&EPA
United States
Environmental Protection
Agency

Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples

October 2004

Disclaimer

The Technical Support Center, Standards and Risk Management Division, of the U.S. EPA Office of Ground Water and Drinking Water, Cincinnati, OH, and the National Exposure Research Laboratory of the U.S. EPA Office of Research and Development, Cincinnati, OH, have prepared this guidance manual. Support for preparation of the manual was provided by the CSC Biology Studies Group under contract number GS-10F-0135K. The manual has been subjected to the Agency's peer and administrative review and it has been approved for publication as an EPA document.

This manual is not a regulation; EPA offers it as guidance for laboratories developing polymerase chain reaction (PCR) based-analyses on contaminants in environmental samples and for decision makers who need to judge the quality of PCR data.

The mention of trade names or commercial products in this manual does not constitute endorsement or recommendation for use.

Any questions regarding this document should be addressed to:

Keya Sen
U.S. EPA Office of Ground Water and Drinking Water
Technical Support Center
26 West Martin Luther King Drive
Cincinnati, OH 45268-1320
sen.keya@epa.gov
(513) 569-7026
(513) 569-7191 (facsimile)

G. Shay Fout
U.S. EPA Office of Research and Development
National Exposure Research Laboratory
26 West Martin Luther King Drive
Cincinnati, OH 45268-1320
fout.shay@epa.gov
(513) 569-7387
(513) 569-7117 (facsimile)

TABLE OF CONTENTS

TABLES AND FIGURES

SECTION 1. INTRODUCTION

From the isolation of specific genes to the sequencing of entire genomes, the polymerase chain reaction (PCR) has become one of the most widely used technologies for conducting biological research. Advances have led to the development of specific and sensitive high-throughput PCR methods for the detection of a variety of microorganisms, and these methods are increasingly being applied to analysis of environmental samples.

The successful application of PCR requires the proper use of techniques and interpretation of results. Many PCR methods offer a level of sensitivity equal to, or greater than, more traditional environmental microbiological methods. Due to the ability to amplify small amounts of nucleic acid, PCR can be used to detect organisms that are difficult to culture *in vitro* or that cannot be cultured. However, the advantages of these techniques can be offset by the demanding assay protocols and the need to follow quality assurance/quality control (QA/QC) procedures carefully. These QA/QC procedures are necessary because the ability of PCR to produce many copies of target DNA creates the possibility of contamination by previously amplified products, which can lead to false-positive results. In addition, environmental samples may inhibit the PCR, which can lead to false-negative results. As efforts are made to standardize PCR protocols for analyses of environmental samples, it is essential to establish standardized QA/QC procedures.

1.1 Purpose

This guidance manual has been developed to serve as a resource and a reference for the following:

- Laboratory QA/QC practices for environmental sample analyses involving PCR techniques

- Laboratory QA/QC practices for new U.S. Environmental Protection Agency (EPA) PCR method protocols

- Laboratory QA/QC practices for EPA grants and assistance agreements involving PCR analyses

- A basis for researchers, managers, and quality assurance officers to evaluate the quality of PCR data of research projects and technical papers

1.2 Scope

This manual is intended to serve as general guidance for the development of laboratory and method-specific QA/QC procedures for PCR analysis of environmental samples. However, because PCR analysis includes a broad range of nucleic acid targets and procedures, all QA/QC procedures necessary for a particular protocol may not be addressed.

This document does not address federal, state, and local regulations governing waste management, hazardous materials, and radioactive material; it is the laboratory's responsibility to comply with relevant regulations. Furthermore, this guidance does not address related safety issues; it is the laboratory's responsibility to establish appropriate safety and health practices.

The following topics are addressed in this manual:

- **Section 2. Laboratory Quality Assurance.** This section provides guidance on general laboratory QA/QC applicable to PCR analysis of environmental samples. It provides recommendations for personnel, facility design, workflow, equipment, disposables, and cleaning.

- **Section 3. Reagents, Kits, Primer Sets, and Enzymes.** This section provides QA/QC guidance for reagents, kits, primer sets, and enzymes used in PCR analysis of environmental samples, including the information for these materials that should be recorded in laboratory logbooks and storage conditions.

- **Section 4. Method Development and Assessment.** This section provides guidance on the development of a PCR method and its component parts, including factors to consider when selecting appropriate PCR techniques and confirmation of amplicons.

- **Section 5. Quality Control Samples for Methods Using PCR.** This section provides a summary of the controls necessary for PCR analysis of environmental samples. Recommended corrective actions for control failures and proficiency testing are also covered.

- **Section 6. Data Recording, Record Keeping, and Data Evaluation.** This section provides guidance on data recording, record keeping, and data interpretation, including variability, equivocal results, and limitations.

SECTION 2. LABORATORY QUALITY ASSURANCE

Laboratories performing PCR analyses of environmental samples should develop a written QA Management Plan that describes how the laboratory conducts its day-to-day routine operations. This plan should describe the laboratory organization and line of authority. It should identify a Laboratory Quality Assurance Manager and describe the Manager's responsibilities and authority. The plan should describe minimal requirements for personnel (Section 2.1), facilities (Section 2.2), sampling and sample handling procedures (Section 2.3), equipment maintenance and quality checks (Section 2.4), laboratory supplies (Section 2.5) and reagents (Section 3), and procedures for maintaining laboratory cleanliness (Section 2.6). It should identify and describe the analytical procedures (see Section 4) and reference appropriate standard operating procedures. The implementation of appropriate QC measures for each method (Sections 4 and 5) should be detailed. The plan should describe how the accuracy of raw data is maintained, how raw data is converted to final data and how records are maintained and stored (Section 6). Finally, it should identify the laboratory's response and corrective actions (Section 5.4) to quality assurance failures.

Section 2 provides guidance on the recommended qualifications and training for personnel who perform PCR analyses and on the use of protective wear by laboratory personnel to prevent sample contamination. Facility design and workflow are considered, as they are essential for preventing both sample contamination and erroneous results. In addition, calibration, maintenance, and cleaning procedures for laboratory equipment and the standards for disposable labware are discussed.

2.1   Personnel

Personnel working in the laboratory performing PCR analysis should meet background and training specifications outlined below and should follow the guidance provided concerning protective outerwear.

2.1.1 Background and Training

Analysts involved in PCR analyses should have undergraduate course work in molecular biology, biotechnology, biochemistry, molecular genetics, or other course work that covers PCR and recombinant DNA theory and practice. However, commensurate job-related training and experience may be substituted. Hands-on training, including the review of standard operating procedures (SOPs) or manuals, should be completed for each technique under the supervision of experienced personnel (e.g., principal investigator, senior analyst, or manufacturers' representative).

Although the amount of time required for training will vary depending on the analyst and the technique, each analyst should demonstrate that they can successfully perform the method through analyses of positive and negative control samples before being allowed to analyze environmental samples without supervision. An initial demonstration of capability should include at least four replicate analyses of seeded reagent water, two replicates of seeded environmental water, and a method blank (see Section 5.2.2), as well as proficiency testing (see Section 5.6). These recommendations for the initial demonstration of capability are based on current requirements for EPA's conventional microbiological methods. Each analyst also is expected to be knowledgeable in laboratory safety and QA/QC procedures.

The laboratory should maintain a training record for each analyst that documents the following:

- Dates and scope of PCR method training

- Initial demonstration of capability

- Proficiency test results for each analysis type

- Dates and scope of laboratory QA/QC training

- Dates and scope of laboratory safety training

2.1.2 Outerwear

Dedicated laboratory coats and powder-free gloves should be available in each laboratory room (see description of laboratory rooms in Section 2.2.1). Laboratory coats should be removed and gloves discarded before leaving each room. Changing laboratory coats and gloves reduces the possibility of contamination with template (the nucleic acid from which the PCR is performed) or amplified nucleic acid. Gloves should be changed after working with seeded or environmental samples, after handling template or amplified nucleic acids, and after contact of the outside of the gloves with skin. The latter prevents introduction of enzymes prevalent on the skin, such as DNases and RNases, that degrade nucleic acids. In the absence of a laminar flow hood or biological safety cabinet (see Section 2.4.7), protective dust masks or surgical masks may be worn to further reduce the risk of contamination of airborne nucleic acids from the analyst.

Laboratory coats should be cleaned regularly to reduce the possibility of contamination of the designated workspace and the PCR reaction. Laboratory coats should be separated from non-laboratory clothing (i.e., laboratory coats should not to be taken home and washed with other clothes), and cleaned only with other laboratory coats that were in the same work area (e.g., laboratory coats from the sample preparation room should not be cleaned with laboratory coats from the amplification and product room). Many regions have specialized companies that are qualified to clean laboratory coats. These companies often clean coats in lots to prevent the mixing of coats from a designated laboratory area with other coats or materials from another area. The frequency of cleaning is dependant on the amount of PCR work the laboratory is performing, and busy laboratories may want to clean coats weekly or daily. Laboratory coats used to prepare positive controls should be dedicated to this task and cleaned more frequently. To eliminate the need for cleaning laboratory coats, disposable (single-use) laboratory coats also may be used.

2.2   Facility Design and Workflow

The high sensitivity of PCR techniques requires that demanding assay conditions be followed. The laboratory should be designed and operated in a way that prevents contamination of reactions with amplified products from previous assays and cross-contamination between samples, both of which can lead to false-positive results. Guidance on laboratory design and workflow to mitigate potential contamination and maximize data quality is provided in Sections 2.2.1 and 2.2.2.

2.2.1 Facility Design

Contamination between samples and from previous PCR amplicons generated in the laboratory is a significant potential source of invalid PCR results. Thus, the separation of work space is critical. A laboratory performing PCR analyses on environmental samples should be divided into at least three physically separate rooms:

- Reagent preparation (using positive pressure to prevent the introduction of contamination)

- Sample preparation (using negative pressure to keep template nucleic acids in the room)

- Amplification and product detection (using negative pressure to keep amplified nucleic acids in the room)

A unidirectional workflow will reduce the opportunity for contamination to occur (see Section 2.2.2 and **Figure 2-1**). No materials, supplies, or equipment from the sample preparation room should be taken into the reagent preparation room. Nothing from the amplification and product detection room should be taken into the sample preparation room or the reagent preparation room. To reduce the threat of sample contamination from sample processing activities, separate biological safety cabinets (see Section 2.4.7) should be dedicated for positive control and test sample processing. Vacant areas in the rooms may be used for non-PCR activities, such as analysis of chemical samples, but the personnel associated with these activities should maintain the unidirectional workflow, and the equipment should not be moved between the rooms used for any PCR sample processing and analysis steps.

Figure 2-1. **Workflow through Laboratory Rooms**

Reagent Preparation Room

| Preparation of reagents for enviromental sample processing | Preparation of PCR reagents | Preparation of master mix (reagents minus nucleic acid) |

Positive pressure room

Sample Preparation Room*

Concentration/ isolation of organism from environmental samples → Isolation of nucleic acids → Addition of sample nucleic acids to master mix

Negative pressure room

Amplification and Product Room

PCR (thermal cycling) → Confirmation (visualization, quantitation, cloning, sequencing, etc.)

Negative pressure room

*   The sample preparation room should be divided into two physically separated areas (hoods) or rooms, one for sample and negative control preparation and the other for positive control preparation.

2.2.1.1 Reagent Preparation Room

The reagent preparation room should be designated for the preparation and storage of PCR reagents, including master mixes (mixtures of all reagents required for PCR except the sample). Addition of master mixes to PCR tubes should be performed in this room. To prevent cross-contamination and to avoid repeated freezing and thawing, reagent-stock solutions should be aliquoted into smaller "working" volumes and stored in this room for later use. It is recommended that the room be under positive pressure to deter contamination.

The reagent preparation room should have dedicated, adjustable pipettes with plugged, aerosol-barrier or positive-displacement pipette tips, laboratory coats, and disposable gloves. Fresh gloves and laboratory coats should be worn at all times to control contamination. Personnel should perform tasks in this room before working in the sample processing or amplification/detection rooms and should not move from these rooms back to the reagent preparation room.

---

*Important: Materials from other rooms (including amplified, template, and target nucleic acid or positive controls, supplies, or equipment) should **not** be brought into the reagent preparation room.*

2.2.1.2 Sample Preparation Room

The sample preparation room should be designated for sample processing and preparation of positive and negative controls. Sample processing may include concentration of target organisms in environmental samples as well as extraction and purification of nucleic acids from these organisms. The processed samples and controls should be added to tubes containing PCR master mix in this room. Whenever possible, PCR tubes should be capped as soon as the sample is added. When using PCR plates and adhesive covers, positive controls and seeded samples should be separated from wells with field samples to avoid contamination during template addition.

The sample preparation room should have dedicated adjustable pipettes with plugged, aerosol-barrier tips or positive-displacement tips. Fresh gloves and laboratory coats should be worn at all times to control contamination from this room to any other location. This room should be kept under negative pressure to prevent contamination outside of the room.

Ideally, two biological safety cabinets (see Section 2.4.7) should be used within the room—one for sample and negative control preparation, and the other for positive control preparation—to protect the samples from cross-contamination and to protect workers from exposure to pathogens. Separate pipettes and laboratory coats also should be designated for work in each hood.

Important: Nothing from this room should be taken to the reagent preparation room.

In addition to separating positive control preparation from sample and negative control preparation, laboratories may want to include a designated enclosed area or room for sample receiving and storage. This room would contain refrigerators for sample storage and should be isolated from any of the other areas. Personnel should not move from this room to the reagent preparation room.

2.2.1.3 Amplification and Product Room

This room should be designated for activities associated with PCR amplification and post-PCR analyses. The thermocycler should be located in this room. Gloves and laboratory coats should be worn at all times

and removed before leaving the room to control amplicon contamination of other locations. All equipment used for amplification and product detection should be dedicated to this room, including adjustable pipettes with plugged, aerosol-barrier or positive displacement pipette tips. This room should be kept under negative pressure. Although PCR amplification and post-PCR analyses may be performed in the same room, laboratories may want to separate these activities in different areas or different rooms to reduce the risk of contamination from amplified products.

Important: Nothing from this room should be taken into either the sample preparation room or the reagent preparation room.

2.2.2 Workflow

A unidirectional workflow should be used to reduce the potential for contamination. Color-coding of equipment, reagents, laboratory coats, and supplies can help maintain the unidirectional workflow by designating colors specific to each laboratory room. Laboratory manuals and notebooks also should not be moved from room to room. Laboratories may want to consider using electronic data reporting to avoid this potential source of contamination.

On a given day, analysts should not return to the reagent or sample preparation rooms after working in the amplification and product room. Similarly, analysts should not return to the reagent preparation room after working in the sample preparation room. If an analyst must unexpectedly return to an upstream room in the workflow process, the analyst should first shower and change clothes. If a breach in this workflow process does occur, the affected laboratory rooms and equipment should be cleaned according the guidance provided in Sections 2.4 and 2.6, and the results should be monitored as defined in Sections 5.2 and 5.4.

A schematic representation of the workflow is presented in **Figure 2-1.**

2.3 Environmental Sample Acceptance Protocol

The laboratory should have a protocol in place for the acceptance of environmental samples for PCR analysis. This protocol should be documented in a laboratory SOP and include sample acceptance criteria and corrective actions for samples that do not meet the criteria (e.g., recollection of the sample or follow-up with the sample collector to obtain missing information). The sample should be assessed when it is received at the laboratory to verify that the sample volume was adequate, the sample was handled and preserved appropriately (e.g., chilled and shipped overnight), the holding time requirement was met, and that all required sample collection information was recorded by the sample collector. Specifications for sample volume, sample handling, and holding times will be method dependent and sample acceptance criteria should be based on the specifications described in an SOP prepared for each method. After the sample is assessed, information on the date and time of sample receipt and sample condition should be recorded. The sample should be marked, logged, and tracked with a unique identifier.

2.4 Equipment

The equipment used to perform a PCR method should function properly to generate reliable data. To verify that equipment is functioning properly, the laboratory should have a schedule for maintaining equipment. The schedule should include the setup, calibration, repair, record keeping, and normal operation of all equipment used in sample analysis, as stated in the SOP for each individual instrument or

method. The results of all tests should be documented in an equipment logbook and/or electronic database. The logbook or database should be checked monthly by QC personnel or the laboratory supervisor, and any problems and corrective actions noted. Equipment should be dedicated to a specific laboratory room, and the instrument manuals from the manufacturer should be available. Individual laboratories should decide which tests to perform to assess the functionality of the instruments and the frequency at which to test them. Recommended QA for specific types of instruments used for PCR analyses are provided in Sections 2.4.1 through 2.4.11.

2.4.1 Thermocyclers and Real-time PCR Instruments

Thermocyclers are essential to all PCR methods, and great care should be taken to ensure that they are well-maintained and reliable. Typically, the manufacturers of the instruments have developed recommended procedures to test and maintain the instrument. The block temperature of a thermocycler should be tested at least twice a year (or at the frequency specified by the manufacturer) by the laboratory or under a maintenance agreement to ensure uniform heating throughout the block. Monthly testing should be performed by laboratories performing a large number of reactions.

