CHAPTER 9

Corroborative Testing of Parasites by Polymerase Chain Reaction (PCR)

SECTION 1 -Corroborative Testing for *Myxobolus cerebralis* by PCR

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SECTION 2 - Corroborative Testing for *Ceratomyxa shasta* by PCR

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I. Introduction

This chapter provides protocols for corroborative testing of *Myxobolus cerebralis* and *Ceratomyxa shasta* by Polymerase Chain Reaction[1] (PCR). PCR is a relatively new molecular tool to the field of fish health and is utilized in the Survey alongside standard detection methods for these two parasites. PCR will increase our knowledge of the presence of Whirling Disease and Ceratomyxosis in natural populations and allow the Service to fully evaluate the potential of this molecular tool for use in fish health diagnostics. As additional DNA sequences are elucidated for other pathogens, PCR may become an invaluable tool for detecting fish pathogens in both hatchery and wild populations.

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

II. General Guidelines for PCR

The specificity of oligonucleotide primers and the shear quantity of DNA that is produced during amplification makes PCR an extremely powerful detection method. These same characteristics, however, can lead to problems with sample and/or laboratory contamination with escaped DNA if protocols are not strictly followed. All extracted and amplified DNA must be confined to the sample tubes to ensure laboratory workspace is free of excess target and non-target DNA. The protocols in this chapter are intentionally very detailed to ensure quality control in using PCR. The following guidelines will be followed for all work with PCR:

- Always place fresh bench coat paper over work areas before starting to do DNA extractions, PCR set-up, and dispensing or allocating PCR reagents. (All three of these activities will be done in physically separated areas.) Dispose of the bench paper when your work is complete to ensure that trace amounts of DNA can be disposed of before they get dispersed around the lab.

- Work surfaces should be decontaminated by washing down the work area with 10% chlorine to hydrolyze possible DNA contaminants. Tube racks will be soaked in 10% bleach for 30 minutes.

- All PCR reagents are stored separately from DNA samples.

- Always use aerosol resistant pipette tips when you are working with PCR reagents and amplified DNA samples.

- Use only pipettors dedicated for pipetting PCR reagents (Master Mix "cocktail") and never use these pipettors for DNA or tissue of any kind.

- Primers and other reagents are to be aliquoted in a clean area separate from the diagnostic area where the PCR is taking place. DNA samples never enter this room. After the PCR "cocktail" has been prepared in this room it can be taken to the diagnostic room for dispensing the DNA template into the reaction tubes.

- Dispense the DNA samples into the reaction tubes in a laminar flow hood to avoid contaminating the lab (the blower need not be running). When you are done clean the work

area in the hood and turn the germicidal UV lamp on for several hours or overnight. This will "UV crosslink" any contaminants making them unable to be amplified.

- Dispense your positive control sample last to minimize handling this sample before diagnostic samples are handled.

- Change gloves frequently especially after handling DNA samples of any kind. Dispose of gloves and bench liner immediately if you suspect any amplified DNA has been spilled on them.

- Dispose of trash containing amplified DNA or contaminated materials frequently to prevent accidental spills that could contaminate the lab.

- When opening and closing tubes containing DNA samples of any kind avoid touching the inside of the cap and the rim of the tube to prevent contamination of the samples.

- Wear gloves that fit snugly to prevent contamination from baggy fingertips dragging inside the sample tubes or across the inside of the caps when opening and closing them. Especially when transferring material from the round 1 reaction to round II.

- Use a heated lid Thermocycler when possible to prevent vapors generated during the PCR cycling from condensing near the lid of the tube where they are more likely to become airborne when the tube is opened.

- Use a mineral oil overly for your PCR reaction to prevent condensation of vapors near the lid during the cycling or after prolonged refrigeration of the PCR sample.

- Have a separate pipettor to use only for loading amplified DNA samples into the agarose gels. This will be contaminated with very high doses of amplified DNA and should never be used for DNA extraction or PCR "cocktail" preparation. The pipettor should be cleaned with chlorine (followed by diH_20 rinse) or a UV lamp on a regular basis.

- Dispose of the agarose gels and running buffer in an appropriate manner keeping in mind they contain amplified target DNA that can get spread about the lab.

- **In general treat any amplified DNA as if it were radioactive!** Don't be careless with it and keep it contained at all times.

II. Corroborative Testing for *Myxobolus cerebralis* by PCR

A. INTRODUCTION

This nested PCR protocol for *Myxobolus cerebralis* (Mc) was developed by Dr. Karl Andree and Dr. Ron Hedrick of University of California, Davis. This highly sensitive and specific detection method utilizes two rounds of PCR amplification and specific primer sequences that target the ribosomal DNA of this fish parasite.

For the Wild Fish Survey, the standard method for detecting this pathogen is the Pepsin-Trypsin Digest Method (PTD) described in Chapter 8 (Parasitology).

Mc-PCR is used as the corroborative technique to positively identify *Myxobolus cerebralis* when spores are detected in the initial PTD method. It may not be necessary to use a nested PCR (two rounds of PCR amplification using two primer sets) for corroborative testing when sufficient numbers of spores (a moderate to heavy infection) are found in the initial detection method (personal communication, Dr. Karl Andree). Single round PCR (Baldwin and Myklebust 2002) has been validated for detection of spores in the digested tissue obtained with the Pepsin Trypsin Digest method.

As previously noted in the PTD protocol, formalin should not be used to preserve the digest material if PCR will be used for corroborative testing. Also an additional heat and microwave step is recommended to disrupt spores and release Mc-DNA prior to PCR testing.
This section is divided into the following major steps for the Mc-PCR assay:

B. SAMPLE PROCESSING
C. DNA EXTRACTION FROM ARCHIVED CRANIAL TISSUE
D. DNA EXTRACTION METHOD FOR PTD SPORE PREPARATIONS
E. PREPARATION OF MASTER MIX (MM) ("Cocktail" for PCR amplification)
F. AMPLIFICATION - ROUND 1 (R1)
G. NESTED PCR - AMPLIFICATION OF ROUND 2 (R2)
H. GEL ELECTROPHORESIS
I. INTERPRETATION OF GEL AND PHOTO DOCUMENTATION
J. REPORTING RESULTS
K. BIBLIOGRAPHY
Appendix A - Equipment, Supplies and Reagents
Appendix B - Mc-PCR DATA SHEET

B. SAMPLE PROCESSING

Tissue dissection can be performed on the bench top in a designated area of the laboratory, or in a bench top hood with a UV lamp for disinfection. Proper disinfection of work surface and tools is necessary to prevent laboratory contamination with specific DNA. Use 10% chlorine solution, a commercial DNA detergent, or 60-minute disinfection with UV light to decontaminate all work surfaces and supplies. All supplies, instruments and sample tubes should be sterile prior to use. All excess tissue should be autoclaved prior to disposal.

