MICROBIOLOGICAL METHODS

Microbiological Water Methods: Quality Control Measures for Federal Clean Water Act and Safe Drinking Water Act Regulatory Compliance

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Quality assurance (QA) and quality control (QC) data are required in order to have confidence in the results from analytical tests and the equipment used to produce those results. Some AOAC water methods include specific QA/QC procedures, frequencies, and acceptance criteria, but these are considered to be the minimum controls needed to perform a microbiological method successfully. Some regulatory programs, such as those at Code of Federal Regulations (CFR), Title 40, Part 136.7 for chemistry methods, require additional QA/QC measures beyond those listed in the method, which can also apply to microbiological methods. Essential QA/QC measures include sterility checks, reagent specificity and sensitivity checks, assessment of each analyst's capabilities, analysis of blind check samples, and evaluation of the presence of laboratory contamination and instrument calibration and checks. The details of these procedures, their performance frequency, and expected results are set out in this report as they apply to microbiological methods. The specific regulatory requirements of CFR Title 40 Part 136.7 for the Clean Water Act, the laboratory certification requirements of CFR Title 40 Part 141 for the Safe Drinking Water Act, and the International Organization for Standardization 17025 accreditation requirements under The NELAC Institute are also discussed.

Quality assurance (QA) and quality control (QC) measures are required in order to have confidence in both analytical test results and the equipment and processes used to derive those results. Essential QA/QC measures for microbiology include, but are not limited to, demonstration of each analyst's capabilities, method blanks and controls, sterility checks, and matrix spikes for difficult matrixes. Some of these parameters have a specific definition with regard to microbiological methods, whereas some QA/QC parameters are standard in the laboratory, such as equipment calibration, control charts, root cause analysis, and corrective actions, which are discussed here. Additionally, there is a requirement to document and record all QA/QC criteria to ensure consistent test results and analyst performance. The details of these procedures, their performance frequency, and expected ranges of results should be formalized in a written quality assurance manual and as standard operating procedures (SOPs; 1).

Some AOAC water microbiology methods already include specific QA/QC procedures, frequencies, and acceptance criteria (2). These are considered to be the minimum quality controls needed to perform the method successfully. Additional QA/QC procedures can and should be used. Regulated testing, such as testing performed according to regulatory requirements, may require additional QC and must be consulted before tests are performed.

Each method used in the laboratory should include acceptance criteria. If these criteria are not readily available, the laboratory should determine its own criteria by control-charting techniques or other documented procedures. In some cases, the laboratory may obtain certified reference materials for such tests or evaluate by analyzing strain-spiked samples. Reference cultures and other materials should come from accredited providers or competent suppliers.

To help verify the accuracy of calibration standards and overall method performance, laboratories may participate in an annual, or preferably a semiannual, program of analysis of blind QC samples, ideally provided by an external source. Such programs are sometimes called proficiency testing (PT) performance evaluation (PE) studies. An acceptable result on a sample of this type is a strong indication that a test protocol is being followed correctly. If an unsuccessful result is obtained, the laboratory should perform corrective action that includes a root cause analysis to determine the cause of any failed PT/PE sample. In many jurisdictions and in some regulatory programs, participation in PT studies is a required part of laboratory certification/accreditation.

Each QA/QC section below references a documentation section. All relevant QC information accompanying the data must be retained. Retention of data must be described in laboratory SOPs and is generally for a minimum of 5 years or as long as required by governing regulations or accreditation/certification programs.

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Definitions

Batch.—Samples that are prepared and/or analyzed together

using the same process and personnel along with the same lot(s) of reagents.

Demonstration of capability (initial or ongoing).—A documented process whereby an analyst uses single-blind sample(s) and performs the QC requirements of the method, laboratory SOP, client specifications, and/or any additional laboratory standards. Test results must be within the limits of the laboratory's QC requirements.

Laboratory fortified blank.—Also referred to as a spiked blank, QC check, or laboratory positive or negative control sample. This sample is the matrix with no target microorganisms present, which is then spiked with a known concentration of a verified microorganism. This sample is then taken through all sample preparation and analytical steps of the procedure.

Matrix.—The substrate of the test sample, in most cases some form of liquid, which could include, but is not limited to, drinking water, bottled water, ambient water, and pool/spa or marine water.