Block temperature should be tested with an external probe that has been calibrated against a temperature standard. For testing, the probe is placed in several of the wells in the periphery and center of the instrument. All temperatures should be within the manufacturers' specifications. The amplification program used in each run should be printed to further verify the conditions of the PCR. This also allows the analyst to determine if there were any instrument malfunctions during the reaction. If the thermocycler does not have software with this capability, then the cycling program used should be documented in the laboratory's SOP with results of monthly calibration checks.

Real-time PCR instruments are equipped to perform fluorescence excitation and detection to monitor amplification throughout the PCR cycles. For this reason, the design is often different from the standard thermocycler, and calibration may be specific to the instrument design and should be carried out according to manufacturers' specifications. For example, several real-time platforms do not have heating blocks but rotor designs, with samples spinning in an air chamber that is heated and cooled. Temperature can be calibrated for this type of instrument using a calibration rotor provided by the manufacturer.

Laser performance, alignment, and safety devices should also be checked and optical systems calibrated. This may involve analyzing fluorescent test trays to confirm consistent detection from each well. For rotor designs, each tube is passed by the same detector, providing greater sample to sample consistency. Spectral calibration files may also need to be recalibrated to confirm that the excitation signals from the range of fluorophores used can be separated, and gain settings adjusted. Analysts can perform part of this calibration, but service technicians trained by the equipment supplier will be required to carry out a full maintenance and calibration service. A full service should be preformed at least annually.

2.4.2 Centrifuges

Separate centrifuges, including microfuges, should be used for pre- and post-PCR procedures. Centrifuges should have speed capabilities that meet the criteria required at different stages in the overall method. The manufacturers' instructions for calibration should be followed. The centrifuge should be balanced before use to increase bearing life and minimize vibrations that can unsettle concentrates.

2.4.3 Gel Electrophoresis Chambers

Chambers should be inspected before each use to ensure that electrodes and buffer tanks are intact, and that power supply electrodes fit snugly. Gel electrophoresis chambers should be rinsed several times with water after each use in the designated product room.

2.4.4 Power Supplies

Power supplies should provide voltage and current readings. Before use, electrodes should be checked to ensure that they fit snugly. Electrical cables, electrode wires, and cable connections should be checked for breaks, fraying, corrosion, or looseness, and replaced if needed.

2.4.5 Hybridization Apparatuses

Hybridization with target sequence-specific oligonucleotide probes is a commonly used post-PCR product confirmation step. Hybridizations can be performed in several different types of apparatus, however, hybridization ovens are becoming increasingly popular. Hybridization ovens should come with detailed instructions from the manufacturer on the cleaning and routine maintenance of the apparatus. General maintenance includes the cleaning of the incubator itself (the interior, exterior, and rotator) with distilled water periodically as well as when any spill has occurred inside the unit (and in accordance with radiation removal protocols, if applicable). Twice a year (or according to the manufacturers' instructions), the temperature and the rotation/rocking speed of the oven should be tested and the unit repaired, if the tested values do not match the registered values indicated on the unit.

Care should be taken with the hybridization bottles. These bottles should be inspected periodically for chips and cracks and for deterioration of the O-ring. Bottles that are chipped or cracked should be discarded and replaced. Deteriorated or damaged O-rings should be replaced immediately. Bottles and O-rings that contact radioactive material should be thoroughly cleaned of any radiation before they are discarded. These bottles also should be cleaned after each use in accordance with manufacturers' specifications and in accordance with radiation removal protocols (if applicable).

2.4.6 Sequencers

Sequencers should be calibrated according to the manufacturers' recommendations, and all calibrations should be documented. For sequencers using capillaries, it may be necessary to perform both spatial and spectral calibrations using standards provided by the manufacturer. Calibrations should be performed at the frequency specified by the manufacturer and according to the manufacturers' instructions. If the sequencer uses sample blocks, the blocks should be cleaned regularly and checked for fluorescence contamination. Instrument performance should be checked regularly using standards provided by the manufacturer. Preventative maintenance should be performed annually by a qualified technician.

2.4.7 Laminar-Flow Hoods / Biological Safety Cabinets

Classification and naming of laminar-flow hoods (hoods) and biological safety cabinets (cabinets) are not consistent between vendors and laboratory users should pay careful attention to the specifications of the hood or cabinet to ensure that it is appropriate for its designated use by the laboratory. General classifications of hoods and cabinets for the purposes of this document are indicated in **Table 2-1** and the text below.

Table 2-1.   Summary of Classification of Hoods and Cabinets

Unit type	UV light present	Air flow system	Use
PCR cabinet (Type A)	Yes	None	Reagent preparation only
PCR cabinet (Type B)	No	Intake filtered	Not recommended for any aspect of PCR preparation
PCR cabinet (Type C)/Laminar-flow hood	Yes	Intake filtered	Reagent preparation only
Class I biological safety cabinet	Yes	Exhaust filtered	Not recommended for any aspect of PCR preparation
Class II or III biological safety cabinet	Yes	Intake and exhaust filtered	All aspects of PCR preparation

Although several types of chambers are available, many may not provide satisfactory contaminant removal for the preparation of reagents and samples for PCR. Laminar-flow hoods (also listed by suppliers as "enclosures" or "chambers") provide air that is cleaner than the laboratory air and reduce the chance of airborne contamination. These hoods provide protection from outside contamination to material inside the hood, but do not protect the worker or the environment unless they have a filtered exhaust. Biological safety cabinets (BSCs) have a classification system that ranges from Class I to Class III (33, 47). Class I cabinets have inward air flow and HEPA-filtered exhaust that provides personal and environmental protection, but no product protection. Class II and III BSCs filter both air intake and exhaust, and prevent contaminants from entering and leaving the hood (reducing the likelihood of sample and work area contamination). Laboratories purchasing Class III BSCs should be aware that they require a larger air intake than Class II cabinets. This can produce problems with air balance if the hood is vented to an outside environment.

Hoods without an air-flow system or those with filtered air intakes may be used for reagent preparation, if they are equipped with UV lights. For all other purposes, laboratories should use Class II or III BSCs with UV lights. These types of hoods and cabinets provide efficient laminar air flow to keep contaminants from entering the work area or the laboratory. Other chambers that lack these qualities increase the likelihood of sample contamination. An alternative to the recommended BSCs is a Fed 209 Class 100 clean room with an appropriate ISO-class HEPA-filtered air intake and UV light.

Before use, hoods should be decontaminated using UV light for at least 8 hours (14, 34) and cleaned with bleach or other effective nucleic acid inactivating agent. After a spill or accident (see Section 2.6 for guidance on cleaning), hoods also should be cleaned with the nucleic acid inactivating agent and then decontaminated further using UV light. The airflow and HEPA filtration in all hoods should be monitored and certified per manufacturers' recommendations on at least annually.

2.4.8 Ultraviolet Lights

The placement of UV lights in hoods is designed to decrease biological and nucleic acid contamination by cross-linking nucleic acids (34). However, it should be noted that UV may not provide adequate protection from contamination for PCR amplicons that are very short. The UV bulb should be wiped with a wet cloth to remove dust every week. UV lights should be checked for intensity loss using a UV light meter once a month and replaced per manufacturers' recommendations.

2.4.9 Pipettes

Due to the numerous small volume transfers involved in PCR methods, automatic pipettes, fixed-volume, adjustable, positive-displacement, and/or micropipettes should be used, and should be calibrated

quarterly by the manufacturer or a technician using the tips commonly used in the laboratory. Documentation on the precision of the recalibrated micropipette should be retained. Calibration records should be kept on file in the laboratory's QC logbook. Each pipette should be sterilized according to manufacturers' recommendation on a regular basis or whenever contamination is suspected. Calibration of the pipette should be performed after sterilization.

If a micropipette calibration problem is suspected, the laboratory should tare an empty weigh boat using an analytical balance and pipette the following volumes of reagent water into the weigh boat using the pipette in question: 100% of the maximum dispensing capacity of the micropipette, 50% of the capacity, and 10% of the capacity (do not go beyond the recommended range of the pipette). Ten replicates should be performed at each weight. The weight of the water (assume that 1.00 mL of reagent water weighs 1.00 g) should be recorded and the relative standard deviation (RSD) calculated for each. The balance used must have sufficient accuracy in the range that will be used for calibration (e.g., for 1-50 µl volumes the balance should be able to accurately weigh 0.0001g). When measuring ultra-low volumes, a moisture source or other device should be used to prevent evaporation.

If the weight of the reagent water is within the manufacturers' recommended limits using the tips commonly used in the laboratory, then the pipette remains acceptable for use. If the weight of the reagent water is outside the acceptable limits, the instruction manual for the pipette should be consulted to determine appropriate adjustments/actions that can be taken and the calibration checked again. If problems with the pipette persist, the laboratory should send the pipette to the manufacturer for recalibration. Spectrophotometric calibration of pipettes also may be performed (1). Kits for this type of calibration are available from commercial vendors.

2.4.10   Temperature-Dependent Equipment

Thermometers should be placed in any equipment for which temperature is critical for proper performance of a parameter involved in the testing method or reagent handling. Calibration of all thermometers should be checked annually, at the temperatures used, against a reference National Institute of Standards and Technology (NIST) thermometer. The calibration factor should be indicated on the thermometer. The laboratory should record the date the thermometer was calibrated and the calibration factor in the QC logbook. If a thermometer differs by more than 1°C from the reference thermometer, it should be discarded. (50). Reference thermometers should be recalibrated every five years and the calibration factor noted on the thermometer. If the reference thermometer differs by more than 1°C, it should be discarded.

For equipment used in PCR analysis, the following temperature ranges should be applied:

- Incubators, water baths, and heating blocks: ± 0.5 °C of the temperature required by the protocol

- Refrigerators: 1°C to 5°C

- Standard laboratory freezers: -20°C ± 5°C

- Ultra-low freezers: -70°C ± 10°C

Temperatures of equipment should be monitored and recorded at least once a day for each workday in use. Alternatively, temperatures may be monitored continuously using a computer-based alarm system.

Important: *"Frost-free" freezers should not be used. Temperature fluctuations lead to reagent degradation.*

Separate refrigerators and freezers for samples, reagents, and final amplification products should be maintained in the appropriately designated laboratory room. Amplified product should always be kept in a separate freezer from reagents (such as master mix), samples, and sample concentrates.

2.4.11 Spectrophotometers, Luminometers, and Fluorimeters

Spectrophotometers, luminometers, and fluorimeters are frequently used in PCR methods to report concentrations (40). NIST-traceable spectrophotometer standards can be purchased commercially and consist of either filters or various dilutions of potassium dichromate or potassium hydrogen phthalate (KHP), and holmium oxide filters. Calibration of both wavelength and transmittance/absorbance can be performed by comparison to these standards. Fluorescent detectors should be calibrated every three to six months or more frequently if a problem is detected. Calibration is carried out through spectral calibration solutions needed to establish the pure dye spectra. These dyes are of known spectra and come with a normalization reference. Standard light plates and tubes can be obtained from the manufacturer for the calibration of luminometers that allow the reproducibility, sensitivity, and linearity of the luminometer to be confirmed. Some luminometers are also equipped with internal calibration protocols.

2.5 Disposables

Disposable materials used in PCR analysis include pipette tips, sample tubes, PCR tubes, and gloves. To reduce the contamination and degradation of the target nucleic acids, disposable materials should meet the standards discussed in Sections 2.5.1 to 2.5.3.

2.5.1 Pipette Tips

Standard pipette tips are not appropriate for PCR use, due to the possibility that contamination may be transferred from the pipette to the sample or vice versa. Vendors offer special tips for PCR analysis that can be used as an alternative to the positive displacement tips and pipettes. Special tips for PCR analysis include barrier tips and aerosol-resistant tips, both of which minimize cross-contamination of samples during pipetting. These tips can be purchased pre-sterilized and pre-loaded in hinged racks to provide tip protection and easy access. Pipette tips for PCR analyses should be lot-certified, RNase-free, DNase-free, and pyrogen-free.

2.5.2 Sample and PCR Tubes

Laboratories should use polypropylene tubes that are lot-certified DNase-, RNase-, and pyrogen-free. The size and style of PCR tubes or reaction plates recommended by the manufacturer for the thermocycler should be selected to ensure that the tubes are compatible with the block and lid height. Thin-walled tubes provide the best heat transfer, ensuring that the reaction volume reaches its specified temperature in the shortest amount of time, thereby improving specificity and reproducibility. Tubes containing stored samples and reagents should be centrifuged briefly before opening to ensure that all liquids are at the bottom of the tubes.

2.5.3 Gloves

Disposable gloves should be available in each section of laboratories used for PCR analysis. Gloves should be changed before leaving and entering each section of the laboratory and each time contaminating DNA is potentially encountered. In addition to reducing potential contamination from samples, wearing gloves may protect the technician from potential chemical exposure and prevent sample contamination due to human DNases and RNase.

2.6   Laboratory Cleaning

All work surfaces should be cleaned after each use with 0.6% sodium hypochlorite (NaOCl). The NaOCl solution should be prepared fresh daily by diluting commercial bleach 1:10 in water and adjusting the pH to 7. This solution inactivates pathogenic agents and destroys nucleic acids. Residual bleach, which may cause pitting in stainless steel counter tops and hoods, may be removed with a 0.1% sodium thiosulfate solution and a 70% ethanol rinse or equivalent. Commercial products that are specifically designed for removing nucleic acids and nucleases also can be used for surface cleaning.

Thermocyclers and centrifuges should be cleaned with the diluted bleach solution (see above) whenever contamination is suspected. Pipettes should be cleaned according to manufacturers' instructions. Racks and trays should be soaked in the 0.6% NaOCl solution and thoroughly rinsed with water after each use. Gel-trays, gel combs, and glassware used for blot hybridization should be rinsed with water or a mild detergent after each use.

SECTION 3. REAGENTS, KITS, PRIMER SETS, AND ENZYMES

This section addresses the general QA procedures that should be followed when working with reagents, kits, primer sets, and enzymes used in PCR analysis. Guidance on the record keeping for reagents, kits, primer sets, and enzymes is provided in Section 6.

3.1   Reagents

The reagents used in PCR amplification can be purchased or prepared in-house. Care should be taken to ensure the reagents are maintained contamination-free. All reagents should be clearly labeled with name, expiration date, and relevant safety information. Reagents from different lot numbers should not be interchanged without prior functional validation (see Section 3.1.1 through 3.4).

Molecular-grade water or its equivalent from commercial sources should be used for all assays. Laboratories also may use water purification systems that produce high-quality pyrogen and DNase/RNase-free water. If a laboratory uses a water purification system, it should be installed in a nucleic acid-free area (such as the reagent preparation area) to reduce the possibility of contamination.

Diethylpyrocarbonate (DEPC) treatment can be used to eliminate RNase from water used in RNA analysis. Addition of DEPC results in the covalent modification of nucleases (such as RNase), causing them to lose their function. Reagent water is treated with a solution of 0.1% DEPC for several hours and then autoclaved to degrade the DEPC completely. Proper autoclaving is necessary, because trace amounts of DEPC in a solution will lead to the modification of the purine residues in RNA by carboxymethylation. This leads to downstream effects in RNA experimentation (e.g., removing the ability of reverse transcriptase to bind RNA and synthesize DNA from an RNA template) (3).

3.1.1 Commercially Prepared

All reagents should be molecular grade, if available. Commercially prepared reagents should be stored according to the manufacturers' recommendations. All reagents from new lots should be tested to ensure that they work properly by running a PCR positive control (Section 5.1.1) using the new reagents. If the PCR positive control fails, new lots should be tested against the old lots.

3.1.2 Prepared In-House

For each type of laboratory-prepared reagent, criteria should be developed for expiration dates, functional acceptability, and storage conditions using product sheets from similar commercial products as guidance. The criteria should be documented in a laboratory SOP. Mixes should be used no later than the earliest expiration date of one of the components. Buffers should be inspected for precipitates or microbial contamination before each use.

3.2   Commercially Available Kits

Many types of commercial kits are available for PCR applications. These products expedite and simplify procedures, such as the isolation of DNA and RNA and the purification of nucleic acids to remove contaminants. A copy of the manufacturers' specifications and procedures should be kept in the laboratory's SOP binder for every commercially available kit used in the laboratory. The effectiveness of

the kits should be evaluated before use for the analysis of field samples by running an appropriate positive control (See section 5.1).

3.3   Primer Sets and Hybridization Probes

PCR analyses require the use of short segments of chemically synthesized DNA (which are called oligonucleotides or, more commonly, "oligos"). Primer sets are oligos with nucleotide sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Hybridization probes are oligos with specific nucleotide sequences that are internal to the sequences of the primers and which are used to confirm the amplification of the target. Guidance on the design of primers and probes is given in Section 4.3.3. Primers and probes, containing a specific sequence of nucleotides, can be obtained from a commercial vendor or can be prepared in-house, if the proper equipment is available.