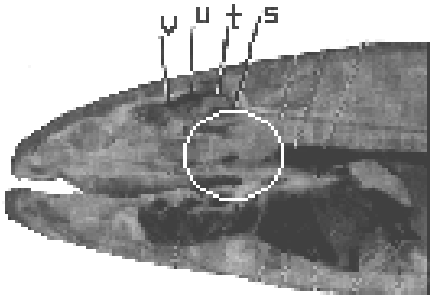
If the archive tissue was correctly labeled with fish ID per 5-pool sample (as described in the PTD protocol), only the single sample that corresponds with the digest sample has to be tested by Mc-PCR to confirm the identification of *Myxobolus cerebralis* spores. If samples were not labeled and tracked with corresponding digest samples, then entire archive sample set for that case history will have to be tested to confirm the presence and identification of *M. cerebralis*.

1. Archived Heads - Dissection Methods (Necropsy or Miltex 6mm Biopsy Punch)
   a. Sample Size - 6mm biopsy punches work well with fish smaller than 6 inches.
   b. For larger fish, use a scalpel to excise the tissue aiming for tissue size of ~ 5-6mm^2.

2. Wear clean gloves and use individual sterilized instruments for each fish tissue

3. Target Tissue – Dissect the cartilage and bone tissue from the caudal ventral portion of the skull; posterior to the optic lobe and include the majority of the auditory capsule.

4. Keep the tissue cold during collection to prevent degradation of DNA.
   (Store fish tissue frozen if DNA extraction will be done at a later time. Avoid repeated freeze thaw cycles to prevent shearing of DNA).



s. Medulla oblongata
t. Cerebellum
u. Optic Lobe
v. Cerebral hemisphere

Figure 1 – Target tissue site detection of *Myxobolus cerebralis* by PCR

C. DNA EXTRACTION FOR ARCHIVED CRANIAL TISSUE (QIAGEN DNeasy Extraction Kit using the Rodent-tail Protocol)

1. Follow the DNA Extraction method in the QIAGEN DNeasy Kit Handbook - using the Rodent Tail Protocol . Follow all manufacturer's instruction for initial preparation of kit solutions.

2. Quantifying extracted DNA for use in PCR:

   Quantification of DNA by spectrophotometry requires that the preparation of DNA be pure, as evidenced by a 260/280 reading of 1.8 or higher by UV spectrophotometry. If the DNA preparations are not pure enough, then the relationship upon which spectrophotometric quantification of DNA is based (1 A_{260} =50ug/mL) is not valid.

   Furthermore, the quantities of DNA template recommended in this protocol are based on DNA preparations with a 260/280 ratio of at least 1.8. If DNA extracted from tissue or spore samples do not meet this criteria, additional steps will be required to further purify the preparations, or to utilize alternate purification methods.

   All samples should be "spected" prior to testing by PCR to minimize the risk of false negative reactions due to poor quality, or inadequate DNA. **See Appendix C for measuring extracted DNA using a UV Spectrophotometer.** Follow the manufacturer's instructions for setting the correct measuring parameters for the UV spectrophotometer being used. The recommended quantity of DNA for Mc-PCR is approximately 300ng per reaction, with a 260/280 ratio of 1.8 or higher (Andree, 1998).

   Using a spectrophotometer to quantify the DNA ensures that the quality and quantity of DNA is sufficient for PCR testing, an important step in quality assurance for the extraction and amplification process.

D. DNA EXTRACTION METHOD FOR PTD SPORE PREPARATIONS

1. Pellet the spores collected from PTD by centrifugation in a microtube at 14000 rpm for 2 minutes.

2. Aspirate the supernatant and dry the pellet for 30 minutes at 50°C.

3. After drying, microwave the spores in the same microfuge tube for 1 minute (standard microwave set on high power).

4. Add 180µL of the ATL (Lysis Buffer) from the QIAGEN Kit and follow the Mouse-tail protocol through completion.

E. PREPARATION OF MASTER MIX (MM) "Cocktail" for PCR amplification :

Preparation of the PCR cocktail, or Master Mix (MM), is done in a designated clean area of the laboratory, preferably in a UV hood. NO TISSUE SAMPLES, EXTRACTED DNA, OR AMPLIFIED PRODUCTS ARE BROUGHT INTO THIS AREA. Dedicated pipettors, racks, tubes, and all supplies should be located in this area to prevent cross-contamination from other areas of the laboratory.

1. Preparing the MM "Cocktail" - always WEAR CLEAN GLOVES when handling MM reagents.
   a. Thaw the frozen MM REAGENTS - Taq (in glycerol) should be kept on ice, thaw the other reagents. Keep the MM tubes (R1 & R2) on ice during preparation.
   b. Determine amount of MM to prepare as follows:
      i. Number of samples to run.
      ii. Enough additional cocktail for 2 reactions (for pipetting loss).
      iii. Prepare cocktail for all positive and negative controls.
      iv. **The TOTAL NUMBER of REACTIONS WILL BE PREPARED TWICE** - one set for each of the R1 and R2 tubes (Specific primers for each round of amplification will be added to the corresponding tube last - all other components and volumes are the same for each round of MM).

      EXAMPLE: You have 6 samples and 2 controls (1 positive and 1 negative control). You'll prepare one extra reaction to cover any lost volume during pipetting.

      You prepare the volumes indicated for 10 reactions by rounding up to the nearest value that allows easy calculations. You'll prepare MM for 10 reactions, but you will REPEAT all dispensing steps (except primers) into the second tube designated as R2 MM. (You simply prepare 20 reactions by repeating each dispensing step, except primers, into the second R2 tube).

   c. Set up a clean rack with TWO, 1.5mL microcentrifuge tubes labeled R1 and R2 MM (Prepare MM for both R1 and R2 amplification steps, however store the tubes for R2 in the freezer for later use). Keep MM solution on ice during preparation.

   d. Place labeled, sterile PCR sample tubes in the rack including tubes for the controls - use clear tubes for R1 samples and color-coded tubes for R2 (or label each tube with both the ROUND NUMBER as well as the Sample ID).

   e. Using dedicated pipettes (labeled MM ONLY) carefully dispense all reagents in order into the R1 & R2 tube, EXCEPT THE PRIMERS and the TAQ.

Table 1 - Master Mix Formulation and Primer Sequences

STOCK Concentration	FINAL Concentration	1 rxn	10 rxn	25 rxn	50 rxn
DNase-free Water		30.6	306	765	1530
Taq Buffer 10X	**1X**	5	50	125	250
$MgCl_2$*	**25mM**	5	50	125	250
dNTPs 10mM	**400µM**	2	20	50	100

***Recommend using Taq Buffer without MgCl2 and adding this as a separate component to achieve the final concentration of 2.5mM per reaction.**

ADD TAQ LAST – AFTER SPECIFIC PRIMERS

Taq Polymerase 5U/µL	**2U/rxn**	0.4	4	10	20

ROUND 1 Primer Sequences:

Tr3-16 5' - GAATCGCCGAAACAATCATCGAGCTA - 3'

Tr5-16 5' - GCATTGGTTTACGCTGATGTAGCGA - 3'

ROUND 1 PRIMERS are added to the R1 TUBE ONLY. Add in numeric order to prevent errors (i.e., Tr3-16, then Tr5-16).

