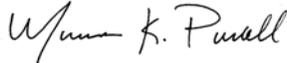


## Standard Operating Procedure (SOP)

### **Title: Nested Polymerase Chain Reaction (nPCR) for the detection of *Renibacterium salmoninarum***

**Number: BACT-6**

**Version: 01 Created April 15, 2011**

Approval:	Date:	Signature:
Bacteriology Supervisor:	April 30, 2011	

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Area of Application:	

**Purpose:**

To detect nucleic acids from *Renibacterium salmoninarum* in DNA extracted from fish tissues or fluids using a nested PCR primer set

**Sections:**

- I. Background
- II. References
- III. Materials
- IV. Procedure

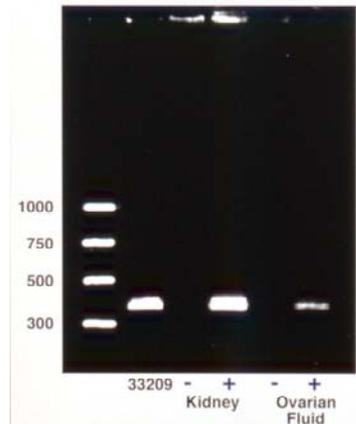
**Disclaimer:**

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## I. Background

The nested PCR (nPCR) test uses two sets of primers that amplify a segment of the *Msa* gene that encodes the 57 kDa major soluble antigen protein. DNA can be extracted from fish tissues, ovarian fluids or broth culture. The recommended DNA extraction procedure is outlined in SOP BACT-5 (“DNA extraction for PCR-based detection of *Renibacterium salmoninarum*”). DNA is subjected to two rounds of PCR amplification. The first round primer set produces a 383 bp band while the nested round primer set produces a 320 bp band (Figure 1). The DNA products from both rounds are visualized by agarose gel electrophoresis.

Figure 1. Typical positive and negative results from nPCR



\*33209 = DNA extracted from a culture of *R. salmoninarum* strain 33209.

Nested PCR is prone to false positives due to contamination by first round products.

Laboratories should have four dedicated areas for each stage of the procedure, including:

- **PCR set-up area:** no nucleic acids in this area (PCR reagents only)
- **DNA template area:** area to handle extracted DNA
- **Amplified template area:** area to handle first round template
- **Gel electrophoresis area:** area to handle second round template

Each area should be equipped with dedicated instruments and supplies (e.g. dedicated pipettors, microcentrifuges, lab jackets, etc.). Work flow should always start in the PCR set-up area and finish in the gel electrophoresis area (never the opposite direction).

## II. References

- Chase, D.M. and R. J. Pascho (1998) Development of a nested polymerase chain reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method of detection of the bacterium in salmonid kidney. *Diseases of Aquatic Organisms* 34: 223-229.
- Pascho, R.J., D. Chase, and C.L. McKibben (1998) Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. *Journal of Aquatic Animal Health* 9: 99-107.

### III. Materials

**Taq PCR Core Kit:** Our laboratory uses the Qiagen *Taq* PCR Core kit (Qiagen Inc<sup>§</sup>, part # 201223). *Taq* polymerase, buffer, dNTPs and loading dyes are also available from a variety of vendors.

**Rediload™:** Rediload™ allows direct loading of PCR reactions into agarose gels. Purchase from Invitrogen Inc<sup>§</sup>.

**Molecular grade water:** DNase- and RNase-Free water can be purchased from a variety of vendors. We recommended using a dedicated stock of water for PCR set-up.

**Nested PCR Primers:** Primers can be synthesized from a variety of vendors. Primers are typically diluted in molecular grade water to a stock concentration of 100 µM (100 pmoles /µL). Primers are subsequently diluted to a working concentration of 20 µM (20 pmoles /µL). Store primers at -20°C.

Primer Round	Primer Name	Primer sequence
First Round	P3	5'-A GCT TCG CAA GGT GAA GGG-3'
	M21	5'-GC AAC AGG TTT ATT TGC CGG G-3'
Second Round	P4	5'-AT TCT TCC ACT TCA ACA GTA CAA GG-3'
	M38	5'-C ATT ATC GTT ACA CCC GAA ACC-3'

\*New lots of primers should be verified using a known positive sample.

**Agarose gel:** Molecular grade low EEO agarose can be purchased from various vendors.

**0.5 X TAE:** 50X concentration Tris-Acetate-EDTA (TAE) electrophoresis buffer can be purchased from a variety of vendors and diluted to the 0.5X working concentration.