3.3.1 Certification of Analysis

Primers and probes should be free from other contaminating sequences and enzymes. Impure oligos will decrease the specificity of the procedure. Certification of the quality of the oligos, including method of purification, purity, and concentration, should be required from all commercial manufacturers.

Purity may be assessed by HPLC or by separation on an acrylamide gel of appropriate concentration (24). Every lot of new oligos should be checked for contamination by being used in a PCR negative control (Section 5.2.1). No positive results should be found. Primers and probes should be added to the PCR master mix in the reagent preparation area.

Functional validation also should be performed on new lots of primers and probes by comparing their performance against older sets of known quality (31). The new oligos should be rejected or the concentration adjusted, if performance is significantly different from the proven lot. When real-time PCR is used, the validation of new sets can be done by comparing the PCR efficiencies of old and new sets. The PCR efficiency (E) is calculated from the slope of a standard curve (S) using the formula, $E=10^{-1/S}-1$ (36). The samples used for generating standard curves should be carefully prepared dilutions of a stock solution containing a known number of purified PCR template nucleic acids or total nucleic acids extracted from the target organism. A PCR efficiency of 1 (or 100%) is obtained when each target sequence present in the PCR reaction doubles during each round of amplification. A high efficiency is needed to obtain accurate and reproducible results. A low efficiency indicates that primers and/or probes are performing poorly. No single efficiency (as a percentage) has been set as a limit for data to be acceptable, although 80% or greater is an accepted norm.

3.3.2 Storage

Most oligos and DNA templates should be stored at -20°C or -70°C in either TE buffer (10 mM Tris-HCl and 0.1mM EDTA, pH 8.0) or molecular grade water. PCR products may also be stored at -20°C or -70°C. RNA templates should be aliquoted and stored at -70°C. TE buffer generally is the preferable storage buffer for oligos and DNA templates, because it may prevent DNA degradation, however, molecular-grade water may be more suitable for certain purposes, such as taking spectrophotometric readings. The pure, concentrated oligos should be stored in the original tube from the manufacturer and labeled with the primer name and concentration. To minimize the chance of contamination and degradation, these concentrated stocks should not be used on a regular basis. Diluted working stocks should be made for each oligo, and these working stocks should be used for all experiments. Before use, oligos should be thawed and mixed completely. Laboratories should either set expiration dates of one year for primers and probes, or check their sensitivity when they are a year old and then on a regular basis to see if any degradation has occurred. The sensitivity of primers can be checked by running a PCR

positive control using the old and new primers as described in Section 5.1.1. The sensitivity of probes can be checked by comparing old and new probes using a blotting technique as described in Section 4.4.2. Alternatively, the purity of the old primers can be checked using HPLC or polyacrylimide gel electrophoresis (PAGE). If degradation has occurred, new primers and probes should be prepared.

3.4 Enzymes

Because the enzyme is the true machinery of all PCR procedures, enzymes should be purchased from a commercial source to ensure purity. Laboratories should select vendors that provide QA information with the enzymes.

3.4.1 Quality

The enzyme manufacturer should provide documentation on the quality control that has been performed to ensure enzyme purity. Information about the minimum activity characteristics and the conditions under which those characteristics are tested should be provided. This documentation should be stored in the enzyme logbook in addition to all other pertinent information.

After receipt from commercial sources, each new lot of enzyme should be compared with old lots using known controls and environmental samples (31). The new enzymes should be rejected or the concentration adjusted, if performance is significantly below that of the proven lot.

Important: The analyst should have only one container open at a time when working with enzymes. Pipette tips should be discarded after each dispense to prevent cross-contamination.

3.4.2 Storage

The manufacturers' instructions on enzyme storage and use should be followed carefully. Enzymes typically are stored at -20℃, and should never be left at room temperature in the laboratory. Insulated bench-top coolers or ice can be used to keep the enzyme cold in the laboratory, when used on the bench top.

Important: "Frost-free" freezers should not be used. Temperature fluctuations lead to reagent degradation.

SECTION 4. METHOD DEVELOPMENT AND ASSESSMENT

When developing a new PCR method, laboratories should design and select the individual components of the method, including nucleic acid isolation and PCR analysis, to optimize the method's ability to recover and detect the target analyte. The laboratory also should consider how samples will be collected and processed for the method, and assess the performance of the individual method components and the entire analytical process before using the method for environmental sample analyses.

The following guidance on method development and assessment is provided in this section:

- The major components that should be considered when developing a PCR method for environmental monitoring (Section 4.1 through 4.4)

- The determination of method sensitivity and precision (Section 4.5)

- The factors that should be considered for validating a method after it has been developed and assessed internally (Section 4.6)

4.1 Environmental Sample Collection and Processing

Sample collection and transport conditions impact the results of PCR analysis. Laboratories should develop a detailed sampling and processing SOP for each new method. The SOP should define the range of acceptable sample volumes, sample handling protocols, and the time samples can be held before beginning sample processing. It also should describe the use, cleaning and sterilization of any sampling apparatus. The procedures described in the SOP should be designed to preserve the integrity of the target nucleic acid sequence in the environmental sample. It may be necessary to test seeded samples and follow their recovery as described in Section 4.5 in order to develop these procedures.

The sample processing component of an environmental PCR method is performed to isolate the organism or nucleic acid of interest from the environmental matrix, while minimizing co-purification of potential contaminants and components of the matrix that may inhibit PCR. The isolation procedure also should stabilize the target nucleic acid from nucleases and reduce the sample to a volume small enough to be analyzed by PCR. Positive controls that demonstrate that the target organism or nucleic acid has been successfully isolated and that the PCR inhibitors have been removed are discussed in Section 5.

4.2 Nucleic Acid Isolation

Some methods require the lysis of the target organism and the isolation of its nucleic acid before proceeding to PCR. If the nucleic acids of the organism are isolated before addition to the PCR, the performance of this part of the method also should be assessed independently and as part of the entire analytical process.

The efficiency of nucleic acid isolation varies with sample type and extraction procedure. Ideally, an isolation technique should meet the following goals:

- Demonstrate a high efficiency of target nucleic acid recovery

- Maintain nucleic acid integrity and minimize fragmentation

- Provide sufficiently pure nucleic acid, free from PCR inhibitors

- Minimize the use of dangerous chemicals

- Be repeatable

4.3   Polymerase Chain Reaction Amplification

When developing methods to analyze environmental samples using PCR, the first step is to select the type of PCR that is most appropriate for the analysis being conducted. The performance of the PCR amplification step should be assessed separately first, then as part of the entire analytical method. A wide variety of PCR techniques and reagents are available for use with environmental samples. Aspects that should be considered in the selection of the appropriate technique include PCR type, enzyme type, primer and probe design, and reaction parameters, such as thermal cycling temperatures and reagent concentrations. These factors are discussed below, in Sections 4.3.1 to 4.3.4.

4.3.1 PCR Type

From its inception in 1985, PCR has been adapted to fit many different applications, including detection of specific DNA, sequencing stretches of DNA, amplification directly from bacterial colonies, and amplification and detection of mRNAs, ribosomal RNAs (rRNA), and viral RNAs after using reverse transcriptase to make DNA copies of RNA templates. Thus there are many different types of PCR that may be used and each is unique to the application for which it was designed. The use of an inappropriate type could compromise the effectiveness of a PCR-based method. For example, the use of nested PCR on a target sequence that does not require secondary amplification can result in an unnecessary increase in the potential for contamination due to the additional round of amplification. There are five common designs or types of PCR used in the analysis of environmental samples: conventional PCR, multiplex PCR, reverse transcription (RT)-PCR, real-time PCR, and nested PCR, each of which are described below. **Table 4-1** describes some of the applications, advantages, and disadvantages of these common types.

Table 4-1. **Applications, Advantages, and Disadvantages of PCR Types**

PCR Type	Target	Application	Advantages	Disadvantages
Conventional	DNA	Amplification and detection of DNA sequences	• Easiest of the PCR types to perform • Low cost of equipment and supplies	• Normally produces only qualitative results • Requirement for post-amplification analyses increases time and labor as well as risk of cross-contamination and human error • Products should be confirmed by probe hybridization or sequencing
Real-time	DNA	Amplification, detection and quantification of initial copy number of nucleic acid target	• Rapid • Potential for relative or absolute target sequence quantification • Usually eliminates requirement for post-amplification analyses • Increased specificity because probes or melting curves are used • Totally closed tube analyses creates less potential for cross-contamination	• Requires more expensive equipment and reagents • Less flexibility in primer and probe selection • Less amenable to other downstream product confirmation analyses, such as sequencing due to small amplicon size
Multiplex	DNA	Simultaneous amplification and detection of two or more different DNA sequences (can be performed as a conventional or real-time procedure)	• Amplification of multiple target sequences in a single reaction reduces time and labor requirements	• Less flexibility in primer selection • Requires significant optimization • Generally has lower sensitivity and specificity
Nested	DNA	Amplification and detection of DNA using external and internal primer sets in sequential steps	• Potentially more sensitive • Decreases the potential for non-specific amplification	• More likely to produce false positives due to carryover of products from first amplification step • An additional room for sample preparation after the first amplification step is needed
Reverse transcription (RT)	mRNA, rRNA, viral RNA	Amplification and detection of RNA	• Amplification of all RNA types	• RNA is sensitive to degradation • Added RT step may increase time and costs as well as potential for contamination

4.3.1.1 Conventional PCR

Conventional PCR uses a thermostable DNA polymerase to amplify a region of target DNA defined at each end by a specific primer. The exponential replication of the same target sequence produces enough DNA product or amplicons for use in subsequent analyses. PCR typically consists of three basic steps:

- During the first step, **denaturation**, the sample is heated to separate or denature the two strands of the DNA. This is usually performed at temperatures between 94°C and 97°C for 15 to 60 seconds.

- Denaturation is followed by the **annealing** step, in which the reaction temperature is lowered allowing the oligonucleotide primers to bind to the separated single strands of the template nucleic acid. This is usually performed at temperatures between 47°C and 60°C for 30 to 60 seconds.

- The last step is **elongation**, during which the temperature is raised, typically to 72°C, allowing specific enzymes to make a complementary copy of the template. The length of the elongation step (30 seconds to three minutes) is determined by the speed of the enzyme, its ability to continue moving down the template DNA (i.e., processivity), and the length of the DNA segment to be amplified.

One repetition or thermal cycle of these three steps theoretically doubles the amount of DNA present in the reaction. The number of repetitions needed for a PCR application is determined by the amount of DNA present at the start of the reaction and the number of amplicon copies desired for post-PCR applications. Typically 25 to 40 cycles are performed. (10)

A schematic representation of the events that occur at different temperatures is shown in **Figure 4-1.**

Conventional PCR normally is not a quantitative assay. However, it is possible to generate quantitative results by using most probable number (MPN) or competitive PCR approaches. The MPN approach is performed by serially diluting a sample to its detection limit (see Section 4.5). Five or more replicates are run from each dilution, and the MPN value is determined using an MPN calculator (a free calculator is available at www.epa.gov/microbes). With the competitive PCR approach, an internal standard is made by cloning a modified version of the amplicon. The modified version usually contains a small deletion or insertion, which produces a different length product, and often contains an alternative hybridization probe recognition sequence that allows the product to be differentiated from the normal amplicon by probe hybridization. After preparation, the modified clone is purified, quantified by spectrophotometry, and serially diluted. Replicate samples containing the target nucleic acids to be quantified are mixed with different concentrations of the clone, and the mixes are amplified by PCR. The products are then run on a gel and visualized by staining. The concentration of the target nucleic acid from the sample is assumed to be equal to the number of copies of the clone in the reaction that produces equal amounts of the clone and the target. In this method, it is important to ensure that the clone and target sequences are amplified at the same rate. This can be facilitated by minimizing the sequence and size differences between the target amplicon and the clone.

Competitive RT-PCR can also be performed, but in this case it is necessary to first generate and quantitate an RNA transcript of the clone. Accurate quantification of the transcript can be difficult because of RNA degradation and because of the presence of the clone used to generate the transcript even after DNase treatment. Transcripts of variable lengths can also be generated, making it more difficult to correlate spectrophotometric reading with the true copy number. The results of competitive RT-PCR must therefore be interpreted with caution.

Figure 4-1.   Conventional PCR: One Cycle of Amplification
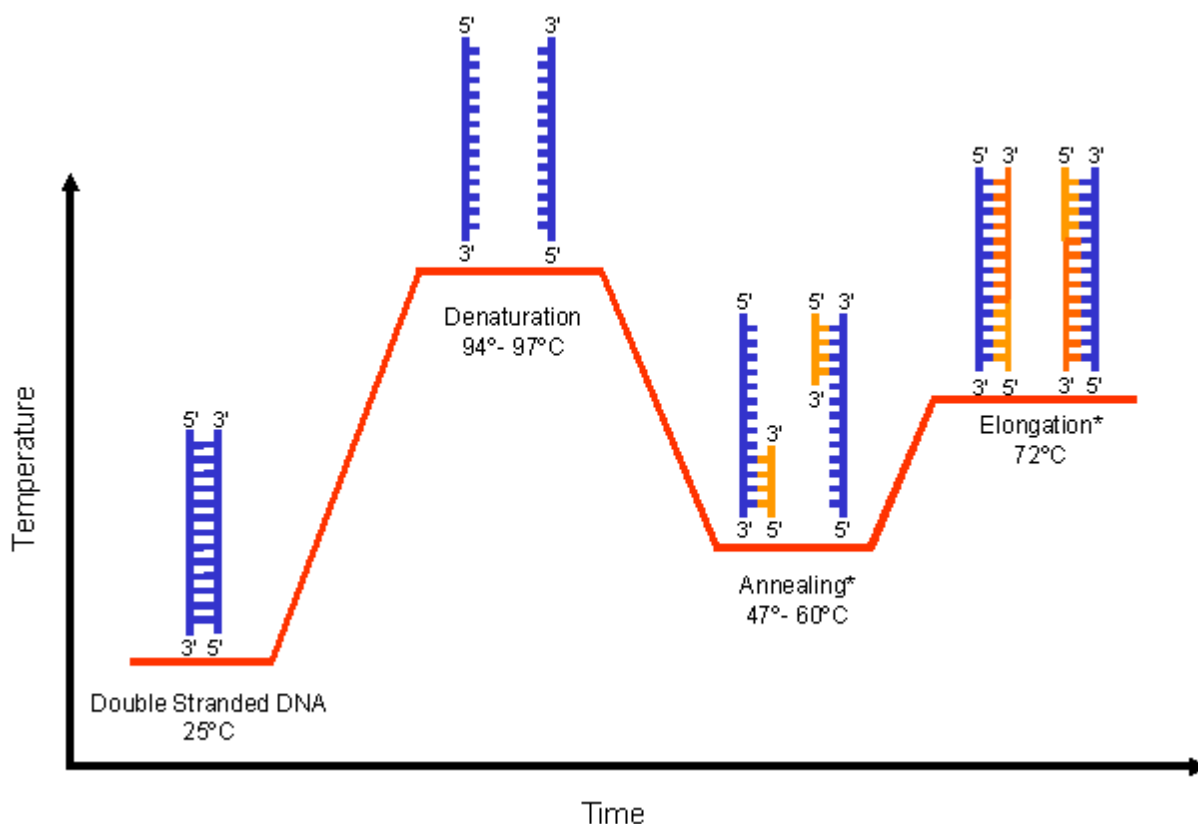
*Annealing and elongation can be combined into one step at about 60℃ for two-step PCR processes.

4.3.1.2 Real-Time PCR

Real-time PCR is so named because it detects and measures the amplification of target nucleic acids as they are produced. Real-time PCR requires the use of primers similar to those used in conventional PCR. However, unlike conventional PCR, real-time PCR uses an oligonucleotide probe labeled with fluorescent dyes or an alternative fluorescent detection chemistry (see below), and a thermocycler equipped with the ability to measure fluorescence. Typically, the binding of a dye-labeled probe to the template sequence causes fluorescence to increase in direct proportion to the concentration of the PCR product being formed. A computer is used to monitor the fluorescence increase and to calculate a cycle threshold (C_T) value. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. In the absence of an absolute standard, the starting copy numbers of nucleic acid targets (or number of target organisms) from different samples can be determined in a relative sense (e.g., sample one has 20 times more target organisms than sample two). If an absolute standard, which contains known quantities of the target nucleic acid or organism and which bracket the concentrations found in test samples, is run to generate a standard curve, the starting copy number in the test samples can be estimated. (8, 46)

Real-time PCR also differs from conventional PCR in that the target selection for real-time PCR is more restricted due to requirements of smaller target fragment and the need to select probes with a higher melting temperature than the primers to ensure that the probe is fully hybridized during primer extension. In addition, the annealing and elongation temperatures are usually combined in a two-step PCR process that is performed at an intermediate temperature (e.g., 60°C) for one to two minutes.

