	Monthly	9*a*
	Repeat counts	Monthly	
Reagent water	Monitor quality	See Table 9020:II	
Refrigerator	Check temperature	Daily	4*i*
Sample bottles	Check sterility	Each batch or lot	5*d*
	Check decholorination agent efficiency	Each batch or lot	
	Check 100 mL line	Each lot	
	Check for autofluorescence if also used for testing	Each lot	
Temperature devices:			4*a*
Working units	Check accuracy	Annually, preferably semiannually	
Reference units	Recertify	Every 5 years	
Timer:			4*h*
Autoclave	Check timing with stopwatch	Quarterly	
Stopwatch	Check against National Time Signal	Annually	
UV lamps, short-wave disinfection	Monitor bulb use	Each use	4*l*
	Test with UV meter or perform plate count check	Quarterly	
Weights:			4*b*
Working	Check with reference weights		
Reference	Recertify	Annually	

Education provides the theory and basic science of microbiology. Training should detail proper techniques and demonstrate the negative consequences (e.g., when SOPs are not followed properly). For specialized testing, such as protozoan or molecular analyses, additional training and bench experience is required. For each analytical method performed, analysts must demonstrate capability in performing laboratory operations before generating reportable data (initial DOC) and periodically thereafter (ongoing DOC) using blind samples (preferred) or known positive samples.

The supervisor should routinely 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 help laboratory personnel obtain additional training and course work to enhance their technical skills and advance their careers. Employee training records and performance scores obtained by analyzing single-blind samples, especially for enumeration methods, and DOCs should be reviewed/monitored and maintained.

2. Biosafety Criteria

Biosafety is a concern for all microbiological laboratories to prevent exposure. There are three elements to consider: laboratory practices, safety equipment, and facility design. Risk assessments of the work performed for each biological agent will determine the appropriate combination of these elements. Personnel must be trained in aseptic techniques and wear personal protective equipment (PPE) (safety glasses, protective clothing, gloves, etc.). For example, PPE clothing and gloves should not be worn outside the laboratory, nor should equipment be routinely moved in and out of the microbiology laboratory. Also, report all accidents and "near-misses."

The U.S. Centers for Disease Control and Prevention (CDC) Public Health Prevention Service divides laboratories handling potentially hazardous biological agents into four biosafety levels (BSLs). Each BSL denotes a combination of laboratory facilities, practices, techniques, and safety equipment appropriate for the laboratory function or activity, the operations performed, the infectious agents' suspected transmission routes, and risk mitigation. A brief discussion of each BSL follows; however, detailed information 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): According to 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 up to the laboratory director to determine which biosafety practices to follow based on the samples and agents involved.

The standard practices and safety equipment for BSL 1 are as follows:

1) Access to the laboratory is limited or restricted at the laboratory director's discretion by posting a sign (e.g., "Restricted Area—Biohazard Laboratory Personnel Only") when experiments or work with samples are in progress. Ensure that doorways and windows are closed when aseptic work is in progress.
2) Wash hands thoroughly with soap and water after handling viable materials, after removing gloves, and before leaving the laboratory.
3) Do NOT eat, drink, smoke, handle contact lenses, apply cosmetics, operate personal cell phones or portable music devices, wear open-toed shoes, or store food for human use in work areas.
4) Do NOT pipet by mouth.
5) Establish and follow policies for safely handling sharp items.
6) Decontaminate work surfaces before and after each use and after any spill of viable material.
7) Decontaminate all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method, such as autoclave sterilization. Keep related decontamination records.
8) Establish and maintain an insect- and rodent-control program.
9) Wear laboratory coats, gowns, or uniforms to avoid contaminating or soiling street clothes. Safety glasses are recommended. Wear gloves, especially if there is a rash or open lesion on the hands. Perform all procedures so no aerosols or splashes occur.

b. Biosafety level 2 (BSL 2): BSL 2 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 the various sections of Part 9000. In addition to BSL 1 practices listed above, BSL 2 requires 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 procedures that could create infectious aerosols be conducted in biological safety cabinets (BSC). BSCs are designed to protect microbiologists from microbial contaminants in samples. If available, appropriate immunizations should be given.

The standard practices and equipment for BSL 2 include all those listed for BSL 1 and the following:

1) Always be very careful with any contaminated sharp items, including needles, syringes, slides, pipets, capillary tubes, and scalpels.
2) Decontaminate work surfaces whenever work is started and completed, at the end of the day, and after any spill or splash of viable material, using disinfectants that are effective against the agents of concern.
3) Place cultures or potentially infectious wastes in a container labeled "Biohazardous Waste" with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.
4) Use BSCs (preferably Class II) or other appropriate PPE whenever conducting procedures that could create infec-

tious aerosols or splashes and whenever using high concentrations or large volumes of infectious agents.
5) Use face protection to avoid splashes or sprays of infectious materials whenever the microorganism must be manipulated outside the BSC.
6) Wear appropriate laboratory coats, gowns, or uniforms; gloves; and safety glasses while in the laboratory. Leave these in the laboratory before exiting to non-laboratory areas.
7) Wear gloves when hands could 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 inhalation and contact. Because *Standard Methods* does not address agents in these categories, the special practices, containment, and facilities for these levels are only outlined here.

1) BSL 3—The standard practices and equipment for BSL 3 include all those listed for BSLs 1 and 2. BSL 3 also requires that personnel be professionally trained in handling infectious materials. The laboratory must be secured and access limited. Work must be conducted within BSCs by personnel wearing appropriate PPE. No one with open lesions should enter the laboratory. There should be passages between the outer hallway and the laboratory entrances where personnel can change into PPE; the doors at each end of these passages should NOT be able to open at the same time or else these safety barriers are compromised. All potentially contaminated material (gloves, laboratory coats, etc.) must be decontaminated before disposal or reuse.

2) BSL 4—BSL 4 is for biological agents, often exotic, that are extremely hazardous both to personnel and/or the environment. The standard practices and equipment for BSL 4 include all those listed for BSLs 1, 2, and 3. BSL 4 also requires that laboratory access be strictly controlled and situated in a clearly marked area removed from normal operations or in a separate building. Personnel must completely disrobe and put on laboratory-specific clothing before entering test areas and be decontaminated before leaving.

3. Facilities

Develop an environmental control policy to ensure that environmental conditions do not invalidate results, impair measurement quality, or harm personnel.[12] Health and safety policies and procedures must be posted or readily available. The factors to be considered and monitoring to be performed are described below. Much of this information applies to any laboratory facility.

a. Ventilation: Design and construct well-ventilated laboratories that can be kept free of dust, drafts, and extreme temperature changes. Install heating, ventilation, and air-conditioning (HVAC) and humidity-control systems to reduce contamination, permit more stable operation of incubators, and decrease moisture problems with media and instrumentation. Adjust HVAC vents so airflow does not blow directly on the working surface areas. Where feasible, ensure that air only flows into (rather than out of) the laboratory to avoid the possibility of contaminating other areas of the building.

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 entrances and exits.

Ensure that there is sufficient workspace available for the volume of work to be performed. For example, maintain separate work areas for sample receipt; media, glassware, and equipment preparation and sterilization, and decontamination of media and glassware; testing and culturing; and data handling and storage. Maintain heat-generating equipment, such as autoclaves, in a room separate from incubators. Using a hood or BSC to dispense and prepare sterile media, transfer microbial cultures, or work with pathogenic materials is recommended. In smaller laboratories it may be necessary, although undesirable, to perform these activities in the same room; however, do not perform them near open doorways or windows. Have sufficient storage space available in the laboratory to store materials (e.g., reagents, glassware, and laboratory supplies) appropriately.

c. Laboratory bench areas: It is optimal to provide at least 2 m (6 ft) of linear bench space per analyst and additional areas for preparation and support activities. Bench height should be reasonable and comfortable for the analysts. For stand-up work, typical bench dimensions may range from 90 to 97 cm (35 to 38 in.) high and 70 to 76 cm (27 to 30 in.) deep. For sit-down activities, such as microscopy and plate counting, benches may range from 75 to 80 cm (29 to 32 in.) high. Specify benchtops of stainless steel, epoxy plastic, or other smooth, impervious surfaces that are inert and corrosion-resistant with minimal seams and NO cracks or crevices. Install even, glare-free lighting with about 1000 lux (100 ft-c) intensity at the working surface; test using a photometer.

d. Walls, floors, and ceilings: Ensure 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 smooth, nonfibrous ceiling surfaces and recessed lights.

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

Monitor air quality routinely—at least monthly or more frequently if area is heavily used or biocontamination risk analysis indicates more frequent monitoring is needed. In aseptic work areas, use air-density settling plates (a passive sampling process wherein particles can settle on the agar surface). If risk assessment indicates the potential for aerosol contamination, use active air samplers.[4] Replicate organism detection and counting (RODAC) contact plates or the swab method[1] can be used weekly or more frequently to monitor bench-surface contamination.

Although uniform limits for bacterial density have not been set, each laboratory can use passive or active air-monitoring systems to establish baselines for specific work areas, evaluate trends, establish alert and action levels, and take appropriate action when necessary. Start by averaging the results obtained from tests over a period of time to determine the typical bacterial density for a given location. In general, airborne bacterial populations should not exceed colonies/plate/15 min exposure, or 1 colony-forming unit (CFU) per minute. Longer exposure times can be used, but water loss may occur and reduce growth potential. In addition to this surveillance system, the laboratory

may wish to identify contaminants recovered with commercially available automated identification systems.

Prevent adverse sound and vibration levels in 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 benchtops 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 benchtops. Eliminate or cover any overhead pipes that cannot be cleaned routinely. Keep liquid hand soap (preferably in a gravity-fed touchless sensor dispenser) and paper towels (touchless paper roll dispenser can be used) available at laboratory sinks. Do not allow smoking or consumption of food or drink in the laboratory. Dispose of laboratory materials properly (e.g., by autoclave sterilization or incineration).

g. Electricity: Ensure a stable source of electricity, a sufficient number of outlets [including ground fault circuit interrupter (GFCI) outlets where needed], and appropriately placed surge protectors. An emergency power backup and alarm system may be necessary where the work is critical.