Matrix spike.—A sample of matrix that is spiked with a known amount of organisms and processed as a typical sample, either quantitatively or qualitatively. Matrix spikes are often performed to determine if the matrix will have an effect on the outcome of the test.

Positive and negative culture controls.—Cultures of known microorganisms that will or will not produce a reaction in known media and under known test conditions. Certified reference cultures should be used, when available.

Proficiency test sample.—A blinded sample with a known concentration and/or population of microorganisms that is provided to test whether the laboratory can produce analytical results within the specified acceptance criteria.

QA.—A management system that includes laboratory activities, such as planning, implementation, assessment, reporting, and quality improvement to ensure that a process or service is of the type and quality needed and expected by the client.

QC.—Technical activities that measure the attributes and performance of a process, item, or service against defined standards to verify that they meet the stated requirements established by the customer.

SOP.—A version-controlled document that outlines all the materials, equipment, steps, and procedures of a process, and is used to train or re-train laboratory personnel. SOPs are part of the QC process within a laboratory to ensure that test procedures are performed consistently and correctly by all trained laboratory personnel.

Sterile or sterility.—Free from viable microorganisms.

QC Practices

Demonstration of Capability (DOC)

Each analyst must demonstrate initial and ongoing capability for each analysis performed. These results must be documented. Any potential problems must be identified, corrected, and documented. The intent is to prove both the reliability and integrity of the laboratory's test results. There are two types of DOC: initial and ongoing.

Initial DOC.—An initial DOC is performed before any test method is used, and any time there is a change in instrument type, personnel or test method. Prior to the use of a new method one of the two following options must be selected.

(**a**) If validation data are available from the manufacturer and/or regulatory agency, analyze four spiked samples in matrixes similar to the normal laboratory samples.

(**b**) If validation data are not available from the manufacturer and/or regulatory agency, analyze 10–20 spiked samples in matrixes similar to the routine laboratory matrix samples.

Analyze at least one PT sample, if available, from a PT provider certified or approved by a regulatory agency or accreditation authority. Observe analyst performance and analysis of known and unknown samples, and confirm that results meet laboratory criteria before allowing analyst(s) to conduct routine samples. Finally, document initial DOC results.

Ongoing DOC.—At least annually, analyze single-blinded samples, which can be a PT sample. For colony count methods, determine analyst colony counting variability. Replicate counts for the same analyst should agree within 5%, and replicate counts between analysts should agree within 10%. Determine the precision of duplicate counts, and repeat counts on one or more positive samples at least monthly.

Documentation.—Write procedures in SOPs, for example, initial and ongoing DOCs. Document initial DOC test results, e.g., analyst(s) name, matrix, microorganism(s) of concern, identification of method(s) performed, date of analysis, summary discussion of results involving conversion to logarithmic values, and comparison to method published results or to established and documented values.

Maintain each employee's training record and performance scores, authorization of employee to perform analysis, and documentation of review by management.

Record data comparability with reference laboratory and other laboratories, e.g., PT statistics. Document analyst colony counting variability, where applicable, and record any investigation and any corrective action taken. Document statistical calculations of data precision, along with any comments on data results, and finally, record comparability of a standard or reference method to laboratory's test results.

Method Blanks and Sterility Checks

Sterility testing and the use of method blanks ensure that unknown samples have not been compromised, contaminated, or invalidated due to improper handling or preparation, inadequate sterilization, or environmental exposure.

Method blanks.—Method blanks demonstrate that equipment, media, reagents, and sample containers were properly sterilized and were not contaminated while in storage or during the testing process.

A method blank is sometimes referred to as a Laboratory Reagent Blank and is typically a sterile sample consisting only of reagent water, or other blank matrix, that is treated and processed exactly the same as an unknown sample to determine if any method-specific reagents or equipment has interfered with the test sample results.

At least one method blank should be run with each batch of samples. In the event that the blank sample shows contamination or unexpected results, discard the affected test sample(s) and request re-sampling.

Sterility checks.—Sterility checks ensure that the processes used for sterilization are valid, and are done before running the method. Sterility checks for all media, reagents, buffers, and dilution/reagent water may be performed using nonselective growth media and should be performed on each new lot of media

or equipment before use (3). In the event that the sterility check sample shows contamination or unexpected results, discard the affected material. These tests may be done by a contract laboratory.