October 2004

There are several different fluorescent detection chemistries used for real-time PCR, including the following:

- **Dual-labeled fluorogenic oligonucleotide probes** are most frequently used. These probes (e.g., TaqMan® probes) have a reporter fluorescent dye at the 5' end and a quencher dye at the 3' end. The probes are added to the PCR master mix along with the PCR primers. During the PCR, if the target sequence is present, the probe anneals downstream from a primer site and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase during polymerization (PCR product formation). This cleavage releases the reporter dye from the probe and away from the quencher dye, resulting in fluorescence that is detected by the instrument. Both the polymerization and 5' nuclease activities of *Taq* polymerase are used in this process.

- **Fluorescent resonance energy transfer (FRET) probes** involve the hybridization of two probes to adjacent sequences within the amplified product. The upstream probe has a fluorescent dye at the 3' end and the adjacent probe has a fluorescent dye at the 5' end. Correct hybridization of these probes brings the two dyes into close proximity. The laser excites the first fluorescent dye, which emits light at a different wavelength. This light then excites the second fluorescent dye by FRET between the adjacent probes. The real-time PCR machine detects the wavelength of light emitted by the second fluorescent dye.

- **Molecular beacon probes** use a variation of this same process, wherein reporter and quencher dyes are held together by a hairpin structure in the probes but become sufficiently separated by linearization of the probe after annealing with the template to allow the reporter dye fluorescence to be detected.

- **SYBR® Green I**, a fluorescent dye, is another frequently used real-time detection chemistry. When this dye intercalates into double-stranded DNA, including PCR products, its fluorescence increases greatly, and this increase can be detected by the real-time PCR thermocyclers. Because this detection chemistry is not target sequence specific, it is more versatile than probe-based detection, but is susceptible to false positives due to the formation of nonspecific PCR products or primer-dimers. Melting curve analyses (Section 4.4.5) are often used as an additional product confirmation for procedures using SYBR® Green.

4.3.1.3 Multiplex PCR

Multiplex PCR is a modification of conventional or real-time PCR in which two or more different PCR products are amplified within the same reaction. This type of PCR consists of the same steps as conventional PCR, except that multiple sets of primers are used in each reaction. Multiplex PCR requires less time and effort in amplifying multiple target templates or regions than individual reactions and may be a useful screening assay. (28)

While multiplex PCR provides a potential time savings by allowing simultaneous detection of multiple targets, significant optimization is required to obtain all of the products with equal efficiency and sensitivity (see Section 4.5). Extra precaution must be taken to design primers that do not have adverse primer interactions (see Section 4.3.3). The best concentration ratios of different primer sets need to be determined carefully. Even with extra precaution and optimization (16, 26), the amplification of non-specific targets and reduced sensitivity for some primer sets can be problematic (24, 28). Optimization of PCR is discussed in Section 4.3.4.

4.3.1.4 Reverse Transcription (RT)-PCR

RT-PCR is used to amplify RNA target sequences (8), such as messenger RNA and viral RNA genomes. This type of PCR involves an initial incubation of the environmental sample or control RNA with a reverse transcriptase enzyme and a DNA primer. DNA primers that are used commonly include oligos dT

(an oligos consisting of only thymidine residues), random hexamers (primers made of six random nucleotides), or a specific primer. Oligos dT will hybridize to the poly A tail of messenger and certain viral RNAs and prime DNA from the 3'-end of the RNA molecule. This approach may not be adequate for amplification of RNA segments near the 5'-end of the molecule. Random hexamers work with any RNA, but require an extra initial incubation at 25°C. Specific primers can be either the PCR primer that hybridizes to the RNA at the 3' side of the amplification region or a primer that hybridizes further downstream from the PCR primers. RNase inhibitor should be added to RT reactions to prevent the degradation of the RNA target sequence by RNase present in the sample or introduced as contamination.

The reverse transcription and the PCR amplification can be performed in a one- or two-step process. In general, the two-step process is more sensitive, while the single-step reactions are less likely to be contaminated, because the tube is not opened after reverse transcription. The determination of which process should be used depends on the level of sensitivity required and the likelihood of contamination.

There are many types of reverse transcriptases available for RT-PCR. The characteristics of the enzymes make some better suited for a one- or two-step reactions and other downstream applications. Some enzyme characteristics that impact the type of reverse transcriptase used for RT-PCR include: the presence or absence of RNase H activity that degrades RNA in an RNA:cDNA hybrid, processivity of the enzyme, divalent ion requirements, specificity and sensitivity, ability to incorporate dUTP for UNG carryover contamination (see Section 5.5.1), and optimum temperature for function. (7)

4.3.1.5 Nested PCR

For analysis of environmental samples with complex microbial populations, conventional PCR may not exhibit sufficient specificity or sensitivity for certain target sequences and organisms. Reduced specificity can lead to false positives if non-specific fragments that are the same size as the product of interest are amplified. Reduced sensitivity can cause false-negatives, if the sample being analyzed contains residual inhibitors that reduce the number of amplicons to levels that cannot be detected. To minimize these problems, nested or semi-nested PCR have been used to increase the specificity and sensitivity of target sequence detection (51).

Nested PCR is a conventional PCR with a second round of amplification using a different set of primers. This second set of primers is specific to a sequence found within the DNA of the initial conventional PCR amplicon. The use of a second amplification step with the "nested" primer set results in a reduced background from products amplified during the initial PCR due to the nested primers' additional specificity to the region. The amount of amplicon produced is increased as a result of the second round of amplification and due to a reduction in any inhibitor concentrations. The presence of the expected second PCR amplicon is usually viewed as confirmation of the presence of the target organism in a sample.

Used correctly, the multiple rounds of nested PCR should increase both the sensitivity and specificity of the PCR. However, there is an increased chance of carryover or cross-contamination when taking product from the first round of PCR and putting it in the tubes for the second round of PCR. Additional steps and precautions may need to be taken to reduce the chance of sample contamination and false-positives, including the following:

- Never opening more than one tube at a time
- Using a separate thermocycler for the first and second amplifications
- Adding additional negative controls beyond those recommended in Section 5.2.1. The additional controls should be interspersed with tubes containing samples
- Including first round negative controls in the second round of amplification to check for false-positives
- Designating a fourth room or separate area for sample preparation after the first amplification

4.3.2 Enzyme Type

Taq DNA polymerase, which is isolated from the thermophilic bacterium *Thermus aquaticus*, is the primary enzyme used in the amplification of DNA in nearly all procedures. Modifications of this enzyme or other DNA polymerases with specific functions and unique properties, including different extension rates, processivity (the ability of DNA polymerase to amplify longer sections of DNA), greater proofreading ability, and different temperature tolerances, generally expressed as a half life at the denaturing temperature, may be more appropriate for some PCR applications. Hot-start DNA polymerases are also commonly used. These enzymes are inactive until a specified temperature is reached (usually greater than annealing temperatures of the primers; see Section 5.5.1.3). Use of hot-start enzymes reduces the production of non-specific products by preventing the elongation of primers that have non-specifically annealed to the template at lower annealing temperatures (which may happen during master mix preparation or in the first step of PCR cycling).

When selecting an enzyme type for a method or study, the analyst should evaluate the different strengths and weaknesses of the DNA polymerases available to determine which individual polymerase, or combination of polymerases, will work with their template nucleic acid. Before switching manufacturer, enzyme type, or lot number, the new enzyme's effectiveness should be tested against the enzyme originally used to validate the PCR using both QC and environmental samples and the results documented.

4.3.3 Primer and Probe Design and Specificity

Design and selection of the specific primer and probe set to be used for an experiment is based on the application, the type of PCR and hybridization that will be performed, and the segment of the target nucleic acid sequence that is known. Primers should be designed to amplify only the DNA or RNA of interest. As a general rule, primers are characterized by the following:

- Length of 18 to 27 base pairs

- No homology within or between primers, especially at the 3'end to avoid primer-dimer formation

- No guanine-cytosine (GC) stretches greater than four base pairs

- GC content of 40% to 70%

- Melting temperatures (Tm) that are as close as possible

- No hairpin loops with an energy of -0.5 kcal/mol or less

A variety of computer programs are available to aid in the creation of the best possible primers and probes. These programs also can help determine the optimum annealing temperature for newly created oligos and check for the formation of intra- and intermolecular dimers and hairpin loops. Laboratories should consider repeating the design process with more than one computer program, because these programs represent a simulated environment that may not include all the variables that affect oligo design. For laboratories that are performing real-time PCR, the software provided with the real-time PCR instrument may be used for primer design. New primers and probes should always be tested experimentally for sensitivity as described in Section 4.5 and specificity before use in any method (10, 22).

The specificity of a chosen sequence should be evaluated using BLAST (Basic Local Alignment Search Tool) (32) or its equivalent. Versions of BLAST are available on the WEB at a number of sites, including www.ncbi.nlm.nih.gov and www.embl-heidelberg.de. BLAST compares the designed oligo sequences to known nucleic acid databases such as GenBank and EMBL. The search determines the potential of hybridization of the chosen oligo with sequences from other organisms. The results of this search should

be used to define any relevant, closely matched sequences for specificity testing. Specificity testing should be conducted as described for the PCR positive control (Section 5.1.1) by substituting the heterologous sample in place of the target organism or nucleic acid. Primers and/or probes should be redesigned if any heterologous sample is positive.

It is not always possible to design perfect primers and probes. Primers and probes should be selected based on the best theoretical considerations, and negative features should be documented, and results interpreted accordingly.

4.3.4 Selection of Procedure Parameters

The exact parameters of the PCR should be selected based on the target nucleic acids that are to be amplified. Parameters that need to be evaluated for successful PCR amplification include thermocycling conditions, reaction volumes, template concentration, and the concentration of PCR reagents, including that of primers. It may be necessary to use conditions that are less than optimal for some individual primer sets (e.g., when using multiple primer sets in a multiplex format). After optimal conditions are established, they should be documented in an SOP for the method and followed thereafter.

4.3.4.1 Thermocycling Conditions

Optimal denaturation, annealing, and elongation times and temperatures should be determined for each primer/template set, although in practice, the use of a single PCR thermocycling condition is more convenient and may often be acceptable for use with different primer sets with slightly varying optima. The PCR cycle number needs to be evaluated carefully. Having too few cycles may result in false negative samples while too many can increase the potential for false positive results and affect quantitation (24).

4.3.4.2 Reaction Volumes

PCR volumes typically range from 10 to 100 µL and may be dependent on the type of thermal cycling instrument being used. Most modern thermocyclers are not designed to accommodate reaction volumes greater than 100 µL due to the difficulty of uniformly heating and cooling throughout the reaction. Increasing the volume of sample added to a reaction may increase the probability of a positive result at low target concentrations because more sample can be tested per reaction, but may also increase inhibition.

4.3.4.3 Primer and Template Concentrations

The primer concentrations used in each newly developed PCR assay should be optimized to obtain maximum amplification efficiency. Optimization of primer concentrations is especially important when performing multiplex PCR (see Section 4.3.1.3). The addition of too much DNA (either template or non-template) to the reaction may be inhibitory (29). Detection of two or more targets in a multiplex format may be problematic, if there are differences in concentration between the targets. When designing multiplex reactions, the effects of different levels of targets should be tested to determine their impact upon the assay.

4.3.4.4 PCR Reagents and Master Mix Preparation

Key reagents that may require optimization include the reverse transcriptase and RNasin concentrations for RT-PCR, and the DNA polymerase, magnesium, and dNTP concentrations for PCR. Buffering capacity, pH, and KCl concentrations also may need to be optimized for some types of samples, but this should be rare. PCR kits containing all reagents, except primers and templates, can be purchased from commercial sources, and these can be used if one is found that matches the results of the laboratory's optimization studies.

In addition to required reagents, enhancers that act on different aspects of the reaction may be added to the PCR master mix. These include dimethyl sulfoxide (DMSO), betaine, tetramethylammonium chloride (TMAC), and bovine serum albumin (BSA) (25, 27). Other proprietary enhancers and additives are available from various manufactures. Some of the enhancers act by increasing thermostability of the polymerase and some by giving the polymerase greater processivity through GC rich regions. The effects of many others are not completely understood, and although they may be beneficial in some reactions, their effect on all reactions cannot be predicted. Each additive should be empirically tested with each combination of template and primers.

A master mix containing optimized reagents should be prepared in the reagent preparation room. The master mix contains all of the reagents necessary for the reaction to occur except the template, thus minimizing the potential for reaction-to-reaction variability associated with the pipetting of these reagents. During master mix preparation, pipette tips should be changed after handling each reagent. The master mix should be prepared on ice, unless the manufacturers' instructions specifically state that the reagents can be mixed at room temperature. Due to the potential interference of the glycerol solution in which some enzymes are stored, the volume of enzyme used in the reaction should not exceed 10% of the total reaction volume (9). The master mix should be aliquoted into reaction tubes in the reagent preparation room. If desired, laboratories can prepare a large quantity of master mix and store aliquots at -20°C after demonstrating that the freeze/thawing of aliquots does not reduce sensitivity.

4.4 Amplicon Detection and Confirmation

PCR results must be confirmed. Confirmation that the specific target was amplified can be accomplished during PCR using real-time quantitative probe-based hybridization or following PCR using gel electrophoresis; blot hybridization; restriction mapping; melting curve analysis; and/or sequencing. Summaries of each determinative technique are provided in Sections 4.4.1 to 4.4.6 and summarized in **Table 4-2**. Recommendations for QC steps and data reporting for each of these procedures are discussed in Sections 5 and 6, respectively.

Although sequencing provides the most reliable confirmation of a PCR result, the use of two or more other determinative techniques increases the confidence in the result. The inherent use of internal hybridization probes in real-time PCR procedures provides a degree of amplicon confirmation that is similar to the use of dot blot hybridizations in conventional PCR (Section 4.4.2.2.). Further confirmation of the products of this method by gel electrophoresis and sequencing may still be required. Some real-time PCR thermocyclers are equipped with capabilities to determine melting point curves of the products that can also be used for their confirmation.

Note: *Due to the small size of some amplicons, especially in real-time PCR detections, confirmation by sequencing may not be possible.*

Table 4-2. **Advantages and Disadvantages of Techniques for Detection and Confirmation of Amplicons**

Technique	Objective	Advantages	Disadvantages
Electrophoresis	Determination of amplicon(s) size on a gel	• Easy • Least expensive • Relatively fast compared to most other techniques	• Confirmation based only on product size • Resolution of multiple products sequences may be poor • Confirmation by other techniques, e.g. sequencing, is usually needed
Southern blot	Detection of a specific sequence of nucleic acid by size on a solid support	• Combines product size and probe-based confirmation	• Requires increased time for transfer of PCR product from gel to membrane and for hybridization • Requires a preliminary electrophoresis step • Confirmation by other techniques, e.g. sequencing, may still be needed
Dot blot	Detection of a specific sequence of nucleic acid on a solid support	• Faster than Southern blot	• Confirmation based only on probe • Confirmation by other techniques, e.g. sequencing, may still be needed
Restriction mapping	Determination of the size of nucleic acid fragments after enzyme digestion	• Relatively easy and well-documented	• Requires the presence of a restriction site in the amplicon • Requires additional enzymes and buffers • Requires additional electrophoresis after digestion with the enzymes • Slow • Confirmation by other techniques may still be needed
Probe-based quantitative PCR (quantitative real-time PCR)	Sequence detection through the use of fluorescence	• Quantitative • Rapid - product confirmation by probe hybridization incorporated into the procedure • Less potential for cross-contamination	• Cost of equipment and reagents • Confirmation by sequencing or other techniques may still be needed
Melting curve analysis	Determination of the melting temperature of an amplicon	• Simple and quick; analysis carried out at the end of real-time PCR	• Resolution of multiple products may be poor, e.g., two different amplicons can have the same melting temperatures • Confirmation by sequencing or other techniques may still be needed
Sequencing	Confirmation of the sequence of the amplicon	• Most reliable confirmation	• Slow • May require an initial cloning step

4.4.1 Gel Electrophoresis

Gel electrophoresis is the most common method used to detect products from conventional PCR. Electrophoresis is the separation of charged molecules in an electrical field. Movement of the nucleic acid through the gel is dependent on electric charge; separation is dependent on shape and size of the amplicon. Nucleic acid molecules carry a uniform negative charge throughout their length, and the shape of all amplified DNA will be linear. Therefore, the major factor resulting in the separation of amplified DNA is size.

The most common medium used for separation of DNA is agarose, a linear polymer that forms a solid gel of uniform density. An alternative medium is acrylamide, which can form a polyacrylamide gel as a result of covalent cross-linking of the acrylamide molecules. This type of medium provides higher resolution than agarose. To visualize the DNA, gels are stained with fluorescent dyes, such as ethidium bromide or SYBR® Green I, after electrophoresis. Ethidium bromide, but not SYBR Green, also can be added to the agarose before electrophoresis. Various running dyes can be added along with the DNA in a loading buffer to monitor DNA migration through the gel.

Many different buffers and concentrations of matrices are used to make and run gels. Different combinations can provide more or less resistance to the DNA being separated to achieve the desired resolution and separation.