4. Laboratory Equipment and Instrumentation

Identify equipment by serial number or unique laboratory reference number. Implement procedures to verify that each identified piece of equipment is installed properly and operating consistently and satisfactorily. Verify by constant monitoring, routine maintenance, and a regular calibration schedule that each item 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, along with relevant QC acceptance criteria (see 9020B.6). Keep manufacturers' manuals available for easy retrieval. Perform equipment standardization/calibration using reference standards and maintain equipment regularly, as recommended by the manufacturer, or obtain preventive-maintenance contracts on autoclaves, balances, microscopes, and other critical equipment. Directly record all QC checks in dedicated logbooks, three-ring binders, or electronic records, and maintain documentation so it is accessible for the time period mandated by law. Develop a system for "flagging" problems and related corrective actions.

Ensure that the laboratory has all equipment and supplies required to perform environmental tests and calibrations. For molecular testing, the laboratory's equipment and supplies need to be dedicated to specific rooms.[9] Keep enough equipment and supplies where needed so they are not routinely moved from one laboratory area to another. When certain equipment is only available offsite, document how the laboratory will ensure that all QC factors will be satisfactory. 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 notebook, e-notebook, or computer file.

Use the following QC procedures for both applied and research laboratories (equipment needed for specialized testing may not be listed here):

a. Temperature-sensing and -recording devices: Historically, microbiology laboratories used mercury-filled thermometers, but many states have discouraged the use of such thermometers due to environmental concerns about mercury's neurotoxicity. The National Institute of Standards and Technology (NIST) stopped calibrating mercury thermometers in 2011 (http://www.nist.gov/pml/mercury.cfm). Instead, microbiology laboratories can use organic fluid-filled analog thermometers or digital sensing devices. Review established thermometer guidance[13] for information on the three types of sensors—platinum resistance sensors, thermistors, and thermocouples—used in digital thermometers. Ensure that the thermometer markings are legible and the liquid column or glass case has no break or change. Discard thermometers with illegible graduation marks.

Use thermometers with temperature increments of 0.5°C or less, as appropriate (e.g., for a 44.5 ± 0.2°C water bath used for incubation of thermotolerant bacteria, use a thermometer with 0.1°C increments). Thermometers used in refrigerators or sample-receipt areas may have temperature increments of 1 or 0.5°C. If using liquid-based thermometers to measure temperatures in air incubators and refrigerators, keep thermometer bulb in water or glycerol. When testing temperatures exceed 50°C (e.g., autoclave spore check functionality—50 to 64°C), place the thermometer bulb in a glass container filled with sand.

Another option is to equip incubators, water baths, etc. with temperature-recording instruments that continuously monitor the operating temperature. These wired or wireless data-logging systems can be downloaded into a computerized or printed record. Data-logging units must meet the same requirements as temperature-sensing devices. Establish a system for recording information from data-logging units so analysts are aware of temperature violations shortly after they occur; can invalidate test samples, as appropriate; and can collect new samples. Also, establish a documentation system of data-logger results so time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessments.

Annually, or preferably semiannually, verify the accuracy of all working temperature-sensing devices (e.g., liquid-in-glass thermometers, thermocouples, and temperature-recording instruments) at the use temperature(s). To do this, compare each device's measurements to those of a certified NIST temperature-sensing device or one traceable to NIST and conforming to NIST specifications. Discard temperature-sensing devices that differ by >1°C from the reference device.

For ambient water-temperature readings, ensure that the water has come to equilibrium by letting it sit for at least 1 h. Use a circulating water bath or beaker of water with stir bar set at the appropriate test temperature. When conducting an ice-point check, use reagent grade water and ice (i.e., the concentrations of minerals, salts, etc. in the water and ice should NOT inhibit reaching the true ice-point determination).

Perform a three-point verification testing (at, below, and above the temperature at which the temperature-sensing device will be used). Record accuracy-check results—along with the date, device identification number, and the technician's signature or initials—in a QC logbook. If a correction calculation is necessary, mark the appropriate correction factor on the device so only

corrected temperature values are recorded. For example, when ice-point temperature determination does not match certificate value, adjust all subsequent temperature readings by the same amount (difference in temperature) or submit the unit for recertification (a new certificate of accuracy). Verify accuracy of the certified reference temperature-sensing device as often as specified on the certificate of accuracy or at least once every 5 years. Some accreditation organizations or federal or state agencies may require more frequent verification/certification.

b. Balances: Locate balances in areas without rapid air movement (e.g., drafts) and level them on firm, even surfaces to prevent vibrations. Re-level balance each time it is moved to a new location. Check balance routinely (preferably daily before use) using at least two working weights that bracket the normal usage range. Before each use, clean balance and tare weight before adding reagents to weigh paper or boats. Clean balance pans after use and wipe spills up immediately with a laboratory tissue. Follow manufacturers' instructions for operation and routine maintenance of analytical and top-loading balances.

Use only plastic-tip forceps to handle weights. Check working weights monthly for accuracy, precision, and linearity against a set of reference weights of known tolerance[14] [e.g., ANSI/ASTM Class 1, 2, or 3 or NIST Class S (redefined as ASTM Class 1 weights and no longer available), accompanied by appropriate calibration certificate]. Record results along with date and technician's initials. If weights are corroded or dropped, have them professionally cleaned and recertified or replace them.

Service balances annually, or more often as conditions change or problems occur, following in-house protocols or through service contracts. Recertify reference weights as often as specified in the calibration certificate, or at least once every 5 years.[15,16] Some regulators or accreditors may require that reference weights be recertified more frequently.

c. pH meter: Use a digital meter, graduated in 0.1 pH units or less, that includes the theoretical slope of temperature compensation because the electrode pH response is temperature-dependent. Use electrodes suitable for a wide temperature range, and use a flat-head electrode to measure solid agar media. Measure test solution's pH at a temperature near that used to calibrate the meter. The most desired temperature range for determining pH is 18 to 25°C (room temperature). Keep the probes clean and store electrode immersed in the manufacturer-recommended solution.

Use only commercial buffer standard solutions for calibrations, and standardize pH meter with at least two certified pH buffers (usually pH 4 and 7 or pH 7 and 10) that bracket the pH of the sample being measured (two-point standardization). Record standardization results, date, and technician's initials. Immediately after use, discard the buffer-solution portions or single-use/ready-to-use solution packets used to standardize meter. Discard all buffer solutions made from packets after 1 d.

Each time a fresh bottle of buffer solution is opened, inscribe the date on bottle and in logbook; thereafter, check the bottled solution monthly against another pH meter, if possible. Replace pH buffer supply containers by the expiration date, preferably 6 months after opening because the solution may absorb carbon dioxide.

To verify that the pH meter is functioning properly, measure and record its slope after standardization daily (or each day it is used). Most meters provide slope values automatically. If the pH meter does not calculate the slope automatically, but can provide the pH in millivolts (mV), use the following formula to calculate the slope:

$$\text{Slope, as } \% = |\text{mV at higher pH} - \text{mV at lower pH}| \times 100/177$$

If the slope is $<95\%$ or $>105\%$, the electrode or meter may need maintenance. If all three buffers are used in sequence to standardize the meter (three-point standardization), analysts may provide either both slopes or an average. For full details of pH meter use and maintenance, see Section 4500-H^+ or follow manufacturer's instructions.

d. Water-purification system: The quality of laboratory-prepared reagent water depends on the quality of the source water and the water-purification equipment used to develop and store it. Commercial systems are available that include some combination of prefiltration, activated-carbon filter, ion-exchange cartridge or cylinder, and reverse osmosis with final filtration to produce reagent-grade water. Such systems tend to produce the same water quality until the ion-exchange resins or activated carbon is near exhaustion; then, the quality abruptly becomes unacceptable. Some deionization components now automatically regenerate the ion-exchange resins.

Do not store laboratory-prepared reagent water unless a commercial ultraviolet (UV) irradiation device is installed and confirmed to maintain sterility. Maintain and monitor the equipment routinely to ensure that the water meets the appropriate standards. Every day laboratory-prepared reagent water is used, monitor it with a standardized conductivity meter (see ¶ *q* below). Each month (or use, as appropriate), determine total chlorine residual and heterotrophic bacteria concentrations, which may provide an early warning of potential problems. Increasing bacteria numbers indicate the possible presence of complex organic compounds, inorganic compounds, and endotoxins that can be nutrient sources for bacteria. At least once a year, analyze reagent water for trace metals. Also, perform the bacteriological water quality test annually, and whenever the water-purification system is modified or repaired. The water quality test described in 9020B.5*f*1) is not required for Type II water or medium-quality water or better, as defined in 20th, 21st, 22nd, and Online Editions of *Standard Methods*, Section 1080C, or in other widely accepted standards.[17] Most systems used today meet or exceed these standards. Perform the use test [see 9020B.5*f*2)] whenever there is a new source of water or new water system employed in the laboratory.

Replace cartridges at manufacturer-recommended intervals 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 unavailable, use a 0.2-μm-pore membrane filter for aseptic final filtration and collect in a sterile container. Monitor treated water for contamination and replace filter as necessary.

e. Water still: Stills produce good water 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), and stored distilled water will absorb more NH_3 and carbon dioxide (CO_2) from the air. Drain and clean still and reservoir according to manufacturer's instructions and usage. To

reduce cleaning frequency, use softened water as the source water.

f. Mechanical media-dispensing apparatus: Check apparatus' accuracy by dispensing a sample volume of medium into a graduated cylinder just after filling/refilling it and periodically throughout extended runs; record results. Before dispensing medium for sample analyses, flush apparatus with a small volume of medium to ensure that evaporation has not blocked the tip or changed the concentration of reagent. Between runs, rinse apparatus by pumping hot reagent-grade water through it. Correct leaks, loose connections, or malfunctions immediately. At the end of use, 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 strips of a spore-forming microorganism (e.g., *Bacillus atrophaeus*) that ideally has a minimum spore density of 1×10^6. Test the strip in glassware similar to the items being sterilized. Measure oven temperature with a thermometer whose bulb is placed in sand, a thermocouple-type probe, or a continuous-read temperature recorder. The temperature-measuring device must be accurate in the 160 to 180°C range. Record results and contents when in use. Use heat-indicating tape, chemical strips, or Diack tubes to identify supplies and materials that have been exposed to sterilization temperatures.

h. Autoclave: For new autoclaves, conduct an initial temperature profile to determine any hot or cold spots throughout the unit, using probes placed in various areas.

