9020

QUALITY ASSURANCE/QUALITY CONTROL

Revised, 2022.

9020 A. INTRODUCTION

1. General Considerations

A quality management system (QS) for microbiological analyses establishes 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;
- ensure compliance with regulatory requirements,
- fulfill customer and project quality objectives and requirements, and
- adhere to applicable standards of accreditation or certification.

The laboratory practices in Section 9020 represent best practices for stand-alone (brick and mortar) and mobile laboratories. These practices may be required by regulations, standard-setting organizations, and laboratory certification or accreditation programs.

Each laboratory develops and documents its QS policies and objectives in a quality management plan or quality manual. The document clearly defines responsibilities and duties to ensure that the data generated are the required type, quality, and quantity.

Staff typically spend about 15% of overall laboratory time on the various aspects of an established QA program. More time may be needed for analyzing and reporting crucial analytical data (e.g., data for enforcement actions). When properly administered, balanced, and conscientiously applied, a QS optimizes data quality, identifies problems early, and increases 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.

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,
- ensure the laboratory follows necessary safety requirements to prevent exposures
- systematically review laboratory functions, analytical results and analyst training records, and
- assume a leadership role by establishing specific responsibilities for all staff, thereby ensuring that all staff are aware of, and implement, a comprehensive program.

Although 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, activities performed by the laboratory staff, and the satisfaction of the customer or end-user.

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 with the authority and operational independence necessary to succeed. The QA officer preferably

- has coursework, job experience, or specialized training in microbiological testing;
- is acquainted with all aspects of laboratory work;
- is aware of and familiar with the laboratory's QA program and QC practices; and
- is familiar with statistical techniques for evaluating data.

The QA officer is responsible for

- implementing the QA program
- providing necessary technical support and training where needed
- signing off on all standard operating procedures (SOPs),
- ensuring that documents are updated routinely,
- conducting frequent (weekly to monthly) reviews (along with frequent audits) with laboratory management and staff to ensure that the program is being followed correctly and to resolve any problems that may arise, and
- reporting 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.

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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 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

The QS manual 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 or operator.

The plan should address the following:

a. Quality policy statement, which describes the specific QS objectives, includes an ethics statement, and notes the commitment of laboratory staff and management 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 indicate specific qualifications, training requirements, and job responsibilities for all analysts and supervisors.

d. Equipment and instrument requirements, which include a list of critical equipment and instruments available (including their serial 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 note 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 establish standards for the laboratory's oversight and acceptance of products.

g. Sampling procedures (if performed by the laboratory) and sample-acceptance criteria, which describe 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 list the laboratory's scope for testing, its validation procedures for nonstandard or new methods, the accreditation or certification status for individual methods and analytes, and the requirements for initial and ongoing demonstrations of capability.

i. Analytical quality control measures, which state 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, 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 the actual practices used by the laboratory; are signed by management, 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 identify the recordkeeping formats (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 describe 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, laboratory information management system (LIMS), and reporting.

2) Onsite evaluations by certification or accreditation agencies to ensure that the laboratory and its personnel are following an acceptable QA program.

3) Proficiency test (PT) studies, in which the laboratory generally participates once or twice a year. These collaborative studies confirm the laboratory's ability to generate acceptable data comparable to both the reference laboratory and other participants. They also 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 multistep 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 9020 B 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

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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 US Environmental Protection Agency (EPA).

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9020 B. Intralaboratory Quality Control Guidelines

Quality control practices are designed to ensure that the laboratory's processes are under control and to mitigate uncertainty around the laboratory's measurements. Given the uncertainty surrounding the detection of living organisms, microbiology laboratories have additional QC not required by other analytical laboratories. QC activities are 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. Key QC practices are listed in Table 9020:1 and discussed below. Ideally, laboratories address all of the QC guidelines discussed herein, as appropriate, depending on the methodologies used by the laboratory. In addition to the QC criteria described here, laboratories must ensure they are meeting the QC required by their regulatory or certification (accreditation) authority. Additional sources of information about laboratory QC practices are available.[1-10]

1. Personnel

Individuals performing microbiological testing require an appropriate level of training and laboratory bench experience in general microbiological techniques. If trained personnel are unavailable, a professional microbiologist (one with college level coursework or degree in microbiology) must provide training and supervision.

Proper microbiological training includes microbiological theories and practices across the many techniques performed in a full-service microbiology laboratory. The procedural descriptions detailed in *Standard Methods* rely on the notion that the users have a thorough understanding of microbiological theories and practices. 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 demonstration of capability; DOC) and periodically thereafter (ongoing DOC) using blind samples (preferred) or known positive samples.

The analyst's skill must be routinely observed, evaluated and documented by the supervisor to demonstrate ongoing proficiency with laboratory practices.

Management must ensure laboratory personnel have appropriate education and assist them in obtaining ongoing additional training and course work to enhance their technical skills and, in turn, advance their careers. Employee training records and performance scores obtained by analyzing single blind samples, especially for enumeration methods, and DOCs must be reviewed, monitored and maintained.

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Table 9020:1. Key Quality Control Practices

Item	Action	Frequency	Further Information in Section 9020 B
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	Monthly, preferably	
	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 9050 C.1*a*
Freezer	Check temperature	Daily	4*j*
	Defrost	Annually	
Glassware	Inspect for cleanliness, chips, and etching	Each use	5*a*
	Check pH with bromothymol 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	
Micropipettors	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*
Multiwell 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-grade water	Monitor quality	See Table 9020:2	…
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	Monitor bulb use	Each use	4*l*
Disinfection	Test with UV meter or perform plate count check	Quarterly	
Weights:			4*b*
Working	Check with reference weights	Monthly	
Reference	Recertify	Every 5 years	

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2. Biosafety

Biosafety is the application of safety precautions that reduces laboratory staff members' risk of exposure to a potentially infectious microbe and limit contamination of the work environment and, ultimately, the community.

The US Centers for Disease Control and Prevention (CDC) Public Health Prevention Service classifies laboratories handling potentially hazardous biological agents into 4 biosafety levels (BSLs), which are described in the Biosafety in Microbiological and Biomedical Laboratories (BMBL).[11] Each BSL defines specific controls (such as laboratory facilities, practices, personal protective equipment (PPE), and safety equipment) for containment of microbes and biological agents. Factors that are considered in the determination of the BSLs are infectivity, severity of disease, and transmissibility. Each BSL level builds on the level before it.

Every microbiological laboratory, regardless of BSL status must follow standards that are considered to be standard microbiological practices. These include (for full listing see BMBL):

1) Institutional policies that control access to the laboratory.
2) Persons must wash their hands after working with potentially hazardous materials and before leaving the laboratory.
3) Eating, drinking, smoking, handling contact lenses, applying cosmetics, and storing food for human consumption are not permitted in laboratory areas.
4) Mouth pipetting is prohibited; mechanical pipetting devices must be used.
5) Policies for the safe handling of sharps, such as needles, scalpels, pipets, and broken glassware must be developed and implemented. Precautions, including those listed below, must always be taken with sharp items.
    a. Careful management of needles and other sharps. Needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal.
    b. Used disposable needles and syringes must be carefully placed in conveniently located puncture-resistant containers used for sharps disposal.
    c. Nondisposable sharps must be placed in a hard walled container for transport to a processing area for decontamination, preferably by autoclaving.
    d. Broken glassware must not be handled directly. Instead, it must be removed using a brush and dustpan, tongs, or forceps. Plastic ware should be substituted for glassware whenever possible.
6) Procedures must be performed so as to minimize the creation of splashes and aerosols.
7) Decontaminate work surfaces after completion of work and after any spill or splash of potentially infectious material with appropriate disinfectant.
8) Decontaminate all cultures, stocks, and other potentially infectious materials before disposal using an effective method.
9) A sign incorporating the universal biohazard symbol must be posted at the entrance to the laboratory when infectious agents are present.
10) An effective integrated pest management program is required.
11) The laboratory supervisor must ensure that laboratory personnel receive appropriate training regarding their duties, the necessary precautions to prevent exposures, and exposure evaluation procedures. Personnel must receive annual updates or additional training when procedural or policy changes occur.

