

## 3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD)

*Renibacterium salmoninarum* are small Gram-positive rods (0.3-1.5µm x 0.1-1.0µm), non-motile, non-acid-fast, and non-spore forming bacteria. *Renibacterium salmoninarum*, which can be detected in both freshwater and marine habitats, has a wide geographic range including countries in North America, Europe, South America, and Asia. This bacterium, which can be transmitted either vertically or horizontally, primarily infects salmonids, but has also been isolated from non-salmonid fishes. *Renibacterium salmoninarum* infections can occur at any life stage in salmonid populations. Clinical signs of disease are uncommon in fish less than six months of age. Mortality has been reported at water temperatures between 4°C and 20.5°C, with the disease progressing rapidly between 15°C to 20.5°C. Acute and sub-acute forms of disease are less common than the more typical chronic form of disease, characterized internally by a large edematous kidney that appears gray and corrugated. The kidney can exhibit off-white lesions that vary in size and number, but lesions sometimes occur in other organs such as liver and spleen (Austin and Austin 1987; Bullock and Herman 1988; Elliot 2014; Thoesen 1994).

According to present knowledge, suggested sampling is as followed: Kidney- sample one fish per sample (FAT, PCR, ELISA); Ovarian fluid- sample up to 5 fish per sample pool (FAT, PCR, ELISA)

### A. Summary of Screening Test

#### 1. Fluorescent Antibody Test (FAT) (Section 2, 3.8.E “Fluorescent Antibody Test (FAT)”)\*

- a. Collect tissues as described in Section 2, 2.2 Sampling and prepare FAT slides.
  - i. Kidney (suggest prepare individually, one fish per sample)  
Prepare kidney smear on a non-coated or acetone-cleaned glass slide.
    1. Place a piece of posterior kidney or homogenized preparation on the slide.
    2. Create a thin smear on the surface of the glass slide.
  - ii. Ovarian Fluid Pellet Smear (suggest up to five fish per sample pool)
    1. After pooled ovarian fluid samples are processed and the appropriate amount of supernatant removed for virology assays (see Section 2, 4.4.C “Processing of Coelomic (Ovarian) Fluid Samples”), the pellet is re-suspended in the remaining ovarian fluid by thorough vortexing or repeat pipetting.
    2. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see **Note**). Freeze the remainder of the sample at -20°C for PCR confirmation.

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3. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see **Note**).
4. The pellet is carefully removed with a small amount of supernatant using a sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

**Note:** Elliott and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e. 2.5 mL from a five-fish-pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

- b. After the tissue smear is heat fixed or air dried, slides are fixed in acetone for five minutes.
- c. Stain slides with FITC-conjugated *R. salmoninarum* antisera or equivalent as described in Section 2, 3.8.E.2 “Direct FAT (DFAT) Staining.”
- d. Examine at least 50 fields using oil immersion at 1000X magnification to detect the 1.0 X 0.5  $\mu\text{m}$  bacterial cells, which should appear equivalent staining as your positive control (fluorescent and bright).
  - i. **Smears which do not show any fluorescent bacterial cells may be discarded and reported as negative for *R. salmoninarum*.**
  - ii. Any smears, which have fluorescent, diplobacilli bacterial cells present measuring approximately 1.0 X 0.5  $\mu\text{m}$ , shall be considered **PRESUMPTIVELY positive** for *R. salmoninarum*.
- e. A positive control is required for each run. It is preferable to use tissue infected with *R. salmoninarum* for a positive FAT control. Positive control culture isolates of *Renibacterium salmoninarum*, however, can be obtained from the American Type Culture collection (ATCC)<http://www.atcc.org>. Suppliers of commercially prepared antibodies for FAT may also provide positive control materials for use in this assay (see Section 2, 3.8.E.6 “Commercial Sources for Antibodies”).

\*Salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT.

