CHAPTER 7

Corroborative Testing of *Renibacterium salmoninarum* by Polymerase Chain Reaction (PCR)

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I. Introduction

The Polymerase Chain Reaction[1] (PCR) assay has been shown to be an effective method for identifying low level infections of *R.salmoninarum* in fish tissue (Brown et. al 1994). The *Renibacterium salmoninarum* Polymerase Chain Reaction (Rs-PCR) technique employs oligonucleotide primers to amplify base pair segments of the gene that codes for the 57 kDa protein of *R. salmoninarum.* DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a primer for a smaller segment of DNA within the larger segment amplified initially. This "nested PCR" step results in extreme sensitivity in detecting the target DNA (Chase 1998, Pascho et.al 1998). The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The PCR assay has been shown to be an effective and accurate method for identification of low-level infections with this bacterial pathogen in various fish tissues (Pascho et.al 2002). The technique is also used to corroborate test results from immunological assays such as Enzyme linked Immunosorbent Assay (ELISA).

The materials and methods described in this protocol are adapted from those developed by Ronald J. Pascho and Dorothy Chase of the Western Fisheries Research Center (USGS) in Seattle, Washington. The Service would like to thank Ron Pascho and Dorothy Chase for training and technical assistance provided to Fish Health Centers for *Renibacterium salmoninarum* testing by both ELISA and nested PCR. A special acknowledgement is in order for Dorothy Chase for the protocols and training that she has provided to numerous Fish Health Centers over the years, her continued research and validation of the Rs-PCR assay, and her overall support of Survey objectives for detection of *Renibacterium salmoninarum* in wild fish populations.

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

II. Nested Primer Sets

	Initial	
Forward P3	A GCT TCG CAA GGT GAA GGG	Product 383 bp
Reverse M21	GC AAC AGG TTT ATT TGC CGG G	
	Nested	
Forward P4	AT TCT TCC ACT TCA ACA GTA CAA GG	Product 320 bp
Reverse M38	C ATT ATC GTT ACA CCC GAA ACC	

III. DNA Extraction using QIAGEN Kit

A. MATERIALS AND REAGENTS

QIAGEN DNeasy Kit (QIAGEN #29304)
Ethanol (Absolute 97-100%)
Proteinase K (Sigma P2308)-(also included in QIAGEN kit)
Lysozyme Lysis Buffer
Pipettor (100-200 µL and 0.5mL)
Aerosol Barrier Tips
1.5mL centrifuge tubes
Micro centrifuge
Heat block(s) (70°C and 95°C)
Latex or nitrile gloves

Lysozyme Lysis Buffer

Lysozyme 2 g
Tris HCl Stock 2 mL
EDTA Stock 2 mL
Triton 1.2 mL
Bring to 100 mL with DI water.

Tris HCl Stock Solution

(100 mL at 1 M pH 8.0)
5.7g trizma base+8.9g Tris HCl into 85.4 mL DI water

EDTA Stock Solution

(100 mL at 0.1 M)
3.72g EDTA - qs distilled water to 100mL

B. GENERAL QUALITY CONTROL CONSIDERATIONS

1. **Wear gloves and change gloves often.**
   This prevents contamination of sample DNA with degrading nucleases and acids that naturally occur on the skin. Frequent changing of gloves also prevents DNA contamination of hands and work surfaces in the laboratory. Steps in this protocol that are underlined indicate significant sources of error and/or potential for contamination should deviation from this protocol occur.
2. Utilize microcentrifuge tubes with locking caps. Heating of extraction solutions causes unlocked caps to pop open, which can cause release of aerosols that can cause cross-contamination between samples and controls.
3. Always run positive *Renibacterium salmoninarum (Rs)* control tissues as well as negative controls (water, or known negative tissue) from the start of the extraction process through nested amplification to final electrophoresis. This is the only means of assuring validity of the assay and its results.

C. PROCEDURE

1. **Weigh 25-50 mg of kidney tissue** into a 1.5 mL Micro centrifuge tube. Kidney tissue can be fresh, previously frozen, or processed ELISA pellet (centrifuge and pour off supernatant). Ovarian fluid can also be used, however do not centrifuge the tissue, and a larger volume of 50µL is recommended (all other steps for OF and KD are similar).

