6.2 PCR – Quality Assurance/Quality Control

A. General Considerations

1. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions outlined in Section 2, Chapter 2 Sampling to avoid cross-contamination.

2. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like "DNA Away") to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray and/or wipe pipettors and working areas with DNA or RNAase -Away and turn UV on for at least 30 minutes after use (UV light damages DNA).

3. **Wear and change gloves often**. This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.

4. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.

5. Mix and aliquot pre-amplification ingredients under bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.

6. **One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents!** Therefore, three separate areas of lab space are necessary to reduce the risk of contamination.

    a. Master Mix (MM) Area with UV Hood
    For mixing and aliquotting master mix reagents. Supply area with dedicated pipetters, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**

    b. Sample Loading Area with UV Hood and Dedicated Pipettor
    For loading of extracted (template) DNA from samples**.**

    c. Amplified DNA Area
    Supplied with pipettor dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA Away" type solutions after working with amplified DNA.

7. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

8. Controls

    a. Extraction controls
    A known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.

    b. PCR Controls
    Sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

    c. Internal Controls
    An internal control DNA, such as ß-actin (commercially available), should be used to verify PCR performance. Internal positive controls that can be multiplexed into QPCR reactions are also commercially available (e.g. TaqMan® Exogenous IPC from Applied Biosystems Inc.). If the internal control band is not produced, there may be inhibition of the PCR reaction. Therefore, the Taq DNA polymerase, storage conditions of reagents, and thermal cycler performance are potential variables to investigate.

9. Enhancing PCR specificity
    a. Uracil N-glycosylase (UNG) and dUTP can be used in PCR amplifications to prevent false positives due to contamination with carry-over PCR products generated in previous reactions. (Commercial kits are available, such as GeneAmp® PCR Carry-over Prevention Kit for conventional PCR or TaqMan® Universal Master Mix with AmpErase® for QPCR (Applied Biosystems Inc.)). In PCR reactions, dUTP is substituted for dTTP so the resultant PCR products contain uracil instead of thymidine. These substituted products are susceptible to degredation by UNG, thus, the products from previous PCR amplifications can eliminated from subsequent reactions by incubation with UNG which excises the uracil residues. The resulting abasic polynucleotide is destroyed by a 95ºC heat treatment at the start of the thermocycler program.

10. Newly received primers should be tested on known positive/negative controls prior to use.

11. Dispose of trash containing amplified DNA products frequently.

B. Sample Processing

1. Tissue samples should be collected on a clean bench top, which has been disinfected using a 10% chlorine (or “DNA Away”) solution if possible. If collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil, etc.).

2. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.

3. Keep samples cold and freeze as soon as possible at or below -20^{o}C until processing can be accomplished.

4. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT-PCR should be frozen immediately and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

C. Extraction of DNA or RNA from Samples

Individual protocols will vary in specific steps for extraction of genetic material; however, the following general considerations should be employed:

1. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

2. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

3. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

4. **Quantification of DNA**
   If the protocol being used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in Section 2, 6.5 Analysis of Extracted DNA using an UV Spectrophotometer.

D. Interpretation of PCR Results

Use of the appropriate controls should allow you to assess the integrity of your PCR result.

1. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR, or human error (e.g. loading errors).

2. False-positive reactions may result from contamination either directly from the sample lot being tested or from previously amplified target DNA.

3. For further help in troubleshooting, see PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).