Note: *Electrophoresis alone usually is not sufficient to confirm PCR products, because unrelated amplicons of similar size to the target of interest may be detected.*

4.4.2 Probe Hybridization (Blots)

The most common procedure for confirming amplicons is to hybridize a labeled probe to PCR products that have been transferred to a solid support, such as nitrocellulose or nylon membranes. Products are transferred to a solid support using one of several blotting techniques. Hybridization probes, which are usually oligos that have a sequence complementarity to the target amplicon, are then hybridized to the bound amplicons. The hybridization probes cannot have sequences that overlap with either PCR primer.

4.4.2.1 Southern Blot

Southern hybridization is a method by which PCR products are transferred from a gel following electrophoresis onto a solid support, such as a nylon membrane. The now membrane-bound PCR products are hybridized to a oligonucleotide probe that contains radioactive, flourescent, biotin, digoxigenin, or other tags. Binding of the probe to the PCR product allows the visualization of the amplicon and determination that the amplicon is of the expected size for the primer/template set. These probes also are capable of detecting amplicons that are present in concentrations that are too low to be visible on a gel.

4.4.2.2 Dot Blot

A dot blot is performed by directly transferring the amplicon from a PCR assay to a solid support without first performing electrophoresis. Dot blot hybridization provides a faster confirmation than Southern blotting, but it does not provide the added confirmation of the size of the amplicon.

4.4.3 Restriction Mapping

Restriction mapping uses inherent restriction sites (specific nucleotide sequences) in the DNA sequence to determine whether the DNA amplified is the target amplicon. Restriction maps are created by digesting the amplicon using restriction endonucleases. Because the sequence of the target nucleic acid is usually known and the sites where the restriction enzyme digests the amplicon are known, the sizes of the resulting pieces of DNA can be predicted. A gel confirming the digestion should be run. The results on the gel should be compared to the predicted restriction map to determine if the cut amplicon produces bands of the predicted size. If the amount of amplicon is limited, the amplicon may be cloned prior to restriction mapping.

4.4.4 Probe-Based Quantitative PCR

Probe-based quantitative real-time PCR is a method of confirmation in as much as the fluorescence detected is directly correlated to the presence (confirmation) of a specific product. As with other probe-based confirmation methods, the accuracy of this method is dependent on the specificity of the probe for the desired target sequence (see Section 4.3.1.2).

4.4.5 Melting Curve Analysis

Some real-time PCR thermocyclers are equipped with confirmation procedures that determine melting point curves. The melting temperature of nucleic acids is affected by length, GC content, and nucleotide base mismatches (if present). If it can be shown that amplicons from non-specific targets do not have the same melting temperatures as the specific targets, confirmation by melting temperature can be considered equivalent to probe-based confirmation. (38)

4.4.6 DNA Sequencing

DNA sequencing determines the nucleotide sequence of a PCR product using either automated or manual protocols. Automated sequencing instruments detect fluorescently modified nucleotides or primers to determine the nucleotide sequence. Manual sequencing (which is not recommended) requires four separate reactions to determine the sequence. DNA sequencing often is performed as a PCR reaction using a single primer. It is usually not adequate to perform sequencing directly on a PCR-amplified sequence, because environmental samples often contain mixtures of the analyte of interest (e.g., non-specific amplicons; multiple, but related species; different sero- or genotypes of a virus). At best, direct sequencing only detects the analyte that was present in the highest concentration. Instead, gel-purified PCR products should be cloned into a known plasmid followed by sequencing of multiple clones (colonies). Sequences of the PCR product should be validated by sequencing both strands of the PCR product using forward and reverse primers. The two sequences are compared to one another, edited, and a consensus sequence is determined. In addition to sequencing both strands, multiple PCR amplifications can be performed and the products sequenced and compared for consistency.

Commercial sequencing facilities that have the necessary equipment and expertise may be used for DNA sequencing. These facilities should be provided with the appropriate forms of amplified DNA for their sequencing platform and technology. If confirmation of an amplicon by sequence analysis is anticipated, the requirements of the sequencing facility should be considered in the development of the PCR application.

4.5 Method Sensitivity, Precision, and Recovery

After the individual components of the method have been optimized, the entire method should be evaluated to determine the method's detection limits (sensitivity), precision, and recovery as discussed in Sections 4.5.1 to 4.5.3.

4.5.1 Detection Limits

Target organisms are often present at low levels in the environment, and some of these may be able to produce infection at low concentrations. Therefore, an acceptable PCR method should be able to detect a level of organisms that is biologically significant. The level may be based upon acceptable risk levels, predicted environmental concentrations, or concentrations that produce an infectious dose. In order to know whether an acceptable level can be detected, a method's detection limit must be determined.

The detection limit of quantitative methods is defined in this guidance as the minimum concentration of a substance that can be measured with a given level of confidence that the analyte concentration is greater than zero (the confidence level should typically be 95% to 99%, but the analytical limitations of some methods may require the use of lower levels). For presence/absence methods, the detection limit as defined in this guidance is the minimum concentration of analyte that produces a positive response with a given level of confidence. The detection limit can be expressed either as the minimum number of organisms or of the target sequence copy number in a given volume. There are many uncertainties that can affect the detection limit. Some of them are:

- The type of target nucleic acid being detected (e.g., DNA, mRNA, tRNA, rRNA, etc.)

- The secondary structure and the GC content of the nucleic acid target molecule

- The matrix from which the organism is isolated

- The detection of microbes that are inactivated by physical and chemical disinfectants

There are two different detection limits that are of interest when analyzing environmental samples using PCR: the detection limit of the PCR procedure and the detection limit of the entire method. For bacteria, protozoa, and fungi these detection limits often are measured in terms of the minimum detectable counts or colony forming units (CFU). For viruses, the detection limit should be measured in terms of the minimum number of detectable physical viral particles, if possible.

The two types of detection limits are determined by analysis of replicate, seeded samples containing increasingly lower levels of the target organism or target nucleic acid. One way of accomplishing this is to prepare serial dilutions of an enumerated stock suspension of the target organism. Cell concentrations in the stock suspension can be determined by direct counting on filters or in a hemacytometer under a microscope; by automated direct counting methods such as Coulter counting or flow cytometry; or, for organisms that can be cultured, by counting of colony forming units (CFU) following plating on a suitable growth medium. Physical particle counts for some viruses can be determined spectrophotometrically (16). If no method is available to determine physical particles, enumeration can be by infectious units (e.g., plaque forming units [PFU]). However, laboratories should recognize that a number of animal viruses have a high physical to infectious particle ratio, and for these viruses, PCR detects more virus particles in a sample than an infectious assay can detect (16). The number of replicates that should be analyzed at each dilution level will depend on the variability of the procedure, the accuracy and variability of the spike enumerations, the desired level of confidence in the detection limit, and the application of the method. Depending on the application of the method, it may be desirable to determine the detection limit for reagent water and a variety of sample matrices to determine the effect of matrix inhibition on the detection limit.

It should be noted that manually enumerated spikes containing low numbers of organisms may be inaccurate, and the results obtained from such spikes should be interpreted with care. Errors associated with serial dilutions should be minimized by using appropriate volumes that can be accurately measured and by thoroughly homogenizing stocks immediately before removing an aliquot for the next dilution. If possible, multiple replicates of each dilution should be enumerated independently of the PCR analysis to assess the accuracy of the dilutions.

The detection limit can also be expressed in terms of copy number of target sequences (41). This requires that the nucleic acid sequence of interest be amplified and cloned in a suitable vector and that the concentration of these molecules be determined, as described below. This is especially helpful when the organism cannot be cultured. The lowest copy number of the nucleic acid fragment that can be detected by the PCR method can be calculated from the lowest concentration of DNA detected in g/mL. Ideally, the detection limit should be in the single copy range.

The following formula can be used to calculate the copy number (CN):

$$CN = \frac{M \times N}{L \times D}$$

Where:
- M = minimum concentration of nucleic acid detected (g/mL)
- N = Avogadro's number (6.022×10^{23} molecules/mole)
- L = length of nucleic acid in kilobase pairs (total length of plasmid + insert)
- D = conversion factor from 1 Kb of nucleic acid to daltons
  - ds DNA = 6.6×10^{5} g/mole/Kb

Nucleic acid concentrations (M) can be measured using three approaches:

- Spectrophotometry

- Fluorimetry

- UV transillumination

Using spectrophotometry, the approximate concentration of the nucleic acid in grams is calculated by measuring the optical density (OD) per 1 cm light path, according to the following relationship:

1 OD of ds DNA at 260 nm = 50 µg/mL (5×10^{-5} g/mL)

Nucleic acid concentrations can be estimated by measuring fluorescence after staining with a fluorescent dye such as Hoechst 33258. Nucleic acid concentrations also can be calculated by measuring UV-induced fluorescence emitted by intercalating dyes such as ethidium bromide. Both the fluorimetry and the UV-transillumination methods require the preparation and measurement of standards (40). A summary of the three approaches is presented in **Table 4-3.**

Table 4-3. **Summary of Methods for Measuring Nucleic Acid Concentrations**

Method	Range	Advantages	Limitations
Spectrophotometry	5 to 90 µg/mL	• Rapid and simple • Non-destructive	• Requires highly purified preparations of nucleic acids • Cannot distinguish between DNA and RNA or between intact and degraded nucleic acids
Fluorimetry	As low as nanogram level	• Simple • More sensitive than spectrophotometry	• Requires careful preparation and measurement of standards
UV-transillumination	As low as one to 5 nanogram(s)	• More sensitive than spectrophotometry • Can determine nucleic acid fragment size - particularly useful for discreet sized fragments	• Sample must be run on a gel • Not effective for heterogeneous sized fragments • Requires careful preparation and measurement of standards

Detection limits may also be expressed in terms of PCR or RT-PCR units (16), especially for analysis of organisms (e.g., certain viruses) that cannot be enumerated. PCR units are determined by performing PCR on serial dilutions of the organism or the nucleic acid from the organism. A unit is defined in terms of the lowest spike level or highest dilution (lowest concentration) that gives a positive result. These limits should be estimated from analysis of at least five replicates at each dilution and expressed using most probable number (MPN) statistics. It should be noted that this method for expressing detection limits can only be used for relative comparisons of samples using the same primer set, rather than the absolute values.

When reporting detection limits for the entire method or the PCR procedure, the procedure for calculating the detection limit should be clearly described, including the units, whether the detection limit is for whole organisms or copy number, and the associated confidence limits. The units used to report the detection limit should be based on the procedure used to enumerate the spikes (e.g., if bacterial plate counts are used to determine CFU, then the method detection limit should be reported in terms of CFU).

4.5.1.1 Detection Limit of PCR

The PCR detection limit reflects the sensitivity of the PCR procedure, which includes the sensitivity of the primers and probes as well as the preparation of the master mix and the optimization of thermocycling conditions. It does not include reductions in sensitivity by any procedures used to concentrate organisms in the sample and isolate nucleic acids. For this reason it is often determined on the basis of nucleic acid concentrations rather than organism concentrations.

4.5.1.2 Detection Limit of Method

The method detection limit is the lowest amount of target that can be reproducibly detected by the entire method. It reflects not only the sensitivity of the PCR, but the efficiency of the procedures used to recover organisms from the environmental sample and the efficiency of the procedures for recovering nucleic acid templates from the organisms. Thus, a method's detection limit will depend on a range of factors, including the efficiency of the collection, concentration, isolation of whole organisms from the environmental sample, and lysis of organisms to release the nucleic acid, in addition to the detection limit of the PCR itself.

October 2004

The method detection limit can be determined by processing reagent water, standardized matrix, or field samples that have been seeded with different concentrations of the target organism prior to any sample processing. It is not uncommon for the method detection limit to be higher than the PCR detection limit, depending on the efficiency of the concentration and purification steps, inhibitors present in the field sample, and other sample processing variables.

4.5.2 Precision

Good analytical methods will have a high precision. Precision is a measure of how closely values from replicate measurements of a sample agree with each other.[1] For quantitative PCR analyses on environmental samples, method precision can be expressed as the relative standard deviation (RSD) or the relative percent difference (RPD). The RSD is the standard deviation from three or more replicate samples divided by the mean and multiplied by 100%. The RPD is the absolute difference of duplicate samples divided by the mean and multiplied by 100%. The RSD or RPD decreases with increasing precision and increases with increased variability.

Variability (i.e., low precision) may result from differences in analyst performance, reagents or equipment, or matrix characteristics. The precision of a method should be tested at the levels at which the target organism will be found in the environment. If environmental levels are unknown, the precision should be tested at levels 10 to 100 times greater than the detection limit. The number, range, mean value, and RSD or RPD of the replicates analyzed to determine precision should be documented, and the nature of any imprecision characterized.

4.5.3 Recovery

In addition to high precision, good analytical methods will have low bias. Bias is a measure of disagreement between the concentration of an analyte as measured by a method and the true concentration in the environmental sample that was tested. A PCR method's recovery of the target sequence of interest is an important factor for characterizing method bias at levels higher than the detection limit. Bias can occur at any component of a PCR method. If a method is designed to detect a group of related organisms, members of the group may have different recoveries and thus different biases through the sampling protocol, nucleic acid extraction, and the PCR steps. Bias at the PCR level can occur from the favoring of the amplification of certain target sequences due to properties of the target, the flanking sequences, or the overall genome (35). PCR bias may be seen for mixed-template PCRs, including reactions using internal controls, and may make interpretation of results difficult, because the final results do not reflect the original makeup of the target templates. Bias usually results when the amplification efficiencies of the target sequences are not the same. Target sequences may amplify at different efficiencies because the sequences are not equally accessible to primer hybridization after denaturation, primer-template hybrids do not form with equal efficiency for all templates, and/or the polymerase acts on the templates with different efficiencies (42). One factor that may effect primer-template hybridization is the GC content of the template (35, 42). PCR bias may be reduced by running a low number of PCR cycles and by using high template concentrations, when possible (35).

---

[1]From a statistical perspective, precision is expressed as the reciprocal of the coefficient of variation, which would *increase* as precision increased and *decrease* as variability increased. However, because this guidance document is designed for use by environmental laboratories, which commonly use RSD or RPD, method precision should be determined using this approach. The use of RSD or RPD also is consistent with the approach used in quantitative EPA microbiology methods; further information on the use of RSD and RPD for environmental measurements is available in the draft *EPA Microbiological Alternate Test Procedure [ATP] Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods* (EPA- 821-B-03-004) (48).

October 2004

Recovery of the PCR analysis and the entire method can be assessed by the analysis of seeded samples. For quantitative PCR analyses, recovery is determined as the total amount of the analyte found in a seeded sample minus the background (i.e., amount detected in the unseeded sample) divided by the amount of the analyte seeded into the sample. To determine recovery for presence/absence methods, serial dilutions of the seeded sample should be performed and five replicates at each dilution analyzed. The amount of analyte in the seeded and unseeded samples should then be calculated using MPN analysis as described in Section 4.3.1.1. The recovery would then be calculated similar to the quantitative analysis by subtracting the MPN-estimated background concentration from the MPN-estimated concentration of the seeded sample and then dividing by the amount seeded (section 5.1).

Results that are obtained using methods that have low recoveries should be qualified as potentially biased. Laboratories should also be aware that recovery of target organisms that are endogenous to a matrix (e.g., enteric pathogens associated with fecal material in river water) may not be the same as that measured by seeded samples (e.g., where the organisms are not associated with other material). Unless it can be shown that the recoveries of endogenous and seeded organisms are similar, sample results should be recorded as biased.

4.6   Method Validation

Although the design and coordination of comprehensive method validation studies are beyond the scope of this guidance document, validation of the optimized method is the final step in the method development process. For PCR methods developed for use in one laboratory and for a single matrix, method validation need only be conducted by that laboratory. If a PCR method is developed for wide-spread use, a coordinated validation study should be conducted at multiple laboratories to adequately evaluate the method's robustness and applicability to a representative range of matrices and ability to be used consistently by multiple laboratories. Regardless of whether single-laboratory or interlaboratory validation will be conducted, the following parameters should be evaluated in characterizing the method's performance:

- Specificity

- Detection limit (sensitivity)

- Intra- and interlaboratory precision

- Recovery (bias)

Guidance on the evaluation of microbiological method performance relative to these parameters is provided in the draft *EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods* (EPA-821-B-03-004) (48). Additional guidance is available through standards from ASTM International, the International Organization for Standardization, AOAC, and other consensus standards organizations.

The results of these studies should be carefully considered before the method is used for environmental sample monitoring. If the validation study data indicate that the method is not consistent enough for routine use in a single laboratory or cannot be performed by other qualified laboratories, the method should not be used until it is standardized. If the validation study data indicate that the method does not perform acceptably in many of the matrices in which it would be used for routine monitoring, additional development work is needed to address matrix interferences. If the results of the single-laboratory or interlaboratory validation study demonstrate that the method can be consistently performed, and is characterized by acceptable accuracy, sensitivity, and specificity, then it may be used for monitoring.