Personnel in microbiology laboratories, regardless of BSL level, use personal protective equipment (PPE) and safety equipment and implement measures to ensure proper containment of biohazardous materials.[11]

1) Use protective laboratory coats, gowns, or uniforms to prevent contamination of personal clothing.
2) Wear protective eyewear when conducting procedures that have the potential to create splashes of microorganisms or other hazardous materials. Persons who wear contact lenses in laboratories must also wear eye protection.
3) Wear gloves to protect hands from exposure to hazardous materials. Base glove selection on an appropriate risk assessment. Wash hands before leaving the laboratory.
    a. Change gloves when contaminated, when glove integrity is compromised, or when otherwise necessary.
    b. Remove gloves and wash hands when work with hazardous materials has been completed and before leaving the laboratory.
    c. Do not wash or reuse disposable gloves. Dispose of used gloves with other contaminated laboratory waste. Hand washing protocols must be rigorously followed.
    d. Laboratories should have doors for access control.
    e. Laboratories must have a sink for hand washing.
    f. The laboratory should be designed so that it can be easily cleaned. Carpets and rugs in laboratories are not appropriate.
    g. Laboratory furniture must be capable of supporting anticipated loads and uses. Spaces between benches, cabinets, and equipment should be accessible for cleaning.
        i. Bench tops must be impervious to water and resistant to heat, organic solvents, acids, alkalis, and other chemicals.
        ii. Chairs used in laboratory work must be covered with a nonporous material that can be easily cleaned and decontaminated with appropriate disinfectant.
    h. Laboratory windows that open to the exterior should be fitted with screens.

A brief discussion of each BSL follows; however, detailed information on special practices, containment, and facilities is included in the BMBL. Because *Standard Methods* does not address BSL-3 and BSL-4 agents, requirements for these laboratories are not included here. For further information and more complete details on all BSLs, review the BMBL.[11]

a. Biosafety level 1 (BSL-1): 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 may be handled under BSL-1 practices include total and thermotolerant

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(fecal) coliform bacteria, *E. coli* (some pathogenic strains, such as O157:H7, require higher BSL levels), enterococci, iron and sulfur bacteria, actinomycetes, and other nonpathogenic microorganisms. The laboratory director determines which biosafety practices to follow based on the samples and agents involved.

b. Biosafety level 2 (BSL-2): BSL-2 is suitable for 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 the laboratory personnel

- have specific training in handling pathogenic agents and are supervised by scientists competent in handling infectious agents and associated procedures;
- restrict access to the laboratory when work is in progress; and
- conduct all procedures that could create infectious aerosols or splashes in biological safety cabinets (BSC).

BSCs are designed to protect microbiologists from microbial contaminants in samples. If available, appropriate immunizations should be given.

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.

a. Ventilation: Design and construct well-ventilated laboratories that can be kept free of dust, drafts, and extreme temperature changes. Properly installed heating, ventilation, and air-conditioning (HVAC) and humidity-control systems 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 there is a negative air pressure in the laboratory (air is pulled into the laboratory from surrounding areas); this mitigates the possibility of contaminants becoming airborne and 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 only compatible functions are performed in the same laboratory workspace.

Ensure sufficient workspace for the volume of work. For example, maintain separate work areas for

- sample receipt;
- media, glassware, and equipment preparation and sterilization,
- 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 preferred. 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 space in the laboratory to store materials (e.g., reagents, glassware, and laboratory supplies) appropriately.

If the laboratory plans to conduct PCR or other nucleic acid amplification procedures, the laboratory should consider implementing unidirectional workflow, where analysts move through areas dedicated to each step of the procedure to minimize the risk of cross contamination by amplified nucleic acid.[9]

c. Laboratory bench areas: Ideally, 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 in height from 90 to 97 cm (35 to 38 in.) and 70 to 76 cm (27 to 30 in.) deep. For sit-down activities, such as microscopy and plate counting, benches may range from a height of 75 to 80 cm (29 to 32 in.). Laboratory benchtops must be 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. Ensure that there are no shadows over the work area and that analysts have sufficient light to accurately view test reactions and discern test results.

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. Air vents must be kept free of rust, dust, and mold.

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, water baths, and refrigerators. Avoid buildup of water in refrigerator drip pans, and clean all vent filters. Avoid allowing dust to accumulate on surfaces.

Monitor air quality routinely, at least monthly or more frequently if an 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 15 colonies/plate/15 min exposure, or 1 colony-forming unit (CFU) per minute. Longer exposure times can be used, but water loss from media 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.

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f. Laboratory cleanliness: Regularly clean laboratory rooms and wash benches, shelves, floors, windows, overhead lights, exposed pipe surfaces, and air vents. Wet-mop floors and treat with a disinfectant solution weekly; do not sweep or dry-mop. Ensure that disinfectants used in the laboratory are adequate for the organisms present. Products designed for home use may not be appropriate for use in the laboratory. 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 9020 B.2). Do not permit the 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 a touchless sensor dispenser) and paper towels (touchless paper roll dispenser preferably) available at laboratory sinks. Do not allow smoking or consumption of food or drink in the laboratory. Handle and dispose of contaminated 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. By constant monitoring, routine maintenance, and a regular calibration schedule, verify 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 9020 B.6). Keep manufacturers' manuals available for easy retrieval. Perform equipment standardization and 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, 3-ring binders, or electronic records, and maintain documentation so it is accessible for the time period mandated by law. Develop a system for noting 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 are ideally 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 are 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. All files must be available for review by auditors, despite the format used.

Use the following QC procedures for 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 these thermometers due to environmental concerns about mercury's neurotoxicity. Review established thermometer guidance.[13]

Thermometers filled with organic fluids (e.g., ethanol) are available for use, with the temperature range determined by the boiling point of the liquid contained in the thermometer. Generally, these thermometers lack the accuracy of mercury-containing thermometers, but they may be used where less accuracy is acceptable.

Thermometer graduation markings must be legible and the fluid in the liquid column must not have any breaks. The thermometer must not have any cracks or breaks. The column of glass thermometers must be examined annually and any changes noted (glass is an amorphic solid and changes may occur over time). Discard if there are any changes that affect the thermometer's ability to be properly used.

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 the thermometer bulb in water or glycerol. When testing temperatures exceed 50 °C (e.g., autoclave spore check or hot air ovens), place the thermometer bulb in a glass container filled with sand.

Many laboratories have begun to use electronic temperature sensors that record data over time that have built-in digital processors called *digital data loggers*. These data loggers have electronically transferable data capture, allowing the lab to quickly and easily see the temperature data on any device.

There are generally 3 types of sensors used in electronic thermometers: platinum resistance sensors, thermistors, and thermocouples. The platinum resistance thermometers (PRTs) generally have the greatest accuracy and the largest range of temperatures for use.

These electronic temperature sensing devices are acceptable for use and some can even be used in harsh environments (e.g., autoclave). Many types of electronic temperature sensing devices are available and although each can be connected to a computer to download the temperature data to a digital file, many are Wi-Fi enabled and can transmit to a server or cloud, making the temperature data easily and instantaneously accessible. Some units are also radio frequency enabled for use when Wi-Fi isn't available (e.g., in remote areas or during emergencies involving power outages).

The programs used to capture the temperature data from the sensor can be set to record temperatures at any time frequency. Reading temperatures frequently can provide a much better understanding of temperature fluctuations in the laboratory environments and facilitates more stable temperature environments. When using the electronic temperature sensing devices, the laboratory must establish a system for frequently checking readings so the analysts are aware of temperature violations shortly after they occur; can invalidate test samples, as appropriate; and can collect new samples.

The electronic temperature monitors must meet the same requirements as traditional thermometers. Establish a system for

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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 and temperature readings can be used to trace a sample and its testing conditions during laboratory assessments. Any records generated using these electronic devices must be made available to an auditor (as traditional log books would be) for assessment of conformity to applicable standards.

Many laboratories use infrared (IR) thermometers to measure the temperature of water samples. When using these, the temperature of the container is actually being measured, not the temperature of the contents. For that reason, it is critical that the procedure followed when using IR thermometers be consistent to ensure accurate temperature measurements.

The manufacturer's instructions for how far to hold the device from the item to be measured is specific to the type of unit. The IR thermometer must be consistently used at the same distance (some have suggested attaching a ruler to the IR thermometer to ensure the same distance is consistently used) to ensure the readings are accurate.

Verify the IR device at least every 6 months using a reference thermometer over the full temperature range that will be used. This includes ambient (20-30 °C), iced (4 °C) and frozen (0 to −5 °C) temperatures. Each day of use make a single check of the IR by checking the temperature of a bottle of water at the temperature of interest that contains a verified thermometer. If the two do not agree within 0.5 °C, recalibrate the device. Annually, or preferably semiannually, verify the accuracy of all working thermometers (e.g., liquid-in-glass thermometers, electronic temperature sensing devices, and temperature-recording instruments) at use temperatures. To do this, compare each device's measurements to those of a reference device that is traceable to the International System of Units (SI) through a national metrology institute. Discard temperature-sensing devices that differ by >1 °C from the reference device.