2. Add **180 µl of Lysozyme Lysis Buffer (LLB)**.

3. Incubate at 37°C for 1 hour, vortexing occasionally.

4. Add **25µl of Proteinase K stock solution and 200 µl of buffer AL.**

5. Mix by vortexing and incubate at 55°C for 30 minutes (vortex occasionally during this incubation period).

6. Incubate at 95°C for another 10 minutes.

7. Add **210µl of ethanol**, mix thoroughly by vortexing.

8. Place a QIAGEN spin column in the 2mL collection tube provided. Pipet sample mixture onto the filter in the spin column being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1minute. (All centrifugation is at ambient temperature).

9. Transfer the spin column (upper section of tube with filter) into a clean 2 mL collection tube and discard lower tube containing the filtrate.

10. Carefully open the spin column and add **500µl Buffer AW** (wash buffer). Centrifuge as above.

11. Repeat steps 9 and 10, centrifuging at 6000 x g for 1 minute, then at full speed for an additional 2 minutes.

12. At this point, **place an aliquot of Buffer AE in the 70°C heat block** to preheat for the next step.

13. Place spin column in clean 1.5 mL Micro centrifuge tube that has a closable cap.

14. Add **200µl of Buffer AE** (elution buffer), which has been **heated to 70°C** (Tris, pH 9.0 or water heated to 70°C can also be used for this step as an elution buffer).

15. Incubate for 5 minutes at 70°C. Centrifuge at 6000 x g for 1 minute.

16. Repeat step 14 so that the total volume of DNA is 400µl.

17. During the last step, the ELUTED DNA SOLUTION in the bottom microcentrifuge tube is retained and the SPIN COLUMN IS DISCARDED.

18. Store extracted DNA solution at -20 or -70^{o}C until use.

III. Initial Amplification of *R.salmoninarum* DNA

A. MATERIALS AND REAGENTS

10X Buffer
dNTP (nucleotides)
Primers (forward and reverse; dilute primers in H_2O to 100 pmole/µl if necessary)
Taq Polymerase
$MgCl_2$ Buffer (comes with some Taq products)
Mineral oil - molecular grade (Only use PCR Grade for overlaying samples. Standard mineral oil can be used for thermocycler equipment.)
Thermocycler
0.5-25µl and 20-200µl pipettors

> **NOTE:** Use only highly accurate micro-pipettors such as positive displacement types with matching displacement tips. Use Aerosol-resistant tips for all other pipettors.

Gloves (latex or nitrile)
Bench top UV cabinet.
Cryo-rack frozen to $^{-}70\ ^{o}$ C

B. GENERAL QUALITY CONTROL CONSIDERATIONS

One aerosol drop of amplified DNA contains approximately 24, 000 thousand strands of DNA which can easily contaminate laboratory surfaces, other samples and reagents used in PCR !

1. To reduce this risk of contamination in the laboratory, it is important to establish three separate work areas for reagent preparation, sample preparation, and amplification steps, as follows:

AREA 1 - **<u>REAGENT PREPARATION AREA</u>**: Mix and aliquot pre-amplification ingredients (Master Mix or "PCR cocktail") in a clean room or under a dedicated UV cabinet. Provide dedicated pipettors, preferably positive displacement type, and all supplies that are required to prepare

Master Mix. **NEVER** contaminate this area with sample material or amplified DNA product.

AREA 2 - **SAMPLE PREPARATION / DNA EXTRACTION AREA:** Extract DNA from tissue samples in an area away from reagent prep or amplification steps. Use strict containment precautions to prevent contamination of surfaces and equipment. Disinfect this area after each use with a commercial DNA-disinfectant or a strong chlorine solution.

AREA 3 - **AMPLIFICATION AREA**: Transfer amplified DNA from Round 1 to Round 2 tubes under a Bench-top UV Hood. Gloves and all other supplies should be stored within the hood for easy access and to prevent amplified DNA from leaving this area.

2. Always wear gloves and change to a fresh pair when leaving or entering the PCR REAGENT AREA. Change gloves whenever contamination from a sample tube is even suspected in the SAMPLE PREP/EXTRACTION AREA (i.e. aerosols may have been released upon opening a tube, liquid appears on the outside edge of a tube or cap, or liquid is visible on gloves after handling an individual tube, etc.)

3. Use only aerosol barrier tips and positive-displacement pipettors for dispensing PCR reagents. Change pipette tips between all reagents and all samples. Discard dirty tips into autoclave bags and discard bags daily.

4. All sample racks and reusable equipment should be washed in DNA-away or chlorine disinfectants and autoclaved after use. Spray/wipe pipettors and working areas with disinfectant and sterilize work within the hood with at last 30 minutes of UV light (UV light denatures DNA).