## B. Confirmatory Tests

1. **Bacterial Isolation and Identification** (Austin et al. 1983)

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- a. At the time samples are collected during the inspection (Section 2, 2.2.E.2 “Collection of Kidney Cultures”), aseptically inoculate samples of tissues onto plates containing selective kidney disease media (SKDM-2) (Section 2, 3.7.A.3 “Selective Kidney Disease Medium-2 (SKDM-2)”).
- b. Incubate for 2 to 3 weeks at 15°C in a humid chamber to prevent dehydration of media.
- c. At 2 to 3 weeks, observe plates for growth of pinpoint bacterial colonies.
  - i. If *R. salmoninarum* is presumptively identified in FAT, corresponding samples inoculated onto SKDM-2 should be examined weekly.
  - ii. If no growth, continue to incubate plates for up to six weeks, and examine them several times per week for growth.
  - iii. **If no growth after six weeks, samples may be discarded and reported as negative for *R. salmoninarum*.**
  - iv. If growth of small (2 mm diam.), smooth, white round colonies is observed, obtain inoculum from colony and confirm identification using FAT or PCR.
    1. **If FAT or PCR results on culture are positive, sample is reported as positive for *R. salmoninarum*.**
    2. **If FAT or PCR results on culture are negative, sample is reported as negative for *R. salmoninarum*.**

**Note:** The slow growth of this organism makes phenotypic characterization of suspect isolates difficult and time consuming. The inspector may consider pursuing phenotypic characterization if the detection of *R. salmoninarum* by these techniques continues to be questionable (consult Austin and Austin 1993).

#### 2. Molecular Assays (Chase and Pascho 1998, Chase et al. 2006, and Elliott et al. 2013).

##### a. Extraction of DNA from Kidney and Ovarian Tissues

(NOTE: The following procedure employs an extraction kit available from Qiagen, Inc. (<http://www.qiagen.com>). DNA Extraction kits of similar efficacy are available from many other sources, and can be utilized as alternatives for extraction of DNA in this protocol. These kits utilize “spin columns” for binding and elution of DNA from tissue lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction). Extracting DNA from the individual kidney or ovarian fluid pool corresponding to the presumptive FAT positive is suggested best practice.

##### i. Procedures

1. Transfer 25 to 50 mg of kidney tissue, or 50 µL ovarian fluid, into a 1.5 mL microcentrifuge tube. Tissue can be fresh, previously frozen, or properly stored in > 95% ETOH.
2. Add 180 µL of lysozyme lysis buffer (Section 2, 3.7.G.1 “Lysozyme Lysis Buffer”). Incubate at 37°C for one hour, vortexing occasionally.

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3. Add 25  $\mu\text{L}$  of Proteinase K stock solution and 200  $\mu\text{L}$  of buffer AL lysis buffer (provided by extraction kit manufacturer), then mix by vortexing and incubate at 70°C for 30 minutes. Vortex occasionally. Tissues should be well lysed by the end of 30 minutes.
4. Incubate at 95°C for another 10 minutes. Vortex occasionally.
5. Add 210  $\mu\text{L}$  of ethanol, mix thoroughly on vortex.
6. Place a spin column in a 2 mL collection tube. Place sample mixture over 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 for 1 minute at room temperature.
7. Place the spin column in a clean 2 mL collection tube and discard the tube containing the filtrate.
8. Carefully open spin column and add 500  $\mu\text{L}$  buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.
9. Repeat steps 7 and 8, using 500  $\mu\text{L}$  buffer AW2. Centrifuge at full speed for three minutes to dry the membrane.
10. Place spin column in clean 1.5 mL micro centrifuge tube and add 200  $\mu\text{L}$  of buffer AE (elution buffer provided by kit manufacturer) for elution of DNA (TE buffer pH 8.0 or water can also be used). Incubate for five minutes at room temperature. Centrifuge at 6000 x g for one minute.
11. Repeat step 10 so that the total volume of DNA is 400  $\mu\text{L}$ . Discard spin column and store DNA solution at -20-70°C until use.
12. Quantify the amount of DNA extracted with a spectrophotometer (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR)).
  - a. 25 to 50 mg of fish kidney tissue should produce between 100 and 300 ng DNA per  $\mu\text{L}$  using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.
  - b. 50  $\mu\text{L}$  ovarian fluid produces a much lower amount of DNA per  $\mu\text{L}$  using this procedure. Dilute the template if DNA exceeds 300 ng/ $\mu\text{L}$ .

