3.2.17.1 Nested PCR to Identify *Nucleospora (Enterocytozoon) salmonis* DNA within Fish Tissue Appendix 1

A. Reference

Protocol modified from: Barlough, J. E., T. S. McDowell, A. Milani, L. Bigornia, S. B. Slemenda, N. J. Pieniazek, and R. P. Hedrick. 1995. Nested polymerase chain reaction for detection of *Enterocytozoon salmonis* genomic DNA in Chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 23:17-23.

B. Primer sets

1st Round

ES1A: 5'-CTTTGTGAACCCAGACGGG-3'

ES2A: 5'-TGCCTTAGTGAGACACTGTTAC-3'

2nd Round

ES3A: 5'-GACATTCTCTGTCCAGCGG-3'

ES4A: 5'-GAGCTAATCCTGCTCATCC-3'

C. Initial amplification: (1st Round) produces 1093 bp product

PCR Reagents	Lot#	Stock Concentration*	Final Concentration	Volume/Reaction (Total volume= 50 µl)	Volume for __ samples
d-H_2O			Add to total 50 µl		
10X Buffer		10X	1X	5µl	
$MgCl_2$		25 mM	1.5 mM	3 µl	
dNTP's		10 mM each	0.1 mM	1µl	
ES-1A Primer		20 pMole/µl	40 pMole	2µl	
ES-2A Primer		20 pMole/µl	40 pMole	2µl	
Platinum TAQ polymerase		5 units/µl	0.5 units	0.1µl	
Extracted DNA				(300 ng)	----
				TOTAL = 50 µl	

* Change "Stock Concentration" parameters as necessary depending on reagent source.

1. Add PCR reagents except the template DNA into the "Master Mix" tube.
2. In PCR tubes, aliquot required volume of Master Mix and close lid tightly.
3. Move to DNA template area and add required volume of extracted DNA to the PCR tubes.
4. Load samples into thermal cycler and use the following PCR parameters:

   Initial denaturation: 95°C for 5 minutes.

   Amplification for 35 cycles:

   a. Denature 95°C for 30 seconds.

   b. Anneal at 55°C for 30 seconds.

   c. Extend at 72°C for 1 min 20 seconds.

   Final extension at 72°C for 5 minutes.

D. Nested amplification (2nd round) produces 407 bp product.

PCR Reagents	Lot#	Stock Concentration*	Final Concentration	Volume/Reaction (Total volume= 50 µl)	Volume for ___ samples
d-H_2O			Add to total 50 µl	35.9 µl	
10X Buffer		10X	1X	5µl	
$MgCl_2$		25 mM	1.5 mM	3 µl	
dNTP's		10 mM each	0.1 mM	1µl	
ES-3A Primer		20 pMole/µl	40 pMole	2µl	
ES-4A Primer		20 pMole/µl	40 pMole	2µl	
TAQ		5 units/µl	0.5 units	0.1µl	
1st Round Product		---	---	1 µl	----
				TOTAL = 50 µl	

* Change “Stock Concentration” parameters as necessary depending on reagent source.

1. Add PCR reagents except the template DNA into the "Master Mix" tube.
2. In nested PCR tubes, aliquot **49** µl of Master Mix. Close cap tightly.
3. Move to amplified DNA area and add **1** µl of first round PCR product to the nested PCR tubes.
4. Load samples into thermal cycler and use the following PCR parameters:

   Initial denaturation: 95°C for 5 minutes.

   Amplification for 35 cycles:

   a. Denaturing at 95°C for 30 seconds.

   b. Annealing at 60°C for 30 seconds.

   c. Extending at 72°C for 30 seconds.

Final extension at 72°C for 5 minutes.

E. Gel electrophoresis

Subject 10 µl of the 2nd round product to gel electrophoresis in a 1.5% agarose gel.