SECTION 5. QUALITY CONTROL SAMPLES FOR METHODS USING PCR

Laboratories using PCR should analyze positive and negative QC samples on a routine basis to demonstrate adequate performance of PCR based methods. General guidance on both positive and negative control types and the frequency in which they should be performed is provided in Sections 5.1 and 5.2. The actual number of controls will depend on the experimental design and expected variability. A summary of these controls is provided in both **Tables 5-1** and **5-2** and **Figure 5-1**. A summary of QC samples for detection and confirmation procedures is provided in Section 5.3. Corrective actions for QC sample failures and false positive/false negative prevention are discussed in Section 5.4 and 5.5, respectively. Guidance for participating in proficiency testing studies is suggested in Section 5.6.

The method protocol should be documented in the laboratory's SOPs, and concise versions of the SOP for a particular part of the method should be posted at the bench where the procedures will be performed. Posting bench SOPs provides technicians with easy access to method procedures, and helps promote consistency between analysts. SOPs for a particular method also should document QA procedures, such as the types and frequency of QC controls, and corrective actions for positive and negative control failures.

5.1   Positive Controls

Positive controls are analyzed to verify that the method is capable of adequately recovering and amplifying the target. The concentration of the sequence of interest in these positive controls should be 10 to 100 times higher than the defined detection limit of the PCR (Section 4.5.1.1). A positive control is considered to be acceptable if the DNA of interest was amplified by PCR, as determined by the same confirmation technique (Section 4.4) used for the analytical samples. Guidance on positive control samples that should be included for PCR analyses is provided in Sections 5.1.1 to 5.1.4. Precautions should be taken to avoid contamination of field samples with the positive control template. Positive control preparation should be physically separated from field sample and negative control preparation, and positive control samples should be handled last. A summary of the recommended positive QC samples is provided in **Table 5-1**.

Table 5-1.   Summary of Positive Controls

Description	Purpose	Frequency
PCR positive control	Verify that the PCR master mix and reagents were prepared correctly to produce amplification of the target nucleic acid	One or more per PCR batch
Inhibition positive control	Verify that interfering constituents from an environmental matrix carried over from the isolation of the organism or nucleic acids do not inhibit the PCR	With every field sample
Method positive control	Verify that the entire method is performing properly and is capable of amplifying the target nucleic acid from the organism of interest	With every sample batch
Matrix spike	Determine the effect of the matrix on the overall method's recovery and verify it does not have an inhibitory effect on the PCR	Per matrix

5.1.1 PCR Positive Control

PCR positive controls are used to verify that the PCR master mix and reagents were prepared correctly in order to produce amplification of the target nucleic acid. This type of positive control is run with each PCR batch. A PCR batch is defined as a group of samples that are processed and amplified at the same time under the same conditions, using the same PCR master mix, and in the same thermocycler.

PCR positive controls are prepared by the addition of an exogenous control to the master mix. Exogenous controls can be any of the following:

- A purified total nucleic acid extract from the organism containing the sequence of interest.

- The whole organism, which may be used when the nucleic acid target of interest can be released from the seeded organism by heating before or during PCR.

- A specific nucleic acid fragment containing the entire sequence to be amplified, including primer binding sites (e.g., a low concentration of a previously amplified, and/or a cloned DNA fragment that has been sequenced for confirmation).

- A cloned DNA fragment containing a modified form of the target sequence (see Section 4.3.1.1). Modified forms produce PCR products that can be distinguished from the target sequence by size, by restriction mapping, and/or by an alternative probe recognition sequence. Modified forms, which also are called "internal controls," are prepared by *in vitro* generation of insertions, deletions or other sequence changes.

Note: Modified forms of the target that differ significantly from the target sequence in length or GC content will have different amplification efficiencies from target sequences, and thus are not appropriate for use as positive controls.

- A heterologous sequence that has been previously shown to be amplified with an efficiency that is comparable to the target sequence.

- RNA transcribed from a cloned DNA fragment containing the target sequence or a modified target sequence (for use in RT-PCR applications).

For quantitative PCR methods, the PCR positive control is evaluated by determining the total amount of the target nucleic acid or organism in the control divided by the amount added to the reaction as a spike. For qualitative PCR methods, the PCR positive control typically is evaluated in terms of 'detect' or 'non-detect' (although a detection signal that is weaker than normal may still indicate a problem).

5.1.2 PCR Inhibition Positive Controls

Inhibition positive controls are used to verify that interfering constituents from an environmental matrix, which may be carried over during isolation of nucleic acids or organisms during sample processing, do not inhibit the PCR. Inhibition positive control templates can be prepared by adding any of the exogenous controls from Section 5.1.1 to a processed sample or by using an endogenous control.

Endogenous controls are target sequences that are expected to always be present in the sample (e.g., ribosomal DNA or RNA). These controls should be used only if it is demonstrated that there are consistent occurrence and recovery of the endogenous control templates in different samples, and if the control and target assays show comparable susceptibility to inhibition in different matrices. They are commonly used for analyses of clinical samples, but are unlikely to be applicable for the analyses of most environmental samples.

Exogenous controls can be used to analyze for PCR inhibition in several ways:

•   In separate aliquots of the same nucleic acid extract from the same sample

•   In extracts from separate, replicate samples processed in parallel

•   In the same aliquot of the same sample using modified internal controls

The absence of detectable PCR product from this control signals PCR inhibition. For quantitative PCR methods, different degrees of inhibition can be assessed directly by comparing the results from the control with the results from the PCR positive control (section 5.1.1). However, this requires that both controls be amended with the same amount of the positive control template. For qualitative PCR methods, the PCR inhibition positive control is evaluated in terms of a detect or non-detect (although a detection signal that is weaker than that of the PCR positive control may still indicate a problem).

Assessing different degrees of inhibition can be difficult using spikes of the template itself as the positive control since this template may be present in varying concentrations in the test sample. Use of a modified form of the target template as the positive control is generally recognized to be the preferred approach, because it is likely that the nearly identical sequences of the target and control template will confer equal susceptibility to PCR inhibition. However, this approach requires the custom design and preparation of a surrogate template for each primer set. Analyses for an added heterologous control template are generally easier to accomplish, but it is particularly important to demonstrate that the susceptibility of these assays to PCR inhibition is comparable to that of the target sequence in all samples. Primer sets for heterologous exogenous controls also should be checked for compatibility with the target sequence primers, when used in same tube reactions. Care should be taken when designing same-tube reactions, because the different assays will be competing for the same reagents (e.g., dNTPs, enzymes, and, in the case of modified target templates, primers), and target detection sensitivity may be affected adversely.

Because environmental matrices, such as river water, are constantly changing, inhibition positive controls should be performed for every sample and every target. If no matrix effects on the targets of interest are detected, then the frequency with which this control is performed can be reduced to a periodic check to assess potential changes in the matrix over time.

5.1.3 Method Positive Control

The method positive control is used to verify that the entire method is performing properly. This control should be performed by analyzing a reagent water sample seeded with known quantities of the target organisms prior the start of sample processing. For quantitative PCR methods, recovery of the method positive control is determined as the total amount of the analyte found in the sample divided by the amount of the control analyte added into the sample as a spike. The recovery should be expressed in terms of the units used to measure the concentration of the spike (e.g., CFU, PFU, counts, etc.). For qualitative PCR methods, the method positive control is evaluated in terms of detect or non-detect (although a detection signal that is weaker than normal may still indicate a problem). At a minimum, method positive controls should be performed for each sample batch. A sample batch is defined as a set of test samples set-up and processed together through all steps of the method leading to PCR.

5.1.4 Matrix Spike

The matrix spike is used to determine the effect of the matrix on the overall method recovery. This control can be performed by the analysis of a duplicate sample collected at the same time and location as the environmental sample and seeded with known amounts of the target organism prior to sample processing (section 5.1). The seeded sample should be processed at the same time and in the same manner as the unseeded environmental sample and the method positive control (Section 5.1.3), if feasible. Laboratories should, however, be cautious when processing seeded matrix samples at the same time as unseeded

environmental samples to prevent cross-contamination. The MS recovery is determined as the total amount of the analyte found in the sample minus the background (determined in the unseeded environmental sample) divided by the amount of the target analyte added into the sample as a spike (see Section 4.5.3). If it is believed that the sample matrix is inhibiting the isolation of target organism or nucleic acid, dilutions of the bulk or processed sample can be performed to determine if the signal becomes stronger as the sample is diluted. It should be noted that, while diluting the sample to reduce inhibition, the concentration of the target is also being reduced. Therefore, this technique may not be appropriate for samples where the concentration of the target is low.

A second approach to the matrix spike is to seed the environmental sample with a different organism than the target organism. This approach eliminates the need to process and analyze duplicate samples, since the target and surrogate organism nucleic acids can be independently analyzed in the same sample. It should only be used if the surrogate has similar properties to the target organism and if it is not present in the matrix tested. The same amounts of surrogate organism should also be added with the target organism to the method positive control (Section 5.1.3) for comparison of results with the matrix spike samples. However, this approach should not be used unless it is shown that the environmental inhibitors of PCR affect detection of surrogate and target organisms similarly. In addition, it should be shown that the recoveries of the surrogate and target organisms throughout the entire method are similar. The use of a different organism than the target for the control may also be limited if the front-end sample processing techniques are specific for the target (e.g., immunomagnetic separation that uses magnetic beads coated with antibodies specific to the target), or if there is competition between the target and the surrogate organism.

The processing and analysis of additional samples required for matrix spikes can be minimized by analyzing this control for only the first sample analyzed from a particular matrix. As described above for inhibition controls, however, some environmental matrices are constantly changing. As a result, MS samples should be performed regularly on a particular matrix to assess if any potential changes in the matrix have affected method performance, or until it can be documented that the variability of the recoveries within a given environmental matrix is acceptable.

5.2   Negative Controls

Negative controls using each primer set should be analyzed to verify that no contaminating nucleic acid has been introduced into the master mix or into samples during sample processing. These negative controls are considered acceptable if no amplification of nucleic acids is detected. Guidance on the two major types of negative controls is provided in Sections 5.2.1 and 5.2.2. A summary of the negative controls is provided in **Table 5-2**.

Table 5-2.   Summary of Negative Controls

Description	Purpose	Frequency
PCR negative control	Verify that no contaminating nucleic acid has been introduced into the master mix	At least 10% of the number of field samples analyzed per PCR batch
Method blank	Verify that no contamination has been introduced throughout the entire sample processing	With every sample batch

5.2.1 PCR Negative Control

PCR negative controls are used to verify that no contaminating nucleic acid has been introduced into the master mix. These controls are prepared when template is added to the master mix. They are prepared as separate samples to which aliquots of molecular-grade water or buffer are added to the master mix in place of target nucleic acid or sample. A negative result with this control indicates that the master mix and final processing reagents are not contaminated. The number of PCR negative controls should be 10% of the field samples analyzed per primer set per PCR batch. A PCR batch is defined as a group of samples set-up and amplified at the same time under the same conditions, using the same PCR master mix, and in the same thermocycler.

5.2.2 Method Blank

The method blank is designed to check for contamination throughout sample processing and PCR analysis. This control is performed on a sterile reagent water sample that is processed with the test samples using the same preparation, extraction, sample transfer, and PCR procedures as the test samples. At a minimum, method blank samples should be performed once per batch. A sample batch is defined as a set of test samples processed together through all steps of the method leading to PCR. Method blank samples may need to be run more frequently, depending on the data quality needs or method and monitoring program requirements.

Figure 5-1.   Sample Flow and Controls of PCR Methods on Environmental Samples
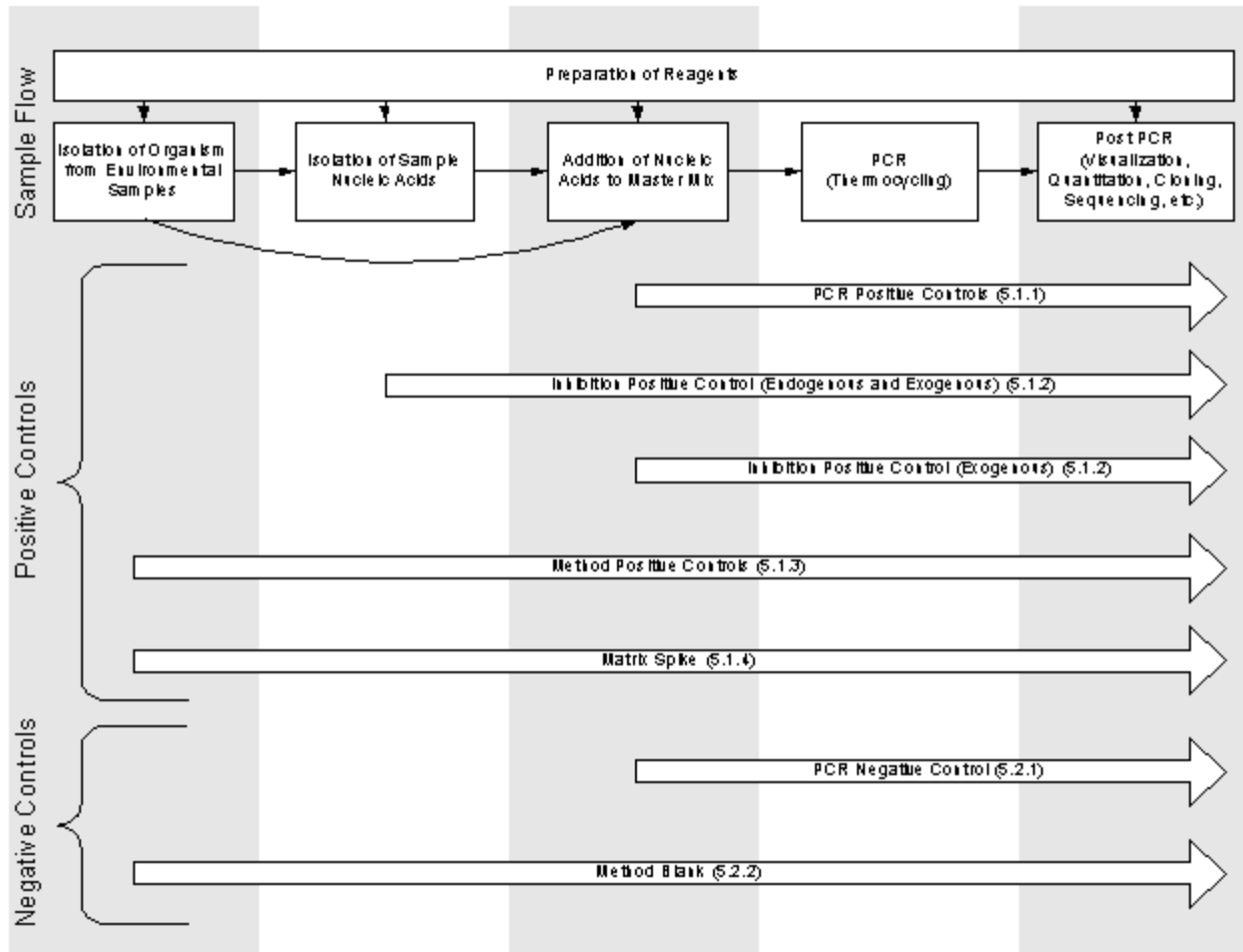
Note: Beginning of arrows indicate introduction of control into sample flow

5.3   Quality Control Samples for Confirmation Procedures

Each of the confirmation procedures used on PCR analysis should have individual QC samples associated with them. Guidance on these controls is provided below in Sections 5.3.1 to 5.3.5.

5.3.1 Gel Electrophoresis Controls

Each gel electrophoresis should contain a positive control and a negative control. The positive control should consist of a segment of DNA of known size (preferably of the same size as the target amplicon). The negative control consists of only the buffers and reagent water. The PCR positive and negative controls from the PCR can be used as the positive and negative gel electrophoresis controls, respectively.

A DNA ladder (a mixture of DNA fragments of known sizes), should also be run on each gel to provide a standardized gauge of the size of DNA fragments seen in the test samples and controls. The size of the expected product should be within the size range covered by the standard. Extrapolation beyond the range of the standard should not be performed.

5.3.2 Hybridization Controls

If probe hybridization is used as the confirmation procedure, the positive and negative controls from the PCR should be transferred to the membrane in the same manner as the test samples, and be used as the positive and negative controls for the hybridization. All positives should produce a positive response, and if performing a Southern blot, the positive should be at the same position as the target amplicon on the gel. All negative controls should produce a negative response for the result to be acceptable. Labeled size markers should be used for Southern blots to determine the amplicon size and can also serve as a detection control for both Southern and dot blots.

5.3.3 Restriction Mapping Controls

If restriction mapping is used to confirm the PCR result, positive controls for each restriction enzyme or combination of enzymes should be performed on DNA of a known sequence or with a pre-made restriction map (other than the amplicon of interest) to verify the activity of the restriction enzyme(s). Digestions should be performed in the buffer and at the temperature recommended by the manufacturer.

A sample of the amplicon, prepared and processed in the same manner as the positive control, but with no addition of the restriction enzyme, should be run to verify that digestion of the nucleic acids resulted from the added restriction enzyme, and not contaminating enzymes.

5.3.4 Probe-based Quantitative PCR and Melting Curve Analysis

Probe-based quantitative PCR and melting curve analysis are both part of real-time PCR, and the same positive and negative controls should be used for these confirmation techniques as are used for any type of PCR (see Sections 5.1 and 5.2).