When filling the autoclave, avoid overcrowding (e.g., do not place racks on top of each other; leave space between racks and flasks so steam can flow past individual test tube racks and flasks). After each run cycle, record the items sterilized, sterilization temperature, total run time (heat exposure), programmed/preset sterilization period, actual pressure readings, and analyst initials.[18,19] New units may print most of this information on tape automatically (i.e., time, temperature, and pressure at selected time interval). For older units, if possible, use a recording thermometer chart or electronic high-temperature data logger (HTDL).

For general sterilization tasks, the recommended autoclave temperature range is 121 to 124°C (at 200 kPa), although higher temperatures (≥121°C) are acceptable for decontaminating laboratory materials. Ensure that the autoclave maintains 121°C with minimal temperature variation at ≥15 lb/in.^{2} (≥103 kPa) for 15 min during the media sterilization cycle and that media are withdrawn from the autoclave in 45 min or less. Autoclave temperature control tolerances may vary, depending on the nature of the media being sterilized. In these cases, follow the relevant recommended procedures. Typically, however, keep temperature within ±2°C of prescribed temperature for media (and ±10 kPa of recommended P).

Some regulatory programs and new media may require a different temperature/pressure/time sequence. Certain media may be heat sensitive and require narrow temperature tolerances. If media contain lactose, for example, excessive heat exposure (i.e., autoclaving too long or at too high a temperature) will result in lactose hydrolysis, rendering the medium unsuitable for its intended use. Other media may need a shorter autoclave cycle. Review all pertinent information for these cases. See 9020B.5*j* for further discussion.

For routine use, verify autoclave temperature weekly with a maximum registering thermometer (MRT) (generally a mercury-filled Teflon-coated device) or accurate HTDL able to withstand 15 to 20 lb/in.^{2} If neither device is available, use a strip or pie chart recorder with interpretations written on the chart. Maintain verification records. Using a chemical steam indicator for each cycle will show if minimum exposure conditions were met but will not indicate whether sterilization was achieved. Heat-indicating tape can quickly identify supplies and materials that have been sterilized.

Maintaining proper autoclave functions is critical. Test monthly for sterilization efficacy at the media's normal sterilization time and temperature using a biological indicator (e.g., commercially available *Geobacillus stearothermophilus* in spore strips, suspensions, or capsules, preferably at a 1×10^6 concentration). Place the indicator in glassware containing a liquid to simulate actual autoclave sterilization performance on media.[20] Some biological indicators may require more time at the sterilization temperature than is used for most carbohydrate media. If this becomes problematic, use biological indicators for autoclave runs that exceed 20 min (e.g., dilution water and contaminated materials).

Each quarter, use a calibrated timer or national time signal to check the timing of all three cycles for a media run (≤15 min conditioning cycle, 15 min sterilization cycle, and ≥15 min exhaust cycle). Keep autoclave clean and free of debris by checking both trap and seals monthly. Service autoclaves annually either in-house or through service contracts.

i. Refrigerator: An initial temperature profile is suggested to determine any hot or cold spots in the unit. Maintain temperature at 2 to 8°C and monitor it using either thermometers whose bulbs are submerged in distilled water or glycerol solution, or digital temperature-sensing devices placed on the top and bottom shelves of each use area. Every day while in use, check and record temperature (corrected, if necessary), also noting date and observer's initials. Identify and date materials stored in refrigerator, and discard outdated materials monthly. Clean units annually, or more frequently if needed.

Frost-free units may dehydrate stored media more quickly because heating is used to prevent ice buildup. Flammable materials should be stored in explosion-proof refrigerators. Volatile organic chemicals should not be stored in the same refrigerator used for microbiological media, reagents, or cultures.

j. Freezer: The freezer temperature range depends on analytical need (e.g., a standard laboratory freezer may range from −15 to −25°C, while an ultra-low freezer may range from −70 to −90°C). A recording thermometer and alarm system are highly desirable. Every day while in use, check and record corrected temperature(s), also noting date, time, and observer's initials. Avoid opening units repeatedly because frost will build up and make the freezer less efficient. Identify and date (e.g., manufacturer- or lab-expiration) materials stored in freezer. Storing materials in insulated boxes with snug-fitting lids and away from freezer walls may be beneficial. Defrost and clean units annually (or more frequently, as needed); discard outdated materials.

Frost-free units may dehydrate stored media more quickly because heating is used to melt ice buildup. Store flammable materials in explosion-proof freezers.

k. Membrane filtration equipment: Before initial use, assemble filtration units and check for leaks. Discard units if chipped or inside surfaces are scratched. Units that leak should be repaired accordingly or discarded. Replace damaged screens on stainless steel units. Wash and rinse filtration assemblies thoroughly after use, wrap in nontoxic paper or foil, and sterilize via autoclave or dry heat oven. When measuring sample volumes using funnels with volumetric graduation marks, initially check the marks' accuracy using a Class A graduated cylinder or volumetric pipet. Record results. For presterilized single-use funnels, check one per lot or a set percentage (e.g., 1 to 4%) to confirm the accuracy of volumetric graduation mark.

l. Ultraviolet lamps:

1) Short-wave ultraviolet lights (254 nm)— Germicidal short-wave UV lights are commonly used to sanitize, not sterilize, such items as membrane filtration units. When in use, disconnect lamps monthly and clean bulbs with a soft cloth moistened with ethanol (70% ethanol/30% reagent-grade water) or with spectroscopic grade 2-propanol in areas where baked-on material is collecting. Test lamps quarterly with an appropriate (short-wave) UV light meter, and replace bulbs when output drops to <70% of initial output. Alternatively, expose spread plates containing 200 to 300 CFU/mL of a selected bacterial suspension for 2 min. Incubate plates at 35°C for 48 h and then count colonies. Replace bulb if colony count is not reduced 99%. It also is advisable to ask the manufacturer for the bulb's expected lifespan and then track hourly usage.

2) Long-wave ultraviolet lights—Long-wave (365–366-nm) UV lights are used to determine fluorescence. Analysts should use 6-W lamps because faint fluorescence may not be visible when using 4-W lamps. Keep units clean, periodically use a light meter to check that the bulb remains at the proper wattage, and replace the UV light yearly.

CAUTION: Although the short-wave (254-nm) UV light is known to be more dangerous than long-wave (365-nm) UV light, both can damage eyes and skin and are potentially carcinogenic.[21] Protect eyes and skin from exposure to UV light. Consider installing 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, along with good microbiological techniques, provide an effective containment system for safely manipulating moderate- and high-risk microorganisms (BSL 2 and 3 agents). Both Class I and II BSCs have inward face velocities (80 to 100 linear ft/min) designed to protect laboratory workers and the immediate environment from infectious aerosols generated inside the cabinet. Class II BSCs also protect the material itself through high-efficiency particulate air (HEPA) filtration of the airflow down across the work surface (vertical laminar flow). Standard operating procedures are as follows:

1) Before and after use, purge air for 10 to 15 min and wipe down unit with disinfectant. Use a piece of tissue to confirm inward airflow.
2) Enter straight into cabinet and perform work slowly and methodically. Place material well within cabinet—not on front grill—and do not disrupt or block laminar airflow. Place discard pan within cabinet.
3) Decontaminate interior of BSC after work is completed and before it is removed. Allow cabinet to run for 10 to 15 min and then shut off.[22]

Provide for testing and certification of Class I and II BSCs *in situ* when they are installed, moved, and at least annually thereafter. Maintain cabinets as directed by the manufacturer. Avoid using a Bunsen burner inside BSCs because it will change airflow and may destroy the HEPA filter. Do not allow workspace to become crowded because objects may disturb airflow pattern, allowing contaminant(s) to exit at the face opening. Place working objects at least 6 in. from the face.

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 an appropriately marked total immersion thermometer if available (¶ *a* above). When incubator is in use (i.e., samples are being incubated), monitor and record corrected temperature twice daily separated by 4 h.

Electronic temperature-sensing devices (i.e., data loggers) may be used so long as the laboratory's system for recording information from the devices also promptly notifies analysts of temperature violations so they can invalidate test samples, as appropriate, and request that new samples be collected. This system also must document the data such that time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessments. Each data logger should be marked with any correction factor needed.

Fill unit only with reagent-quality water. Maintain water level so it is above the upper level of the medium in either tubes or flasks. 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 (gravity convection or mechanical forced hot-air, water-jacketed, or aluminum block): Place incubators in an area where room temperature is maintained between 16 and 27°C (60 and 80°F), or else in a separate, well-insulated room with forced air circulation. Clean and then sanitize incubators routinely. Determine that incubators maintain appropriate, uniform spatial test temperatures. It may take longer for media to reach the set incubation temperature in gravity convection hot-air incubators.

While in use, check and record corrected temperature twice daily (morning and afternoon, separated by at least 4 h) on the shelves in use, or at least on the top and bottom shelves to ensure consistency throughout unit. If using a glass thermometer, submerge bulb and stem in water or glycerin to the immersion mark. For best results, use a recording thermometer and an alarm system that promptly notifies analysts of temperature violations so they can invalidate test samples, as appropriate, and request that new samples be collected. Maintain a logbook or digital records of temperature readings and alarms. Electronic temperature-sensing devices (i.e., data loggers) may be used so long as the laboratory's system for recording information from the devices also promptly notifies analysts of temperature violations and documents results such that time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessment. Each data logger should be marked with

a correction factor, as needed. Allow sufficient space between items to permit unobstructed airflow; do not overload nor stack Petri dishes more than four plates high.

Incubator humidity may be a concern if Petri agar media are dehydrated because less water is available for metabolic growth and cells may lyse. Incubated agar plates should be evaluated for the percent moisture weight loss for the method's incubation period. Moisture weight loss should be performed annually. If agar weight loss is >15%, moisture needs to be added to the environment by either using humidified incubators or enclosing the media in tight-sealing containers or bags. If additional moisture is needed, wet paper towels can be added to container or a large shallow pan filled with water can be added to the incubator, refilling as necessary. If there is no weight loss and smearing of colonies on media is evident, humidity needs to be reduced accordingly.

p. Microscopes: Before each use, check Kohler illumination to confirm that alignment is correct. When available on binocular microscopes, adjust ocular lenses for diopter (difference of visual acuity between an analyst's eyes) to reduce or eliminate headaches, motion-sickness symptoms, and the potential for personal errors. After each use, clean optics and stage with lens paper. Cover microscope when not in use.