Primer #1 (Tr3-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-16) 20 pmole/µL	4**0 pmole/rxn**	2	20	50	100

ROUND 2 Primer Sequences:

Tr3-17 5' - GGCACACTACTCCAACACTGAATTTG - 3'

Tr5-17 5' - GCCCTAT TAACTAGTTGGTAGTATAGAAGC - 3'

ROUND 2 PRIMERS are added to the <u>R2 TUBE ONLY</u>. Add in numeric order to prevent errors (i.e., Tr3-17, then Tr5-17).

Primer #1 (Tr3-17) 20 pmole/µL	**40 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-17)	**40 pmole/rxn**	2	20	50	100

f. Carefully add the appropriate primer sets to their corresponding tube, i.e., R1 tube receives primers Tr3-16 and Tr5-16 and R2 tube receives primers Tr3-17 and Tr5-17.

g. Vortex all tubes gently. ADD THE TAQ LAST.

h. Dispense 47µL of R1-MM to each labeled tube for R1 amplification.

i. Close the caps, vortex gently by hand.

j. Repeat this process for the **<u>R2</u>-MM tubes if they will be used the same day*.** Freeze the R2 tubes (-20°C) for use following R1 amplification.

***NOTE on ADDING TAQ**:
It is recommended that the Taq not be added to R2 MM tubes unless they will be used within 3-4 hours, i.e. immediately following completion of R1.

If R2 will not be completed until the next day, do not add Taq to R2 MM tubes. In this case, it is recommended that the Taq be added to the R2 MM just prior to aliquoting into PCR tubes and as soon as possible prior to beginning R2 amplification.

k. Take the R1 tubes to the appropriate workspace designated for TEMPLATE DNA (extracted DNA from test samples).

F. AMPLIFICATION - ROUND 1 (R1):

All transfers of DNA material, both extracted DNA from tissues, and amplified products following amplification should only be done in an UV HOOD to prevent cross-contamination of samples and laboratory space. Again, dedicated pipettors should be used for this purpose only and labeled clearly for their specific use. Always disinfect the hood area with UV (30-60 minutes) between transfers and overnight at the end of the day.

1. ADDING DNA TEMPLATE (extracted DNA from samples) - WEAR CLEAN GLOVES.

a. Without touching the rim or upper edges of the tubes, carefully add 3µL of DNA Template (150-300ng/rxn) to each corresponding sample tube containing the MM.
b. Add 1-2 drops of sterile mineral oil (molecular grade only) to each tube. Use caution in controlling how mineral oil is applied to prevent cross-contamination of samples (drop from above the tube, avoid aerosols, close caps carefully).

2. ADDING THE NEGATIVE CONTROL:
Sterile, DNase-free water is used as the negative control by using the same volume as the DNA template in each amplification round (3µL for R1 and 1µL for R2).

3. ADDING THE POSITIVE CONTROL:
The positive control is plasmid DNA p18Tr29, or another known positive tissue for Mc. The plasmid contains a 1934 bp insert of 18s rDNA from *M.cerebralis*. The stock concentration of this positive control is 15ng/µL and is approximately equal to $2.9x10^9$ plasmid molecules/µL. You can dilute a control of this concentrate at least ten-fold and still get a good strong signal.

For dilution of the positive control plasmid, use Tris-EDTA (TE) Buffer (10mM Tris, 1mM EDTA final concentration). You can prepare a stock 10X TE Buffer for more accurate measure of the small weights used in this formula, then perform an additional 1:10 dilution with DNase-free water for the working concentration. Alternatively, DNase-free water can be used as a positive control diluent, but the control will be less stable for long-term storage (approximately 2-3 months). The Positive Control, diluted in TE Buffer, can be stored in the refrigerator (4°C) for 1 year.

USE EXTREME CARE IN HANDLING THE POSITIVE CONTROL, especially when transferring amplified product from R1 to R2 tubes. Risk of laboratory and sample contamination is highest with this reagent.

a. Carefully add 1 µL of positive control plasmid DNA to the appropriate MM tube labeled for positive control (1 µL is adequate volume for both R1 reaction tube).
b. Add 1 drop of sterile, PCR-grade mineral oil (NOTE: A separate tube of mineral oil can be designated for use with positive controls only).
c. Close the cap immediately.
d. Change gloves immediately after handling the positive control tube.
e. Disinfect the work area with the UV lamp for 30 minutes.

4. Amplification using a Thermocycler:
a. Most thermocycler units must run ~ 30 minutes prior to use (follow the manufacturer's recommendation for machine warm up).
b. Place one drop of standard grade mineral oil in each well of the machine block (if recommended by manufacturer). Oil aids in transfer and uniformity of heat within the sample tube during the processing cycles.
c. Add sample tubes to the block.
d. If the thermocycler includes a heated lid, place it over the tubes and close the chamber.

e. Select a program for Mc-PCR (pre-programmed) or set the thermocycler program as follows:

STEP	TEMPERATURE (°C)	TIME (minutes)	
Preamplication	95	5	
Denaturing	94	1	**35 CYCLES**
Annealing	65	2.5	
Extension	72	1.5	
Post-amplication	72	10	
Hold	15	(hold temp at 15°C until unit can be turned off. Colder hold temperatures can be used but are not necessary and cause un-due wear on the unit.)	

This program takes approximately 3.5 hours to run; it can be initiated in the afternoon and left overnight - the amplification will stop after 35 cycles, the machine will run through the post-amplification step and hold at the designated temperature.

G. NESTED PCR - AMPLIFICATION OF ROUND 2 (R2)

After R1 amplification is complete, DNA is transferred from R1 tubes to R2 tubes for the nested PCR. Again, all transfers are done in the UV hood with dedicated pipettors, followed by complete disinfection of the work surface.

1. Transfer R1 PCR products to R2-MM tubes:
   a. Thaw the R2 MM tubes that were prepared earlier and frozen.
   b. Wear clean gloves, remove tubes from thermocycler and wipe excess oil from tube bottom with Kim-wipes. Place sample tubes in a rack and place in the UV hood.
   c. Use a dedicated pipettor for amplified DNA only.
   d. Carefully place pipette tip under the mineral oil interface and mix the solution by gently pipetting up and down 1-2 times. Withdraw 1µL of the R1 PCR PRODUCT.
   e. Without touching the rim or upper edges of the R2-MM tubes, transfer each PCR product from R1 to the appropriate sample tubes and negative control tube containing the R2-MM.
   f. Add 1 drop of mineral oil to each tube without cross-contaminating samples or the mineral oil.

2. ADD THE POSITIVE CONTROL R1 PCR product to the R2 MM labeled "Mc+"
   a. Pipette the positive control product last and immediately CHANGE GLOVES.
   b. Add 2 drops of mineral oil to the tube, being extremely careful (use the dedicated source of mineral oil for this purpose).
   c. Turn on UV lamp for 30 minutes to disinfect the hood area.

3. The same thermocycler program is used in R2 amplification of the nested primer sequence.
   a. Place one drop of mineral oil in each well and use heated lid if available.

b. Add sample tubes to the block and close the chamber.
c. Select the program for **Mc PCR.**

Program takes approximately 3.5 hours to run, can be left overnight - machine will stop and hold at the programmed temperature.