Documentation.—Write testing procedures in SOPs and mention the need for method blanks and sterility checks. The SOPs should include corrective action steps for nonconforming materials.

Document all method blank and sterility test results. If a contract laboratory is used for sterility testing, documentation must be obtained from them and maintained by the laboratory. Retain sterility data for each lot of laboratory-prepared or purchased material.

QC Samples/Laboratory Fortified Blank (LFB)

LFBs may also be referred to as QC samples, or negative and positive controls. They are used to ensure that growth media or other method reagents/materials are capable of supporting proper growth and/or analytical results.

LFB samples may be used to establish intralaboratory or analyst-specific precision and bias, or to assess the performance of all or a portion of the measurement system. They may also be used for initial DOC and ongoing DOC.

A QC sample/LFB is typically a sterile aliquot of reagent water or blank matrix to which a known quantity of a single verified microorganism is added. Use a low concentration inoculum level to duplicate normal environmental conditions. The added organism may be either typically positive or negative for a specific method. Add only one type or strain of organism to one sample. These samples are processed and analyzed exactly as a test sample.

Organisms to be used must be.—(**a**) Reference cultures obtained from an accredited reference culture provider, a recognized national collection, organization, or manufacturer recognized as an industry reference.

(**b**) Microorganisms may be single-use preparations, e.g., impregnated onto disks or strips, quantitative lyophilized cultures, or strain cultures (live or lyophilized), which are maintained in the laboratory following documented procedures that demonstrate the continued purity and viability of the organism. *Note*: If a facility does not have the capability to maintain stock cultures, the testing can be outsourced.

Add a known amount of organism to sterile reagent water or blank matrix. This sample may be used for initial and/or ongoing demonstration of capability or to assess multiple method attributes, such as selectivity, sensitivity, growth promotion, and growth inhibition.

Documentation.—The SOP must be written, outlining the process for creating and utilizing the QC samples/LFB. SOPs must denote the process for maintaining reference cultures and/or the use of single use preparations, and all results must be documented.

Matrix Spike and Matrix Spike Duplicate for Difficult Matrixes

The matrix being tested can have a profound and often unknown effect on resulting data. To mitigate unusable data, suspected difficult matrixes should be spiked with known concentrations of organisms to determine recoverability. Some methods may routinely require a matrix spike and matrix spike duplicate (4).

Matrix spike.—Add a known concentration of microorganism(s) at an anticipated ambient level to a field sample collected from the same site as the original. Process using the same conditions and criteria as a typical sample. Invalidate any sample if organisms are not recovered at the expected level from the matrix spike, then re-evaluate processes. Follow this process for any required matrix spike duplicate.

Documentation.—Describe the process for analyzing a matrix spike for difficult matrixes in a laboratory's SOP. Record all conditions and materials or strains used in the laboratory, including test results.

Calibration of Microbiological Equipment (Initial and Continuing) Performance Qualification

The laboratory must demonstrate that it has sufficient equipment and instrumentation of appropriate quality for each analytical method it conducts. Test equipment and instrumentation before initial use and during continual usage in the laboratory to demonstrate that they perform consistently (continued qualification), thereby meeting user's needs and suitability for their intended purpose.

Calibration.—Determine performance capability of all major equipment and instrumentation before first use. Monitor performance capability on an ongoing basis as determined by SOP, and **s**chedule regular calibration activities.

Use reference standards; e.g., National Institute of Standards and Technology (NIST) traceable thermometers, NIST Class S/ American Society for Testing and Materials Class 1 weights, and certified or otherwise qualified personnel, to perform calibrations.

Conduct equipment maintenance on a routine basis to ensure continued performance as directed by standards and/or manufacturers' recommendations, using internal staff experts or experienced experts obtained by contract. Review these activities to detect any deviations from accepted protocol.

Documentation.—Record written procedures on the use and operation, calibration, maintenance, and acceptance limits on all relevant equipment or instrumentation in the form of SOPs. Retain all critical manufacturers' manuals and document their location for easy retrieval.

Record reference standards used and their calibration if applicable. Document initial and ongoing calibration and ongoing maintenance activities and results. Finally, document any problems found and its resolution.