Permit only trained technicians to use fluorescence microscope and light source. Monitor fluorescence lamp and replace when a significant loss in fluorescence is observed, according to manufacturer recommendations, or when maximum hour usage specified by a rule or laboratory guidance document has been reached, 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.

Establish an annual service contract. Review the microscope-manufacturer's manual; for further information, visit the manufacturer Web site or refer to Section 9030.20[20] and elsewhere.[23]

q. Conductivity meter: A conductivity meter measures the presence of dissolved ions, such as aluminum, calcium, chloride, iron, magnesium, nitrate, phosphate, sodium, and sulfate. Conductivity measurements are temperature-dependent, and the temperature's effect is solution-dependent. Follow manufacturer's instructions for meter check and calibration procedures. Check daily before use and calibrate, if needed. Every month, standardize meter or determine cell constant using certified low-level standards that bracket the expected sample conductivity (e.g., 10 μS/cm) at 25°C. For on-line conductivity meters that cannot be calibrated, remove a portion of reagent water and measure its conductivity with another meter. When solutions must be measured at another temperature, use a meter with automatic temperature compensation or take solution's temperature, record reading, and then correct reading to 25°C using the formulae in Section 2510B.5*b* (usually 2%/°C). Open sample bottle as rarely as possible because conductivity measurements will change when the sample is exposed to ambient air.

r. Microwave ovens: Microwave ovens vary in power and acceptable placement of material, but they have been used successfully to melt presterilized agar media. Set microwave power and time to minimum settings. Check unit's performance and undertake comparison studies to confirm that microwaving is comparable to standardized melting procedures. Take care to avoid media bubbling over.

s. Micropipetters: Micropipetters are high-precision laboratory instruments for dispensing extremely small volumes. Use with sterile precision tips supplied by manufacturer or equivalent that securely fix to the nose cone to ensure a tight seal. Maintain technique consistency in pipetting action, such as pre-wetting, plunger release, 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 micropipetter's recommended range, which can cause mechanical damage. Follow manufacturer's instructions to perform routine maintenance, such as cleaning, seal replacement, and re-lubrication. Check accuracy and precision of volume dispensed by each pipetter before first use after purchase, maintenance, or repair, and at a frequency related to its usage (e.g., quarterly or sooner if pipetter is showing overt signs that it is inaccurate or if tip manufacturer changes). Calibrate at least annually either in-house or by the manufacturer. Record results of calibration. If water is used to calibrate or check accuracy of pipetter, remember that changes in liquid viscosity can affect the volume dispensed.

5. Laboratory Supplies

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

a. Glassware: Here, the term *glassware* refers to both borosilicate glass and heat-resistant plastic materials. Markings must be legible. Volumetric glassware, pipets, graduated cylinders, and beakers with calibration marks should be accurate to the specified volumetric tolerances. See established standards[24] for calibration of laboratory volumetric apparatus. Volumetric glassware is generally either Class A or Class B (undesignated); Class A is more precise. Determine tolerance once per lot or at a set percentage (e.g., 1 to 2.5%). Graduated cylinders should be accurate to within ≤2.5%.

Before each use, examine glassware and discard items with chipped edges or etched inner surfaces—especially screw-capped dilution bottles and flasks with chipped edges that could leak and contaminate the sample, analyst, and area. After washing, inspect glassware for excessive water beading, stains, and cloudiness, and rewash or discard if necessary. Replace glassware with excessive writing if markings cannot be removed. Store glassware either covered or bottom up to prevent dust from settling inside it. If glassware is being used for fluorescence detection [i.e., with EC + 4-methylumbelliferyl-β-D-glucuronide (ECMUG) medium], check it for autofluorescence before use.

Perform the following tests for clean glassware:

1) pH—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. (A *batch* is all glassware washed in the same load.) To test clean glassware for an alkaline or acid residue, add a few drops of 0.04% bromothymol blue (BTB) or other pH indicator to dry glassware and observe the color reaction. If there is no residual, the reaction should be neutral (blue-green for bromothymol blue). However, if the indicator turns yellow (acid residual) or deep blue (alkaline residual), then the glassware must be rewashed and tested again. If the re-test indicates a problem, review the washing equipment, procedures, and detergent used.

Use commercially or laboratory prepared reagents for this pH check. To prepare 0.04% BTB solution, add 16 mL 0.01*N* NaOH to 0.1 g BTB and dilute to 250 mL with reagent water.

2) Inhibitory residues—The main objective of this test is to determine whether the laboratory's washing procedure leaves an inhibitory substance on the glassware. Certain wetting agents or detergents may contain bacteriostatic, inhibitory, or stimulatory substances that may take 6 to 12 rinses to remove. If each batch of glassware is pH tested, then this test is only needed when changing washing compounds or procedures. However, if glassware is not consistently pH-tested or the detergent is not laboratory-grade, then conduct the inhibitory residue test just before first use and annually thereafter. Record results. The following procedure is suitable for both Petri dishes and other glassware.

a) Procedure—Wash and rinse six Petri dishes (Group A) according to usual laboratory practice. Wash six more Petri dishes (Group B) as above, and then rinse 12 times with successive portions of reagent water. Rinse six more Petri dishes (Group C) with water containing the detergent (in use concentration), and air-dry without further rinsing.

Sterilize dishes in Groups A, B, and C by the usual procedure. For presterilized plasticware, set up six plastic Petri dishes (Group D). Prepare and sterilize 200 mL plate count agar and temper in a 44–46°C water bath. Prepare a culture of *Enterobacter aerogenes* ATCC® 13048 known to contain 50 to 150 CFU/mL. Preliminary testing may be necessary to achieve this count range. Inoculate three dishes from each test group with 0.1 mL culture and the other three 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—The averaged counts on plates in Groups A through D should differ by <15% if there are no toxic or inhibitory effects. If averaged counts differ by <15% between Groups A and B and >15% between Groups A and C, then the cleaning detergent has inhibitory properties that are eliminated during routine washing. If averaged counts differ by >15% between Groups A and B, then inhibition is occurring because more colonies grew when there was additional rinsing. If the difference between B and D is ≥15%, then an inhibitory residue is present after the normal washing process 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 and autoclavable borosilicate glass or plastic with screw caps that are either linerless or have inert liners. Clean before use. Commercially available bottles prefilled with dilution water are acceptable. Before using each batch or lot, conduct sterility test (9020B.9*d*); check one per lot or a set percentage (e.g., 1 to 4%) for pH and volume (99 ± 2 mL). 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 bottles from a different source. Recheck volume at regular intervals to determine volume loss rate under holding conditions. Discard by expiration date.

d. Sample bottles: Use wide-mouth reusable, nonreactive, autoclavable borosilicate glass or plastic bottles with screw caps that are either linerless or have inert liners, or else commercially prepared sterilized plastic bottles or bags with ties of sufficient size. The bottles or bags must be large enough to collect the needed sample and still have an adequate headspace (1 in.) to allow sample to be shaken in the container.

Clean and sterilize bottles before use and, depending on use, add sufficient dechlorination agent to neutralize residual chlorine (Section 9060A.2). Sample container may be purchased with added dechlorination agent. Test for sterility at least one or a set percentage (e.g., 1 to 4%) of each batch sterilized in the laboratory or of each presterilized lot purchased from a vendor. Document results. If growth occurs, discard entire batch or lot. Also, check one per batch or lot for efficacy of dechlorination agent, accuracy of 100-mL mark (if present), and auto-fluorescence properties (if used for fluorescence testing). Record results.

e. Multi-well trays and sealers:* When using multi-well trays for growth studies, check one per lot for sterility beforehand by aseptically adding 100 mL of sterile tryptic soy broth or other non-selective medium, sealing, and incubating at 35 ± 0.5°C for 24 and up to 48 h. No growth indicates sterility. If the wells become very turbid (indicating nonsterile condition), there could be gas production and concomitant blowout between wells. See 9020B.9*d*.

Every month, evaluate the heat sealer's performance by adding one to two drops of a food-color dye to 100 mL deionized water sample, run the multi-well tray through the sealer, and visually check each well for leakage. Clean and conduct 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. Examine the tray wells for consistency and perform appropriate QC controls, as indicated by the manufacturer. Use controls from an approved certified vendor; these may be labeled for the system being tested. The laboratory may need to detoxify or sterilize the plates if their use requires this.

f. Reagent-grade water: Use reagent-grade water to prepare solutions and media, and for final glassware rinses. The water must be proven to be free from inhibitory and bactericidal substances. The quality of water obtainable from a water-purification system depends on the system and how it is maintained (see 9020B.4*d* and *e*). See Table 9020:II for recommended reagent water-quality limits for the microbiology laboratory. If these limits are not met, investigate and correct or else change water source. NOTE: Reagent water's pH tends to drift, but extreme readings indicate chemical contamination.

1) Test for bacteriological quality[25]—This test, also called the *water suitability test*, is based on the growth of *Enterobacter aerogenes* ATCC® 13048 in a chemically defined minimal-growth medium. The presence of a toxic agent or a growth-promoting substance will increase or decrease the 24-h population by 20% or more, compared to a control. Perform the test at least annually, whenever the reagent-water source is

* 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	Continuously or usage day	<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†	Monthly	<500 CFU/mL or MPN <500/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

* Or more frequently if there is a problem.
† See Section 9215.
‡ This water-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, 22nd, and Online Editions), Section 1080C.

changed, and whenever an analytical problem occurs. This bacteriological quality test is not needed for Type II water or better [as defined in *Standard Methods* Section 1080C (18th and 19th Editions)] or medium-quality water or better [as defined in *Standard Methods* Section 1080C (21st, 22nd, and Online Editions)]. Test to ensure continued quality of this water to meet the above ATCC growth standards. Because of its complexity and because most laboratories use Type II water or better, this test is seldom performed but may be used when an analytical problem occurs.

The test is complex, requires skill and experience, and is not easily done on an infrequent basis. It requires work over 4 d, ultrapure water from an independent source as a control, high-purity reagents, and extremely clean culture flasks, Petri dishes, test tubes, pipets, and other equipment. A contract laboratory familiar with the test can be used.

a) Apparatus and material—Use borosilicate glassware; presterilized plastic Petri dishes may be used in plating steps. Rinse glassware 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 the 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 ACS-grade reagents and chemicals. Test sensitivity is partly controlled by reagent purity. Use medical-grade water or water freshly redistilled from a glass still; the water can be purchased (see Table 9020:III). Prepare reagents 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.