H. GEL ELECTROPHORESIS

The Anticipated Product (AP) from R2 of the nested Mc-PCR is 410 base pairs. The R1 AP is 1300 bp, but normally will NOT be present in diagnostic PCR because insufficient copies are produced in R1 amplification. However for corroborative testing of PTD-positive samples with moderate to heavy infection levels with Mc spores, this band should be present. As noted in the introduction, a single amplification PCR, rather than the nested PCR, may be more appropriate for corroborative testing of known-positive samples.

If nested PCR is used, amplified products from both R1 and R2 are run on the gel as this may help with interpretation of positives and troubleshooting if anticipated products are not resolved.

TABLE 2 - Size of DNA fragments (kb) optimally resolved

(modified from Sambrook, Fritsch, and Maniatis**)**

AGAROSE CONCENTRATION	Size of DNA fragments (kb)
0.7%	0.8 to 12
1.0%	0.5 to 10
1.2%	**0.4 to 3**
1.5%	**0.2 to 3**
2.0%	0.01 to 1

1. **DETERMINE THE GEL SIZE** needed by the number of samples to be run. Select the appropriate gel apparatus. The following instructions and tables are provided as a guide only for the equipment specified in this protocol. Consult the manufacturer of the gel unit to determine the optimum conditions for gel electrophoresis.

   a. Determine the number of samples for both rounds of PCR products and DNA LADDERS and select the gel unit and comb (Fischer Mini-EC370 unit has combs with 8-12 wells; the larger unit, Midi-EC350 has combs with 22-40 wells).
   b. Determine the quantity of agarose gel needed by following the recommended gel volumes for each unit. A consistent gel thickness of 1/2 to 2/3 cm is recommended for all Mc-PCR gels.

TABLE 3 - AGAROSE VOLUMES needed for gel apparatus (and buffer volumes)
Tris Acetate-EDTA (TAE Buffer) is used in this protocol. See Appendix A for more information on buffers.

GEL UNIT	GEL THICKNESS	Volume OF 0.5X-TAE Buffer to mix with Agarose	Agarose (grams)	0.5X- TAE Buffer in Gel Chamber
MIDI-EC350	1.0 cm	150 mL	2.25 g	1200 mL
(10x20cm)	**.7 cm**	**110 mL**	**1.65 g**	**1050 mL**
8x4"	.5 cm	85 mL	1.28 g	1000 mL
Mini- EC370	1.0 cm	50 mL	.75 g	425 mL
(6.5x9cm)	**.7 cm**	**35 mL**	**.53 g**	**350 mL**
2¾ x 3"	.5 cm	25 mL	.38 g	300 mL

2. **SET UP THE GEL APPARATUS**
   a. Level the gel apparatus using leveling screws (clockwise lowers corner, counter-clockwise raises corner)
   b. Place gel tray with rubber dams on each end in chamber.
   c. Place COMB near the cathodal end (NEGATIVE ELECTRODE) of gel chamber. The mnemonic phrase "RUN TO RED" is used to remind the operator of the correct orientation of the gel tray in regard to samples running towards the red (positive) electrode.
   d. Use the COMB SPACER placed under the COMB to set the height of the wells and ensure uniform depth of wells across the entire gel. The .75mm spacer is used (1.5mm
   used for very fragile gels with a low agarose content).
   e. Tighten the COMB HOLDER SCREW when the comb is in place and adjusted correctly.

3. **PREPARE A 1.5% AGAROSE GEL:**
   a. Weigh out agarose and add to an erlenmeyer flask (oversized for volume needed).
   b. Add appropriate volume of buffer and swirl to dissolve.
   c. Microwave on HIGH heat watching carefully to prevent boiling over or burning of unmelted agarose (approximately 2 minutes). Stop and swirl contents periodically to distribute heat evenly and dissolve completely. CAUTION: USE A HEAT MIT AND EXTREME CARE AS FLASK AND AGAROSE ARE VERY HOT.
   d. Once agarose boils, gently heat an additional 30-45 seconds to dissolve completely. Agarose should appear completely dissolved and clear when held up to light; no clumps or semi-dissolved agarose should be present.
   e. COOL GEL to approximately 55°C (usually 10-15 minutes at RT). The gel can also be cooled by swirling in cool water, or placing in a water bath at 55°C for several minutes (If the gel sets up prior to pouring, it can be remelted).
   f. Pour gel in gel tray carefully avoiding bubbles.

g. Let gel cool completely in tray (approximately 30 minutes). Remove the comb by gently pulling it directly up (avoid side to side or uneven motions that will distort the well chambers).

4. **TEST THE GEL UNIT**

Test unit prior to committing samples to the gel. Follow manufacturer's directions.

a. Add appropriate buffer volume to unit chamber. Buffer should cover the gel with ~ 1-2 mm of buffer over the surface of the gel.
b. The gel tray should be oriented so that the combs are closest to the black/negative electrode, and running towards the red/positive electrode. The mnemonic phrase "RUN TO RED" is helpful to ensure proper orientation of the gel in the electrophoresis unit.
b. Carefully slide the interlocking safety cover onto the chamber and make sure that both banana plugs are securely attached.
c. Connect the attached leads to the Power Supply making sure that the polarity of the leads agrees with the polarity of the output.
d. Refer to Table 4 to select appropriate voltage, switch the Power Supply "ON" and adjust the output to the desired level.
e. Check that the system is functioning properly - the voltage and current levels should correspond to the expected levels and should not fluctuate. Bubbles should rise from the electrodes in the chamber when current is passing through the unit.
f. After verifying proper operation, switch the power supply "OFF" and disconnect the leads from the Power Supply. Remove the cover from chamber.

TABLE 4 - MINUTES REQUIRED BY MARKER SIZE (for fragments to move 6.2 cm)
(Conditions given for a 1% agarose gel at 0.5cm thickness in 1X TAE buffer)

		50V	100V	150V
Marker/Fragment Size				
100 bp	**102**	**49**	**30**	
500 bp	**121**	**60**	**36**	
1000 bp	**145**	**72**	**42**	

NOTE: The Mc-PCR recommended gel is 1.5%, 0.5cm thick agarose gel, therefore this table is a guideline only. Refer to the section RUNNING THE GEL for recommended voltage and time.