The laboratory must record in a QC record book the following information:

- serial number of laboratory thermometer
- serial number of SI-traceable thermometer (or other reference thermometer)
- temperature of laboratory thermometer
- temperature of SI-traceable thermometer (or other reference thermometer)
- correction factor and adjusted (corrected) temperature
- date of verification
- analyst's initials

If using a traditional (liquid-in-glass) thermometer as a reference thermometer, perform an annual ice point verification to ensure that there were no changes to the thermometer column. Maintain records showing the verification was done and record the ice point in a log book for use when verifying the laboratory's working thermometers.

To conduct an ice point verification, suspend the reference thermometer in an insulated container containing an ice and water slurry. Use ice prepared from reagent-grade water to ensure there are no additives that could result in temperature inconsistencies. Break the ice into small pieces to ensure consistent temperature and so it does not interfere with the set up. The bulb must not touch the bottom of the container or any other surface. Read the ice point (or 0.0 °C) mark after a few moments. Record the temperature after there is no change in the reading for 3 to 5 minutes.

If the ice-point temperature determination does not match the 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).

When performing annual thermometer verifications, suspend the reference thermometer alongside the test thermometer (thermometer being verified) in a container at the temperature of use. Styrofoam works to suspend the thermometers and can also be used to insulate and help preserve temperature consistency. The thermometer bulbs must be adjacent and not touching any surface. Use a stir bar to gently stir the water to ensure an even temperature environment. Record the temperatures of the two thermometers when they have reached the environment temperature and there is no change for 3 to 5 minutes.

When verifying the temperature of thermometers to be used at ambient temperatures, ensure that the water has come to equilibrium before testing.

Perform 3-point verification testing (at, below, and above the temperature at which the temperature-sensing device will be used). Record verification results, the date, device identification number, and the analyst's signature or initials. If a correction calculation is necessary, mark the appropriate correction factor on the device so only corrected temperature values are recorded. For example, if a thermometer reads 0.2 °C lower than the reference thermometer, mark the device with a +0.2 so staff know to add 0.2 °C to each reading.

If electronic sensing temperature devices are used, check with the manufacturer to gauge how often the battery needs replacing. Document the procedure for how temperatures will be recorded while the unit is being serviced. Some manufacturers recommend replacement (to mitigate fears of contamination that could occur when sending the unit off for repair), while other manufacturers allow the unit to be returned to them for temperature calibration, certification, and battery replacement.

Verify the accuracy of certified reference temperature-sensing devices as often as specified on the certificate of accuracy or at least once every 5 years.

b. Balances and weights: Locate balances in areas without rapid air movement (e.g., drafts) and level them on firm, even surfaces to prevent vibrations. Re-level a balance each time it is moved to a new location.

Each day the balance is used, perform a verification using reference masses at approximately the same nominal mass to be determined. Perform verifications each weighing session unless it can be shown that fluctuations in the environment do not affect the calibration. Before each use, clean the balance and tare it before adding reagents to weigh paper or boats. Clean balance pans after use and wipe spills up immediately with a laboratory tissue. Follow the manufacturer's instructions for operation and routine maintenance of analytical and top-loading balances. Use balances that provide a sensitivity of at least 0.1 g for a load of 150 g, and 1 mg for a load of 10 g or less.

Use only plastic-tip forceps or cotton gloves to handle weights. Check working weights monthly for accuracy, precision, and linearity against a set of reference weights of known tolerance[14] (e.g., ASTM Class 1, 2, or 3, accompanied by a verification certificate). Record results, along with correction factors, the date

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and analyst's initials in a log book. The correction factors must be on file and used.

If the weights are corroded or dropped, have them professionally cleaned and recertified or replace them. If a laboratory does not wish to have two sets of weights, they can use a single set of weights and have them certified annually.

Service balances annually, or more often as conditions change or problems occur, through service contracts. Recertify reference weights as often as specified in the calibration certificate, or at least once every 5 years.[15,16]

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 the pH of solid agar media. Measure a 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 electrodes immersed in the manufacturer-recommended solution. Record the date the electrode is put into service.

Use only commercial buffer standard solutions for calibrations, and standardize the pH meter with at least 2 certified pH buffers (usually pH 4 and 7 or pH 7 and 10) that bracket the pH of the sample being measured (2-point standardization). Record the standardization results, date, and analyst's initials. Immediately after use, discard the buffer-solution portions or single-use, ready-to-use solution packets used to standardize the 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 the bottle and in the logbook; thereafter, check an aliquot of the bottled solution monthly against another pH meter, if possible. Replace the pH buffers by the expiration date marked on the container because the solutions may absorb carbon dioxide. A pH 10 buffer is especially susceptible to degradation; minimize its exposure to air. If practical, purchase this buffer in small quantities.

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:

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

If the slope is <95% or >105%, the electrode or meter may need maintenance. If all 3 buffers are used in sequence to standardize the meter (3-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 the 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. These 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 automatically regenerate the ion-exchange resins.

Do not store laboratory-prepared reagent water unless a commercial 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 (See Table 1080:2, but note that the specification for SiO_2 content is not required for Part 9000 methods.) Every day that 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, or endotoxins that can be nutrient sources for bacteria.

At least once a year, analyze reagent water for trace metals. If a laboratory's reagent water does not meet the requirements for medium quality water (as described in Section 1080 C), the bacteriological water quality test (described in 9020 B.5*f*1) must be performed annually. The bacteriological water quality test is also performed whenever the water-purification system is modified or repaired.[17] For systems meeting the requirements for medium quality water, perform the use test (see 9020 B.5*f*2) whenever there is a new source of water, the water purification system is repaired (including cartridge changes) or for a new water system in the laboratory.

Replace cartridges at manufacturer-recommended intervals based on the estimated usage and source water quality. Do not wait for column failure. Record any maintenance (such as the replacement of consumables) or service performed in a logbook. 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 the water in a sterile container. Monitor treated water for contamination and replace filters as necessary.

e. Water still: Water distillation units are capable of producing high quality water, but the unit must be properly maintained and cleaned to prevent deterioration from corrosion, leaching, and fouling. Drain and clean the still and reservoir according to manufacturer's instructions and usage. To reduce cleaning frequency, use softened water as the source water.

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 absorbs NH_3 and carbon dioxide (CO_2) from the air.

f. Mechanical media-dispensing apparatus: Check apparatus' accuracy by dispensing a sample volume of medium into a graduated cylinder just after filling or refilling and periodically throughout extended runs; record the results. Before dispensing medium for sample analyses, flush the apparatus with a small volume of medium to ensure that evaporation has not blocked the tip or changed the concentration of the reagent. Between runs, rinse the apparatus by pumping hot reagent-grade water through it. Correct leaks, loose connections, or malfunctions immediately. At the end of use, break the apparatus down into parts, wash, rinse with reagent-grade water, and dry. Lubricate parts according to the manufacturer's instructions or at least once per month.

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g. Hot-air sterilizing oven: Ovens must maintain a uniform temperature of 170 °C or greater for 2 hours, with a temperature tolerance of 10 °C (160 to 180 °C). Record the date, contents, sterilization time, temperature, and analyst's initials for each cycle.

Test oven 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 the oven temperature with a thermometer whose bulb is placed in sand, an electronic temperature data logger, 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: Typically autoclaves have an internal heat source, a temperature gauge with a sensor on the exhaust, a pressure gauge, and an operational safety valve. The autoclave must maintain a sterilization temperature during the sterilizing cycle and complete an entire cycle (i.e., time between starting the autoclave and removing items from the autoclave) within 45 minutes when a 12- to 15-minute sterilization period is used. It should depressurize slowly enough to ensure that media do not boil over and bubbles do not form in inverted tubes. 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), because sterilization only occurs when air is displaced by steam, which cannot effectively occur in an overcrowded autoclave. For each run cycle, record:

- the date
- the contents of the load
- sterilization time and temperature (might be maximum temperature reached if using maximum temperature registering device)
- total run time
- programmed or preset sterilization time
- actual pressure readings, and
- analyst initials[18]

Newer autoclaves may print most of this information on tape automatically (i.e., time, temperature, date, and pressure at selected time interval). The printouts can be used as a record of the cycle if the required information is included. For older units, if possible, use a recording thermometer chart or electronic high-temperature data logger. Copies of the service contract or internal maintenance protocol and maintenance records must be kept. Conduct maintenance at least annually. Retain a record of the most recent service performed on file, available for inspection.

In general, an effective sterilization cycle is one in which an autoclave maintains 121 °C (with minimal temperature variation) at ≥15 lb/in.^{2} (≥103 kPa) for at least 15 min. Although the time required for an effective sterilization cycle may vary due to a number of factors (e.g., size of load, type of load, cycle used), the temperature necessary to have an effective autoclave cycle is generally considered to be 119 to 123 °C (at 200 kPa). As steam is added to an autoclave, temperature fluctuations occur that may exceed 123 °C. Such brief temperature fluctuations are expected, but the autoclave must not maintain an elevated temperature for extended times.