Control Charts and Trend Analyses of QC Results

The laboratory must demonstrate equipment, instrumentation, or analytical changes over time. These trends in process control are best demonstrated in tabular form, graphs, or charts and show that the laboratory is operating under control and with the expected variations of the analyses. If trends exceed control limits, corrective action must be initiated.

Steps to manage and trend QC results.—Follow SOPS for critical equipment and instrumentation, e.g., autoclave performance for timing, temperature, pressure and usage, and temperature recording device(s) calibration; glassware washing, including inhibitory detergent residue checks; balance calibrations, etc.

Follow SOPs for each analytical method being used, and follow all QC checks for steps of the analytical process, e.g., sample, dilution, sample bottle checks, volumetric checks, media preparation, and culture control testing.

Calculate precision of replicate analyses for each different type of sample examined, e.g., drinking water, ambient water, or wastewater by performing duplicate results on the first 15 positive samples of each matrix type, with each set of duplicates analyzed by a single analyst.

If there is more than one analyst, include all analysts regularly running the tests, with each analyst performing an approximately equal number of tests. Thereafter, analyze 10% of routine samples in duplicate or one per test run. Develop control charts with the initial 20 assays, then measure changes over time after developing mean and upper and lower control limits.

Documentation.—Record results for calibration, verification, and QC of all critical equipment and instrumentation and analytical method activities. Record routine analyst(s) performance:

(**a**) For routine performance evaluation, compare counts between analysts testing the same samples.

(**b**) Replicate counts for the same analyst should agree within 5% (within analyst repeatability of counting) and those between analysts should agree within 10% (between analysts reproducibility of counting). If they do not agree, initiate investigation and necessary corrective action.

(**c**) Record duplicate analyses of the first 15 positive samples of each matrix type and record as D1, or D2 if a second analyst is also conducting these tests. Calculate the logarithm of each result. If either of a set of duplicate results is <1, add 1 to both values before calculating the logarithms. Calculate the range (R) for each pair of transformed duplicates and the mean ($\bar{R}$) of these ranges.

(**d**) With the routine samples run in duplicate or one per batch or test run, transform the duplicates and calculate their range as above. If the range is $>3.27(\bar{R})$, there is greater than 99% probability that the laboratory variability is excessive; in such a case, discard all analytical results since the last precision check. Identify and resolve the analytical problem before making further analyses. For additional information *see Standard Methods for the Examination of Water and Wastewater* (5).

Corrective Action and Root Cause Analysis (RCA)

The objective of a QA manual is to ensure that the laboratory produces data of known and documented quality, thus ensuring a high quality of laboratory performance. Both internal and external audits of the laboratory operations and procedures allow early identification of any weaknesses, including training needs, opportunities to improve documentation and record-keeping, review of reporting systems, and ensuring compliance with regulations and client requirements. However, events that result in either incorrect or questionable data results can still occur. When this happens, it is important to have established and implemented a systematic process to uncover the root cause of the issue and a plan of action to prevent the situation from occurring again. These two processes are defined here, but will require modification depending upon the type and severity of the initial problem.

RCA.—RCA is a structured problem-solving process that involves identification of a specific procedural step or process that led to a faulty or unexpected outcome. The purpose of performing an RCA is to address, correct, or eliminate root causes, as opposed to merely addressing the obvious symptoms.

Corrective actions.—Corrective actions are directed corrective measures aimed at preventing specific issues uncovered during RCA. It is likely that recurrence can be prevented if specific, measurable, corrective actions are put in place after a root cause is identified.

General Process of RCA and Corrective Actions

The following steps and questions can be used to help the laboratory develop and implement both a RCA and corrective action plans. Not all parts will pertain to every laboratory, and other processes not mentioned here may be worthy of adding. The RCA and corrective action development will be specific to a laboratory and the processes and steps that are followed there. Be prepared to document your investigations and elicit a team to help ensure objectivity.

Define the problem factually.—Include the quantitative and qualitative properties of the outcome or issue, the nature of the issue, and the magnitude, locations, and timing.

Classify and document.—What are the steps that must be taken to get to an end result similar to the current issue? List these steps and any associated training or other requirements for each step. Classify causes into causal factors that relate to an event in the sequence and root causes, that if eliminated or changed, probably interrupted that step of the sequence chain.