Filter sterilize or 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. When solutions become turbid, discard them and prepare new ones.

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 two flasks or tubes A and B. Add water samples, media reagents, and redistilled water to each flask as indicated in Table 9020:III. Add a suspension of *E. aerogenes* ATCC® 13048 (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 are less sensitive to nutrients in the test water.

e) Preparation of bacterial suspension—On the day before the distilled-water suitability test, inoculate a strain of *E. aerogenes* ATCC® 13048 onto an approximately 6.3-cm-long nutrient agar slant 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—After incubation, pipet 1 to 2 mL sterile dilution water from a 99-mL water blank onto the culture. Emulsify growth on slant by vortexing, gentle sonication or swirling; 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 third bottle into a fourth water blank, shaking vigorously 22 times after each transfer. Pipet 1.0 mL of the fourth dilution (1:105) into Flasks A and B. This procedure should produce a

†ATCC 13048, or equivalent.

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

final dilution in the range of 30 to 80 viable cells per milliliter of test solution.

h) Verification of bacterial density—Variations among strains of a given organism, different organisms, media, and surface area of agar slopes may require that the dilution procedure be adjusted to achieve an appropriate cell density. To determine the bacterial density for a specific organism and medium, make a series of plate counts from the third dilution. Then, choose the proper volume from this dilution, which when diluted by the 30 mL in Flasks A and B, 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 reproduce results on repeated experiments with the same strain of microorganism. Run tests in triplicate.

i) Procedural difficulties—Problems in this method may be due to:

- test water sample stored in soft-glass containers or glass containers with linerless metal caps;
- reagents prepared with chemicals that are not analytical-reagent grade or of recent manufacture;
- reagent contaminated by distilled water containing background levels of bacteria (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);
- bacterial density outside 30- to 80-viable cells/mL range (e.g., incorrect dilution chosen for 24-h plate count);
- inconsistent mixing;
- delay in pouring plates; or
- samples incubated for longer than 26 h, thereby desensitizing growth response.

j) Calculation—For growth-inhibiting substances:

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

If the ratio is 0.8 to 1.2 (inclusive), no toxic substances are present; if the ratio is <0.8, there are growth-inhibiting substances in the water sample.

A value >1.2 indicates an available nutrient source for bacterial growth; however, the test is very sensitive and the ratio could go as high as 3.0 without any undesirable consequences. Do not calculate if the first ratio indicates a toxic reaction.

k) Interpretation of results—The colony count from the control, Flask A, after incubation will depend on the strain of *E. aerogenes* used and the number of organisms initially inoculated in the flask. Therefore, run Flask A for each individual series of tests. If the strain of *E. aerogenes* ATCC® 13048, initial inoculum, and environmental conditions are the same, the terminal count should be reasonably constant. On the other hand, a difference in initial inoculation of 30 to 80 will result in a final count about threefold larger for the 80 organisms if the growth rate remains constant. Thus, it is essential that initial colony counts on Flasks A and B be approximately equal.

Specific corrective measures cannot be recommended for every instance of defective distillation apparatus. Carefully inspect the distillation equipment and review the distilled-water production and handling processes to help locate and correct the cause of difficulty. Feed water 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, then feed water quality may be 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.

2) Use test for evaluating reagent water—Before using a new reagent-water source, analysts should compare it for equivalence with the current lot in use (reference lot). Note: It may not be possible to compare reagent-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, spread, or membrane-filter plate tests on both reference and test lots (see Sections 9215 and 9222). At a minimum, analyze five different water samples known to be positive for the target organism or culture controls of known density. Replicate analyses and additional samples can be tested to better detect any differences between reference and test lots.

When analyzing reagent water, perform the quantitative bacterial tests in parallel using a known high-quality water as the

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 both lots for size and appearance. If colonies on the test lot plates are atypical or noticeably smaller than those 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 or 100 mL. Transform the count to decimal logarithms and enter the log-transformed results for both 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, *sd*, of these differences (see Section 1010B).

Calculate Student's *t* statistic:

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

where n = the number of samples.

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

c) Interpretation—Compare the calculated *t* value to the critical *t* value from a Student's *t* table. At the 0.05 significance level, Student's *t* is 2.78 for five samples (four degrees of freedom). If the calculated *t* value is ≤2.78, the test lot is acceptable (i.e., the two lots' results are not significantly different). If the calculated *t* value is >2.78, the test lot is unacceptable.

If the colonies are atypical or noticeably smaller on the test lot or Student's *t* exceeds 2.78, then review test conditions and repeat the test or else 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 ACS-grade chemicals or equivalent because impurities can inhibit bacterial growth, provide nutrients, or fail to produce the desired reaction. Maintain any safety data sheets (SDS) provided with reagents or standards and have them available to all personnel.

Date chemicals and reagents both 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, preferably in volumetric flasks, and store them in 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, preparer's name, 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 microbiological dyes must be strong and stable enough to produce correct reactions, only use those certified by the Biological Stain Commission. Prepare minimal quantities and before use, test dyes using at least one positive and one negative control culture. Record results. For fluorescent stains, test for positive and negative reactivity each day of use. Do not freeze dyes or stains. Read and follow manufacturer's information for storage time and temperature.

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

1) Specifications—Manufacturers of membrane filters and pads for water analyses must meet standard specifications for flow rate, retention, percent recovery, and inorganic and organic chemical extractables.[28,29] Some manufacturers also report pore size, sterility, and pH, and certify that their membranes are satisfactory for water analysis. Although the standard membrane filter evaluation tests were developed for 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 use tests—When each lot of membranes arrives at the laboratory, record lot number and date received. Inspect each lot before use and during testing to ensure that membranes are round and pliable. If lot is held for one or more years, carefully check for brittleness and discard lots that appear brittle. Confirm sterility prior to first use of the lot by placing a membrane filter on a pad saturated with tryptone glucose extract broth (or equivalent non-selective broth or agar) and incubating it at 35 ± 0.5°C for 24 h; the filter is sterile if no growth occurs. Alternatively, run a sterility control with 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 culture methods depend on properly prepared bulk media, use the best available materials and consistent techniques to prepare, store, and use media, and prepare the correct medium for the intended application. For QC, use commercially prepared bulk media whenever available, but note that the quality and ingredient composition of such media may vary both from lot to lot and from manufacturer to manufacturer. Before first use, compare the growth recovery of newly purchased lots of bulk media to those of proven lots, using positive and negative reference cultures (preferably), recent pure-culture isolates, or natural samples [see ¶ *f*2) above]. This is known as a *use test* as applied to media. Test using cultures whose estimated density is similar to samples normally tested in the laboratory. Observe media for growth promotion, inhibitory properties, physical appearance, and pH.

File any SDS accompanying media.

Order media in quantities expected to be used within 1 year (preferably within 6 months) after opening. Order commercially prepared media in quantities expected to be used by the manufacturer's expiration date. Use media on a first-in, first-out basis. When practical, order media in smaller containers (e.g., 0.25 lb or 125 g) rather than 1-lb or 500-g bottles so most of the supply remains sealed as long as possible. Keep written or digital records of the type, amount, and appearance of media received,

lot number, expiration date, and dates received and opened; also, mark containers with the expiration date and date opened. Check inventory quarterly for reordering.

Each lot of media for detecting fluorescence should be checked for autofluorescence before use. This can be done by dissolving the medium in reagent water and examining with UV light.

1) Preparation of media—Prepare media in clean containers that are at least twice the volume of the medium being prepared. Use reagent-grade water. Measure both water and media with graduates or pipets that conform to NIST and APHA standards, respectively. Use TD (to deliver) pipets, NOT blowout ones. 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.

After sterilization, check and record pH of a portion of each medium because the specified pH of the medium is the actual pH required for adequate growth. If pH adjustment is needed, use filter-sterilized 1*N* NaOH or 1*N* HCl solutions to make minor adjustments so medium's pH meets that specified in the formulation. (Commercially available media will seldom need pH adjustment.) If medium is known to require pH adjustment, adjust it appropriately before sterilization and record final pH. If the pH difference is >0.5 units, discard the batch and check both preparation instructions and reagent water's pH to resolve the problem. Incorrect pH values may be due to reagent water quality, deterioration of medium, or improper preparation. If reagent water's pH is unsatisfactory, prepare a new batch of medium using water from another source (see 9020B.4*d* and *e*). If water is satisfactory, remake medium and check pH; if pH is still incorrect, prepare medium using a different lot or source of media. NOTE: Certain specific isolation media prepared with organic or fatty acids will have marked changes in pH following sterilization. Discard media if crystal formation or color variations are found. NOTE: A precipitate is normal in Endo-type media.

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 whether variations in sterilization time and temperature could be the cause of problems. If any of the above occurs, discard the medium.

2) Sterilization—Sterilize media at ≤121°C with minimal temperature variation for minimum time specified. Follow manufacturer's directions for sterilizing 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 in small units (e.g., test tubes and small flasks). Do not expose media containing carbohydrates to elevated temperatures for >45 min; some media cannot be exposed to heat for that long. For example, presence–absence media cannot be exposed to heat for >30 min. *Exposure time* is the period from initial heat exposure to removal from the autoclave. Overheating media can result in nutrient degradation. Maintain autoclave printout records.

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.

NOTE: Where possible, avoid sterilizing large amounts of media in containers because it will take longer for the media to reach sterilization temperature. Use a temperature probe in a media flask to determine the length of time needed to reach sterilization temperature.

Remove sterilized media from autoclave as soon as chamber pressure reaches zero or, if using a fully automatic model, as soon as the door opens. Use extreme care to avoid boiling over due to superheated liquids. Do not re-autoclave media.