#### **b. *Renibacterium salmoninarum* Real-Time PCR**

##### **1. Principle/Scope**

The purpose of this Standard Operating Procedure (SOP) is to describe the reagents, materials and procedures for real-time polymerase chain reaction (PCR) detection of *Renibacterium salmoninarum* DNA in fish tissues, ovarian fluids or bacterial isolates. This real-time PCR targets a 69 base pair region of the major soluble antigen (msa) gene. This bacterium causes bacterial kidney disease (BKD) in salmonid fishes. The assay was developed, optimized and evaluated for purpose by the USGS Western Fisheries Research Center (Chase et al. 2006; Elliott et al. 2013).

## 2. Equipment and Materials Required

- a. Powder-free disposable gloves
- b. Lab coat
- c. Vortex mixer
- d. Biohazard waste disposal receptacles
- e. Ultra-low freezer set at  $\leq -60^{\circ}\text{C}$
- f. Frost-free freezer set at  $-20^{\circ}\text{C} \pm 10^{\circ}\text{C}$
- g. Refrigerator set at  $4^{\circ}\text{C} \pm 3^{\circ}\text{C}$
- h. Ice buckets
- i. Real-time PCR detection system
- j. Microcentrifuge tube rack
- k. Pipettes: 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 100  $\mu\text{L}$ , 200  $\mu\text{L}$ , 1000  $\mu\text{L}$ , 8 or 12 channel pipettes
- l. Aerosol filtered RNase & DNase free pipette tips: 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 100  $\mu\text{L}$ , 200  $\mu\text{L}$ , 1000  $\mu\text{L}$
- m. Repeat pipette (optional)
- n. 500  $\mu\text{L}$  repeat pipette tips (optional)
- o. Sterile polypropylene conical tubes
- p. Sterile polypropylene 1.5 mL tubes
- q. Absorbent wipes
- r. Autoclave bags
- s. Appropriate real-time PCR reaction plates or strips
- t. Appropriate optical adhesive film to cover plates
- u. Plate sealer (optional)
- v. Plate centrifuge (optional)
- w. Ice
- x. RNA-free water
- y. BKD forward primer: 5'- GTGACCAACACCCAGATATCCA -3'<sup>\*</sup>
- z. BKD reverse primer: 5'- TCGCCAGACCACCATTTACC -3'<sup>\*</sup>
- aa. BKD probe: 5'-(6-FAM)- CACCAGATGGAGCAAC -(NFQ/MGB<sup>1</sup>)-3'<sup>†</sup>
- bb. Real-time PCR Master Mix (typically sold in 2X concentrations)<sup>§</sup>
- cc. Extracted DNA and appropriate controls (positive extraction control (PEC), negative extraction control (NEC), no template control (NTC), positive amplification control (PAC))
- dd. TE buffer 1X (Tris EDTA pH 8.0)<sup>\*</sup>

<sup>\*</sup>Primers and probe should be ordered from a commercial oligonucleotide synthesis company such as Invitrogen or Integrated DNA Technologies Inc. (IDT). They are reconstituted in water, Tris-EDTA (TE) or per manufacturer's instruction, followed by dilution to working concentrations with water. Primers and probe are aliquoted into smaller volumes to minimize freeze-thaw cycles and minimize the probe exposure to light.