5. LOAD SAMPLES ON GEL:
   a. The Gibco 100bp DNA Ladder has bands at 100-1500 bp, with a brighter band at 600bp. - See Sambrook, Fritsch and Maniatis or other reference for preparing loading buffer.
   b. Prepare the LOADING BUFFER/DYE for the samples:
   Loading buffer has two purposes; it adds color to samples for placement into wells and monitoring of band electrophoretic movement across the gel. Secondly, it adds

weight to the sample so the sample solution is heavier than the TAE buffer. Generally the sample is mixed with buffer at a ratio of 5:1, hence the designation of loading buffer at "6X" concentration.

c. Pipette 3µL of 6X LOADING BUFFER for each sample you will load; place in rows on parafilm in same order as samples (use spacers to help keep track of different samples or wells you plan to skip, for example if leaving empty wells around the positive control).
d. Add 15 µL of the sample, mix by pipetting up and down 1-2 times in the dye solution. Load 10-15 µL in the correct well. Loading volumes are dependant on the size of the wells prepared in the gel.
e. When loading sample, hold pipette tip <u>just above well,</u> this prevents tearing the well (dye is heavy and sample will sink into the well as it is loaded).
f. Load the POSITIVE CONTROL last, leave wells on either side if extra space is available on the gel.
g. Place the chamber cover on in the correct direction (RUN TO RED).
h. Plug in corresponding leads into the Power Supply and turn power "ON".
i. Run at corresponding voltage for gel unit, and gel size as follows:

6. RUN THE GEL - Power Supply Settings:

The voltage used for the gel is determined by the size and resistance of the gel unit, the thickness of the gel, and the desired resolution of the bands - generally the slower the electrophoresis rate, the "cleaner" the resolution of DNA on the gel (faster rates can create smears and/or band artifacts).

Each unit has a maximum voltage that should not be exceeded per manufacturer recommendation. The following are recommendations by EC (manufacturer) and a "rule of thumb" based on the LINEAR DISTANCE (LD) of the gel apparatus (LD = Distance between electrodes on the gel chamber.)

EC Recommends	DO NOT EXCEED	(LD)	"Rule of Thumb" is 3.5 - 4.0 Volts per LD (cm)
Mini-EC370	15 WATTS	16.5 cm	60 - 70 VOLTS
MIDI-EC350	30 WATTS	21.5 cm	75 - 85 VOLTS

NOTE : Watts = volts x amps {1 amp = 1000mA}.

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Migration Rate: A 410bp band migrates at a distance of 4 cm (1.5 inch) in a gel system with the following parameters:

- **1.5% Agarose gel, at 1/2 to 2/3 cm thickness;**
- **Gel apparatus - linear distance between electrodes is 15 cm;**
- **Run at 4 v/cm = 60 VOLTS for 45 minutes.**

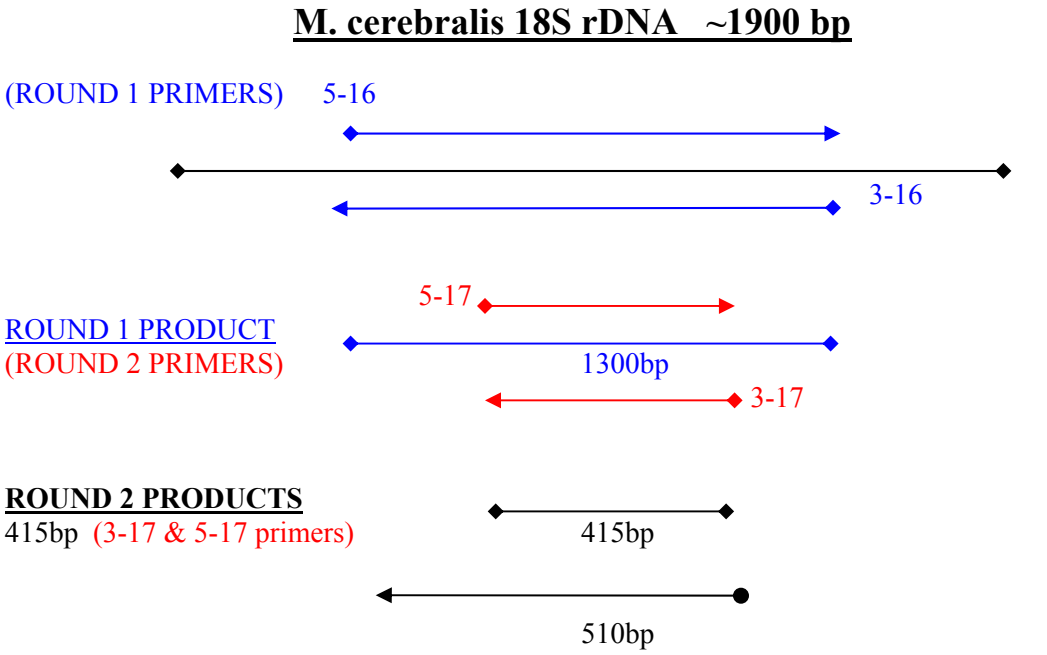
I. INTEPRETATION OF GEL AND PHOTO DOCUMENTATION

1. Stain the gel with Ethidium Bromide for 10 minutes. Destaining for 20-40 minutes is an optional step; longer destaining can produce clearer photographs of the gel.

2. **Anticipated product from nested PCR is 410 bp**. The 415bp product is diagnostic and must appear in the positive control and any test samples if they are to be rated positive. A light band at 510 bp may also be present.

   The larger 1300bp product should be present when a single amplification using the Round 1 primers is performed on samples with significant quantities of Mc DNA (as recommended for corroborative testing of spores detected by PTD).

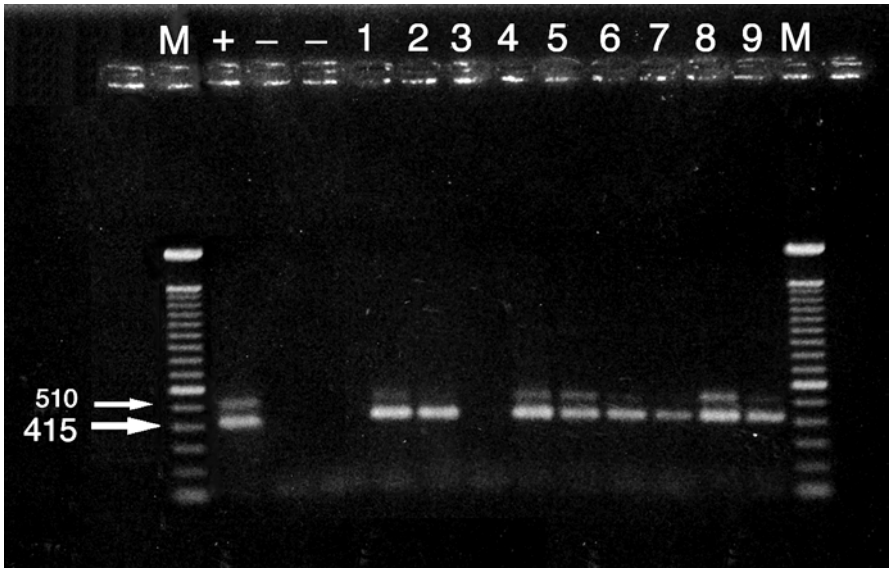
Figure 2 – Schematic of Anticipated Products based on primer sequence.



NOTE: Normally get a small amount (light band) of the 510bp product.

Figure 3. Anticipated Products for Mc Nested PCR

Photo courtesy of Dr. Karl Andree, University of California at Davis (UCD).



J. REPORTING RESULTS

1. Document all procedures for each assay using the MC PCR DATA SHEET (Appendix 2).

2. Report positive bands (415bp for nested and 1300bp for single round PCR) as positive for *Myxobolus cerebralis.* All positive and negative controls must also support the findings or the results of the assay/gel are equivocal.