5. All reagent batches should be marked and recorded for each test run so they can be checked if problems occur with the assay.

C. PROCEDURES

1. Using **Worksheet A - DNA Sample Data**, record appropriate data for each sample to be tested by PCR.
2. Using **Worksheet B - Initial Amplification of R. salmoninarum DNA by PCR**, record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples to be processed. (Add 4 to the total number of samples to be tested - this allows enough extra reactions to run both positive and negative controls in the assay).

3. In REAGENT PREPARATION AREA (Area 1), add PCR reagents (**except for sample DNA)** to the MM tube in the order listed on Worksheet B, adding water first and Taq last. Keep all reagents cold in a cryo-rack during mixing, and return

unused reagents to the freezer immediately after use.

4. Place 40µL of MM into each 0.5mL PCR tube, then overlay the Master Mix with one drop of PCR grade mineral oil per tube to prevent condensation within the reaction tube. Close caps tightly. Move Master Mix tubes to sample loading area (Area 2).

5. In Area 2, label the top of the MM tubes with sample identification. Load 10ul of each sample DNA into the appropriate tube being careful to expel the sample beneath the layer of mineral oil. Close caps tightly. Change gloves and transfer the sample tubes to the thermocycler. Clean the SAMPLE PREP AREA as described above.

6. Add regular mineral oil into each reaction well of the thermocycler, and load the sample tubes into the machine (Follow manufacturer's recommendations regarding use of mineral oil and sample placement in machine).

7. Thermocycler should be programmed for 30 cycles of the following cycling regime. Record the thermocycler program on Worksheet B:

   Preheat or "Jumpstart" sample to 94°C for two minutes.
   a. Denaturing at 94°C for 30 seconds.
   b. Annealing at 60°C for 30 seconds.
   c. Extending at 72°C for 60 seconds.
   d. Post dwell at 4-16°C for holding samples after cycling is complete.

IV. Nested PCR - Secondary Amplification of *R.salmoninarum* DNA

A. MATERIALS, METHODS, AND GENERAL QA/QC CONSIDERATIONS of Section II also apply to the nested PCR process.

B. PROCEDURES

1. Using **Worksheet C - Nested PCR**, record assay date and perform calculations to determine the amount of each reagent to use in the Round 2 Master Mix. This is based on the number of samples and controls to be processed (usually the same number of samples and controls that was used for Round 1 Master Mix).
   NOTE: The water volumes are increased in Round 2 MM to offset the smaller volume of DNA Template used in the Nested amplification step.

2. Add PCR reagents, except the Template DNA, into the Master Mix tube. Return unused reagents to the freezer.

3. In 0.5 mL PCR tubes, place 49µL of MM and overlay samples with one drop PCR grade mineral oil. Close caps tightly. Transfer tubes to AMPLIFIED DNA AREA (Area 3).

4. Load 1µL of amplified sample DNA into the appropriately labeled PCR tubes for Round 2 amplification. Be sure to expel PCR product beneath mineral oil layer. Gentle mixing with the pipet tip by withdrawing and expelling within the MM solution may help mix the DNA more thoroughly with the Round 2 Master Mix.

5. Add mineral oil to each reaction well of the thermocycler. Load PCR tubes into wells.

6. Program thermocycler for 20 cycles of the following regime:

   a. Jump start thermocycler to 94°C for two minutes.
   b. Denaturing at 94°C for 30 seconds.
   c. Annealing at 60°C for 30 seconds.
   d. Extending at 72°C for 60 seconds.
   e. Post dwell at 4-16°C for holding samples after cycling is complete.

   NOTE: PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

V. Visualization of PCR Product by Electrophoresis

A. MATERIALS AND REAGENTS

GEL ELECTROPHORESIS
SeaKem Agarose
0.5X TAE Buffer Solution
Clean glass flask (200 mL)
Hot Plate or Microwave Oven
Gel unit with Power Supply
Dedicated pipettor for Amplified DNA
Gel Loading Dye
1kbp DNA Ladder (in 100bp increments preferably)

VISUALIZATION OF BANDS
Ethidium Bromide Staining Solution
Staining dishes
Protective UV Eyewear
Transilluminator
Photo documentation Camera

B. PROCEDURES

1. Prepare 0.5 X TAE buffer for both the agarose gel and to fill the gel unit chamber (it's important to use the same buffer for both since small differences in ionic strength can affect migration of DNA). Use sterile deionized water to prepare buffer, following manufacturer's recommendations.

