

6.3 PCR Protocols

A. Preparation of Amplification Reaction Mixture

Specific amplification protocols may require one or two amplification reactions.

Note: samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures. QPCR master mix is typically assembled at room temperature because the *Taq* polymerase is inactive until heated to 95°C (check manufacturer's recommendation for set-up temperature).

1. Using [Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet](#) or [Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet](#), record appropriate data for each sample to be tested by PCR.
2. Under a UV cabinet, prepare “Master Mix” (MM) using pathogen-specific protocols in the worksheets in each chapter. Calculate the amount of each reagent to go into the MM according to the number of samples to be processed. Add PCR reagents, **except for sample DNA**, in the order listed on the worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing and return them to freezer immediately after use. Commercial QPCR master mix is typically stored at 4°C and should not be frozen (check manufacturer's recommendations).

Note: Prepare enough MM for three more samples than actually being tested to compensate for retention of solution in pipette tips and tube.

3. Place specified volume of MM into each PCR tube or plate wells. Close caps tightly. Move PCR tubes or PCR plate to sample loading area.
4. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes or plate wells. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly or seal PCR plate.

B. Running the PCR

All general considerations should be employed including the following:

1. Load the sample tubes or PCR plate into the machine wells (follow manufacturer's recommendations).
2. Program thermocycler for appropriate cycle conditions and run reaction.
3. Before loading into thermocycler, give tubes or PCR Plate a “quick-spin” to ensure that all reagents and sample are drawn down from sides of tube.

4. Thermocycler should be programmed for the specific PCR condition used for each pathogen (details under pathogen).
5. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes or unsealing plates, perform a “quick-spin” to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.
6. PCR products can be refrigerated for up to a month following amplification (or for longer storage they may be frozen at -20°C).

C. Detection of Product

Procedures for preparing the gel (refer to specific manufacturer’s guidelines for preparation of gels and electrophoresis chambers):

1. Assemble the gel tray and position well comb in the tray according to manufacturer’s recommendations.
2. Prepare 1 X TAE ([Section 2, 6.4 Reagents](#)) buffer with distilled water to volume adequate for gel and running buffer.
3. Prepare 1.5 to 2% agarose gel according to the volume recommended for specific gel forms used.
 - a. Weigh appropriate amount of agarose and add to proper volume 1X TAE buffer.
 - b. Heat solution to near boiling until agarose is completely dissolved.
 - c. Allow solution to cool to about 65°C, then pour agarose solution into gel tray. Avoid the formation of bubbles.
 - d. Allow gel to cool completely for about 30 minutes and then carefully remove the comb.
 - e. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.
 - f. Slowly fill the chamber with the remaining 1 X TAE buffer solution until the top of the gel surface is submerged.
4. Load samples into wells as indicated for each assay
 - a. For each tube of PCR product to be visualized, mix 2 µL of gel loading dye ([Section 2, 6.4 Reagents](#)) to every 10 µL of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading.
 - b. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight

standard (one with bands at 100 bp increments in the 100 to 1,000 bp range) for base pair (bp) reference and positive and negative controls

- c. Document gel lane assignments for each sample and control on [Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR](#) or [Section 2, 4.A1.B Worksheet B – Amplification of Nucleic Acid by PCR](#) and allow for at least one lane for a DNA ladder reference.

D. Electrophoresis

Approximately 80 volts for 35 minutes or until tracking dye front approaches the edge of the gel (this is dependent on gel width, so refer to manufacturer's recommendations).

E. Staining the Gel

Remove gel and tray and place in ethidium bromide (EtBr) solution ([Section 2, 6.4 Reagents](#)) for 15 to 20 minutes.

Note: EtBr solution can be reused and stored in a dark plastic tray container with a secure lid. **EtBr is very toxic** and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

F. De-Staining the Gel

In water for 5 to 60 minutes. De-stain water should be handled and disposed of appropriately (see [Section 2, 6.3.E “Staining the Gel”](#)).

G. Visualize the DNA

1. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used.

Note: use UV protective goggles or face shield.

2. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.
3. Photo document all PCR gels and attach to [Section 2, 3.A3.G Worksheet G – Photodocumentation of PCR Product Gel](#) or [Section 2, 4.A1.C Worksheet C – Photodocumentation of PCR Product Gel](#) (or provide reference for finding the photo documentation).

H. Quantitative PCR Detection

1. Fluorescence produced during the QPCR reaction is monitored in real-time using QPCR instrumentation. There are a large number of QPCR instruments on the market. Instrument-specific algorithms are used to determine the fluorescence threshold. The cycle at which the sample crosses the threshold is called the cycle threshold (C_t) or crossing-point (C_p). The fewer cycles it takes to reach the threshold, the higher the initial target copy number.
2. Analysis of QPCR data varies depending on the instrument and manufacturer's instructions should be consulted. There is no manipulation of post-PCR amplified products.