Once the feasibility studies are completed and the decision has been made to switch to qPCR, a fully equipped laboratory has to be set up. One advantage of setting up a qPCR laboratory is that it can be used for additional analytical testing including source tracking or other public health related medical testing. In this chapter, we will focus on the details for setting up a laboratory that is capable of conducting qPCR assays.
3.1. Facility Design

The laboratory workflow should be designed to prevent contamination of current qPCR reactions with products from previous assays, cross-contamination between samples, and contamination from other analytical testing; all are potential sources of invalid qPCR results. Therefore, it is recommended that there should be strict separation between workstations and/or different steps of the qPCR method. Thus, a laboratory performing qPCR analyses on environmental samples should be divided into several physically separate workstations.

Examples for workstation designations are as follows:

• Membrane filtration workstation

• Nucleic acid extraction workstation (This station can be combined with the membrane filtration station)

• Mastermix preparation workstation (ideally using positive pressure to prevent the introduction of contamination). This station has to be strictly separated from other workstations and preferably should be set up in a separate room.
• Reaction preparation workstation (ideally using negative pressure to keep template nucleic acids in the station). This station can be on a clean bench top if there is limited space close to the amplification and product detection workstation.

• Amplification and product detection workstation (This workstation has the thermocycler).

3.1.1. Designation of workstations

Separate workstations for each step in the qPCR analysis should be designated in order to reduce cross-contamination and to create an efficient workflow (Figure 3.1). All stations should be clearly labeled and have necessary warnings on them. Micropipettors should be dedicated to a particular station and labeled appropriately. If your micropipettors are autoclavable, you should routinely sterilize them. If the micropipettors are not autoclavable, wipe them down with a freshly prepared 10% bleach solution and UV sterilize for 20 minutes.

All materials, supplies, or equipment belonging to one specific workstation should remain at their respective area at all times.

Membrane Filtration Workstation

This workstation should be designed for the preparation and storage of the filters obtained by filtering the environmental water samples. Fresh gloves and dedicated laboratory coats should be worn at all times to control contamination. Personnel should perform tasks at this workstation before working in the crude DNA extraction, mastermix preparation, reaction preparation or amplification and product detection workstations.
ons to avoid contamination. Samples, equipment or supplies should not move from these workstations back to the membrane filtration workstation.

**Crude DNA extraction workstation**

Crude DNA extraction workstation should be designed for crude DNA extraction from the prepared filters and preparation of calibrators and controls. *This station can be combined with the membrane filtration station.* Fresh gloves should be worn at all times to control contamination. Personnel should perform tasks at this workstation before working in the master mix preparation, reaction preparation or amplification and product detection workstations and should not move from these workstations back to the crude DNA extraction workstation.

**Mastermix Preparation Workstation**

Extra care needs to be taken to avoid reagent contamination in this workstation. Ideally, this station should be in a separate room, if physical space allows. If this can not be met, a dedicated bench top with a PCR hood is required.
The mastermix preparation workstation should be designated ONLY for the preparation and storage of qPCR reagents (master mixes, primers and probes, water, bovine serum albumin).

Addition of master mixes to PCR tubes or 96 well plates should be performed at this workstation.

To prevent cross-contamination and to avoid repeated freezing and thawing, reagent-stock solutions should be aliquoted into smaller “working” volumes and stored in a dedicated refrigerator at this workstation for later use. It is recommended that the workstation be under positive pressure.

The mastermix preparation workstation should have dedicated adjustable pipettes (preferably autoclavable) with plugged, aerosol-barrier or positive-displacement pipette tips, laboratory coats, and disposable gloves. Fresh gloves and dedicated laboratory coats should be worn at all times to control contamination and should not be worn in other sections of the laboratory. Personnel should perform tasks at this workstation before working at the membrane filtration, crude DNA extraction, reaction preparation or amplification and product detection workstations and should not move from these workstations back to the mastermix preparation workstation.

All workstations should have labels describing the workflow in the workstation and decontamination procedures.

Figure 3.2. Example label for the Mastermix Preparation Workstation.

*Materials from other workstations should not be brought to the mastermix preparation workstation.*
**Reaction Preparation Workstation**

The reaction preparation workstation should be designated for adding sample DNA extracts, positive and negative controls and calibrators to the mastermix. The processed samples and controls should be added to tubes containing PCR master mix in this workstation.

Whenever possible, PCR tubes should be capped as soon as the sample is added. When using PCR plates and adhesive covers, positive controls and calibrators should be separated from wells with field samples to avoid contamination during template addition. This workstation 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 workstation to any other location. This workstation should ideally be kept under negative pressure to prevent contamination outside of the workstation.