Examples of steps and processes that should to be captured, classified, and documented for RCA include, but are not limited to: sampling, including hold time and temperatures; sterility checks; equipment checks; training requirements and updates; performing methods correctly; and supplier documentation. If there are multiple root causes, which is often the case, document these clearly for later optimum selection. Identify all other harmful factors that have equal or better claim to be called root causes.

Identify corrective action(s) that will with certainty prevent recurrence of each harmful effect, including outcomes and factors. Check that each corrective action would, if implemented before the event, have reduced or prevented specific harmful effects.

Identify effective solutions that prevent recurrence, and with reasonable certainty and consensus agreement of the group, are within your control, meet your goals and objectives, and do not cause or introduce other new, unforeseen problems.

Implement the recommended root cause correction(s), and ensure effectiveness by observing the implemented recommendation solutions in action, typically by internal audit.

Documentation.—All steps in the determination of root cause, the corrective actions identified, corrective steps taken, and success of these changes should be documented. Modify any internal documents, such as SOPs or work instructions, to reflect changes made upon RCA and corrective action.

QC Acceptance Criteria

QC acceptance criteria are used to determine if test results are acceptable, and must be established to monitor the daily operation during laboratory testing processes.

Establishing criteria.—QC acceptance/rejection criteria are established for the following: tests for clean glassware; tests for

reagent water quality, also known as the water suitability test, membrane filters, and laboratory-prepared and purchased media; sterility checks of sterile reagent or dilution water; and water sample integrity and holding conditions. Established analytical methods include: variability of colony counting between analysts; precision of quantitative methods; and verification of results, including both positive and negative control. The purpose of verification is to determine if the analytical method is performing as expected. For example, positive samples on M-Endo agar may be tested periodically in lauryl tryptose broth to see if the colonies ferment lactose.

Follow manufacturers' or regulatory acceptance criteria when possible. When no method or regulatory criteria exists, the laboratory should have procedures for the development of acceptance/rejection criteria. Any new method must be validated to establish if the performance criteria provide reliable data.

(**a**) *Qualitative test methods*.—The selection of criteria should ensure the accuracy, precision, specificity/selectivity, detection limit (1 CFU/100 mL for presence/absence samples), robustness, and repeatability of the test (1, 6).

(**b**) *Quantitative test methods*.—The selection of criteria should ensure the accuracy, precision/repeatability, precision/reproducibility, recovery/sensitivity, detection limit, upper counting limit, and range of the test (1, 6).

Determine in advance what action is needed if QC acceptance criteria fails. Possible actions include repeating test, recalibrating, rejection of test batch, RCA, and corrective action.

Documentation.—Document QC acceptance/rejection criteria for established tests in SOPs. Record criteria results and pertinent information for all SOPs with acceptance/rejection criteria.

QC results are reviewed on an ongoing basis by the laboratory manager or designee. QC acceptance/rejection criteria for new methods should be documented.

Document actions to be taken if acceptance criteria are not met. When criteria are not met, record the root cause and corrective action(s).

Batch

A batch is typically an uninterrupted series of analyses on a single matrix type using a single method (1, 7). Generally, an analytical batch describes a group of samples that are processed and/or analyzed as a unit. It is expected that batched samples will behave similarly with respect to both the sampling and testing procedures being used and are processed as a unit. A batch, with appropriate QC samples, is processed together using the same method, the same lots of reagents, and at the same time or in continuous, sequential time periods by the same personnel.

For QC purposes, the maximum number of samples in a preparation batch is generally 20/matrix. If more than 20 samples are to be processed in a 12 h shift, the samples must be separated into preparation batches of 20 or fewer samples. Each analytical batch may contain more than the 20 samples prepared in one preparation batch. Samples must be accompanied at least by a positive and negative control resulting in a minimum of three analyses. The negative control may be a method blank.

Documentation.—Document batch numbers as typically done in the laboratory setting. Record lot numbers from materials used for each batch, including, but not limited to, media used, reagents, and controls.

Record incubation temperature and time for each batch, and record results, including confirmations, if required, for each batch along with any additional observations for each batch.

Minimum Frequency QC Checks of Laboratory Equipment

To ensure precise and consistent results, laboratory equipment must be installed, maintained, and calibrated properly. Critical equipment requires a higher frequency of testing; some examples are listed below (1, 3).