<sup>†</sup>The minor groove binder (MGB) probe is proprietary technology of Applied Biosystems Inc. (1 800-327-3002; [www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

<sup>§</sup>A range of commercial master mixes are likely suitable, but the master mixes should be tested empirically for effects on PCR sensitivity during internal laboratory validation to implement the protocol. The assay

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validation protocol of Elliott et al. 2013 utilized the TaqMan® Gene Expression Master Mix from Applied Biosystems Inc. (containing uracil-N-glycosylase UNG or UDG). The authors reported a 10-fold decrease in assay sensitivity when the TaqMan® Universal Master Mix was substituted. For in-house validation or verification guidance, see 5.4.3 Validation of test methods in AAVLD Requirements of an Accredited Veterinary Medical Diagnostic Laboratory. American Association of Veterinary Laboratory Diagnosticians, INC. AC1, Version 2018-07.

#### 3. Specimens

- a. Specimen DNA should be extracted using an appropriate DNA extraction method, such as the QIAamp DNA Mini Kit (Qiagen Cat No. 51306) following the manufacturer's instructions. Extraction protocols should include a lysozyme digestion step for the Gram positive cell wall, as described above (2.a). Appropriate specimens include:
  - i. DNA extracted from fresh homogenized kidney.
  - ii. DNA extracted from kidney supernatants.
  - iii. DNA extracted from ovarian fluids.
  - iv. DNA extracted from bacterial culture

#### 4. Procedure

For this procedure, it is critical to have separate preparation areas and equipment for nucleic acid extraction, DNA transfer, amplified nucleic acids, and "clean" procedures such as master mix preparation. Never introduce amplified DNA or sample DNA into the clean area. Designate a single biological safety cabinet (BSC) or PCR workstation for this "clean" work only. Similarly, designate a set of calibrated pipettes, tips, RNase-free water, tubes, ice buckets, and racks for "clean" use only to be used for the preparation of clean reagents and to never leave the area. In addition, designate a -20°C frost-free freezer for storage of "clean" reagents. The extraction procedure requires the dedication of a separate BSC, set of calibrated pipettes, equipment and reagents. If possible, use and assign a third BSC or PCR workstation and calibrated pipettes for transfer of DNA to amplification tubes or plates. Powder-free latex/nitrile or equivalent gloves must be worn throughout the procedure and must be changed frequently. Always wear fresh gloves when working with "clean" reagents. DNA is very labile and easily degraded by DNases. Supplies such as pipette tips and micro-centrifuge tubes as well as all reagents should be certified DNase-free. Always change gloves after working with sample DNA or amplified DNA. Wear protective eyewear, gloves, and lab coats as some reagents used are toxic.

- a. Fill out plate map and determine the total number of reactions that you will need to prepare master mix for. Consider the number of replicate PCR wells being used; Elliott et al. 2013 used two replicate wells per sample. Each plate must have a no template control (NTC), positive amplification control (PAC), negative extraction control (NEC), and a positive extraction control (PEC). Include 1 extra reaction for every 15 reactions when calculating reagent volumes to account for reagent loss associated with pipetting. Fill out the worksheet for reagent volumes and lot numbers (Appendix 1).
- b. Thaw and mix the 2X master mix (if stored at -20°C), primer and probe solutions, and RNase-free water. Keep on ice.
- c. Determine the number of reactions and prepare reaction mix according to Table.

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Reagent	Stock concentration	Volume per reaction (μL)
Water*	NA	5.04
2X master mix	2X	12.00
Forward primer	45 μM	0.48
Reverse primer	45 μM	0.48
Probe	6 μM	1.00
Total Volume*		20.00

\*A commercial exogenous internal positive control (Exo IPC) can be used to detect PCR inhibition that can lead to false negative results. The IPC from Applied Biosystems Inc. (part # 4308323) has been evaluated and does not reduce assay sensitivity and is suitable for use in this protocol. The IPC can be incorporated following manufacturer's instructions; reduce water volume as needed.