Autoclave temperature control tolerances may vary, depending on the nature of the media being sterilized. Although higher temperatures (≥121 °C) are acceptable for decontaminating laboratory materials, they are not acceptable for media sterilization because the exposure of media to consistently high temperatures can cause the degradation of medium components. Ideally, keep the temperature within ±2 °C of the prescribed temperature for media (and ±10 kPa of recommended P). Generally, when autoclaving lactose-containing media, lower autoclave temperatures are preferred because prolonged heat exposure can cause degradation of the lactose. Follow the relevant recommended procedures and review all pertinent information for lactose-containing media. See 9020 B.5*j* for further discussion.

Use a maximum-temperature-registering thermometer, data logger, electronic temperature readout device, or continuous recording device during each autoclave cycle to ensure that the proper temperature was reached. Using a chemical steam indicator for each cycle shows whether minimum exposure conditions were met but does not indicate whether sterilization was achieved. Heat-indicating tape can quickly identify materials that have been sterilized.

Maintaining proper autoclave function 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.[19] 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., large volumes of dilution water and contaminated materials).

Each quarter, use a calibrated timer or national time signal to check the timing of all 3 portions of a complete sterilization cycle used for media (≤15 min conditioning cycle, 15 min sterilization cycle, and ≥15 min exhaust cycle). Record and initial the results. Keep the autoclave clean and free of debris by checking the trap and seals monthly.

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

Ideally, use refrigerators that do not have a defrost cycle. Frost-free units may dehydrate stored media and reagents more quickly because heating is used to prevent ice buildup. Store flammable materials in explosion-proof refrigerators. Do not store volatile organic chemicals in the same refrigerator used for microbiological media, reagents, or cultures.

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j. Freezer: The freezer temperature range depends on analytical need (e.g., a standard laboratory freezer may range from –15 to –25 °C, whereas an ultra-low freezer may range from –70 to –90 °C). A recording thermometer and alarm system are highly desirable, especially in ultra-low temperature freezers. Every day in use, check and record corrected temperatures, also noting date, time, and observer's initials. Avoid opening units repeatedly because frost builds up and makes the freezer less efficient. Identify and date (e.g., manufacturer- or lab-expiration) materials stored in the 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.

Freezers that do not have a defrost cycle are preferred over frost-free units, which may dehydrate stored reagents more quickly because heating is used to melt ice accumulation. Store flammable materials in explosion-proof freezers.

k. Membrane filtration equipment: Membrane filtration units must be stainless steel, glass, porcelain, autoclavable or disposable plastic, not scratched or corroded, and must not leak. Discard units if they are chipped or the inside surfaces are scratched. Replace damaged screens and gaskets on stainless steel units. Wash and rinse filtration assemblies thoroughly after use, wrap in nontoxic paper or foil, and sterilize via autoclaving or in a dry heat oven.

If graduation marks on clear glass or plastic funnels are used to measure sample volume, check their accuracy with a Class B graduated cylinder or better (or other Class B glassware), and retain a record of this calibration check. When measuring sample volumes using funnels with volumetric graduation marks, initially verify the accuracy of the marks using a Class A graduated cylinder or volumetric pipet and record the results. For presterilized single-use funnels, check one per lot or a set percentage (e.g., 1%-4%) to confirm the accuracy of volumetric graduation mark and sterility. A 2.5% error tolerance is acceptable in the graduated marks on the vessel.

Confirm the sterility of disposable funnels at least once per lot.

l. Ultraviolet lamps:

1) Short-wave UV lights (254 nm)—Shortwave UV lights are commonly used to sanitize, not sterilize, items such as membrane filtration units. When in use, disconnect the lamps monthly and clean the 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 the lamps quarterly with an appropriate short-wave UV light meter, and replace bulbs when the output drops to <70% of the initial output. Alternatively, expose spread plates inoculated with 200 to 250 CFU/mL of a selected bacterial suspension for 2 min. Incubate the plates at 35 °C for 48 h and then count the colonies. Replace the bulb if the colony count is not reduced 99% compared to an inoculated, unexposed plate. It also may be useful to ask the manufacturer for the bulb's expected lifespan and then track hourly usage.

2) Long-wave UV lights—Long-wave (365-366-nm) UV lights are used to determine fluorescence in test samples. 6-W lamps must be used 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 or as needed.

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.[20] 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 1090 B).

m. Biological 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 (used in BSL-2 and higher laboratories). 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 the unit with disinfectant. Use a piece of tissue to confirm inward airflow.
2) Enter straight into the 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 a discard pan or bag within the cabinet.
3) Decontaminate the interior of the BSC after work is completed and before materials are removed. Allow the cabinet to run for 10 to 15 min and then shut it off.[21]

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 the workspace to become crowded because objects may disturb the airflow pattern, allowing contaminants to exit at the face opening. Place working objects at least 6 in. from the face.

n. Incubator (mechanical forced hot-air, water-jacketed, or aluminum block): Incubator units must have an internal temperature monitoring device and maintain the temperature specified by the method used. Place incubators in an area where room temperature is maintained between 16 and 27 °C (60 and 80 °F), or in a separate, well-insulated room with forced air circulation. Clean and then sanitize incubators routinely. Determine whether incubators maintain appropriate, uniform spatial test temperatures. Gravity convection hot-air incubators must not be used. Load studies may also need to be done to determine how effectively incubators can maintain temperature and how quickly they rebound after the door is opened or samples are added. As a general practice, while in use, open incubator doors as infrequently as possible to prevent temperature fluctuations.

Place thermometers on the top and bottom shelves of the use area. Walk-in incubators may require more than 2 thermometers to confirm consistent temperatures. At least twice per day during each day the incubator is in use, record calibration-corrected temperatures for each thermometer being used, with readings separated by at least 4 hours. Document the date and time of the reading, thermometer identification, temperature, and technician's initials. If using a glass thermometer, submerge the bulb and stem in water or glycerin to the immersion mark. For best results, use a recording thermometer and an alarm system that

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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 as 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 and temperature readings can be used to trace a sample and its testing conditions during laboratory assessment. Allow sufficient space between items to permit unobstructed airflow; do not overload or stack petri dishes more than 4 plates high.

Incubator humidity may be a concern, especially for procedures that use pre-dried plate media (e.g., HPC spread plates), because the ingredients in the medium become more concentrated if dehydrated, which may have a deleterious effect on the colony counts. Annually, evaluate agar plates for the percent moisture weight loss during the method's incubation period. This can be accomplished by weighing the plates before and at the end of the incubation period. If agar weight loss is >15%, add moisture to the environment by either using humidified incubators or enclosing the plates in tight-sealing containers or bags. If additional moisture is needed, wet paper towels can be added to the 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 the media is evident, reduce the humidity accordingly.

o. Water bath incubator: Verify that water bath incubators maintain the set temperature, such as 35 ± 0.5 °C or 44.5 ± 0.2 °C. When the incubator is in use (i.e., samples are being incubated), monitor and record corrected the temperature twice daily separated by at least 4 h.

Electronic temperature-sensing devices (i.e., data loggers) may be used as 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 and temperature readings can be used to trace a sample and its testing conditions during laboratory assessments. Mark each data logger with any correction factor needed.

Fill the water bath only with reagent-grade water. Maintain the water level so it is above the upper level of the medium in either tubes or flasks, but below the lids to avoid potential contamination. Equip the 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 the bath as needed to prevent the buildup of salts and microbial growth, and disinfect it before refilling.

p. Microscopes: Before each use, check Köhler illumination to confirm that the 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 the optics and stage with lens paper. Cover the microscope when not in use.

Permit only trained analysts to use a fluorescence microscope. Monitor the fluorescent lamp and replace it when a significant loss in fluorescence is observed, according to manufacturer recommendations, or when the maximum hour usage specified by a rule or laboratory guidance document has been reached, whichever occurs first. Record lamp operation time, efficiency, and alignment. Always realign the lamp after the bulb has been replaced and as needed. Use known positive fluorescence slides as controls.

Establish an annual service contract. Review the microscope-manufacturer's manual, Section 9030, and elsewhere.[22]

q. Conductivity meter: A conductivity meter measures the presence of dissolved ions, such as aluminum, calcium, chloride, iron, magnesium, nitrate, phosphate, sodium, and sulfate. Meters must be suitable for checking laboratory reagent-grade water and readable in units of either microsiemens per centimeter (µS/cm) or micromhos per centimeter (µmho/cm). Conductivity measurements are temperature-dependent, and the temperature's effect is solution-dependent. Check daily before use and calibrate, if needed, following the manufacturer's instructions. Every month, standardize the meter or determine the cell constant using certified low-level standards that bracket the expected sample conductivity (e.g., 10 µS/cm) at 25 °C. Do not use in-line units that cannot be calibrated to check reagent-grade water. When solutions must be measured at another temperature, use a meter with automatic temperature compensation or take the solution's temperature, record the reading, and then correct the reading to 25 °C using the formulae in Section 2510 B.5*b* (usually 2% per °C).