2. Prepare 2% Agarose Gel -
   a. Assemble the Gel tray and position 12 µl well volume comb in the tray according to

manufacturer recommendations.

Gel units come with various comb sizes. The final volume of sample loaded has to be adjusted to the comb size by following manufacturer recommendation.

Some guidelines follow :

4 place comb.....50µl
8 place comb.....20µl
12 place comb.....12µl

b. Level the chamber using a built-in bulls eye level, or with a small level. Place assembled tray into chamber.

c. Weigh appropriate amount of agarose for 2% gel and add to proper volume 0.5XTAE buffer.

d. Heat solution to boiling while stirring to completely dissolve the agarose.

e. Allow solution to cool to about 65^{o}C before pouring into gel tray (approximately 10 minutes or until still quite warm to touch).

f. Pour agarose solution into gel tray filling to the 1cm mark. Avoid the formation of bubbles.

g. Allow gel to cool completely, then carefully remove the comb by lifting straight up (avoid side to side motions). Remove the rubber ends also.

h. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode. 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.

i. Slowly fill the chamber with the remaining 0.5 X TAE buffer solution until the top of the gel surface is submerged about 1mm.

3. Loading the gel -
   a. Prepare a map of sample placement before loading the gel to ensure that samples, ladders, and controls are placed correctly.

   b. For each PCR product to be visualized, pipet 2µl of gel loading dye onto a clean strip of parafilm. Place the dye with adequate space between each droplet to prevent cross contamination. (Clean 0.5mL tubes can also be used).

   c. Withdraw 10µl from the first PCR product tube and wipe the excess mineral oil from the tip (dispose of each Kimwipe® after each sample). Carefully mix the sample and

the dye by repeated expulsion.

d. When the sample and the dye are adequately mixed, carefully place the pipet tip containing the mixture over an individual well of the 2% agarose gel, and carefully load the sample into the well. Repeat steps b & c for all the samples to be tested on the gel (both Round 1 and Round 2- Nested PCR products).

e. Be sure to load the DNA ladder(s) for base pair (bp) reference.

f. Load the positive and negative controls last.

g. Place the safety cover on the chamber and secure shut. Connect the attached leads to the power supply, making sure the black lead is connected nearest to the samples (use the mnemonic"Run to Red" to remember the correct orientation and direction of current).

h. Turn on the power supply and run the gel at 60-80 volts for at least one hour. Follow manufacturer's recommendations, as gel units will vary in their conductivity characteristics. Small bubbles will form in the buffer at the electrodes, and the loading dye will begin to migrate through the gel after a short period, indicating proper electrophoresis.

4. Staining the Gel:
   a. Prepare a 0.5µg/mL solution of ethidium bromide with 0.5x TAE Buffer.

      CAUTION: Ethidium bromide is a strong mutagen. Wear gloves and follow all MSDS precautions when handling or using this chemical.

   b. Turn off power supply to chamber and remove power leads. Remove safety cover.

   c. Remove gel and tray and place in ethidium bromide solution for 15 to 20 minutes.

   d. Destain gel in water for 5 to 60 minutes.

      NOTE: Ethidium Bromide solution can be reused and stored in a plastic tray container with a secure lid. Be sure to label and store this solution appropriately. Also see the Reagent list for an Ethidium Bromide extraction kit that allows filtration and proper disposal of used staining solutions.

5. Visualizing the DNA:
   a. Carefully carry the gel to the transilluminator and place the gel over the light source.

   b. Be sure to wear UV protective eyewear when visualizing the gel with UV light.

   c. Carefully record locations of base pairs on positive control bands in relation to DNA

ladder bands. Band locations of positive controls should be at anticipated locations according to primer sequence used in both the first and second (nested) round PCR assays.

Anticipated Products: 1st round primer M21 = **383bp**
2nd round (nested) primer M38 = **320 bp**
Bands from the second (nested) round will be brighter than the initial PCR products.

d. Note any unusual band occurrences (bands at 750 bp can occur as a PCR artifact). Negative controls (water used as template) should not have any visible bands. If suspected contamination occurs in negative controls, or test samples, the assay should be re-run from the extracted DNA tubes.

6. Photographing the gel:
   a. Assemble the Polaroid Photo documentation camera: Use an orange filter and appropriately sized snap-on camera hood. Adjust shutter speed and aperture as recommended by the manufacturer. May need to experiment with aperture and shutter speed to obtain the best resolution and exposure.

   b. Position the hood over the gel on the transilluminator and take the picture using the remote shutter release button. You can photograph directly on the gel tray, however adjust the level of the hood to the top edge of the tray, otherwise the image will be slightly out of focus.

   c. **Photo document all gels** and attach the developed photo to the PCR Data sheet and case history information.

