3.2.6.1 Proliferative Gill Disease Methods Appendix 1

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Protocol for the Molecular Detection of *Henneguya ictaluri* in Gills Using Polymerase Chain Reaction Assay
(Hanson et al. 2001; Whitaker et al. 2001)

A. Sample Collection

DNA contamination is the major problem that must be avoided in diagnostic PCR. The highest risk is from PCR products. All tissue processing, PCR product work, and PCR set-up should be performed in physically separated lab spaces. Pipette use should be similarly designated and aerosol resistant pipette tips used. Mix master mixes under a UV pretreated work space, aliquot the master mix, and add the DNA template last in a separate location from the master mix preparation site. Use gloved hands for opening tubes and change the gloves between samples of DNA template. Using new or stringently cleansed chlorox and treated or baked lab utensils, remove a sample of gill filaments.

B. DNA Extraction

1. Suspend 40 to 50 mg of fresh gill tissue in cell lysis solution (Puregene DNA Isolation Kit; Gentra Systems, Inc., Minneapolis, Minnesota) with 100 µg proteinase K/mL.

2. Incubate for two hours at 55°C.

3. Purify according to manufacturer's instructions.

4. Note: modifications of this protocol for formalin fixed tissue include incubating fixed tissue in cell lysis solution at 65°C for 15 minutes, homogenizing the tissue with a microfuge pestle, followed by the proteinase K digestion and extraction as outlined above.

C. PCR Procedure (Hanson et al. 2001; Whitaker et al. 2001)

The *A. ictaluri* specific PCR reaction mixtures contains reaction buffer (15 mM Tris, pH 8.0; 50 mM KCl; 1.5 mM MgCl 2), 20 pmol of each primer (upper 5' CAAAAGTTTCTGCTATCATTG 3' and lower 5'AGCGCACAGATTACCTCA3'), 200 µM of each of dATP, dCTP, dGTP, and dTTP, 1.25 units *Taq* Gold DNA Polymerase (Perkin-Elmer), and 2 µL of template DNA containing 1µg of DNA in a total volume of 50 µL. The PCR incubations consist of three steps.

1. Step 1 is one cycle of 94°C for 10 minutes, 50°C for one minute and 72°C for 30 seconds. This activates the TAQ Gold.

2. Step 2 consists of 20 cycles of 92°C for one minute, 50°C for 15 seconds, and 72°C for 15 seconds.

3. Step 3 is an extension cycle at 72°C for five minutes.

All diagnostic PCR should include positive (10 ng of plasmid clone containing the *H. ictaluri* rRNA gene) and negative controls (water) as template. The 104-bp PCR amplification product is expected and can be visualized by electrophoresis on a 10% polyacrylamide gel and staining using ethidium bromide or Gel Star (FMC BioProducts, Rockland, ME). We recommend the use of a 20 bp ladder such as that produced by Bio-Rad (Hercules, CA) as a size standard. For subclinical infection use 35 cycles in Step 2 instead of the 20 cycles described above. Because the subclinical infection detection method is much more sensitive than the method for confirming clinical infection, stringent measures should be observed to avoid DNA cross contamination. The use of dUTP and uracil N-glycosyalase (Applied Biosystems Foster City, CA) in the PCR reactions as well as known negative control tissue in the extraction process are quality control measures that should be considered. Confirmation of PCR product can be done by direct sequencing (Whitaker et al. 2001) the product will match nucleotides 1515 to 1619 of GenBank Accession AF195510.

ACAAAAGTTTCTGCTATCATTGTTGAAGGCATCGTATAGTTGCCAAGGGTGTATTGGTTGAG
GTGGCAACATCAAGGCTGATGCATTTGAGGTAATCTGTGCGCT

Figure 1. Nucleotide sequence of *H. ictaluri* PCR product.