- d. Mix the reaction components thoroughly and dispense 20 μL into real-time PCR reaction plate or strips.
- e. In the area dedicated for DNA transfer, add 5 μL template DNA, controls, or nuclease-free water to the appropriate individual PCR wells containing the reaction mix. Load samples first, add control templates last. When using plates, cover with the plate sealing film ensuring a good seal on the plate before moving the plate outside of the BSC or PCR workstation.
- f. Briefly centrifuge the plate, ensuring the reaction mix is pulled down to bottom of the wells and air bubbles are eliminated.
- g. Set up and run the real-time PCR program with the following parameters:
  - i. Reaction volume: 25 μL
  - ii. Reporter dye/ quencher: FAM (select appropriate channel)
  - iii. Real time program outline below:

Step #, # of cycles: purpose	Temperature	Time
Step 1, 1 cycle: deactivation	50°C	2 min
Step 2, 1 cycle: activation & denaturation	95°C	10 min
Step 3, 40 cycles:		
denaturation	95°C	15 sec
annealing and amplification*	60°C	60 sec

\*collection of fluorescence

- h. Place the PCR plate in the real-time PCR instrument and start the cycling program.
- i. Once the program is completed, use appropriate methods to export, save and archive all data.

## 5. Data analysis

- a. Refer to the user guide for your real-time PCR instrument for instructions on how to analyze the data, using the recommendations below. Consider data analysis settings when conducting your internal laboratory validation to implement the procedure.
- b. The Auto threshold setting is recommended. The Auto setting minimizes subjectivity when setting the threshold. Review baseline and threshold settings.

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- c. If the baseline is set too low (high background), use the manual threshold and manual baseline settings with a typical default of 3 to 15 cycle range.

#### 6. Acceptance Criteria

- a. Verify that all cycles were completed and the run is valid before analyzing the test sample results.
- b. All PCR and extraction controls must fall within the acceptable positive or negative cycle threshold (Ct) range in order for the test to be considered valid.
  - i. The NEC and NTC have an undetermined value.
  - ii. The PEC is established by the testing lab. Laboratories aim for an DNA sample with a Ct range of 25-30.
  - iii. A PAC should contain several DNA samples representing a range of DNA concentrations and Ct values.
- c. If the NEC yields a Ct value, the extraction and run are not valid and should be repeated. Evaluate the reagents, equipment and procedure for introduction of contaminating nucleic acid.
- d. If the PEC yields an undetermined Ct value, all samples tested on the plate or run must be re-extracted and these DNA samples must be re-tested for a valid test result.

#### 7. Interpretation of Results

- a. Check the component plot for samples and controls.
- b. If a sample has a Ct value less than your defined Ct cut-off value, with a sigmoidal curve, the sample is **POSITIVE**. Elliott et al. 2013 used a Ct cut-off value of 38 for assay validation.
- c. If a sample has Ct value greater than the defined Ct cut-off value, the sample is reported as **NEGATIVE**.
- d. If a sample has a non-sigmoidal curve that crosses the threshold, check the component values. If the FAM component has a straight line, with no upward curves the sample is reported as **NEGATIVE**.

#### c. *Renibacterium salmoninarum* Nested PCR (nPCR)

The polymerase chain reaction technique employs oligonucleotide primers to amplify segments of the gene that codes for the 57 kDa protein of *R. salmoninarum* (Chase and Pascho 1998). DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a “nested PCR” technique. The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The following procedures have been adapted from those of Chase and Pascho (1998), and have been reviewed and approved by the authors.