VI. Equipment / Reagent Source List

DNA Extraction:

Reagents/ Supplies	Source
QIAGEN Tissue Kit	QIAGEN #29304
microcentrifuge tubes (1.7mL, locking caps)	ISC #C-3251-1
Ethanol (Absolute 97-100%)	Spectrum Chem. #ET107
Proteinase K (also comes with QIAGEN kits)	Sigma # P2308
Lysozyme Lysis Buffer	Sigma # L-7651
Trizma (Tris base/Tris Hydrochloride)	Sigma # T1503/T3253
Pipettor, Oxford Benchmate (10-200µl autoclavable)	Thomas Sci. #7733F10
Oxford Benchmate (100-1000µl autocl.)	Thomas Sci. #7733F13
Aerosol barrier tips (1-300 µl)	Thomas Sci #7740-F78
Aerosol barrier tips (1-1000µl)	Thomas Sci #7740-F92
Micro centrifuge (MicroV)	Fisher Sci. #05-090-724
or Var.speed Microcentrifuge	Daigger #YX4241A

Heater - Dry bath incubator	Fisher Sci. # 11-718 series
Blocks for Fisher Dry Baths (1.5 mL mc tubes)	Fisher Sci. # 11-718-9
Gloves (nitrile : non-latex not necessary)	Fisher Sci. # 11-395-19C
Tube storage boxes (100 mc-tubes)	Daigger # YX4280C

PCR Amplification:

Reagents/Equipment	Source
QIAGEN PCR Kit (includes Taq, dNTPs and buffers)	QIAGEN #201223

Or master mix ingredients can be purchased separately:

Taq DNA Polymerase (comes with $MgCl_2$)	Sigma # D1806
PCR Buffer II Kit ($MgCl_2$ and 10X)	Sigma #PCR-II
dNTP (nucleotides)	Sigma # D7295
microcentrifuge tubes (0.65 mL)	ISC #C-3249-1

Primers (forward and reverse; dilute primers in H_2O to 100 pmole/µl if necessary)

PRIMER	DNA Sequence 5' to 3'
Forward P3	**A GCT TCG CAA GGT GAA GGG**
Reverse M21	**GC AAC AGG TTT ATT TGC CGG G**
Forward P4	**AT TCT TCC ACT TCA ACA GTA CAA GG**
Reverse M38	**C ATT ATC GTT ACA CCC GAA ACC**

Primers can be synthesized by:	Great American Gene CO. GIBCO, BRL Life Technologies
Mineral oil (PCR grade for overlay)	Sigma # M8662
PCR Grade water (1 mL quantities)	Sigma # W1754
Molecular Grade Water (Genemate gallon)	ISC #C-553-1
Finnpipette, positive displacement 0.5-25µl	Fisher Sci. # 21-377-9
Positive Displacement tips/plungers 0.5-25µl	Fisher Sci. # 21377-53
Finnpipette, positive displacement 20-200µl	Fisher Sci. # 21-377-10
Tips/Plungers (20-200µl)	Fisher Sci. # 21377-54
Cryo-rack IsoFREEZE Flipper , -holds 0.5 and 1.5 mL microcentrifuge tubes	ISC #R-2020-2
Thermal cycler ,Thermolyne Amplitron II	Fisher Sci. # DB66925 or 35

Electrophoresis:

Reagents/Equipment	Source
Tris-Acetate EDTA Buffer 10x pre-made solution....	Gibco #1558-026
(Available in other forms) 25X Liter powder packs...	ISC #C-5553-2
(can also be made from scratch - see Buffer Recipes)	Sigma # T4038
Agarose, Seakem	ISC # 50003
RediLoad Gel Loading Buffer	Research Genetics
(can be loaded with Master Mix) or,	# 750026
Agarose Gel Loading Dye 6X	ISC # C5400-5
DNA Ladder (100 bp)	GIBCO # 15628-019
Ethidium Bromide (10mg/mL solution)	ISC #C-5515-10
Extractor-Ethidium bromide waste reduction system	Sigma # Z36,156-9
Submarine Gel Systems :A variety are available from Fisher Scientific	
Power Supply EC105	Fisher Sci. #FB-105
Photo-Documentation Camera	Fisher Sci. # FB-PDC-34
(Snap on hoods sized according to gel size)	
Lens Filter (Tiffen) Orange for ethidium bromide	Fisher # FB-PDF15
Film -black and white Polaroid	
- Type 667 (20 exp.)	Fisher Sci. #04-441-91
or Type 107 (8 exp.)	Fisher Sci. #04-441-19
Transilluminator: Fisher Model 88, 302 nm	Fisher Sci. #FB-TI-88
UV Protection spectacles	Daigger #YX11090U