Sterilize heat-sensitive solutions or media by filtration through a 0.2-μm-pore-diameter filter in a sterile filtration and receiving apparatus. Filter and dispense medium in a laminar-flow hood or safety cabinet, if available. Sterilize glassware (e.g., pipets, Petri dishes, sample bottles) in an autoclave or hot-air sterilizing oven (170 ± 10°C for ≥2 h). Sterilize equipment, supplies, and other heat-sensitive solid or dry materials 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 for ≤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. Ideally, prepare media ≥2 d before tests to allow sufficient time for sterility and positive- and negative-control culture testing to be completed. If agar medium is solidified for later use, then melt in boiling water bath or beaker or a unit with a flowing stream of steam (e.g., an autoclave set at 100°C for 5 to 10 min, or low-wattage microwave[30]), use, and then discard any remainder. Because microwaves vary, run comparison tests to ensure that medium integrity has not been compromised. Some media are not suitable for melting in the microwave (i.e., M-Endo/Endo LES). Do not re-autoclave media. Agar may be melted only once, and some media cannot be melted in the microwave without destroying their selective nature.

The volume dispensed depends on 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. (*Durham tubes* are very small test tubes

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.

inverted in larger test tubes to entrap any gas produced.) Examine freshly prepared tubes to determine that there are no gas bubbles in the Durham tubes.

4) Storage of media—Store all media under controlled conditions to maintain quality until expiration date. Dehydrated media are hygroscopic; avoid excessive humidity. 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 opened bottles of media preferably within 6 months. Immediately after use, close bottles as tightly as possible. Store opened bottles in desiccator, if available.

Prepare media in amounts that will be used within holding time limits given in Table 9020:V. Fresh medium is required to ensure that target microorganisms are isolated properly, especially bacteria stressed or injured during treatment. Protect laboratory- and commercially-prepared media containing dyes from light; if color changes, discard the media.

If prepared ready-to-use commercial medium has an expiration date later than that noted in Table 9020:V, have the manufacturer supply evidence of medium quality for that entire period. Verify usability weekly by testing recoveries with known densities of culture controls that will also meet QC check requirements.

Controlling moisture content is important because prolonged storage and subsequent dehydration may alter recovery and selectivity. 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 any poured agar plates not used on preparation day. To prevent dehydration, seal agar plates in plastic bags or other sealed container if they will be held >2 d. Store plates inverted to prevent condensation from falling on medium. If 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 liquid loss by weight or volume when stored for >2 weeks. If loss is 10% or more, discard batch. Discard all Petri dishes with solid media that have been stored for >2 weeks; discard earlier if they are dried out (e.g., wrinkled, cracked, or pitted).

If media are refrigerated, bring to room temperature before use and reject batch if growth or false-positive responses occur. Commercially prepared sterile broths and agars may offer advantages when analyses are done intermittently, staff is unavailable for preparation work, or cost can be balanced against other laboratory-operation factors. Check performance of these media as described in ¶s 5)–7) below.

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

6) Quality control of laboratory-prepared media—Compare new lots and previously acceptable ones [¶ 5) above] for their quantitative recoveries of 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, as well as monitor analyst technique. Maintain information in a bound book.

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

7) Quality control of commercially prepared media—Shipping this 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. However, the laboratory should perform its own enumerative test by challenging media with low numbers of an appropriate microorganism. Record receipt and expiration dates, lot number, and then measure and record medium used. Store as directed by manufacturer and discard by expiration date. Comparing quantitative recoveries with laboratory-prepared media, as directed in ¶ 5) above, is recommended. Test each new lot for sterility and with positive- and negative-control culture checks (suggested control organisms can be found in Table 9020:VI). If commercially prepared medium has a longer shelf life than the laboratory-prepared version, perform these tests more frequently.

6. Standard Operating Procedures (SOPs)[31–33]

The operational backbone of an analytical laboratory, generic and specific SOPs are designed to prevent deviations due to a misinterpreted process or method. Each specific SOP describes, step by step, the details of a routine task or procedure tailored to the laboratory's own equipment, instrumentation, and sample types. Such tasks include preparing reagents, reagent water, standards, and culture media; using balances properly; sterilizing media; washing items; disposing of contaminated material; collecting and analyzing samples; maintaining a chain of custody, keeping records, performing appropriate QC, and confirming that QC acceptance criteria are met. Simply citing a published analytical method is not an SOP, although that information can be included in the laboratory's own SOP.

SOPs are laboratory-specific, written by the person who does the work, and approved in writing by the supervisor (with the effective date indicated). Follow SOPs as written, keep them current via routine reviews, and have them accessible to all necessary personnel. When changes are needed, document them and have the supervisor approve the updated SOP. Keep a file of outdated SOPs for future reference, as needed. If maintained in electronic form (eFiles), SOPs may need to be password pro-

Table 9020:VI. Suggested Control Cultures for Microbiological Tests*

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

* Use appropriate ATCC strains. Note: Other cultures may be used.
- *Enterococcus faecalis* ATCC 11700
- *Enterococcus faecium* ATCC 6057
- *Enterobacter aerogenes* ATCC 13048
- *Escherichia coli* ATCC 11775 or 25922
- *Klebsiella pneumoniae* (thermotolerant) ATCC 13883 or 4352
- *Proteus vulgaris* ATCC 13315
- *Pseudomonas aeruginosa* ATCC 27853
- *Serratia marcesens* ATCC 14756
- *Staphylococcus aureus* ATCC 6538

† *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.

tected to prevent unauthorized changes. Also, the electronic system used to develop and store such files must be retained when no longer in use (e.g., when replaced by a new system).

Consistent use of SOPs helps ensure uniform operations. They also are an effective training tool and a means for determining competency when conducting an assessment.

7. Sampling

Although microbiology-laboratory personnel generally do not collect samples themselves, they need to be familiar with the sample-collection process.

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, depths, and points; the number of samples and analyses needed; workload; and supplies. For natural waters, their knowledge of probable microbial densities and the effects of season, weather, tide and wind patterns, known sources of pollution, and other variables is needed to formulate the most effective sampling plan. Microbiologists also 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: Sample-collection guidance generally addresses the factors that must be considered for each site. Sample-collection SOPs describe sampling equipment and its cleaning, techniques, frequency, handling, chain of custody, holding times and conditions, safety rules, etc., that will be used under various conditions at different sites to ensure sample integrity, the avoidance of cross-contamination, and representativeness. Use the information in these SOPs to draw up sample-collection plans, which must be site-specific and based on appropriate statistical sampling designs. Sample-collection techniques for detecting and recovering microorganisms should be validated.[34]

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. Sample-receipt information should include names or identifiers of both sampling site and sampler, turbidity, and date and time of sample collection. Sample-receipt records also must include date and time received, name or initials of individual accepting the sample, temperature of sample upon receipt, and any deficiencies noted (e.g., frozen, heated, or leaking samples). Note: The number of recoverable microorganisms can increase or decrease over time after sample collection.

d. Sample analysis: The laboratory is responsible for ensuring that analyses are initiated within an acceptable holding time.

8. Analytical Methods

The essential QC elements for microbiology laboratories are described in 9020A. Calibration and maintenance of equipment and supplies, and sterility tests are critical to the successful operation of an analytical method. Conduct appropriate QC checks with each batch or test run of samples. When a matrix changes and analysts anticipate that isolating a particular microorganism may be difficult, analyze matrix spike and matrix spike duplicate samples. This is particularly important for recreational

water programs. Ensure that documentation can successfully follow a sample from receipt in the laboratory to the final data report.

a. Method selection: Microbiological methods are used for a variety of matrices, including drinking water, municipal wastewaters, recreational waters, ground waters, marine waters, storm waters, and direct discharges. Some regulatory monitoring programs specify which analytical methods are approved for monitoring and they may differ for drinking water programs. Factors such as media compatibility with sample matrix, temperature, time at incubation temperature, and minor variations in techniques must be applied consistently to ensure appropriate microbial recovery for qualitative and quantitative determinations. Also, microbiological methods must be standardized so multiple laboratories produce uniform data. 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 multi-laboratory study and approved by regulatory authority if used for compliance monitoring with the sample types of interest. Validate any new method or nonstandard method that the laboratory intends to use, as well as any method being used for a matrix it was not specified for. See the discussion in 9020B.11.

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 there is a high potential for false-positive or -negative results that could affect water-quality decisions.

c. Internal QC: Published analytical methods must contain the required QC checks to ensure 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. These must be part of a laboratory's internal QC program with any additional internal requirements, such as the frequency of QC analyses and verification requirements for new sample types.

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

9. Analytical Quality Control Procedures for Established Methods[6–8,19,35]

In order to estimate uncertainty in analytical measurements, analysts must determine a method's repeatability, reproducibility, and false positive and negative rates. Therefore, replicate analyses, reference cultures, blanks (sterility tests), intra- and inter-laboratory tests and spiked samples become necessary.

General quality control procedures:

a. Colony-counting variability: For routine performance evaluations, analysts should repeat counts on one or more positive samples at least monthly and record results. Only one count is made during official sample testing. When comparing two analysts, each should count the same plate once. When comparing three or more analysts, use a statistical evaluation method. (See 9020B.13*b* for a statistical calculation of data precision.) Replicate counts by one analyst should agree within 5% (repeatability); counts made by two or more analysts should agree within 10% (reproducibility). If counts do not agree within the acceptable margin, determine why and correct as needed. Chart these results in a QC chart.

b. Positive and negative control cultures: Use certified reference cultures obtained from nationally or internationally recognized sources. The reference cultures must come from established commercial sources and be impregnated onto discs or strips or in liquid culture. Subculture the reference culture to develop one or more primary working stocks,[36] but make no more than five transfers (i.e., to a fresh medium to promote growth) from the original culture. Minimize subsequent transfers to ensure that working stocks retain phenotypic and genotypic identity and to reduce potential contamination. Test strains periodically to ensure their viability and that performance remains unchanged. If a laboratory lacks the facilities to maintain a pure culture, its personnel should use single-use culture strips or submit the relevant samples to another laboratory for testing. NOTE: Proficiency test (PT) samples [also called *performance evaluation (PE) samples*] are unknowns and should not be considered replacements for positive and negative culture controls.

For each lot of medium received, each laboratory-prepared batch of medium, and each lot of commercially prepared medium, verify appropriate response by testing with known positive and negative control cultures for the organism(s) under test. See Table 9020:VI for examples of test cultures. Record results.

c. Duplicate analyses[37,38]: Precision (repeatability) of quantitative analytical results when counting plate colonies is evaluated through replicate analyses. Note that the three dilutions are not to be considered replicates for the purpose of determining precision. Replicate analyses are particularly important when a laboratory or analyst is new to a method, or a method or matrix is expected to generate considerably variable results. The results can be charted in a control chart.