**100 bp DNA Ladder:** DNA ladder for agarose gel electrophoresis can be purchased from a variety of vendors.

<sup>§</sup>Vendors:

Qiagen Inc.: 1-800-426-8157; [www.qiagen.com](http://www.qiagen.com)

Invitrogen Inc.: 1-888.584.8929; [www.invitrogen.com](http://www.invitrogen.com)

## IV. Procedure

### A. First Round Amplification of *R. salmoninarum* DNA by PCR

1. In PCR set-up area, add all PCR reagents except the template DNA into one tube; this is the “Master Mix”. See Table 1 for formulation.
2. Aliquot 45µL of Master Mix into 96 well PCR plate.
3. Move to the DNA template area and add 5µL of extracted DNA into the PCR plate.
4. Load samples into thermalcycler. Program thermalcycler for an initial denaturing step at 94°C for 2 minutes.
5. Program the thermalcycler for 30 cycles.
  - a. Denaturing at 94°C for 30 seconds.
  - b. Annealing at 60°C for 30 seconds.
  - c. Extending at 72°C for 1 minute.

Table 1: Master mix formulation for first round amplification

PCR Reagents	Lot	Final Concentration	Stock Concentration	Volume/Reaction (Total volume= 50 µL)	Volume for samples
Extracted DNA*				5	
10X Buffer		1X	10X	5	
MgCl <sub>2</sub> <sup>§</sup>		1.5 mM	25 mM	-	
dNTP's		0.2 mM	mM each	1	
<b>P3</b> Primer		20 pMole	pMole/µl	1	
<b>M21</b> Primer		20 pMole	pMole/µl	1	
TAQ		2 units	units/µl	0.4	
RediLoad		1X	10X	5	
d-H <sub>2</sub> O				31.6	

\*Always include one ‘no template control’ that has only water and no DNA

<sup>§</sup>Only add MgCl<sub>2</sub> to the Master Mix if the 10X Buffer does not contain MgCl<sub>2</sub>.

B. Second (nested) round amplification of *R. salmoninarum* DNA by PCR

1. In the PCR set-up area, add PCR reagents except the template DNA into the "Master Mix" tube. See Table 2 for master mix formulation.
2. Aliquot **49**  $\mu\text{L}$  of Master Mix into 96 well PCR plate.
3. Move to amplified DNA area and add **1** $\mu\text{l}$  of first round PCR product to the nested PCR tubes.
4. Load samples into thermalcycler. Program thermalcycler for an initial denaturing step at  $94^{\circ}\text{C}$  for 2 minutes.
5. Program thermalcycler for 20 cycles.
  - a. Denaturing at  $94^{\circ}\text{C}$  for 30 seconds.
  - b. Annealing at  $60^{\circ}\text{C}$  for 30 seconds.
  - c. Extending at  $72^{\circ}\text{C}$  for 1 minute.

Table 2: Master mix formulation for second (nested) round amplification

PCR Reagents	Lot	Final Concentration	Stock Concentration	Volume/Reaction (Total volume= 50 $\mu\text{L}$ )	Volume for samples
First round template*				1	
10X Buffer		1X	10X	5	
$\text{MgCl}_2^{\S}$		1.5 mM	25 mM	-	
dNTP's		0.2 mM	mM each	1	
<b>P4</b> Primer		20 pMole	pMole/ $\mu\text{l}$	1	
<b>M38</b> Primer		20 pMole	pMole/ $\mu\text{l}$	1	
TAQ		2 units	units/ $\mu\text{l}$	0.4	
RediLoad		1X	10X	5	
d- $\text{H}_2\text{O}$				35.6	

\*Always include one 'no template control' that has only water and no DNA

$\S$ Only add  $\text{MgCl}_2$  to the Master Mix if the 10X Buffer does not contain  $\text{MgCl}_2$ .

## C. Visualization of Amplified DNA on a 2% Agarose Gel

1. Move to gel electrophoresis area.
2. Make a 2% (w/v) agarose gel (2g per 100 mL of 0.5X TAE).
  - a. Total volume will be dependent on electrophoresis apparatus size.
  - b. Microwave to dissolve agarose, cool agarose to ~50°C, pour into electrophoresis tray, and insert comb.
  - c. Allow gel to cool completely before removing comb.
3. Load samples into wells (volume dependent on well size).
4. Subject agarose gel to electrophoresis following manufacturer instruction's for the electrophoresis apparatus.
  - a. Voltage and time is dependent on gel size.
  - b. Run samples until the pink dye (~25 bp) has migrated at least 2/3 of the way through the gel.
5. A band at either 383bp or 320bp is considered a positive.
  - a. The positive processing control should have amplification.
  - b. The negative processing control and no template control should lack amplification.
  - c. See BACT SOP-5 (DNA extraction) for further information on the positive and negative processing controls.