3. Photograph the gel and keep for a permanent laboratory record.

4. Positive PCR products can be archived as a laboratory stock or for future reference.

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Appendix 9.A - Equipment, Supplies and Reagents

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service and/ or the United States government. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

EQUIPMENT:

EC Mini-submarine Gel	Fischer
EC MIDI-submarine Gel	Fischer
Hoefer Power Supply, model	Hoefer
Thermocycler (suggest MJ Research, Perkin-Elmer, or Thermolyne)	
Finnpipettes - digital, various volumes	VWR
(Dedicated to Tissue DNA, Amplified DNA, PCR-MM ONLY, and Gel-Loading).	
Aerosol-resistant tips, 200 & 1000µL sizes.	VWR
Positive-displacement tips, 1-25µL & 200-1000µL sizes	VWR
Micro-7 Minicentrifuge	Fischer
Standard Heatblock (55C incubation)	VWR
Polaroid DS34 Camera	Fischer
Polaroid 667 B&W Film	Fischer
Camera Hood -	Fischer

SUPPLIES / REAGENTS:

Description	Catalog #	Source
PCR Marker with darkened 400/500 bands	15628-019	Gibco
PCR MARKER (DNA bp Ladder)	P-9577	Sigma
Taq Polymerase	D-1806	Sigma
DNase-free Water	W-4502	Sigma
dNTPs	D-7295	Sigma
Tetramethyl Ammonium Chloride (TMAC)	T-3411	Sigma
DNA/RNA-free mineral oil	M-8662	Sigma
Tris-Acetate-EDTA Buffer (TAE 10X)	T-4038	Sigma
Ethidium Bromide(EtBr)*	E-7637	Sigma
***CAUTION:** Strong mutagen, wear gloves and use hazardous chemical precautions.		
S&S Extractor (EtBr Decontamination Kit)	448031	Intermountain Scientific
(Manufacture is Schleicher & Schuell)		
SeaKem GTG (Genetic Technology Grade)	50070	Intermountain Scientific
(Manufacturer is FMC Bioproducts)		
Miltex Biopsy Punches (6mm)	21909-144	VWR
(other sizes are available)		

FORMULAS FOR PREPARING REAGENTS USED:

REAGENT:	REFERENCE:	DIRECTIONS TO PREPARE:
TAE BUFFER 10X	Sigma T-4035	Comes in prepared packets, add Gibco DI water & qs to 1L Label as 10X- STOCK (store at RT). Also can prepare a 50X stock (see Sambrook, et al.)
TAE BUFFER 1X WORKING SLN	TAE-1X	Diluted 1:10 from 10X stock Label as TAE - 1X (store at RT)
PCR LOADING BUFFER -OR- LOADING DYE	Sigma P-7206 LOADING DYE 6X	Pre-made 6X concentrate, ready to use (store -20C) Prepare in-house (per Sambrook, et al -store at 4C): Bromophenol blue 0.25% Xylene cyanol 0.25% Glycerol 30.0 %

USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN".

ETHIDIUM BROMIDE - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

EtBr STOCK SOLN - 10mg/mL	Ethidium Bromide 100 mg Gibco DI water 10 mL Label as EtBr STOCK (10mg/mL) Protect from light (store at RT)
EtBr-WORKING SOLN - 4.0ug/mL* 1X Buffer WORKING STAIN SLN	Add 200µL STOCK SLN to 500mL TAE- Label EtBr - Working Sln (store at RT)

*References may suggest weaker working solutions (0.5ug/mL) and staining periods of 45-60 minutes. Most researchers prefer to use at a stronger working concentration (8 X stronger in this example) and stain gels for 10 minutes. Karl Andree recommends the 4.0ug/mL concentration and staining for 10 minutes.

OTHER BUFFER FORMULAS:
An excellent reference for PCR is Sambrook, Fritsch, and Maniatis (2nd edition, 1987). This reference describes how the electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. For example, in the absence of ions (e.g., if electrophoresis buffer is omitted from the gel by mistake), electrical conductance is minimal and

DNA migrates very slowly. In buffers of high ionic strength (e.g., if 10X electrophoresis buffer is used by mistake), electrical conductance is very efficient and significant amounts of heat are generated. In the worst case, the gel melts and the DNA denatures.

According to Sambrook, several different buffers are available for electrophoresis of double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50mM (pH 7.5-7.8). For historical reasons, TAE is the most commonly used buffer. However, its buffering capacity is rather low, and it tends to become exhausted during extended electrophoresis. Both TPE and TBE are slightly more expensive that TAE, but they have significantly higher buffering capacity. Double-stranded linear DNA fragments migrate approximately 10% faster through TAE than through TBE or TPE, but the resolving powers of these systems are almost identical, with the exception that the resolution of supercoiled DNAs is better in TAE than in TBE. (See Sambrook, Fritsch, and Maniatis for more information on the various types and uses of electrophoresis buffers).

NOTE: The use of 0.5X TAE in this protocol is a non-standard and more dilute concentration of this buffer than that which is normally used for electrophoresis. TAE is normally used at a concentration of 1.0X or 50mM as noted above. The 0.5X TAE works well for Mc-PCR in this researchers hands, however this concentration is only a recommendation and individual researchers may prefer to use the standard concentration of 1.0X, or 50mM.

Appendix 9.B **Mc-PCR DATA SHEET:** **ASSAYDATE:_____**

I. SAMPLE INFORMATION

Case Number	Collection Date	Sample ID	NOTES

II. CONTROL INFORMATION

POSITIVE CONTROLS		NEGATIVE CONTROLS	
Extraction Positive Control		Extraction Negative Control	
Plasmid + Control		R1-MM	
		R2-MM	

III. PRIMER SETS

Initial Amplification		Nested Amplification	
Forward	Reverse	Forward	Reverse

IV. MASTER MIX PREPARATION **Total number of reactions (R1&2):_____**

STOCK CONCN.	FINAL CONCN.	1 rxn	10 rxn	25 rxn	50 rxn
DNase-free Water	--	30.6	306	765	1530
Taq Buffer 10X	**1X**	5	50	125	250
$MgCl_2$	**2.5mM**	5	50	125	250
dNTPs 10mM	**400uM**	2	20	50	100
TMAC 50uM	**5uM**	5	50	125	250
Taq Polymerase 5U/μL	**2U/rxn**	0.4	4	10	20

ROUND 1 PRIMERS are added to the R1 TUBE ONLY.
Add in numeric order (Tr3-16, then Tr5-16).

Primer #1 (Tr3-16) **40 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-16) **40 pmole/rxn**	2	20	50	100

ROUND 2 PRIMERS are added to the R2 TUBE ONLY.
Add in numeric order (Tr3-17, then Tr5-17).

Primer #1 (Tr3-17) **40 pmole/rxn**	2	20	50	100
Primer #2 (Tr5-17) **40 pmole/rxn**	2	20	50	100

V. AMPLIFICATION (THERMOCYCLE PROCESS)

Round Number (Date & time)	Program #	NOTES

VI . GEL PREPARATION

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (mL)

VII. GEL TEMPLATE (SAMPLE PLACEMENT MAP)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

INITIAL PCR PRODUCTS (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

NESTED PCR PRODUCTS (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**

VIII. PHOTO DOCUMENTATION

CAMERA SETTINGS:

F-stop setting _____ Aperture _____ Exp time _____

Attach photo

RESULTS SUMMARY :

Appendix 9.C - Analysis of Extracted DNA using an UV Spectrophotometer.