Use an extremely clean borosilicate glass beaker for calibration that is rinsed several times with the water to be tested before collecting the test sample. Swirl the sample gently to eliminate air bubbles. Perform conductivity measurements on-site because water quality decreases shortly after exposure to both air and the sample container. Open the sample container minimally as conductivity measurements change when the sample is exposed to ambient air.

r. Microwave ovens: Microwave ovens vary in power, but they may be used to melt some presterilized agar media. Media containing antibiotics or other heat labile inhibitors may not be microwaved. Set the microwave power and time to the minimum settings required to melt the agar. Undertake comparison studies to confirm that microwaving is comparable to standardized melting procedures. Take care to avoid media bubbling over.

s. Micropipettors: Micropipettors are high-precision laboratory instruments for dispensing extremely small volumes. Use them with sterile precision tips supplied by the manufacturer or equivalent that securely fix to the nose cone to ensure a tight seal. Maintain technique consistency in pipetting action, such as prewetting, plunger release, and tip immersion depth (between 1 and 3 mm). Operate them only in a vertical position and have both the sample and equipment at an equivalent temperature. Avoid overdialing the micropipettor's recommended range, which can cause mechanical damage. Follow the manufacturer's instructions to perform routine maintenance, such as cleaning, seal replacement, and relubrication. Check the accuracy and precision of volume dispensed by each pipettor before first use after purchase, maintenance, or repair, and at a frequency related to its usage (e.g., quarterly or sooner if the pipettor is showing overt signs that it is inaccurate or if the tip manufacturer changes). Calibrate at least annually and maintain the results of the calibration. If water is used to calibrate or check the accuracy of pipettor, remember that changes in liquid viscosity can affect the volume dispensed.

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5. Laboratory Supplies

Retain records and manufacturer certificates of analysis, purity, and 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. Plastic items must be clear and nontoxic to microorganisms. Volumetric glassware, pipets, graduated cylinders, and beakers with calibration marks must be accurate to the specified volumetric tolerances. See established standards[23] for the 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 must 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, or 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 (EC-MUG) medium], check it for autofluorescence before use.

Perform the following tests for clean glassware:

1) pH—Check each batch of glassware used for microbial analysis for pH reaction, especially if glassware is 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 wet glassware and observe the color reaction. If there is no residual, the reaction is neutral (blue-green for BTB). However, if the indicator turns yellow (acid residual) or deep blue (alkaline residual) when using BTB, then the glassware must be rewashed and retested. If the retest 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 M NaOH to 0.1 g BTB and dilute to 250 mL with reagent-grade 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 (including new lots) 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 the results. The following procedure is suitable for both petri dishes and other glassware.

a) Procedure—Wash and rinse 6 glass petri dishes (Group A) according to usual laboratory practice. Wash 6 more glass petri dishes (Group B) as above, and then rinse 12 times with successive portions of reagent-grade water. Rinse 6 more glass 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. Use 6 plastic petri dishes as a control (Group D). Prepare and sterilize 200 mL plate count agar and temper it in a 44 to 46 °C water bath. Prepare a culture of *Klebsiella aerogenes* ATCC 13048 known to contain 50 to 150 CFU/mL. Preliminary testing may be necessary to achieve this count range. Inoculate 3 dishes from each test group with 0.1 mL culture and the other 3 with 1 mL culture. Pour tempered agar on top of the inocula and swirl to evenly distribute, as described in Section 9215 B. Incubate at 35 ± 0.5 °C for 48 h. Count plates with 30 to 300 colonies and record the results as CFU/mL.

b) Interpretation of results—The averaged counts on plates in Groups A through D will 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 even after additional rinsing and glassware washed using the routine procedure 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 9030 B.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 a sterility test (9020 B.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 a precipitate repeats, procure bottles from a different source. Recheck the volume at regular intervals to determine the volume loss rate under the holding conditions. Discard by the expiration date (3 months if tightly capped and stored at <30 °C).

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 volume and still have an adequate headspace (1 in.) to allow the sample to be shaken in the container. Do not use graduations on bags to measure sample volume.

Clean and sterilize bottles before use and, depending on use, add sufficient dechlorination agent to neutralize residual chlorine (Section 9060 A.2). Sample containers 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 by adding 25 mL of a sterile nonselective broth (e.g., tryptic soy, trypticase soy, or tryptone broth) and incubating at 35 ± 0.5 °C for 24 to 48 hours. Document the results. If growth occurs, discard the entire batch or lot. Also, check one bottle per batch or lot

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for efficacy of dechlorination agent, accuracy of 100-mL mark (if present), and autofluorescence properties (if used for fluorescence testing). Record the results.

e. Multiwell trays and sealers: Check one tray per lot for sterility by aseptically adding 100 mL of sterile nonselective medium (e.g., tryptic soy, trypticase soy, or tryptone broth), sealing, and incubating at 35 ± 0.5 °C for 24 to 48 h. Document the results. If growth occurs, discard the entire batch or lot. See 9020 B.9*d*.

Every month, evaluate the heat sealer's performance by adding 1 to 2 drops of a food-color dye to 100 mL reagent-grade water, run the multiwell tray through the sealer, and visually check each well for leakage. If dye is observed outside the wells, either perform maintenance or use another sealer. Clean and conduct preventive maintenance on sealers annually, or more frequently if needed or recommended by the manufacturer.

Microtiter plates are used in a variety of analytical procedures (e.g., 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: Only satisfactorily tested reagent-grade water from stills or water purification systems may be used to prepare media, reagents, dilution and rinse water, and for final glassware rinses for performing microbial analyses. The water must be proven to be free from inhibitory and bactericidal substances. The quality of water obtained from a water-purification system depends on the system and how it is maintained (see 9020 B.4*d* and *e*). See Table 9020:2 for reagent water-quality limits for the microbiology laboratory. If these limits are not met, investigate and correct or else change the water source. Note: Reagent-grade water's pH tends to drift, but extreme readings indicate chemical contamination.

1) Test for bacteriological quality—This test, also called the *water suitability test,* is based on the growth of *Klebsiella aerogenes* (ATCC 13048) in a chemically defined minimal-growth medium (Table 9020:3).[24] The presence of a toxic agent or a growth-promoting substance increases or decreases the 24-h bacterial population by 20% or more, compared to a control. Perform the test at least annually, whenever the reagent-water source is changed, and whenever an analytical problem occurs. This bacteriological quality test is not needed for medium-quality water or better.

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 (double-distilled) and then sterilize it with dry heat (steam sterilization recontaminates these specially cleaned items). Test sensitivity and reproducibility depend in part on the cleanliness of sample containers, flask, tubes, and pipets. It is convenient to set aside new glassware for exclusive use in this test. Use any strain of coliform with IMViC type – – + + obtained from an ambient water or wastewater sample or reference culture *(*e.g., *K. aerogenes,* ATCC 13048*)*.

b) Reagents—Use only ACS-grade reagents and chemicals. Test sensitivity is partly controlled by reagent purity. Use water freshly redistilled from a glass still; the water can be purchased (see Table 9020:3). 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 9050 C.1*a*) 1:25 in water.

Filter sterilize or boil all reagent solutions 1 to 2 min to kill vegetative cells. Store solutions refrigerated in sterilized glass-stoppered bottles in the dark for up to 3 months, provided that they are tested for sterility before each use. Because the salt-mixture solution develops 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,

Table 9020:2. Quality of Reagent Water Used in Microbiology

Test	Monitoring Frequency	Maximum Acceptable Limit
Chemical tests:		
Conductivity	Continuously or usage day	<2 µmho/cm (µS/cm) at 25 °C
Total organic carbon	Monthly	<1.0 mg/L
Heavy metals, single (Cd, Cr, Cu, Ni, Pb, and Zn)	Annually[a]	<0.05 mg/L
Heavy metals, total	Annually[a]	<0.10 mg/L
Total chlorine residual	Monthly or with each use	<0.1 mg/L
Bacteriological tests:		
Heterotrophic plate count[b]	Monthly	<500 CFU/mL or MPN <500/mL
Use test [see 9020 B.5*f*2)]	For a new source	Student $t \leq 2.78$
Water quality test [see 9020 B.5*f*1)][c]	Annually	0.8–3.0 ratio

[a] Or more frequently if there is a problem.

[b] See Section 9215.