Perform duplicate analyses at least monthly, or more often as needed (e.g., 10% of samples when required by the analytical method or regulations, one sample per batch or test run, or one sample per week for a laboratory that conducts <10 tests/week). A *batch* or *test run* is defined as an uninterrupted series of analyses, generally 20 samples including appropriate QC controls. Evaluate and record results. An adequate sample volume is essential. Balance frequency of replicate analyses against the time, effort, and expense incurred. 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 to ensure that there is no potential interference, and record results. Incubate at least one aliquot per lot or a set percentage (e.g., 1 to 4%) of laboratory- and commercially-prepared medium, broth, or agar at an appropriate temperature for the same time period as the actual test (e.g., 24 to 48 h for coliforms) and observe for growth. For enzyme-defined substrate tests, check for sterility by adding a media packet to 100 mL sterile deionized water and incubating at 35°C for the time specified in the method. Certain granulated ready-to-use enzyme-substrate media may be free of coliforms but not sterile; using nonselective broth could result in growth and turbidity but should not produce a positive reaction when compared to the positive sample tube supplied by the vendor.

TABLE 9020:VII. CALCULATION OF PRECISION CRITERION

Sample No.	Duplicate Analyses		Decimal 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:

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

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

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

Check each new batch (or lot, if commercially prepared) of buffered water for sterility before first use by adding 50 mL of it to 50 mL of a double-strength non-selective 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 48 h and observe for growth. Record results.

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

Check sterility of process methodology as follows:

1) For each manifold used in membrane filter tests, check sterility of the entire process by using sterile dilution water as the sample at the beginning and end of each filtration series of samples and test for growth. If a processing interruption lasts >30 min, use new sterilized funnels and repeat sterility test. Record results.
2) For multiple-tube and presence–absence procedures, check sterility of prepared media and dilution water as outlined above.
3) For pour plate procedures, check sterility by pouring at least one uninoculated plate per batch or lot of media and record results.
4) If any contamination is indicated, determine the root cause. Invalidate analytical data from sample(s) tested. Document both cause/problem and corrective action taken. Request resampling. Laboratories interested in contaminant identification can use either standardized phenotypic testing systems or genotypic procedures.

e. Precision of quantitative methods[37,38]: For plate-based analyses [e.g., membrane filtration and some heterotrophic plate counts (HPC)], calculate precision of duplicate counts using the best dilution for reading each type of sample examined (e.g., drinking water, ambient water, or wastewater) according to the following procedure, and record results. NOTE: SimPlate for HPC does not require duplicate counts in the EPA-approved method.

1) Perform duplicate analyses on first 15 positive samples of each matrix type, with each set of duplicates analyzed by one analyst. 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). If more than one analyst regularly runs the tests, include them all, with each analyst performing an approximately equal number of tests.

2) 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 >3.27 R, there is >99% probability that the laboratory variability is excessive, so discard all analytical results since the last precision check (see Table 9020:VIII). Identify and resolve the analytical problem before making further analyses. If sample test results have already been reported, it may be impractical to discard all test results. Resampling may have already been performed.

3) Update by periodically repeating the procedures using the most recent sets of 15 duplicate results. Using software can make these calculations easier to handle.

10. Verification

Verification is a general process used to determine whether the method and the analyst are both performing as expected to provide reliable data (i.e., determining false-positive and false-negative rates). If the verification percentage for a certain water supply or matrix is low, either another test method or more training is needed. For the most part, the confirmation/verification procedures for drinking water differ from those for other waters because of specific regulatory requirements. Microorganisms often are defined via method or operation, not taxonomy. A false positive occurs when a positive well, fermentation tube, or colony counted as the target bacterium is transferred to a confirmation medium and has a negative result. A false negative is determined when atypical colonies or media from a negative well or fermentation tube gives a positive confirmation result. The following is a brief summary; further information may be

Table 9020:VIII. Daily Checks on Precision of Duplicate Counts*

	Duplicate Analyses		Logarithms of Counts			
Analyses	D_1	D_2	L_1	L_2	Range of Logarithms	Acceptance of Range†
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.

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.

If positive results have not normally occurred within a quarter, analyze at least one positive source-water sample to confirm that the media, laboratory procedures, and equipment produce appropriate responses (for both QC purposes and maintenance of analyst proficiency). 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 (e.g., 10% of positive samples, one sample per test run, or a certain percentage of normal laboratory workload). For large laboratories analyzing a significant number of samples daily, 10% of positive samples may be an unnecessary burden; choose an appropriate lower percentage.

2) Fecal streptococci and enterococci procedures—Verification can be performed as outlined in Section 9230C.5 at a frequency established by the laboratory.

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 Endo LES agar medium and verify as directed in Section 9222B. Also verify any positives for thermotolerant (fecal) coliforms as described in ¶ *b*2) below. Adjust counts based on percent verification. If there are no positive samples, test at least one known positive source-water sample quarterly or, if the laboratory is running positive and negative culture controls, consider that this confirms the analysts are competent to determine a positive sample result.

b) Other water types—Verify positives monthly by picking at least 10 typical and atypical colonies from a positive water sample, as directed in Section 9222B. Adjust counts based on percent verification.

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

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 directed in Section 9221E.1. Adjust counts based on percent verification. To determine false negatives, pick representative atypical colonies of different morphological types and verify as directed in Section 9221B.3.

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 indole test and the citrate test as described in Sections 9225D.4 and 7, or other equivalent identification procedures or systems. Incubate the indole test at 44.5°C. *E. coli* are indole-positive and yield no growth on citrate. Adjust counts according to verification percentage.

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

4) Fecal streptococci procedure—Monthly, pick at least 10 isolated red colonies from m-Enterococcus agar to brain–heart infusion (BHI) media and verify as described in Section 9230C.5. Adjust counts based on percent verification.

5) *Enterococcus* procedures—Monthly, pick at least 10 well-isolated pink to red colonies with black or reddish-brown precipitate from EIA agar. Transfer to BHI media and verify as described in Section 9230C.5. Adjust counts based on percent 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. A positive result is based on the presence and reaction of a specific enzyme, and these tests use a defined substrate with inhibitors for noncoliform bacterial growth. The following is a brief description for those who want to conduct verification testing.

For total coliform analyses, aseptically transfer material from a certain percentage (e.g., 5%) of enzyme-substrate-positive wells and enzyme-substrate-negative wells to M-Endo or Levine EMB or other suitable media. Streak for isolation. For confirmation, test for lactose fermentation (note that a number of coliforms can be either slow lactose fermenters or may not ferment lactose at all) or for β-D-galactosidase by the *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, no confirmation/verification is normally required; a positive result is based on the presence of a specific enzyme. Use of a comparator and negative culture control assists in the determination of a weak fluorescence. If verification is desired, aseptically transfer material from a certain percentage (e.g., 5%) of MUG-positive and MUG-negative wells to MacConkey or Levine EMB or other suitable media. Streak for isolation. Verify by confirming 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 verification percentage.

3) Simultaneous detection of total coliform bacteria and *E. coli*—Review the information in Section 9222J for the dual-chromogen MF procedure and Section 9222K for the flurogen/chromogen MF procedure. As noted above for *E. coli* analyses, verification typically is not required for drinking water samples; a positive result is based on the presence of a specific enzyme. For other water types, verify at the laboratory-established frequency based on need and sample type.

11. Validation of New or Nonstandard Methods[39–46]

The laboratory must validate all nonstandard methods, laboratory-developed methods, and standard methods applied to new test conditions (e.g., matrix) before using them to gather data. *Validation* is the process of demonstrating that a method, when properly performed, provides data that are accurate and reliable for their intended use. Although historically limited to the field of chemistry, validation now also applies to microbiology, using the same terms. The main difference is that when discrete variables (e.g., plate counts) are involved, analysts use different statistics and probability distributions.

For culture-based analyses, validation focuses on whether and how well a test method can detect and/or quantify a specific microorganism or group of microorganisms with set characteristics in the matrix of concern. For culture-independent methods (e.g., immunoassays and molecular genetic techniques), the same need exists to demonstrate process control and confidence in the information's reliability. This is essentially a proof of concept.

For compliance methods, obtain validation data from the manufacturer and/or regulator. Before adopting a new method, conduct parallel tests with the standard or reference procedure to determine the new method's suitability and to compare its performance to the standard's stated performance criteria. Obtain at least 30 positive data points over a period of time (e.g., 4 to 8 months) so analysts can statistically determine equivalence before replacing an established method with the new one for routine use. This can be called a *secondary* or *cross-validation*.

For methods in development (e.g., research methods), establish confidence in the analytical method by conducting full intralaboratory validation studies on a statistically significant number of samples in the applicable matrix or matrices to ensure reliability before making a final determination of usability. Conduct interlaboratory studies (also called *collaborative studies* or *round robin tests*) to validate the method for wider use. The following is a brief discussion of microbial method validation and the desired quality of performance criteria. Review the cited references for further information and for programs involved with microbial method validation.[39–46]

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 laboratory-prepared cell suspensions of the target microorganism from a reputable source. The supplier should provide third-party evidence of competence and compliance with global standards. Microorganisms should be traceable to a culture collection, which can be verified through a license agreement.

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, analysts would need an extremely large number of replicates to statistically evaluate comparability, so these data-quality indicators generally are not determined.
2) Specificity/selectivity—These indicators show how well a test method can preferentially select or distinguish target organisms from nontarget ones in the matrix of choice under normal laboratory sample-analysis conditions (i.e., a method's fitness for use). For qualitative methods, the indicator is growth of the target organism and is determined by verifying all responses (e.g., by microbial identification testing).
3) Detection limit—This indicator reveals the lowest microbial density that can be determined under the stated conditions. Analysts do this by using dilutions of reference cultures and measuring recoveries among replicates of each dilution.
4) Robustness—This indicator measures how well a test method can perform under changing conditions. This test is conducted by the method's initial developer; it is determined by changing variables (e.g., sample holding time or conditions, incubation temperature, medium pH, and incubation time) and determining how much the resulting data vary.
5) Repeatability—This indicator shows 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 enough responses can be detected[47] for either a quantitative or qualitative test. This can serve as one measure of uncertainty.

b. Quantitative test methods: Validation of a method 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—This indicator notes the degree of agreement, or lack of uncertainty, between the observed and true values. Accuracy is estimated by using known reference cultures at the anticipated range of environmental densities and then comparing the new method's results to those of the reference or standard method. It is usually expressed as the percentage of recovery.
2) Precision/repeatability—This indicator reveals 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 enough responses can be detected.[46] This can serve as one measure of uncertainty.