5.3.5   Sequencing Controls

In the event of unacceptable results from sequence analyses (e.g., the fluorescent signal is weak throughout the sequencing ladders of all samples), a sequencing reaction control using a template and primer provided by the manufacturer should be run according to the manufacturers' instruction. The source of the problem (e.g., poor PCR amplification, expired fluorescent dyes) should be determined and corrected, and any affected samples reanalyzed.

5.4   Corrective Actions

If any positive control failures occur, all samples associated with the control should be considered invalid, and negative field samples should be listed as potentially false-negative samples. If amplification of a positive control fails to produce the specific amplification product, the integrity of the control and the PCR design should be examined to determine the reason for the failure. When determined, the reason for the failure should be documented and the controls and samples re-run. Samples associated with MS or inhibition positive controls that have low or no recovery should be qualified as potentially biased or false-negatives, as appropriate, and the matrix should be evaluated to determine the cause of the interference, if possible. For some samples, it may be possible to correct for bias in test results based upon the recovery as determined by the matrix spike. Corrections cannot be made if the PCR inhibition positive control shows evidence of inhibition. Corrections also cannot be extrapolated to samples for which matrix spike data are not available.

If PCR negative controls or method blanks produce specific amplification products, all samples associated with the failed controls should be considered invalid, and all positive samples should be listed as potentially false-positive samples. The source of contamination should be identified and eliminated. For recommendations on cleaning, see Sections 2.4 and 2.6. Once determined, the source of the contamination should be documented, and the samples in the batch should be recollected and reanalyzed, if possible. If the source of the contamination cannot be identified, additional types of negative controls should be added at various steps in the method to determine where the contamination is being introduced. Three such controls are the following.

- **Equipment blank.** This control is performed by passing sterile reagent grade water or buffer through the equipment and processing the water as if it were a PCR negative control. If this control is found to be positive, all analysis should cease until the source of the problem is identified. Equipment blanks should then be run more frequently until it is shown that the problem has been corrected.

- **Wipe test.** This control is performed by wiping an area, pipette, or other equipment with a sterile, nucleic acid-free, gauze or q-tip, then suspending the wipe in molecular-grade water and processing it as a PCR negative control. To determine whether nucleic acids detected are part of the normal background or the source of sample contamination, wipe tests should be performed routinely to establish a baseline for comparison.

- **Room QC controls.** This control is prepared by adding sterile water in place of template to a master mix in the reagent preparation room. These tubes are placed in working areas in the reagent preparation and sample preparation rooms and opened for 15 minutes. The tubes are then closed and run as a PCR negative control. These controls may be run on a monthly basis. If any of these controls are positive, all work should cease until the source of the contamination can be identified and corrected.

5.5   False-Positive/False-Negative Prevention

In addition to the QA/QC guidance discussed in Sections 2 and 3, specific procedures should be implemented to prevent false-positive and false-negative reactions from the techniques used (18). Details on preventing false positives and false negatives are provided in Sections 5.5.1 and 5.5.2.

5.5.1   False Positive Prevention and Detection

False positives can occur from contamination and non-specific amplification. Contamination introduced by personnel, positive controls, or positive samples (i.e., cross-sample contamination) can be minimized by following the guidance provided in Sections 2 and 3, and especially by physically separating work areas as described in Section 2.

October 2004

5.5.1.1 Product Carryover Reduction

Product carryover (contamination with DNA that has been previously isolated and/or amplified) can result in contamination of reagents or samples. Several approaches are available to prevent false-positive reactions through carryover contamination including the following:

- Enzymatic inactivation

- Photochemical inactivation (addition of psoralen after the PCR links the double-stranded DNA and prevents amplification in subsequent PCRs)

- Hydroxylamine treatment (hydroxylamine is added after the PCR and prevents cytosine from bonding with guanine in subsequent PCRs)

The most widely used approach, enzymatic inactivation, involves the introduction of dUTP (the nucleotide deoxyuradine) in lieu of TTP (the nucleotide thymine), along with the enzyme uracil N–glycosylase (UNG, also known as uracil DNA glycosylase, UDG), into the PCR master mix. As a result of this substitution, all amplified product will contain deoxyuradine instead of thymidine. The deoxyuradine incorporated into the DNA becomes a target for site-specific digestion by UNG. Prior to amplification, an incubation step is performed to activate UNG and enzymatically degrade carryover amplified DNA from previous amplifications. The UNG is then inactivated by heating to 94°C, and the actual PCR cycles are begun. This procedure cannot be used with nested PCR, because it would destroy the product of the first amplification. Similarly, it normally cannot be used for the reverse transcription phase of a RT-PCR assay because it would destroy the cDNA produced by reverse transcriptase. However, recently there have been reports of the use of a heat labile UNG that may overcome this problem (43).

5.5.1.2 Detection of False-Positive Reactions from Non-Specific Amplification

False positives can also result from the non-specific amplification of cross-reacting nucleic acids. This type of false positive can be determined using the confirmation protocols identified in Section 4.4. The redesign of primer sets and/or hybridization probes should be used to reduce this type of false-positive reaction.

5.5.1.3 Hot-Start PCR

"Hot-start" PCR is a modification of conventional PCR (Section 4.3.1.1) that reduces non-specific product amplification. In this procedure amplification cannot occur until the reaction temperature is above that where non-specific annealing of primers to targets occurs. This block in amplification is usually accomplished by using a DNA polymerase that is inactive until higher temperatures are reached. (37)

5.5.1.4 Touchdown PCR

Touchdown PCR is another modification of conventional PCR that may result in a reduction of non-specific amplification. It involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles. The annealing temperature is decreased by 1°C every cycle or every second cycle until a specified or 'touchdown' annealing temperature is reached. The touchdown temperature is then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product. (37)

5.5.2 False Negative Prevention

False negatives can result from inhibitors from the environment, poor experimental design of the PCR, poor primer design, the variability of the environmental processing, or contaminants like DNase and RNase that destroy the nucleic acid.

There are several procedures that are reported to reduce inhibition, including the incorporation of 200 to 400 ng/µL of BSA (bovine serum albumin) to the PCR (27). However, there are no procedures that work with all applications, which is why the rigorous use of each positive control discussed in Section 5.2.1 is necessary. Good laboratory practices, as discussed in Sections 2 and 3, should be followed to prevent contamination with DNase.

5.6   Proficiency Testing

Proficiency testing (PT) programs provide another means for laboratories to assess their ability to perform a particular method and may be an appropriate part of some laboratory approval programs. Laboratories should participate in PT programs for PCR analysis of environmental samples, if such programs are available. Samples should be analyzed with the laboratory's regular workload, and be performed by the personnel who routinely perform the method and in the same manner as the laboratory routinely analyzes environmental samples.

PT samples should be composed of a matrix similar to that normally analyzed by the method and should be seeded with whole target organisms. Currently there are few PT programs for molecular methods. If PT programs or samples are unavailable for a particular assay, laboratories should implement other procedures to test for internal consistency in their results, such as testing duplicate or split seeded samples. If possible, the identification of one of the duplicates should be blind so that the technician performing the analyses does not know that the two samples are duplicates. Another alternative to approved PT programs is sending a duplicate or split sample to an outside laboratory for comparison with the laboratory's own results.

SECTION 6. DATA RECORDING, RECORD KEEPING, AND DATA EVALUATION

The generation of all environmental data should be completely and consistently documented, so the results can be assessed by an independent party. Guidance for data recording and record keeping are provided in Section 6.1. A discussion of issues related to the evaluation of PCR data from the analysis of environmental samples is provided in Section 6.2.

6.1   Data Recording and Record Keeping

Laboratories should retain copies of all data records for a minimum of five years after the project is completed. For highly visible projects, records may need to be retained for a longer period of time in the event that results are challenged or questioned. If data are maintained electronically, data should be backed up on a regular basis and stored in a separate location from the original data, if possible. All data recording should be checked by the laboratory supervisor for correctness and completeness, and each entry should be checked for accuracy of transcription.

6.1.1 Equipment

All equipment calibration and maintenance should be documented in appropriate logbooks. A schedule of equipment calibration and maintenance should be established with the procurement of each new piece of equipment, and a copy of the schedule should be posted in the laboratory near the equipment. Each of these activities should be documented in the laboratory's SOPs and followed by laboratory staff involved in the use of the equipment.

6.1.2 Reagents, Kits, Primer Sets, and Enzymes

Logbooks should be maintained for all reagents, kits, primer sets, and enzymes, and should document all pertinent information needed to identify possible sources of contamination, including the following:

- Product name or name of the primer

- Manufacturer

- Product number

- Formulations (reagents)

- Sequence (primers and probes)

- Lot number or in-house lot number

- All associated dates (receipt, preparation, open, expiration, when passed internal laboratory assessments, etc.)

- Receipt or preparation analysts' name and initials

- Storage location and location of components

- Location of the commercially available kit specifications and procedure

- Concentration of original primer stocks and working solutions

- Storage buffer

- Number of units (enzyme)

- Associated buffer (enzyme)

The reagent logbooks should be centrally located and readily accessible.

6.1.3 Sample Processing and Analysis

Laboratories should carefully document all steps involved in the analysis of environmental samples, including the handling, processing, and examination of the samples. Information that identifies the sample should be recorded, including the date, time, and location of sample collection, the date and time the sample was received at the laboratory, and the sample condition upon receipt. Detailed information should be recorded concerning the analysis of the sample, including the date and time each analytical step was performed, as well as the analysts' initials. Lot numbers of reagents, enzymes, primers, and other materials used to analyze the sample also should be recorded. The use of standardized bench sheets for a particular method is recommended for ensuring that all necessary data is recorded in a consistent manner.

For all amplicon confirmation procedures, data should be presented in a clear and concise way to ensure that results are interpreted properly. All laboratory SOP procedures for the method should be followed, including appropriate composition of the gel, buffers, voltage, and length in time of run. Any differences to the SOP should be noted. Specific results for each amplicon confirmation should be recorded as indicated in Sections 6.1.3.1 to 6.1.3.5.

Before being reported to the end user, all data should be reviewed by an analyst familiar with the method to ensure that all method requirements were met, including use of appropriate testing conditions (e.g., reagents, thermocycler conditions), analysis of appropriate controls, and analysis of samples within method holding times. Any information that impacts the quality or validity of the data should be noted on the data report.

6.1.3.1 Electrophoresis

A photograph or digital image should be taken of the gel under UV light, and reactions associated with each lane identified.

6.1.3.2 Blots

The following data should be recorded for blot procedures:

- **Southern blot**. A photograph or digital image of the gel should be taken and labeled prior to the transfer of the DNA to the membrane. A graduated scale should be included in the picture to compare it to the membrane. After the DNA is transferred to the membrane and probed, the x-ray film is taken and compared to the gel picture to verify that the hybridized band is in the same position as the amplicon band on the gel. The x-ray film should be a part of the record. Many laboratories have replaced the x-ray film with other equipment such as the phosphorimager. In such cases, the computer-generated image of the hybridization signal should be compared to the gel picture, and be included as part of the record.

- **Dot blot**. The exposed x-ray film or digital image should clearly show the positive and negative hybridization.

6.1.3.3 Sequences

All raw fluorescent chromatographs of the sequences should be archived and the associated sequences recorded. The sequencer and the manufacturer of the sequencing reaction should be recorded in addition to the date, time, and analyst. A comparison should be conducted to ensure that the determined nucleotide bases match between the sequenced strands for both the forward and reverse sequences, and a combined edited sequence should be reported.

6.1.3.4 Restriction Mapping

A diagram of the target amplicon should be developed, including where the restriction enzymes will cut and the sizes of the pieces. Each lane of the gel should be labeled with its reaction, and a photograph or digital image should be taken of the gel under UV light.

6.1.3.5 Quantitative Real-time PCR

Raw data from quantitative real-time PCR instruments, including fluorescence growth curves, the fluorescence threshold values used for cycle threshold calculations and melting curve analyses, if performed, should be maintained. This information is generally included in run files generated by these instruments. It should be saved and backed up by paper copy or in an electronic form other than the real-time instrument.

6.2   Data Evaluation

Laboratories should have interpretive criteria documented in the SOP for the confirmation used in the method. These criteria should include provisions for determining whether the amplicon is correct, and what to do if borderline results are obtained. For example, in cases where weak positives (by gel or real-time PCR) or weak hybridizations are obtained, sequencing of the amplicon would be a required step, rather than an option as defined in Section 4.4.

6.2.1 Equivocal Results

As with traditional microbiological methods, molecular methods may generate unclear results that reflect differences in the quality and composition of the sample, as well as the performance of the test. Procedures for interpreting test results that are not straightforward should be established and may depend on the application of the test results. For example, in the case of a real-time PCR experiment, the C_T value around the equivocal range should be defined. If numbers are obtained beyond this value, additional confirmation techniques should be performed to confirm the identity of the amplicon or the sample should be recollected and the analysis repeated.

6.2.2 Limitations of Test Results

In addition to equivocal results, data may be limited due to the results of positive and negative QC samples associated with field samples. These limitations are discussed in Section 5.4, and data qualifiers, including potential biases and potential false-positive and false-negative results, should be reported with the field sample results.

Accuracy—The degree of agreement between an observed value and an accepted reference value. Accuracy includes random error (precision) and systematic error (bias or recovery) that are caused by sampling and analysis.

Aliquot—A smaller volume withdrawn from a greater volume.

Amplicon—Amplified fragment of DNA obtained through PCR.

Amplification—The process by which extra copies of a gene or a DNA sequence are formed.

Annealing—In PCR, the step at which primers (oligonucleotides) bind to complementary segments on template/sample DNA (typically done at 47°C - 60°C).

Base pair—The pair of nitrogenous bases, consisting of a purine linked by hydrogen bonds to a pyrimidine, that connects the complementary strands of DNA or of hybrid molecules joining DNA and RNA. The base pairs are adenine-thymine and guanine-cytosine in DNA, and adenine-uracil and guanine-cytosine in RNA.

Bias—A systematic or a persistent distortion of a measurement process that deprives the result of representativeness; i.e., the expected sample measurement is different than the sample's true value expressed in terms of recovery. A data quality indicator.

Blot—Transfer of nucleic acids to a membrane. The bound nucleic acid is often then detected by probe hybridization.

Carry-over contamination—The contamination of a sample, or series of samples or reagents from nucleic acids from previous amplification reactions (usually carried by the equipment or analyst).

Cloning—The use of an organism to produce more copies of a specific fragment of nucleic acid. Normally done using vectors.

Confirmatory test—A test used to validate the results obtained (e.g., the presence or absence of a PCR amplicon).

Complementary—A strand of DNA or RNA that is capable of forming specific pairing of the purine and pyrimidine base sequences with those of a second strand.

Quality control sample—A standard sample included in an assay used to determine the validity of the test based on a predetermined outcome or range of outcomes.

Denaturation—In PCR, breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce single-stranded nucleic acid molecules (typically done at 94°C - 97°C).

Detection limit—See limit of detection

DNA—Deoxyribonucleic acid. A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication, and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix and joined by hydrogen bonds between the complementary bases adenine and thymine or cytosine and guanine. The sequence of nucleotides determines individual hereditary characteristics.

DNase—An enzyme that catalyzes the hydrolysis of DNA.

dNTPs—Deoxyribonucleotide triphosphates. dNTPs are incorporated into DNA during elongation.

Electrophoresis—A method of separating substances (e.g. DNA/RNA/proteins) based on size, charge, or sequence composition, and by the rate of movement of each component in a colloidal suspension through a polymer gel while under the influence of an electric field.

Elongation—In PCR, function of an enzyme (polymerase) that synthesizes a complementary copy of a nucleic acid strand (typically done at 72℃) by extending primers.

Endogenous—A region of DNA or RNA that always is found naturally in a test sample and which differs from the target sequence of a PCR method.

Enzyme—Any of numerous proteins or conjugated proteins produced by living organisms and functioning as biochemical catalysts.

Exogenous—A region of DNA or RNA that is added to a test sample as a control.

Functional validation—Testing that demonstrates that a method or procedure works the way it was intended to work.

GC content— Guanosine-cytosine content.

Gene—A hereditary unit consisting of a sequence of DNA that occupies a specific location on a chromosome and determines a particular characteristic in an organism. Genes undergo mutation when their DNA sequence changes.

Hairpin loops—A secondary structure of a nucleic acid that can be formed due to inverted repeats in the sequence.

Heterologous Sequence—A sequence that differs from another gene's sequence.

Homology—Genes that share a similar sequence and are functionally equivalent.

Hybridization—The binding of one nucleic acid to another by the formation of hydrogen bonds between the bases on the two molecules. The sequences must be completely or nearly complementary to each other in order for this type of bonding to occur. This process is usually used in conjunction with a probe to determine the presence of a specific sequence of DNA.

Hybridize—To form a double-stranded nucleic acid by the pairing of complementary regions of two strands of DNA that were not originally paired.

Inaccuracy—The deviation from the true result.

Inhibition—The reduction in efficiency in a PCR caused by elements in the environmental sample that interfere with the normal reaction.