[c] This water-quality test is not needed for Type II water or better, as defined in *Standard Methods* (18th and 19th Editions), Section 1080 C, or medium quality water or better, as defined in *Standard Methods* (20th, 21st, 22nd, 23rd and Online editions), Section 1080 C.

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Table 9020:3. Reagent Additions for Water Suitability 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

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-grade water to be tested 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 2 flasks or tubes A and B. Add water samples, reagents, and redistilled water to each flask as indicated in Table 9020:3. Add a suspension of *K. 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 and densities above 100 cells/mL are less sensitive to nutrients in the test water.

e) Preparation of bacterial suspension—On the day before the water suitability test, inoculate a strain of *K. aerogenes* (ATCC 13048) onto an approximately 6.3-cm-long nutrient agar slant in a 125- × 16-mm screw-cap tube. Streak the 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 dilution water blank (see 9050 C.1) onto the culture. Emulsify growth on the slant by vortexing, gentle sonication, or swirling; then pipet the suspension back into original 99-mL water blank.

g) Dilution of bacterial suspension—Make a 1:100 dilution of the original bottle into a second dilution water blank, a further 1:100 dilution of second bottle into a third dilution water blank, and a 1:10 dilution of third bottle into a fourth dilution water blank, shaking vigorously 22 times after each transfer. Pipet 1.0 mL of the fourth dilution (10^{-5}) into flasks A and B. This procedure should produce a 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 one or more of the following:

- test water sample was stored in soft-glass containers or glass containers with linerless metal caps;
- reagents were prepared with chemicals that are not analytical-reagent grade or of recent manufacture;
- reagent was 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 was 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 were incubated for longer than 26 h, thereby desensitizing growth response.

j) Calculation—For growth-inhibiting substances:

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

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 be as high as 3.0 without any undesirable consequences. Do not make this calculation if the first ratio indicates a toxic reaction.

k) Interpretation of results—After incubation, the colony count from the control (Flask A) depends on the strain of *K. aerogenes* and the number of organisms initially inoculated in the flask. Therefore, run Flask A for each individual series of tests. If the strain of *K. aerogenes* (ATCC 13048), initial inoculum, and environmental conditions are the same, the terminal count will be reasonably constant. However, a difference in the initial inoculation of 30 to 80 results in a final count about 3-fold 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.

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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, 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 reagent-grade water (redistilled or distilled water polished by deionization), glassware, membrane filters, media, 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 5 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 water, rinse water, and media with the new source of reagent and the control water. Test water for all uses.

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, s_d, of these differences (see Section 1010 B).

Calculate the Student 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 t table. At the 0.05 significance level, Student t is 2.78 for 5 samples (4 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 t exceeds 2.78, then review test conditions and repeat the test or reject the test lot and obtain another one.

g. Reagents: Use only ACS-grade chemicals[25] or equivalent because impurities can inhibit bacterial growth, provide nutrients, or fail to produce the desired reaction. Maintain any safety data sheets (SDSs) 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 [including, e.g., date prepared, preparation details (i.e., concentration), expiration date, pH, sterilization time, positive and negative control reactions]. 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 nontoxic 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 the expiration date. Record positive and negative control culture reactions 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 the results. For fluorescent stains, test for positive and negative reactivity each day of use. Do not freeze dyes or stains. Read and follow the 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, and lot due to differences in manufacturing methods, materials, QC, storage conditions, and application.[26]

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.[27,28] Manufacturers typically 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 must 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 the procedure, see ¶ *f*2) above.

3) Standardized use tests—When each lot of membranes arrives at the laboratory, record the lot number and date received. Inspect each lot before use and during testing to ensure that membranes are round and pliable. If a lot is held for one or more years, carefully check for brittleness and discard lots that appear brittle. Confirm sterility before first use of the lot by placing a membrane filter on a pad saturated with tryptone glucose extract broth (or equivalent nonselective 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, make sure that colonies are well-developed with appropriate color and shape, as defined by the test procedure. The gridline ink must not channel growth along the ink line or restrict colony development. Colonies must be distributed evenly across the membrane surface. Reject the membrane lot if these criteria are not met, and inform the 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. Use

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commercially available dehydrated media when available, but note that the media may vary slightly both from lot to lot and from manufacturer to manufacturer. Before the first use of a new lot of media, perform a use test to compare the growth recovery of the new lot to that of proven lots, using positive and negative references [see ¶ *f*2) above]. Test using cultures whose estimated density is similar to samples typically tested in the laboratory. Monitor media for growth promotion, inhibitory properties, physical appearance, and pH.

File any SDS accompanying the 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 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 at least quarterly for reordering.

Check each lot of media used for detecting fluorescence for autofluorescence before use. This is done by dissolving the medium in reagent-grade 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 graduated cylinders or pipets that conform to NIST and APHA standards, respectively. Use TD (to deliver) pipets. Stir media, particularly agars, while heating. Avoid scorching or boil-over by using a boiling water bath for small batches of media and by continually attending to larger volumes being heated. Preferably, use a hot plate and magnetic stirrer. Label and date the prepared media.

After sterilization, check and record the final pH of a portion of each medium. If pH adjustment is needed, use filter-sterilized 1 M NaOH or 1 M HCl solutions to make minor adjustments so the medium's pH meets that specified in the formulation. (Commercially available media seldom need pH adjustment.) If a medium is known to require pH adjustment, adjust it appropriately before sterilization and record the 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 the reagent-grade water's pH is unsatisfactory, prepare a new batch of medium using water from another source (see 9020 B.4*d* and *e*). If the water is satisfactory, remake the medium and check the pH; if the pH is still incorrect, prepare the medium using a different lot or source of media. Note: Certain isolation media prepared with organic or fatty acids will have marked changes in pH after sterilization.

Document preparation activities, such as the name of the medium, volume produced, format, initial pH, final pH, date prepared, expiration date, 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, crystal formation, 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.

Table 9020:4. Time and Temperature for Autoclave Sterilization[a]

Material	Time at 121 °C (min)
Membrane filters and pads	10
Carbohydrate-containing media (lauryl tryptose, BGLB broth, etc.)	12–15[b]
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

[a] Except for media, times are guidelines.
[b] Certain media may require different sterilization conditions.

2) Sterilization—Sterilize media at ≤121 °C with minimal temperature variation for the minimum time specified. Follow the manufacturer's directions for sterilizing specific media. The required exposure time varies with the form and type of material, type of medium, presence of carbohydrates, and volume. Table 9020:4 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 must not be exposed to heat for that long. For example, presence-absence media (9221 D) must not 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.

Remove sterilized media from the autoclave as soon as the 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 biological 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) Preparation of agars and broths—Temper melted agars in a water bath at 44 to 46 °C until used, but for ≤3 h. To monitor agar temperature, use a temperature control flask, which is a bottle of water or medium of the same size and type that is exposed to the same conditions as the agar. Insert a thermometer in the temperature control flask to determine when the agar arrives at the correct temperature (44 to 46 °C). 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 it in a 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,[29] use, and then discard any remainder. Because microwaves vary, run comparison tests to ensure that the medium integrity has not been compromised. Some media are not suitable for melting in the

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microwave (i.e., m-Endo, Endo LES, or media containing antibiotics or other heat sensitive selective agents).

The volume dispensed depends on the size of the petri dish and its intended use. Invert plates as soon as the 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 inverted in larger test tubes to trap any gas produced by fermentation.) Examine freshly prepared tubes to determine that there are no gas bubbles in the Durham tubes.

4) Storage of dehydrated media—Store all media under controlled conditions to maintain quality until the 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 the 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 a desiccator, if available.

5) Storage of prepared media—Prepare media in amounts that will be used within holding time limits given in Table 9020:5. Fresh medium is required to ensure that target microorganisms are isolated properly, especially bacteria stressed or injured during treatment. Protect media containing dyes from light; if the color changes, discard the media.

Controlling moisture content is important because prolonged storage and subsequent dehydration may alter recovery and selectivity. Store agar plates refrigerated and inverted in plastic bags or a sealed container. If condensate has formed, consider placing plates briefly in a 35 to 37 °C incubator. For media in test tubes or bottles, tighten the caps before storage. Mark the liquid level in several tubes (10% of each batch) after sterilization and monitor for liquid loss by volume when stored for >2 weeks. If the loss is 10% or more, discard the 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), unless ongoing QC procedures show that there is no loss in selectivity or growth promotion.

If media are refrigerated, bring them to room temperature before use and reject the batch if growth or false-positive responses are observed before use. Check the performance of media as described in paragraphs 6–7 below.

6) Quality control of laboratory prepared media—Do not prepare media in the laboratory from basic ingredients when commercially prepared bulk dehydrated media is available.