Linda Vannest, Bozeman Fish Health Center

The GeneQuant II (Pharmacia Biotech) is a spectrophotometer specific for obtaining concentrations of either Double Stranded DNA (dsDNA); Single Stranded DNA (ssDNA) or RNA in units of weight , molar fraction, moles of phosphate and total molecules. The instrument is capable of measuring the RNA or DNA using UV cells at 230nm, 260 nm, 280 nm, and 320 nm simultaneously. The 230, 260 and 280 wavelengths provide the readings for quantification and purity and the 320 wavelength provides a reading for background compensation.

1. Instrument Set Up

The instrument has a set of default parameters that can be altered to meet the needs of the molecular biologist. For >spec readings= of dsDNA the standard settings on the instrument are as follows:

Using the >setup= and >enter= key enter the following parameters:

Cell pathlength (mm) ----------5(OD_{260} range---5-0.2;ug/mL range---250-10)
Printer----------off (on if using a printer)-press >enter=
Sample Number----------enter specific sample number-press >enter=
Date----------key in date-press >enter=
Month----------key in month-press >enter=
Year----------key in year-press >enter=
Use 320 background compensation----No (unless suspect background interference)
Dilution Factor----------1 (unless samples have a dilution factor)
Factor----------press select to key in dsDNABpress >enter=
Molecular Weight----------this displays default calculations of instrument

The remaining parameters are basic default parameters that are entered specifically for the instrument when dsDNA was keyed in.

2. Reference Measurement

A reference measurement must be taken prior to obtaining any sample readings. This is the base reading and will be stored for all samples measured until a new reference is read. DNase free water can be used for setting the reference. If the DNA to be Aspect=d@ was extracted and purified using a commercial kit (such as QIAGEN) the reference background can be set using the final elution reagent from the kit, since that is the reagent solution containing the purified DNA.

To set the reference background;

Add 7 µL of reference reagent or DNase free water to the Ultra microvolume cell
Press >set ref=----------->Please wait= is displayed
When the instrument beeps-------->Insert reference= is displayed
Insert the Ultra microvolume cell
Wait for the beep----------Remove reference is displayed

Remove the cell and the absorbance will display: 260 nm----------0.000AU

3. Sample Measurement

After all the above parameters are set and the reference background has been measured, samples can be quantitated. Add 7 µL of purified DNA to a clean (rinsed with DNase free water) ultra microvolume cell.

Press sample---------->please wait= will be displayed
At the beep---------->insert sample= will be displayed
Insert the sample cell and wait for the beep and >remove sample display=
After the cell is removed in a few seconds the absorbance will be displayed
press >conc= and select to obtain the reading in units of choice:

Conc 1-------ug/mL (range 1C4000 ug/mL)
Conc 2-------ug/µL (range 0.001B0.2 ug/µL)
Conc 3-------pmol/µL (range 0.001-200 pmol/µL)
Conc 4-------phosphate concentration

4. For measurement of oligos (if "spec-ing" primers) follow the same instructions listed in instrument set up except at the setup factor, key in either oligo RNA or oligo DNA (depending on the primers used).

5. Maintenance:
The instrument can be kept clean with a soft damp cloth. A drain hole in the sample compartment allows for drainage of any spillage during sample measurement. This also can be cleaned with a soft damp cloth and should be immediately cleaned with a spillage occurrence.

Fuses can be replaced as recommended by the manufacturer in the Instrument User Manual

6. Troubleshooting:
For instrument problems it is recommended to contact the manufacturer's technical support.

IV. Corroborative Testing for Ceratomyxa shasta by PCR[1]

A. INTRODUCTION

Review of the known geographical range of *Ceratomyxa shasta*, susceptible host species, epizootiology, clinical signs of disease, and diagnostic procedures can be found in Fish Disease Leaflet 80 (Bartholomew 1989) and in the American Fisheries Society Bluebook (Hendrickson and Bartholomew 1994).

[1]The PCR Process is covered by Patents owned by Hoffman-LaRoche, Inc.

B. SAMPLING METHODS

Wet mount and Sample Collection for PCR - *C. shasta* has an affinity for the lower gastrointestinal tract, however, spores may be found in the pyloric caecae, gall bladder, kidney, liver, spleen, and in ascites of severely infected fish. Samples should be taken from living, moribund, or freshly killed fish. Samples intended for visual examination should be held at low temperatures or on ice until assayed. Freezing (-20°C) intestine samples for the PCR procedure is acceptable and will not affect the extraction of *C. shasta* DNA.

1. Expose viscera of fish by making a lateral incision along the length of the abdominal cavity.

2. Excise a 1 - 2 cm long section of the lower intestine using sterile forceps and scalpel.

3. Place sample on a suitable sterile surface such as a section of Parafilm® or disposable petri dish.

4. For PCR, aseptically section a 25 - 50 mg sub-sample of intestine (approximately 1 - 3 mm in length) and place in a sterile 1.5 or 2.0 mL screw cap microtube. Samples from 5 fish may be pooled. The use of a separate blade for each fish or each 5 fish pool is necessary to prevent cross contamination. Inexpensive, non-sterile razor blades (Fisher # 12-640) can be used instead of expensive scalpel blades. Products such as DNA-AWAY can be used to remove DNA if the same blade must be used between samples.

5. If processing several fish at once, place remaining sample for wet mount in a sterile tube or bag and hold at low temperature (<10°C) or fix tissue in 10% neutral buffered formalin.

6. To prepare wet mount, cut along length of intestine to expose inner wall. Using the scalpel blade, scrape intestinal wall and smear onto a sterile glass slide. Dilute smear with 1 - 2 drops PBS, add coverslip, and examine with phase contrast or bright field microscopy at 400X.

Intestinal Lavage - A lavage technique (Coley et al. 1983) can be used to sample the posterior intestine for *C. shasta* without sacrificing the fish. This procedure may be desirable when sampling fish that are listed as threatened, endangered, or species of special concern. The diameter of tubing, syringe size, and volume of PBS needed to perform intestinal lavage successfully will need to be determined for each particular size class of fish. The technique of Coley et al. (1983) was developed for adult salmon. A separate lavage apparatus (syringe and tubing) would be necessary for each fish or sample site to prevent cross contamination. The sample taken with lavage is examined in a wet mount as above. Lavage samples may also be suitable for the PCR although this has not been tested.

C. PRESUMPTIVE DIAGNOSIS

Presumptive diagnosis of *C. shasta* is accomplished by examining wet mounts of tissue scrapings and or fluids collected from the posterior intestine of suspect fish. Wet mounts are examined using a systematic search pattern with phase contrast or bright field microscopy at 400X magnification. Presumptive diagnosis requires the observation of multicellular myxosporean spores consistent with the size and shape of *C. shasta*.