Minimum Guidelines for Maintaining Some Types of Equipment

(**a**) *Autoclaves*.—Initially establish functional properties and performance. Use temperature sensitive tape with each run. Keep records of each cycle including date, contents, sterilization time and temperature, total time in autoclave, and analyst's initials. Use continuous temperature recording device or maximum recording device. The maximum recording device should be used weekly to verify that 121°C has been reached. At least once a month use a biological indicator. Check timing device quarterly, and perform maintenance annually.

(**b**) *Balance*.—Zero with each use. Clean pans after each use. Check with at least two reference weights at least on each day of use or as defined in an SOP. Reference weights used for checks must be from an accredited calibration laboratory or a National Metrology Institute, such as NIST. Service balances annually or sooner if conditions change (for example, if the balance is moved), and recertify weight as specified in the certificate of calibration at least every 5 years.

(**c**) *Biosafety cabinet*.—Before each use, purge air, disinfect before and after use, and certify annually.

(**d**) *Conductivity meter*.—Calibrate meter on day of use.

(**e**) *Freezer*.—Check temperature daily and defrost annually.

(**f**) *Incubator units*.—Verify that uniform temperature is maintained throughout each incubator by recording temperature at least twice a day, at least 4 h apart. If a calibration correction is required, record both the corrected and uncorrected temperature readings.

(**g**) *Glassware*.—Inspect for cleanliness, chips, and scratches before using. Verify volumetric markings before use for non-Class A glassware before use. Check pH with bromothymol blue with each washed batch. Conduct inhibitory residue test with initial use of each new batch of detergent and if the washing procedure is revised.

(**h**) Check for auto-fluorescence with each new batch of purchased bottles.

(**i**) *Hot air oven*.—Use spore strips monthly to confirm sterility.

(**j**) *Membrane filter units*.—If graduation marks on the funnel are used to measure sample volume, check their accuracy with a Class A graduated cylinder and record and maintain results.

(**k**) *Micropipettors*.—Test equipment with movable parts for accuracy and precision on a regular basis, as determined by the laboratory. A quarterly check is commonly used. Calibrate on a regular basis, as determined by the laboratory. An annual calibration is commonly used.

(**l**) *Microscopes*.—Clean optics and stage after each use.

(**m**) *Multiwell sealer*.—Check performance monthly.

(**n**) *pH meter*.—Standardize pH meters before each use with

pH 7.0 and either pH 4.0 or 10.0 standard buffers. Record date, calibration results, and analyst's initials. Measure and record pH meter slope with each use; verify that the slope meets manufacturer's criteria.

(**o**) *Refrigerators*.—Record calibrated-corrected temperature at least once a day.

(**p**) *Spectrophotometer or colorimeter*.—Analyze a calibration standard, or standards, plus a method-specific blank each day before analyzing samples.

(**q**) *Temperature recording devices*.—Glass, dial and electronic thermometers must be graduated in 0.5°C increments unless they are used in tests that are incubated at 44.5°C, in which case, they should be graduated in 0.2°C increments.

At least annually, check glass and electronic thermometers; check dial thermometers quarterly. Check thermometers at the temperature used against a reference thermometer from an accredited calibration laboratory or a National Metrology Institute such as NIST. Discard any thermometer that differs by more than 1°C from the reference thermometer. Recalibrate reference thermometers every 5 years.

(**r**) *Timers*.—Check timing with stopwatch annually.

(**s**) *Water bath incubator*.—Verify that the water bath maintains the set temperature. When the water bath is in use, monitor and record calibration-corrected temperature twice daily, at least 4 h apart.

(**t**) *UV instruments* (short-wave).—Test UV instruments with a UV meter or perform a quarterly plate count check.

Documentation.—There should be written procedures on the use and operation, calibration, maintenance, and acceptance limits on all relevant equipment. Retain manufacturer's manuals and document their location for easy availability. Document reference standards used and calibration certificates, initial and ongoing calibration activities and results, ongoing maintenance activity, such as temperature readings, and any problems found along with corresponding resolution.

Summary

The generation of quality microbiological data requires laboratories to pay particular attention to factors than can negatively influence results. The appropriate handling and processing of samples that may contain living organisms are directly linked to the laboratory's submission of data that reflect water quality and, thus, the regulatory body's ability to assess protection of human health. Following the QA and QC steps detailed here, in addition to requirements specified in a method, will help ensure quality data outcomes.

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