Periodically challenge prepared media with low numbers of an appropriate microorganism. Growth may be affected by media quality, preparation, sterilization, storage time, and storage conditions. Perform QC on all media, whether prepared in-house or commercially, to demonstrate acceptable performance. If contamination is found, determine the root cause and invalidate analytical data from the samples tested. Document both the cause or problem and the corrective action taken. Laboratories interested in contaminant identification can use either standardized phenotypic testing systems or genotypic procedures.

For all media, (both laboratory and commercially prepared) record:

Table 9020:5. Holding Times for Prepared Media

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

[a] Hold under refrigerated conditions (2–8 °C).
[b] Hold at <30 °C.

- dates of receipt, opening, and expiration;
- lot number;
- results of QC testing (sterility, positive and negative controls; see 9*d*); and
- quantitative results (if applicable)
- results of use test (see ¶5*f*2)

7) Quality control of commercially prepared media—Hold commercially prepared media according to the times specified in Table 9020:5 from the preparation date. Often, manufacturers of commercially prepared ampouled broth specify much longer expiration dates than those recommended here. In these cases, the laboratory may hold those media as recommended by the manufacturer if quantitative QC testing demonstrates no loss in selectivity or growth promotion. For dehydrated media (that does not require additional preparation by the laboratory, such as enzyme substrate media), hold the media as described by the manufacturer and discard it after the expiration date.

6. Standard Operating Procedures

The operational backbone of an analytical laboratory, SOPs are designed to prevent deviations due to a misinterpreted process or method. Each SOP describes the stepwise details of a routine task or procedure tailored to the laboratory's own equipment, instrumentation, and sample types.[30–32] These are prepared in a manner that includes what is required by the standardized method being used by the laboratory (as applicable) but also reflect the actual practices used by the laboratory. They are signed by management, 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.

SOPs are prepared for laboratory tasks including:

- preparing reagents, reagent-grade water, and culture media;
- use and care of laboratory equipment (including, e.g., pH meter, balances, autoclaves);
- glass and plasticware washing;
- disposal of waste;
- sample collection;
- sample receipt;
- sample analysis (an SOP for each method used); and
- QC procedures (e.g., thermometer verifications).

SOPs are laboratory-specific, written by the person who does the work, and approved in writing by the supervisor (with the effective date indicated).

SOPs must be:

- made available to all laboratory personnel

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- followed by all laboratory personnel, and
- kept current via routine reviews.

If maintained in electronic form (eFiles), SOPs may need to be password protected 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).

7. Sampling

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

a. Planning: Microbiologists can provide valuable expertise in the development of sample collection programs, including the selection of sample collection sites and depths; the number of samples, sample volume 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 are 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).

b. Methods: Sample-collection guidance generally addresses the factors that must be considered for each site. Sample-collection SOPs describe, for example, sampling equipment and its cleaning, techniques, frequency, handling, chain of custody, holding times and conditions, and safety precautions, 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. Ideally, sample-collection techniques for detecting and recovering microorganisms are validated.[33]

c. Sample acceptance: Upon sample receipt, laboratory staff 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 must include names or identifiers of both sampling site and sampler, temperature, disinfectant residual, turbidity, and date and time of sample collection, as applicable. 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 using analytical methods: Only analyze samples that are within the acceptable holding time and temperature. Samples outside of the acceptable holding time and temperature must be rejected.

Bacteria are known to clump together and attach to particulate matter that may be in a water sample. Proper sample mixing is critical to ensure an even distribution of bacteria throughout the sample. This is especially important when enumerative methods (e.g., multiple-tube or multiwell) procedures are used. MPN results are based on a Poisson (random) distribution of cells in the sample. Failure to properly mix the sample before an analysis may result in an MPN result that underestimates the actual bacterial density.

Shake samples approximately 25 times in a 7 second timeframe, using a 1-foot arc. Mechanical shakers may be used, but they should mimic this shaking. Orbital shakers may not mix adequately; wrist action shakers are more likely to give more accurate results.

If a bottle lacks sufficient headspace for adequate mixing, pour the sample into a larger sterile vessel so it can be mixed properly. Measure the desired sample volume and proceed with the analysis. Do not remove a sample portion until proper sample mixing has occurred.

8. Analytical Methods

The essential QC elements for microbiology laboratories are described in 9020 A. 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 collection to the final data report.

a. Method selection: Microbiological methods are used for a variety of matrices, including drinking water, municipal wastewaters, recreational waters, ambient waters, ground waters, marine waters, storm waters, and direct discharges. Factors such as regulatory requirements, media compatibility with the sample matrix, and the ability to comply with holding time, must be studied to ensure the laboratory is using an appropriate method for sample analyses. Ideally, laboratories perform parallel testing between a new method and another approved procedure for enumerating total coliforms for at least several months and over several seasons to assess the effectiveness of the new test. During this testing, spiking the samples occasionally with sewage or a pure culture may be necessary to ensure that the results are comparable.

b. Data objectives: Review the available literature and determine which methods 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 false-negative results that could affect water-quality decisions.

c. Internal QC: As described in the opening of this section, the laboratory must have a robust QS to ensure that data are of known quality. Required QC checks are an integral part of a QS because they ensure data quality. Refer to Table 9020:1 for key quality control practices. These practices must be part of a laboratory's internal QC program in addition to any laboratory-specific 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. See 9020 B.6.

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Table 9020:6. Suggested Control Cultures for Microbiological Tests[a]

Group	Control Cultures	
	Positive	Negative
Total coliforms	*Escherichia coli*	*Staphylococcus aureus*[b]
	Klebsiella aerogenes[c]	*Proteus vulgaris*[d]
	Klebsiella pneumoniae (ATTC 4352)	*Pseudomonas aeruginosa*[b]
Thermotolerant coliforms	*Escherichia coli*	*Klebsiella aerogenes*
	Klebsiella pneumoniae (thermotolerant)[e]	
Escherichia coli	*Escherichia coli (MUG-positive strain)*	*Klebsiella aerogenes*
		Klebsiella pneumoniae (thermotolerant)
Enterococci[f]	*Enterococcus faecalis*	*Staphylococcus aureus*[g]
	Enterococcus faecium	*Escherichia coli*[h]

[a] Use appropriate ATCC strains. Note: Other cultures may be used.
- *Enterococcus faecalis* ATCC 11700
- *Enterococcus faecium* ATCC 6057
- *Klebsiella 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 marcescens* ATCC 14756
- *Staphylococcus aureus* ATCC 6538

[b] *S. aureus*, *P. aeruginosa*—not lactose fermenter.
[c] *K. aerogenes*—ferments lactose, but is not typically thermotolerant.
[d] *P. vulgaris*—not lactose fermenter, uses hydrolyzed lactose, indicating "overcooked" medium.
[e] *K. pneumoniae*—ferments lactose at 44.5 °C, but does not hydrolyze MUG.
[f] Do not use closely related strains from genus *Streptococcus* as a positive control.
[g] *S. aureus*—sensitive to sodium nalidixic acid medium.
[h] *E. coli*—sensitive to sodium azide in medium.

9. Method-Specific Analytical Quality Control Procedures

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 interlaboratory tests and spiked samples become necessary.[6–8,34,35]

General quality control procedures include the following:

a. Colony-counting variability: For routine performance evaluations, analysts must 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 counts between 2 analysts, the same plate is counted by each analyst once. When comparing 3 or more analysts, use a statistical evaluation method. See 9020 B.13*b* for a statistical calculation of data precision. Replicate counts by 1 analyst must agree within 5% (repeatability); counts made by 2 or more analysts must agree within 10% (reproducibility). If counts do not agree within the acceptable margin, determine the reasons and correct with additional training as needed. Calculate the 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 procedure in paragraph *c*, and record results.

b. Control cultures: Use certified reference cultures traceable to nationally or internationally recognized sources. Follow the culture collection instructions for strain maintenance. Subculture the reference culture to develop one or more primary working stocks,[36] but make no more than 5 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 their performance remains unchanged. If a laboratory lacks the facilities to maintain a pure culture, the use of single-use culture strips is allowed.

Note: Proficiency test (PT) samples are unknowns and should not be considered replacements for positive and negative culture controls.

For each lot of medium received (whether laboratory-prepared or purchased), verify an appropriate response by testing with known positive and negative control cultures for the organisms under test. See Table 9020:6 for examples of test cultures. Record the results

c. Precision of quantitative methods: Precision (repeatability) of quantitative analytical results when counting colonies on plates is evaluated through replicate analyses.[37,38] Use replicate counts from only 1 dilution 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.

1) Perform duplicate analyses on the first 15 positive samples of each matrix type, with each set of duplicates analyzed by one analyst. Record duplicate analyses as *D1* and *D2*. Calculate the logarithm of each result. If either 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 9020:7). 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 9020:8). 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.