##### 1. Initial Amplification of *R. salmoninarum* DNA

- a. General QA/QC considerations must be considered before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR)).
- b. Procedures for initial round:

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1. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
2. Using Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, 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 and the amount of MM needed per reaction (40  $\mu$ L). Add 4 to the number of samples so that there is enough to run controls.
3. Under UV cabinet, add PCR reagents **except for sample DNA** to the MM tube in the order listed on Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, adding water first and Taq last. Keep all reagents cold during mixing, and return them to freezer immediately after use. Do not expose enzymes, Primers, or dNTP's to UV light.
  - a. Water to make a 40  $\mu$ L total volume per reaction.
  - b. PCR Buffer mix (1X).
  - c. MgCl<sub>2</sub> (1.5 mM per reaction).
  - d. dNTP mix (0.2 mM per reaction).
  - e. Primers (20 pmole each per reaction).
    - i. Forward 5' – A GCT TCG CAA GGT GAA GGG – 3'
    - ii. Reverse 5' – GC AAC AGG TTT ATT TGC CGG G – 3'
  - f. TAQ polymerase (2 units per reaction).
4. Place 40  $\mu$ L of MM into each 0.5 mL PCR tube and close caps tightly. Move PCR tubes to sample loading area.
5. In sample loading area, load 10  $\mu$ L of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.
6. Load the sample tubes into the thermocycler wells.
7. Thermocycler should be programmed for 30 to 40 cycles of the following temperature regime and recorded on Section 2, 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*:
  - a. Preheat sample to 94°C for two minutes.
  - b. Denaturing at 93°C for 30 seconds.
  - c. Annealing at 60°C for 30 seconds.
  - d. Extending at 72°C for one minute.

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- e. Post dwell at 4 to 16°C for holding samples after cycling is complete.

#### 2. nPCR-Secondary Amplification of *R. salmoninarum* DNA

- a. Materials, methods, and general QA/QC considerations of this section and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process:
- b. Using Section 2, 3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, 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 and controls to be processed and the amount of MM needed for each reaction (49 µL).
- c. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to freezer.
  1. Primers
    - a. Forward 5’ – AT TCT TCC ACT TCA ACA GTA CAA GG – 3’
    - b. Reverse 5’ – C ATT ATC GTT ACA CCC GAA ACC – 3’
  2. In PCR tubes (0.5 mL), pipette 49 µL of MM. Close caps tightly. Remove tubes from UV cabinet to amplified DNA area.
  3. Load 1 µL of amplified sample DNA into the appropriate PCR tubes.
  4. Load PCR tubes into thermocycler wells.
  5. Program thermocycler for 10 to 20 cycles of the following regime:
    - a. Preheat sample to 94°C for two minutes.
    - b. Denaturing at 93°C for 30 seconds.
    - c. Annealing at 60°C for 30 seconds.
    - d. Extending at 72°C for one minute.
    - e. Post dwell at 4 to 16°C for holding samples after cycling is complete.

**Note:** PCR Products can be stored at 4 - 8°C for one month or frozen at < -20°C for long-term storage.

#### 3. Visualization of PCR Product by Electrophoresis

See Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures.

- a. Visualization of amplified products resulting from PCR for detection of *R. salmoninarum* DNA is best accomplished after electrophoresis on a 1.5 or 2% agarose gel (Section 2, 6.3.C “Detection of Product”).

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- b. Using Section 2, 3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*, record location of each sample on the agarose gel at the time samples are loaded.
  - c. After electrophoresis, stain gel with ethidium bromide or other suitable stain and visualize on an UV transilluminator.
  - d. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.
    1. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays (first round primer M21=383bp; nested primer M38=320 bp). **Bands occurring at these locations are confirmatory for *R. salmoninarum* and are reported as POSITIVE.**
    2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates PCR should be re-run on samples from template DNA tube.
- 4. Document the electrophoresis results** (Section 2, 6.3.G “Visualize the DNA”). Photograph all gels and attach the photo to Section 2, 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel. Attach to case history information.

#### d. Associated Documentation

- b. Chase, D. M., D. G. Elliott, and R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. *Journal of Veterinary Diagnostic Investigation* 18:375-380.
- c. Elliott, D.G., L.J. Applegate, A.L. Murray, M.K. Purcell, and C.L. McKibben. 2013. Bench-top validation testing of selected immunological and molecular *Renibacterium salmoninarum* diagnostic assays by comparison with quantitative bacteriological culture. *J. Fish. Dis.* 36: 779-809.