3) Precision/reproducibility—This indicator shows the degree of variability when the same method or process is conducted under changed conditions (e.g., more than one analyst following the method in another area or room in the laboratory and/or using different equipment). This serves as another measure of uncertainty.
4) Recovery/sensitivity—This indicator notes a test method's ability to recognize or detect the target microorganism or a component thereof in the matrix of choice. Determine by analyzing enough samples using at least two added suspension levels of the target microorganism or by increasing or decreasing the sample volume or dilution analyzed, followed by determination of statistical confidence.
5) Detection limit—This indicator shows the lowest microbial density that can be determined. Determine by using dilutions of reference cultures and measuring recovery among replicates of each dilution.
6) Upper counting limit—This indicator reveals the level at which quantitative measurements become unreliable (e.g., due to overcrowding of typical and atypical colonies, which may mask target organisms on an agar plate). Determine as above.
7) Range—This indicator notes 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. See 9020B.1.

b. Sampling records: A written SOP describing sample-handling records composed of the laboratory's procedures for sample collection, acceptance, transfer, storage, analyses, and disposal is necessary. Records associated with sample handling (i.e., chain-of-custody forms) should be completed for each sample entering the laboratory. Such records should be maintained long term, because it is critical that this record be exact and complete if there is any chance litigation may occur. Some federal or state programs may require chain-of-custody forms in order to ensure sample integrity. Details on chain-of-custody are available in Section 1060B.2 and elsewhere.[1] A laboratory system that uniquely identifies samples in 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, medium and temperature used to conduct the test, date and time analyses were initiated and completed, QC results, 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 appropriate analyst(s) and supervisor(s). The preferable record form is a bound and page-numbered notebook, with entries in ink, or a computer file (e.g., an e-notebook). Any change will be indicated by a single line drawn through the original text, the corrected text inserted adjacently, with the date of change and the recorder's initials next to the correction. Keep records of microbiological analyses for at least 5 years in a secure location. Offsite 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 so long as the following information is included:

- date, place, and time of sampling;
- name of sample collector;
- sample identification;
- 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. Also, verify that no errors occurred when copying the data to the LIMS. Back up all laboratory data on disk or hard-copy system to meet the customer and laboratory needs for both data management and reporting. Verify data on the printouts. Always back up electronic data by protected tape or disk or hard copy.[45] 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.[48–50]

13. Data Handling

a. Distribution of bacterial populations: Microbiological data can have wide uncertainty ranges due to non-homogeneous samples and bacteria's variable growth characteristics. In most chemical analyses, the distribution of analytical results follows a normal (Gaussian) curve, which has a symmetrical distribution of values about the mean (see Section 1010B). Microbial distributions, on the other hand, are not necessarily symmetrical and rarely fit a normal distribution curve. Bacterial counts often have a skewed distribution due to many low values and a few high ones, leading to an arithmetic mean that is considerably higher than the median. The frequency curve of this distribution has a long right tail (see Figure 9020:1), which is referred to as *positive skewness*.

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

The microorganism distribution in a sample may be natural and unique to the sample and matrix, rather than a function of laboratory performance.[51] Also, microbial counts represent colony-forming units (CFUs), which may have resulted from one or more bacterial or fungal cells or filaments,[52] leading to variations in colony counts in replicate plates or multiple dilutions. In addition, the number of CFUs on the agar surface depends on the type of medium used, its growth potential, and incubation conditions. Simply using the same medium produced by different manufacturers may result in different colony counts.

The more common statistical techniques assume data symmetry (e.g., the normal distribution), so skewed data usually must be converted to a more symmetrical distribution before such techniques can be applied. An approximately normal distribution may be obtained from positively skewed data by converting numbers to their decimal logarithms, as shown in Table 9020:IX. A 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: Analysts use two calculations to determine the central tendency (if any) of microbiological data: Poisson distributions and geometric means. A Poisson distribution indicates the likelihood of observing the organism(s) of interest, and the geometric mean indicates the most likely number of such organism(s) to be found in a given sample.

A multiple Poisson distribution indicates the probability of observing organisms via multiple dilutions.[53,54] The resulting curve appears skewed to the right, much like a log-normal distribution curve, because individual Poisson distribution curves indicate colony counts for different organisms—including those not of interest, which further skew the overall distribution curve. When the maximum likelihood approach[55,56] is used, the maxima of these organisms are spread out under the overall distribution curve because different organisms respond differently to the same nutrients, media, temperature, pH, and incubation time. Analysts should study the maximum-frequency data to ensure that they select the correct organism for colony counting.

When analysts examine the most probable number (MPN) curves for 1, 2, 3, and 4 positive tubes out of 5 total tubes incubated, the log-normal probability graph is close to being linear (thus indicating approximate normality) but bows upward. The bowing could indicate kurtosis (a sharpness) brought about by measuring the cumulative probability on the low and high ends of the distribution curve, which is difficult to do and, therefore, more error-prone. The log-normal probability assumption is confirmed when analysts plot the log of values against colony-count MPN on log-normal–cumulative probability graph paper.

The geometric mean best estimates the central tendency of log-normal data; it is used when a probability distribution is anticipated. The term *mean* in geometric mean is misleading; what a geometric mean determines is the maximum likelihood estimate, which is based on the mode (maximum frequency) of the distribution curve (i.e., both frequency of n observations and the count of a random sample on n observations). It is calculated as the nth root of the product of all the data values.[57]

The geometric mean of the maximum likelihood estimates is a better estimate than the arithmetic average for living organisms because the geometric mean considers both frequency and variability in colony counts. When deriving the maximum likelihood[58] for a Poisson probability distribution, the log of the products of MPN can be shown to be a function of the log of frequency, thereby justifying the use of geometric mean. The geometric mean is the log of the inverse of the average log of likelihoods of the measured parameter. This value is generally lower than the arithmetic average of MPNs.[58]

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.[57] By means of the log-likelihood ratio, product properties are converted into summation properties, which are easy to understand and deal with.

c. "Less than" (<) values: There has always been uncertainty as to the proper way to include "less than" values when calculating and evaluating 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

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

assigning the "less than" value the value itself (i.e., changing <1 values to 0, 1/2, or 1, respectively).[59–61]

There are valid reasons for not including "less than" values, whether modified or not. If the database is fairly large and contains few such values, then their influence would be minimal and of no benefit. If the database is small or contains a relatively large number of "less than" values, then they would exert an undue influence and could artificially bias results either negatively or positively. 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 (e.g., 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 publications[1–6] and organizations* address interlaboratory programs.

A *certification program* is one in which an independent authority issues a written assurance or certificate that a laboratory is managed in compliance with that authority's standards. An *accreditation program* is one in which a specialized accredita-

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

tion body sets standards and then a certification body determines whether the laboratory exhibits competence in following the standards. If so, the laboratory 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 help laboratories address continual-improvement efforts.

2. Uniform Criteria

Interlaboratory QC 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 with either common interests or common regulatory requirements. Often one group or person may agree to draft the criteria. If the participants are regulated, the regulator may set the criteria for compliance-monitoring analyses.

Uniform sampling and analytical methods and QC 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 managers, supervisors, and technical staff are described in 9020A.2. In large laboratories, the QA officer is a staff position, but a supervisor or other senior person may assume the role in smaller laboratories.

Once the QA program has been incorporated into laboratory operations and confirmed to be in routine use, the laboratory supervisor and QA officer jointly conduct an internal program review of all operations and records for acceptability, to identify possible problems and help resolve them. 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, managers inform the certifying or accrediting organization and request an external quality assessment. The choice of assessor and type of assessment will depend on a number of variables, such as accreditation request(s) and whether the sample analyses 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 can generate valid, defensible data. Such onsite evaluations are periodic and may be announced or unannounced.

4. External Proficiency Testing

Laboratories applying for certification or accreditation must participate in routine proficiency testing for the analytical, technological, or matrix-specific procedures that they intend to use. On a set schedule, the accrediting authorities send challenge samples (unknowns) to the laboratories for analyses. Each unknown must be processed as a routine sample by the analyst who typically runs the related method, and the results are reported back for evaluation. The certifier/accreditor codes the results for confidentiality, evaluates them according to an agreed-upon scheme, and summarizes them for all laboratories. Each participant then receives an individual report that indicates how well its personnel conduct routine analyses compared to the rest of the group. Also, the overall group's results characterize the performance that can be expected for each analytical method tested. Failure to evaluate unknowns successfully can result in loss of certification/accreditation.

Laboratories not applying for certification/accreditation can purchase unknowns for their own use.

5. Maintenance

After passing an external evaluation and analyzing a set number of unknowns successfully, the laboratory will be formally notified that it has been certified/accredited. To maintain this status, the laboratory must continue to analyze proficiency-testing samples successfully on an annual or semi-annual basis (established by the certifier/accreditor) and pass an onsite assessment about once every 3 years.

6. Example Programs

In the U.S. Drinking Water Laboratory Certification Program, public water supply laboratories must be certified according to minimal criteria, procedures, and QA described in the EPA manual on certification:

- criteria are established for laboratory operations and methodology;
- the certifying state agency or its surrogate must conduct onsite inspections to verify that such criteria are met;
- laboratories must perform acceptably on annual proficiency tests; and
- if problems are identified during inspections or proficiency testing, the certifying state agency must follow up and require corrections within a set timeframe.

Individual state programs may exceed federal criteria.

In addition, there are several Clean Water Act (CWA) programs that monitor recreational water quality, assess impaired waters, and develop total maximum daily loads (TMDLs) for discharges through the National Pollutant Discharge Elimination System (NPDES). The CWA program also requires laboratory certification through either state programs or the National Laboratory Accreditation Institute (TNI). To maintain accreditation by TNI, laboratories must have performed acceptably during two of the last three proficiency tests and successfully pass routine onsite assessments.

Previous onsite inspections of drinking water laboratories indicate that the primary causes of discrepancies have been inadequate equipment, improperly prepared media, incorrect analytical procedures, and insufficiently trained personnel.

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