4) Perform precision measurements of replicate MPN analyses in a similar manner.

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 the results. An adequate sample volume is essential. Balance the 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.

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Table 9020:7. Calculation of Precision Criterion

	Duplicate Analyses		Decimal Logarithms of Counts		
Sample No.	D_1	D_2	L_1	L_2	Range of Logarithms (R_{log}) ($L_1 - L_2$)
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:

$$\Sigma \text{ of } R_{\log} = 0.0981 + 0.0483 + 0.0627 + \ldots + 0.0669 + 0.0414 = 0.71889$$

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

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

Table 9020:8. Daily Checks on Precision of Duplicate Counts[a]

	Duplicate Analyses		Logarithms of Counts			
Analyses	D_1	D_2	L_1	L_2	Range of Logarithms	Acceptance of Range[b]
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

[a] Precision criterion = ($3.27\,\bar{R}$) = 0.1566.
[b] A = acceptable; U = unacceptable.

d. Sterility checks: Test media sterility before first use to ensure that there is no interference, and record the 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 reagent-grade 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.

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 nonselective broth (e.g., tryptic soy, trypticase soy, or tryptose broth). Alternatively, aseptically pass 100 mL or more dilution water (see 9050 C.1) through a membrane filter and place the filter on nonselective medium. Incubate at 35 ± 0.5 °C for 48 h and observe for growth. Record the results.

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

Check sterility of process methodology as follows:

- Membrane filtration procedures: 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. Ideally, filter a blank for each funnel set used during a filtration series. Membrane filter equipment must be autoclaved before the beginning of a filtration series. A filtration series ends when 30 minutes or longer elapses after a sample is filtered. Record results.
- For pour plate procedures, check sterility by pouring at least one uninoculated plate per batch or lot of media and record results.

If any contamination is indicated, determine the root cause, and invalidate analytical data from samples tested. Document both the cause of contamination and the corrective action taken.

10. Verification

Verification, or confirmation, is a general process to determine whether the method and the analyst are performing as expected to provide reliable data (i.e., determining false-positive and false-negative rates). If the confirmation 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 or verification procedures for drinking water differ from those for other waters because of specific regulatory requirements. (Microorganisms sometimes 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

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well or fermentation tube gives a positive confirmation result. The following is a brief summary; further information may be found in the appropriate discussions of the specific microorganism or microbial group.

a. Multiple-tube fermentation (MTF) methods:

1) Total coliform procedure (Section 9221 B)

a) Drinking water—Carry tests through the 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—Confirmation 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 high number of samples daily, 10% of positive samples may be an unnecessary burden; choose an appropriate lower percentage.

2) *Enterococcus* procedures—Perform confirmation as outlined in Section 9230 C.5 at a frequency established by the laboratory.

b. Membrane filter methods:

1) Total coliform procedures

a) Drinking water—Swab the entire membrane or pick up 5 typical and 5 atypical (nonsheen) colonies from positive samples on m-Endo or Endo LES agar medium and verify as directed in Section 9222 B.4*g*. Also verify any positives for thermotolerant (fecal) coliforms as described in paragraph *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 9222 B. 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 9222 B.

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 9221 E.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 9221 B.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 9225 D.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 3b above.

4) *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 9230 C.5. Adjust counts based on percent verification or report as presumptive enterococci.

c. Enzyme defined substrate tests:

1) Total coliform test (Section 9223)

a) Drinking water—Confirmation is not required.

b) Other water types—No confirmation 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 laboratories that want to conduct confirmation 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, 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 9225 D for test descriptions or use other equivalent identification procedures or systems.

2) *E. coli*—For *E. coli* analyses, confirmation is not usually 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 confirmation 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 a MUG reaction using EC+MUG or NA+MUG media or *E. coli* biochemical identification (as described in Section 9225 D) 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 9222 J for the dual chromogen MF procedure and Section 9222 K for the fluorogen and 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

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

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the field of chemistry, validation 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 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 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 validation* or *cross validation.*

For methods in development (e.g., research methods), establish confidence in the analytical method by conducting full collaborative 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 collaborative studies (also called *interlaboratory 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 publications for further information and for programs involved with microbial method validation.[39–46]

To determine the effect of matrix on recoveries, perform a matrix spike: add a known concentration of the target organism (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 must provide third-party evidence of competence and compliance with global standards. Microorganisms must 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 and 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 confirming all responses (e.g., by microbial identification testing).

3) Detection limit—This indicator reveals the lowest microbial density that can be measured 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, analyst and equipment). Use a target microorganism or microbial group density such that at least 75% of the results will be positive (i.e., growth) so enough responses can be detected[44] for either a quantitative or qualitative test. This can serve as one measure of uncertainty.

b. Quantitative test methods: Validation of a method 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) Repeatability precision—This indicator reveals the degree of agreement between replicate analyses or measurements conducted under the same conditions (e.g., laboratory, analyst and equipment). Use a target microorganism or microbial group density such that at least 75% of the results will be positive, so enough responses can be detected.[46] Repeatability precision can serve as one measure of uncertainty.

3) Reproducibility precision—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 or using different equipment). Reproducibility precision 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 determining statistical confidence.

5) Detection limit—This indicator shows the lowest microbial density that can be measured. 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).

7) Range—This indicator notes the interval between the upper and lower detection limits.

12. Documentation and Recordkeeping

a. QA Plan: The laboratory's QA Plan or Quality Manual documents management's commitment to a QA policy and the

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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 9020 A.

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 must be completed for each sample entering the laboratory. Maintain exact and complete records long term, in case of litigation. Details on chain-of-custody are available in Section 1060 B.2 and elsewhere.[47] A laboratory system that uniquely identifies samples in the laboratory and that is tied to the field sample number ensures 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 analysts and supervisors. 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 is 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 as 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;
- persons 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, supervisor, or both.

When a 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 a disk or hardcopy 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 nonhomogeneous 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 1010 B). 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.*

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., a normal distribution), so skewed data usually must be converted to a more symmetrical distribution before these 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 9020:9. A comparison of the frequency tables for the original data (Table 9020:10) and their logarithms (Table 9020:11) 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 organisms of interest, and the geometric mean indicates the most likely number of such organisms 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

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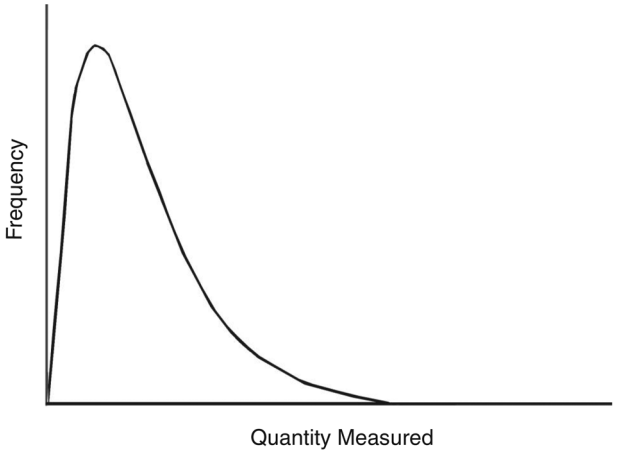

Figure 9020:1. Frequency curve (positively skewed distribution).

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.

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 n^{th} root of the product of all the data values.[57]

Table 9020:9. 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

Table 9020:10. 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 9020:11. 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

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: The proper way to include "less than" values when calculating and evaluating microbiological data is uncertain because such values cannot be treated statistically without modification. Proposed modifications involve changing such numbers to zero, choosing values halfway between zero and the "less than" value, or assigning the "less than" value 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 may be minimal and of no benefit. If the database is small or contains a relatively large number of "less than" values, then they could exert an undue influence and artificially bias results either negatively or positively. Including "less than" values is particularly inappropriate

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if the values are <100, <1000, or higher because the unknown true values could be anywhere from, for example, 0 to 99 or 0 to 999. When such values are first noted, adjust or expand test volumes. The only exception to this caution is 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 or needs. A number of publications[1–6] and organizations (American Association for Laboratory Accreditation and National Cooperation for Laboratory Accreditation) 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 accreditation 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 3 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 sample collection practices 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 is mandatory. A formal document is prepared and provided to all participants.

The QA/QC responsibilities of managers, supervisors, and technical staff are described in 9020 A.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

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review of all operations and records for acceptability, to identify possible problems and help resolve them. If this is done properly, there is 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 depend on a number of variables, such as accreditation requests 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 or 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 or accreditation.

Laboratories not applying for certification or 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 is formally notified that it has been certified or 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 or accreditor) and pass an onsite assessment about once every 3 years.

6. Example Programs

In the US 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 2 of the last 3 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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