> **Nothing from this workstation should be taken back into the reagent preparation workstation.**

Ideally, two biological safety cabinets should be used within the workstation—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.

**Amplification and Product Detection Workstation**

This workstation should be designated for activities associated with qPCR amplification. The thermocycler should be located at this workstation. Gloves and laboratory coats should be worn at all times and removed before leaving the workstation to control amplicon contamination of other locations. All equipment should be dedicated to this workstation.

All workstations should be labeled with instructions for operating. In addition to these workstations, laboratories may want
to include a designated enclosed area or workstation for sample receiving and storage. This workstation would contain refrigerators for sample storage and should be isolated from any of the other areas. Personnel should not move from this workstation to the mastermix preparation workstation. A list of qPCR equipment and supplies organized by station is provided in Chapter 4.

In the absence of a laminar flow hood or biological safety cabinet, protective dust masks or surgical masks may be worn to further reduce the risk of contamination of airborne nucleic acids from the analyst.

### 3.2. Instrument Selection, Calibration and Standardization

There are many different models of qPCR instruments available on the market. Depending on the user’s needs, several types of blocks are available which accept a variety of PCR reaction vessels (48-well plates, 96-well plates, 384-well plates, 384-microwell cards or 3072-through-hole plates).

Although there are several options, all qPCR instruments need to provide:

- An excitation source, which excites the fluorescent dyes,
- A detector to detect the fluorescent emissions (specifically FAM),
- And software for data collection and analysis.

The equipment used to perform qPCR methods should function properly and generate reliable data. To verify that equipment is functioning properly, the laboratory should have a schedule for equipment maintenance. 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 the QA/QC officer or the labora-
tory supervisor, and any problems and corrective actions noted.

As previously stated, equipment should be dedicated to a specific laboratory workstation, 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 of calibration. Examples of PCR quality assurance measures are provided in the 2004 USEPA guidance document, “Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples” Sections 2.4.1 through 2.4.11.

Calibration and Standardization of Instruments

- Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.

- Check thermometers at least annually against a “National Institute of Standards and Technology (NIST)” certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check columns for breaks.

- The spectrophotometer should be calibrated each day of use using OD calibration standards. Follow manufacturer instructions for calibration.

- Micropipettors should be calibrated at least annually and tested for accuracy on a weekly basis.

- Follow manufacturer’s instructions for all calibrations. It is recommended that thermocyclers be re-calibrated every 1-3 years.

3.3. Laboratory Quality Assurance and Quality Control

The successful application of qPCR requires proper use of techniques and interpretation of results. This method can amplify small amounts of nucleic acid and therefore, it is essential to establish standardized quality assurance and quality control procedures.

QA/QC procedures are necessary because the ability of qPCR to produce many copies of target DNA creates the possibility of contamination by previously amplified products, which can lead to false-positive results. A good QC program will also detect false negatives resulting from inhibition or underestimation of the target.

Laboratories performing qPCR analyses on environmental samples should develop a written QA Management Plan that describes how the laboratory will conduct its day-to-day routine operations in order to ensure accurate result reporting.
The main components of a QA Management Plan include: plans for personnel training, facility design, sampling forms, list of equipment, list of reagents and kits, handling and storage of reagents and kits, sample collection and processing protocols, method sensitivity, precision and recovery records, documentation of controls, data reporting and plans for corrective action measures. The plan should further include procedures for proficiency testing, data recording, data evaluation and the limitations of test results.

Quality control should include descriptions and procedures for positive and negative controls (method blank, filter blank, no template control, calibrator, salmon processing control), the development of calibration curves and the frequency they need to be performed. Inhibition must also be defined within the context of the method. For example, the US EPA defines inhibition a 3 cycle threshold difference between the calibrator-SPC pair and unknown-SPC pair. Also, acceptable levels of detection limits within the negative control (no-template control, NTC) must be determined:

- >35 CT in more than a single NTC
- <45 CT in a third of NTC reactions for a single mastermix

3.4. Personnel Training

Personnel working in a laboratory performing qPCR analysis should have previous training in aseptic techniques and handling of molecular reagents and equipment. Strict adherence to guidelines concerning personal protective equipment outerwear is a must. In addition, method specific training is required.

Personnel should use dedicated laboratory coats and powder-free gloves should be available in each workstation. Laboratory coats should be removed and gloves discarded before leaving each workstation. 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.

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 workstation.

Gloves should be changed after:
- working with seeded or environmental samples
- handling template or amplified nucleic acids
- contact of the outside of the gloves with skin or surfaces