D. CORROBORATIVE DIAGNOSIS AND DETECTION OF SUBCLINICAL *C. shasta* INFECTIONS USING POLYMERASE CHAIN REACTION ASSAY

Amplification of *C. shasta* DNA by means of the polymerase chain reaction (PCR) were developed by Bartholomew et al. (1997a and 1997b), Oregon State University (OSU), Corvallis, Oregon.

1. PREPARATION OF EXTRACTION BUFFER

Table 1 - Reagents for DNA extraction buffer.

Stock Reagent	Final Concentration*	Recipe for 100 mL (mL)
NaCl, 5M	100 mM	2.0
Tris-HCl pH 7.8, 1M	10 mM	1.0
EDTA, pH 8, 0.5M	25 mM	5.0
SDS, 10%	1.0%	10.0
H_2O, tissue culture grade		82.0

*NOTE: The buffer recipe is based on molar concentration which is dependent on the concentrations of the stock solution purchased. To calculate the quantities needed, follow the formula $C_1V_1=C_2V_2$, solving for $V_1=C_2V_2/C_1$ as given in the examples below:

Stock Reagents	Catalog Number	Volume for 100 mL	Molar calculation
NaCl 5M	Sigma: S5150	2.0 mL	(100mM) (100 mL)/ 5000mM = 2.0 mL
Tris-HCl pH 7.8 1M	Sigma: T2913	1.0 mL	(10mM) (100 mL) / 1000mM = 1.0 mL
EDTA pH 8.0 0.5M	Sigma: E7889	5.0 mL	(25mM) (100 mL) / 500mM = 5.0 mL
SDS 10X	Gibco: 15553-035	10.0 mL	(0.01) (100 mL) / 0.10 = 10 mL
		qs w/ d-H2O	

a. Prepare DNA Extraction buffer using sterile disposable pipettes, combine reagents in a sterile container and aliquot to 25 mL volumes. The extraction buffer is stable at room temperature so a working stock can be stored at the bench.

b. Store extra buffer at -20°C until needed. Stock reagents (molecular biology grade) can be purchased ready to use from commercial suppliers such as Sigma and Life Technologies-Gibco.

c. **Proteinase K**. This enzyme can be purchased from commercial suppliers such as Boehringer Mannheim (800-262-1640; Cat# 1-373-196) as a solution (14-22 mg/mL) which is stable for > 1 year when stored at 4°C.

d. **RNAse A**. This enzyme can be purchased from commercial suppliers such as 5' - 3' (800-533-5703; Cat# 5-888777) at a concentration of 10 mg/mL. The enzyme is available in a 50% glycerol solution that remains liquid at -20°C.

2. DNA EXTRACTION

a. Collect intestine samples according to methods above. Use all necessary procedures to prevent carry-over and cross contamination.

b. Add 500 µL of DNA extraction buffer to microtube with sample. Add Proteinase K solution to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL).

c. Incubate microtubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4-5 hours, but overnight incubation does not affect the quality of the DNA and is recommended.

d. After samples are completely digested, add 5 µL of RNAse A (10 mg/mL stock) to each microtube and digest for 1 hour at 37°C with rocking.

NOTE: Use care when opening microtubes. Digested sample material may be stuck to the walls and cap of the tube. To minimize the possibility of cross contamination, either by aerosol or contact, centrifuge tubes briefly before opening. Do not touch edge or inside of microtube cap.

e. Place tubes with digested samples in a floating rack and then place in a water bath at 100°C for 5 minutes.

f. Remove rack and cool at room temperature. Upon cooling samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C until needed.

3. PREPARATION OF MASTER MIX

a. Dilute the DNA template (sample) 1:100 with ultrapure sterile water. Less diluted samples are not recommended and will likely produce variable results because the "crude" DNA preparation contains PCR inhibitors. At OSU, very low level infections were still positive by PCR when samples were diluted 1:1000.

b. Calculate the amount of master mix (MM) required for sample assays (Table 2). Prepare MM without sample in a sterile area separate from any DNA/RNA preparations. Add Taq to MM last. Keep enzymes and reagents on ice during MM preparation.

Table 2. Reagents for PCR Master Mix for 20 μL reactions (19 μL MM + 1 μL sample).

Reagent	Stock concentration	Final concentration	Vol/reaction (μL)
Sterile d-H_2O			12.8
10X PCR buffer (supplied with Taq)	10X	1X	2.0
$MgCl_2$, 25mM (supplied with Taq)	25 mM	2.0 mM	1.6
dNTPs (Sigma D7295, diluted 1:5)	2.0 mM	0.2 mM	2.0
CS1 primer	50 pmol/μL	0.5 pmol/μL	0.2
CS3 primer	50 pmol/μL	0.5 pmol/μL	0.2
Taq polymerase (Perkin Elmer N808-0161)	5 units/μL	1 unit/reaction	0.2
DNA template (sample diluted 1:100)			1.0
Total			20.0

4. ADDING TEMPLATE DNA

a. In an area separate from MM preparation, assemble 20 μL reactions, 19 μL MM + 1μL diluted template DNA. Keep reactions on ice during assembly.

Note: Relatively large volumes of MM (5.0 mL = 250 reactions), without Taq or sample,

can be made-up in advance, aliquoted to convenient volumes (500 µL = 25 reactions), and stored frozen (-20°C) until needed. For each reaction combine 18.7 µL MM + 0.2 µL Taq + 1 µL sample following steps 1 - 3 above. Repeated freeze/thaw of MM is not recommended, but 1-2 times is acceptable.

b. Overlay samples with approximately 25 µL (1 drop) sterile mineral oil. The use of mineral oil to prevent condensation is critical, even with heated lid Thermocycler, because of the small reaction volume.

5. AMPLIFICATION

a. Load thermocycler and run the following program:

95°C	3 min	initial denaturation	
94°C	60 sec	denature	} 35 cycles
58°C	30 sec	annealing	
72°C	60 sec	extension	
72°C	10 min	final extension	
4°C		hold PCR products chilled	

6. ANALYSIS OF PCR PRODUCT

a. Prepare a 1% agarose gel solution and cool to casting temperature. Just before casting, add 1 µL ethidium bromide (EtBr, 10 µg/mL stock) for each 10 mL of agarose gel solution.
(Note: staining gels with EtBr solution after electrophoresis is also acceptable).

b. Remove tubes from thermocycler and add 2 µL gel loading solution (Sigma, G-7654) to each reaction. Centrifuge tubes briefly allowing gel loading solution to migrate below the oil overlay.

c. Prepare 100 bp DNA ladder.

d. Load gel with 10 µL of each product and run electrophoresis at 70 - 100 V until products and DNA ladder have sufficient separation.

e. Visualize DNA with UV light. *C. shasta* positive samples will have DNA amplicons of 638 bp from the CS1-CS3 primer set (Table 3).

Table 3. Primers used in the *C. shasta* PCR assay.

Primer	Sequence (5' - 3')	Amplicon size with (reverse primer)
CS1	GGGCCTTAAAACCCAGTAG	(CS3) 638 bp
CS3	CCGTTTCAGGTTAGTTACTTG	

E. REFERENCES

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