Inhibition positive control—A positive control sample used to verify that the constituents of the matrix carried over from the isolation of the organism do not inhibit either the isolation of the nucleic acid or the PCR.

Interference—Endogenous or exogenous substances that can cause false-positive or false-negative results in a test system.

Limit of detection—The minimum concentration of a substance that can be measured (or gives a positive response) with a given level of confidence that the analyte concentration is greater than zero.

Master mix—Solution containing all reagents (except for the test or sample nucleic acid) that is required to perform PCR.

Matrix—The component or substrate that contains the analytes of interest.

Matrix spike—A QC sample prepared by adding a known amount of target analyte to a specified amount of sample matrix for which an independent estimate of target analyte concentration is available. A matrix spike is used, for example, as a positive control determining the effect of the matrix on the overall method's recovery and to verify it does not have an inhibitory effect on the PCR.

Method blank—An aliquot of reagent water that is treated exactly as a sample, including exposure to all glassware, equipment, solvents, and procedures that are used with samples. The method blank is used as a negative control to verify that no contamination is present in the laboratory environment, the reagents, or the apparatus.

Method positive control—A positive control used to verify that the entire method is performing properly and is capable of amplifying the target nucleic acid from the organism of interest.

Molecular-grade water—Water that is DNase and RNase free as well as de-ionized/distilled.

Nested PCR—PCR in which two pairs of PCR primers are used for a single locus. The first pair amplifies the locus. The second pair of primers (nested primers) bind within the first PCR product and produce a second PCR product that will be shorter than the first one. The specificity of the assay is increased because the inner primers amplify only if the first PCR reaction yielded a specific product.

Nucleic acid—Any of a group of complex compounds found in all organisms, composed of purines, pyrimidines, carbohydrates, and phosphoric acid. Nucleic acids in the form of DNA and RNA control cellular function and heredity.

Oligo—See oligonucleotide.

Oligonucleotide—A short segment of single stranded nucleic acid or a short sequence of nucleotides (e.g., PCR primers and real-time probes).

PCR—Polymerase Chain Reaction. A technique for amplifying DNA sequences *in vitro* by separating the DNA into two strands and incubating it with nucleotides, oligonucleotide primers, and DNA polymerase. It can amplify a specific sequence of DNA by more than a billion times (e.g., 40 cycles = 2^{40} = one trillion amplicons).

PCR Batch—A group of samples set-up and amplified at the same time under the same conditions, using the same PCR master mix, and in the same thermocycler.

PCR negative control—A negative control used to verify that no contaminating nucleic acid has been introduced into the master mix.

PCR positive control—A positive control used to verify that the PCR master mix and reaction were designed correctly in order to produce amplification of the target nucleic acid.

Polymerase—Any of various enzymes, such as DNA polymerase, RNA polymerase, or reverse transcriptase, that catalyze the formation of DNA or RNA molecules using an existing strand of DNA or RNA as a template.

Polymerase chain reaction—See PCR

Precision—The degree to which a set of observations or measurements of the same property, usually obtained under similar conditions, conform to themselves; a data quality indicator. Precision is usually expressed as standard deviation, variance, or range, in either absolute or relative terms.

Primer—A segment of DNA or RNA that is complementary to a given DNA sequence and that is needed to initiate replication by DNA polymerase.

Primer binding sites—Sequences in the DNA that are complementary to the primer used in the PCR.

Primer dimer—Two primer sequences that form a small region of double stranded DNA due to complementary sequences being present on both primers. This formation inhibits the primers use in the polymerase chain reaction.

Probe—A substance, such as DNA, that is fluorescently, radioactively, or otherwise labeled and used to detect or identify another substance in a sample.

Processivity—The ability of DNA polymerase enzyme to amplify DNA, often referring to the amplification of longer sections of DNA.

Proficiency testing (PT) samples—Samples qualitatively and quantitatively unknown to the analyst that can be obtained commercially or by agreement from another laboratory.

Proofreading—Capability of a polymerase to be able to check for errors in the nucleotide sequence that it synthesizes as well as being able to repair any errors that occur.

Reagent—A substance used in a chemical reaction to detect, measure, examine, or produce other substances.

Real-time PCR—Detection of specific nucleic acid amplification products as they accumulate during PCR in real-time using various fluorescence-based chemistries.

Recovery—The total amount of the analyte found in the sample, corrected for background, divided by the amount of the analyte added into the sample.

Relative percent difference (RPD)—An estimate of the variability of two numbers expressed in relative terms. Calculated as the absolute value of the difference of the two numbers, divided by their mean:

$$RPD = \frac{|A - B|}{\frac{1}{2} \times (A + B)} \times 100\%$$

Equal to the relative standard deviation of the two numbers multiplied by the square root of 2.

Relative standard deviation (RSD)—The standard deviation expressed as a percentage of the mean ($100\sigma/X$); i.e., the coefficient of variation.

Restriction endonuclease—Class of bacterial enzymes that cut DNA at specific sites.

Restriction mapping—Use of restriction endonucleases to analyze and generate a physical map of genomes or genes.

Reverse transcriptase—A polymerase that catalyzes the formation of complementary DNA (cDNA) from an RNA template.

RNA—Ribonucleic acid. A polymeric constituent of all living cells and many viruses, consisting of a long, usually single-stranded chain of alternating phosphate and ribose units with the bases adenine, guanine, cytosine, and uracil bonded to the ribose. The structure and base sequence of RNA are determinants of protein synthesis and the transmission of genetic information.

RNase—An enzyme that catalyzes the hydrolysis of RNA.

RT-PCR—Reverse Transcriptase-Polymerase Chain Reaction. A technique for amplifying RNA, such as 16S rRNA or mRNA, *in vitro* by using reverse transcriptase and RNA as a template to create a cDNA that is then be used in a PCR.

Sensitivity—The sensitivity of a test can be described as the proportion of all positive results detected that were truly positive. All positives are the sum of (detected) true positives (TP) and (undetected) false negatives (FN). Sensitivity is therefore: TP / (TP + FN) × 100%

Sample batch—A set of test samples set-up and processed together through all steps of a PCR method.

Sequencing—Determining the order of nucleotides in a nucleic acid.

SOP—Standard operating procedure.

Specificity—The specificity of a test can be described as the proportion of all negatives it detects that truly were negative. All negatives are the sum of (detected) true negatives (TN) and false positives (FP). Specificity is therefore: TN / (TN + FP) × 100%

Target nucleic acid—The specific sequence of nucleic acid that the PCR is designed to amplify.

Template—A molecule of a nucleic acid, such as DNA, that serves as a pattern for the synthesis of replicate copies of DNA amplicons.

Thermocycler—Device which can be programed to raise and lower the temperature of reaction mixes and is used to perform PCR.

Ultraviolet (UV)—Of or relating to the range of invisible radiation wavelengths from about four nanometers, on the border of the x-ray region, to about 380 nanometers, just beyond the violet in the visible spectrum.

Uracil N–glycosylase (UNG; a.k.a. uracil-DNA-glycosylase, UDG)—An enzyme that hydrolyses DNA strands with deoxyuradine incorporated in its sequence. It can be used to eliminate carryover contamination from previous PCR.

Vector—A DNA molecule that is inserted into a host cell that allows replication of itself and any inserted sequence as the organism lives and reproduces.

SECTION 8. REFERENCES AND RECOMMENDED READING

1. **AACTG.** 1999. General Pipetting Operational Considerations/Tips. The Adult AIDS Clinical Trials Group. [Online.] http://aactg.s-3.com/pub/download/imm/pipettecalibdocmay99.doc. Accessed 31 December 2003.

2. **American Public Health Association, American Water Works Association, and Water Environment Federation.** 1995. Standard Methods for Water and Wastewater, 20th Edition. Sections: 9020, 9221, 9222.

3. **Ambion.** 2003. The Basics: RNase Control. Ambion, Inc.®. [Online.] http://www.ambion.com/techlib/basics/rnasecontrol/index.html. Accessed 30 December 2003.

4. **Barker, K.** 1998. At the Bench a Laboratory Navigator. Cold Spring Harbor Laboratory Press, Plainview, NY.

5. **Basic Laboratory Incorporated.** 2000. Quality Assurance Plan, revision 4.0. Basic Laboratory Inc. [Online.] http://www.basiclabinc.com/qapp/qapp2000.pdf. Accessed 30 May 2003.

6. **Becker, S., P. Böger, R. Oehlmann, and A. Ernstet.** 2000. PCR bias in ecological analysis: A case study for quantitative taq nuclease assays in analyses of microbial communities. Appl. Environ. Microb. **66**(11):4945-4953.

7. **Biocompare.** 2003. Properties of Reverse Transcriptases. Biocompare (Roche Applied Science). [Online.] http://www.biocompare.com/techart.asp?id=768. Accessed 31 December 2003.

8. **Bustin, S. A.** 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. **25**:169-193. 2000.

9. **Choi J. S., J. S. Kim, C. O. Joe, S. Kim, K. S. Ha, and Y. M. Park.** 1999. Improved cycle sequencing of GC-rich DNA template. Exp Mol Med. **31**(1):20-24.

10. **Coyne, V. E., M.D. James, S. J. Reid, and E.P. Rybicki.** 2003. Molecular Biology Techniques Manual, 3rd ed, University of Cape Town. [Online.] http://www.mcb.uct.ac.za/manual/MolBiolManual.htm. Accessed 30 December 2003.

11. **Dahllöf, I.** 2002. Molecular community analysis of microbial diversity. Curr. Opin.Biotech. **13**:213-217.

12. **DeLeon, R., P. Hacker, and P. A. Rochelle.** 2002. Development of stringent verification procedures for molecular detection of enteric viruses in water. American Water Works Association - Water Quality Technology Conference.

13. **Di Giovanni, G. D., M. R. Karim, M. W. LeChevallier, S. N. Boutros, J. S. Chandler, F. A. Abrams, M. L. Spinner, and J. R. Weihe.** 2002. Overcoming molecular sample processing limitations: Quantitative PCR. Project 00-HHE-2b. Water Environment Research Foundation, Alexandria, VA.

14. **Fairfax, M. R., M. A. Metcalf, and R. W. Cone.** 1991. Slow inactivation of dry PCR templates by UV light. PCR Meth. Appl. **1**:142-143.

15. **Federal Bureau of Investigation.** 2000. Quality Assurance Standards for Forensic DNA Testing Laboratories. Federal Bureau of Investigation. [Online.] http://www.fbi.gov/hq/lab/fsc/backissu/july2000/codis2a.htm. Accessed 27 February 2004.

16. **Fout, G. S., B. C. Martinson, M. W. Moyer, and D. R. Dahling.** 2003. A multiplex reverse transcription-PCR method for detection of human enteric viruses in groundwater. Appl. Environ. Microb. **69**:3158-3164.

17. **Francy D. S., R. N. Bushon, C. Kephart, A. Gifford, E. Granger, K. Mauch, and D. M. Stoeckel.** 2004. Quality Assurance/ Quality Control Manual. Ohio District Microbiology Laboratory. [Online.] http://www-oh.er.usgs.gov/micro/qcmanual/manual.html. Accessed 29 February 2004.

18. **GeneScan.** 2003. Quality control/Quality Assurance. GeneScan USA Inc. [Online.] http://www.gmotesting.com/qa.html. Accessed 30 May 2003.

19. **Glick, B. R. and J. J. Pasternak.** 1998. Molecular Biotechnology Principles and Applications of Recombinant DNA, 2nd ed, ASM Press, Washington, D.C.

20. **Greer, C. W., L. G. Whyte, J. R. Lawrence, L. Masson, and L. Brousseau.** 2001. Genomics technologies for environmental science. Environ. Sci. Technol. **35**:360A-366A.

21. **Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams.** 1996. Real time quantitative PCR. Genome Res. **6**(10):986-994.

22. **Heuvel, J. V.** 2004. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Other PCR Procedures. Penn State University. [Online.] http://www.cas.psu.edu/docs/CASDEPT/VET/jackvh/jvhpcr.html. Accessed 27 February 2004.

23. **Hochmeister, M.** 1995. DNA technology in forensic Applications. Mol. Aspects Med. **16**:315-473.

24. **Innis, M. A., D. H. Gelfand, J. J. Sninsky, T. J. White.** 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

25. **Johannes Gutenberg-Universität Mainz.** 2004. PCR Additives. Johannes Gutenberg-Universität Mainz. [Online.] http://www.uni-mainz.de/~lieb/additiva.html. Accessed 27 February 2004.

26. **Kousuke, I. and F. Manabu.** 2001. Optimization of annealing temperature to reduce bias caused by primer mismatch in multitemplate PCR. Appl. Environ. Microb. **67**(8):3753-3755.

27. **Kreader, C. A**. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene protein. Appl. Environ. Microb. **62**(3):1102-1106.

28. **Markoulatos, P., N. Siafakas, and M. Moncany.** 2002. Multiplex polymerase chain reaction: A practical approach. J. Clin. Lab. Anal. 16:47–51.

29. **Millar, D. S., S. J. Withey, M. L. V. Tizard, J. G. Ford, and J. Herman-Taylar.** 1995. Solid-phase hybridization capture of low-abundance target DNA sequences: Application to the polymerase chain reaction detection of *Mycobacterium paratuberculosis* and *Mycobacterium avium* subsp. *silvaticum*. Anal. Biochem. **226**:325–330.

30. **Muir, P., A. Ras, P. E. Klapper, G. M. Cleator, K. Korn, C. Aepinus, A. Fomsgaard, P. Palmer, A. Samuelsson, A. Tenorio, B. Weissbrich, A. M. van Loon.** 1999. Multicenter quality assessment of PCR methods for detection of enteroviruses. J. Clin. Microbiol. **37**(5):1409-1414.

31. **National Committee for Clinical Laboratory Standards (NCCLS).** 1995. Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline. NCCLS MM3-A.

32. **NIH.** 2004. BLAST. National Institutes of Health, NCBI. [Online.] http://www.ncbi.nlm.nih.gov/BLAST/. Accessed 27 February 2004.

33. **NuAire.** 2004. Selecting a Biological Safety Cabinet. NuAire, Inc. [Online.] http://www.nuaire.com/bscs/selectbsc.html. Access 27 February 2004.

34. **Ou, C., J. L. Moore, and G. Schochetman.** 1991. Use of UV irradiation to reduce false positivity in polymerase chain reaction. BioTechniques. **10**(4):442, 444, 446.

35. **Polz, M. F. and C. M. Cavanaugh.** 1998. Bias in template-to-product ratios in multitemplate PCR. Appl. Environ.Microb. **64**(10):3724-3730.

36. **Pfaffl M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**(9):e45.

37. **Prilusky, J (ed.).** BioGuide - PCR. Weizmann Institute of Science. [Online.] http://bip.weizmann.ac.il/mb/bioguide/pcr/contents.html. Accessed 1 March 2004.

38. **Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer.** 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. **245**:154–60.

39. **Rochelle, P. A.** 2001. Environmental Molecular Microbiology: Protocols and Applications. Horizon Scientific Press, Norfolk, England.

40. **Sambrook, J. and D. W. Russel.** 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, lainview, NY.

41. **Sen, K.** 2000. Rapid identification of *Yersinia enterocolitica* in blood by the 5' nuclease PCR assay. J. Clin. Microbiol. **38**:1953-1958.

42. **Suzuki, M. T. and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16s rRNA genes by PCR. Appl. Environ. Microb. **62**(2):625-630.

43. **Taggart E. W., K. C. Carroll, C. L. Byington, G. A. Crist, D. R. Hillyard**. 2002. Use of heat labile UNG in an RT-PCR assay for enterovirus detection. J Virol Methods. **105**(1):57-65.

44. **Theron, J. and T. E. Cloete.** 2000. Molecular techniques for determining microbial diversity and community structure in natural environments. Crit. Rev. Microbiol. **26**:37-57.

45. **Weaver, R. F.** 1999. Molecular Biology, 1st ed. WCB/McGraw Hill, Boston, MA.

46. **Wilhelm, J. and A. Pingoud.** 2003. Real-time polymerase chain reaction. Chembiochem. **4**(11):1120-1128.

47. **University of Victoria.** 2003. Laminar Flow Hoods & Biological Safety Cabinets. University of Victoria. [Online.] http://ohs.uvic.ca/biosafety/biosafetycabinets.html. Accessed 1 March 2004.

48. **USEPA.** 2003. EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods. EPA- 821-B-03-004. Office of Water, Washington, DC.

49. **USEPA.** 2003. Workshop to Develop a Protocol for Reliable Genetic Methods for the Detection of Viruses, for Use in EPA's Water Programs. Office of Water, Cincinnati, OH.

50. **USEPA.** 1997. Manual for the Certification of Laboratories Analyzing Drinking Water. EPA-815-R-97-001. Office of Water, Cincinnati, OH.

51. **Zimmermann, K. and J. W. Mannhalter.** 1998. Comparable sensitivity and specificity of nested PCR and single-stage PCR using a thermally activated DNA polymerase. BioTechniques. **24**:222-224.