Manufacturers and Phone Numbers:

A. Daigger & Co., Inc.	**800-621-7193**
Fisher Scientific	**800-766-7000**
Gibco	**800-828-6686**
Intermountain Scientific Corp.(ISC)	**800-999-2901**
QIAGEN, Inc.	**800-426-8157**
Sigma	**800-325-3010**
Thomas Scientific	**800-345-2101**
VWR Scientific Products	**800-252-1234**

VII. References and Bibliography

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Appendix 7.A – Worksheet for DNA Sample Data

Case Number_____ **Sample Site**_____ **Species** _____

Tissue Sample Type*	PCR Number	Mean ELISA OD Value	Notes
1.			
2.			
3.			
4.			
5.			
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19.			
20.			

***Record "E" for ELISA processed kidney, or "F" for frozen or fresh kidney.**

Appendix 7.B - Worksheet for Initial Amplification of Rs DNA by PCR

Case Number _____ **Date**_____

PCR Reagent	**Lot#**	**Final Concentration**	**Stock Concentration**	**Volume per Reaction (to total 50µl)**	**Volume for ____samples**
10XBuffer		1X	___ X		
$MgCL_2$		1.5mM	___ mM		
dNTPs		0.2mM	___ mM		
(+)Primer		20pM	___ pM/µl		
(-)Primer		20pM	___ pM/µl		
TAQ		2 units	___ units/µl		
d-H_2O*		**Add to total 50µl**	**including DNA =**		
DNA		-	-	10 µl	

*Add water to Master Mix first, TAQ last. (= 50 µl)

Thermocycler Program: Number of Cycles_____
Denature at _____^{0} for _____ seconds.
Anneal at _____^{0} for _____ minutes.
Extend at _____^{0} for _____ minutes.

Table 1 - Master Mix Formula for Rs-PCR

PCR Reagents	**Lot #**	**Stock Concentration***	**Final Concentration**	***Volume/Reaction (Total reaction volume = 50µL)***
Extracted DNA		--	--	*10.0* µL
10X Buffer		10X	1X	*5.0* µL
dNTPs		*(10*)* mM/µL	0.2 mM	*1.0* µL
(+) Primer	P3	*(70*)* pMole/µL	20 pMole	*1.0* µL
(--) Primer	M21	*(78*)* pMole/µL	20 pMole	*1.0* µL
[1]RediLoad Gel Dye™		10X	1x	*5.0* µL
TAQ		*(5*)* units/µL	2 units	*0.4* µL
d-H_20			*(qs to 50µL)*	*Example only: 23.4* µL *subtotal* *Add 26.6*µL *H_20 per rxn*
[2]$MgCl_2$ (if not included in 10X Buffer)		25mM	1.5 mM	*3.0* µL

*** NOTE: Stock concentrations will vary – those provided here are examples only. Check the specific reagents prior to use.**

[1]RediLoad Gel Dye™ - optional.

[2]$MgCl_2$ - only add not included in 10Xbuffer (or if concentration in buffer is insufficient for final concentration).

Appendix 7.C – Worksheet for Nested (Second Round) Rs PCR

PCR Reagent	**Lot#**	**Final Concentration**	**Stock Concentration**	**Volume per Reaction (to total 50µl)**	**Volume for ____samples**
10XBuffer		1X	___X		
$MgCL_2$		1.5mM	___mM		
dNTPs		0.2mM	___mM		
(+)Primer		20pM	___pM/µl		
(-)Primer		20pM	___pM/µl		
TAQ		2 units	___units/µl		
d-H_2O*		**Add to total 50µl**	**including DNA =**		
DNA		-	-	1 µl	-
*Add water to Master Mix first, TAQ last.				(= 50 µl)	

Thermocycler Program: Number of Cycles____
Denature at _____° for _____ seconds.
Anneal at _____° for _____ minutes.
Extend at _____° for _____ minutes.

Appendix 7.D - Photodocumentation and Report of Results

Case Number _____ Date:_____

Samples : _____

Affix gel photo documentation to this sheet and make notes on results:

Notes

CONFIRMED: □ Yes □ No Initial _____ Date:_____