

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

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 (Austin and Austin 1987; Bullock and Herman 1988; Thoesen 1994).

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 (**Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT).
 - i. Kidney
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
 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.
 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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 2

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 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 µm bacterial cells, which should appear stained as an “apple green” fluorescence.
 - 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 “apple green” fluorescent, diplobacilli bacterial cells present measuring approximately 1.0 X 0.5 µm, shall be considered **PRESUMPTIVELY positive** for *R. salmoninarum*.
- e. 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). The Internet location for ATCC is <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”).

B. Confirmatory Tests

1. Bacterial Culture (Austin et al. 1983)

- 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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 3

- 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. **Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA**

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.

- 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).
- i. Procedures
 1. Transfer 25 to 50 mg of kidney tissue, or 50 μ L ovarian fluid, into a 1.5 mL microcentrifuge tube. Tissue can be fresh or previously frozen.
 2. Add 180 μ L of lysozyme lysis buffer (Section 2, 3.7.G.1 “Lysozyme Lysis Buffer”). Incubate at 37°C for one hour, vortexing occasionally.
 3. Add 25 μ L of Proteinase K stock solution and 200 μ 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 μ L of ethanol, mix thoroughly on vortex.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 4

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 μ L buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.
 9. Repeat steps 7 and 8, using 500 μ 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 μ 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 μ 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 μ L using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.
 - b. 50 μ L ovarian fluid produces a much lower amount of DNA per μ L using this procedure. Dilute the template if DNA exceeds 300 ng/ μ L.
- b. Initial Amplification of *R. salmoninarum* DNA
- i. General QA/QC considerations must be considered before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR)).
 - ii. Procedures for initial round:
 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 μ 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.

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 5

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 μ L total volume per reaction.
 - b. PCR Buffer mix (1X).
 - c. $MgCl_2$ (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 μ 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 μ 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.
 - e. Post dwell at 4 to 16°C for holding samples after cycling is complete.
- c. “Nested” PCR-secondary amplification of *R. salmoninarum* DNA
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:
 - i. 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 the number of samples and controls to be processed and the amount of MM needed for each reaction (49 μ L).

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 6

- ii. 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'
- iii. In PCR tubes (0.5 mL), pipette 49 µL of MM. Close caps tightly. Remove tubes from UV cabinet to amplified DNA area.
- iv. Load 1 µL of amplified sample DNA into the appropriate PCR tubes.
- v. Load PCR tubes into thermocycler wells.
- vi. Program thermocycler for 10 to 20 cycles of the following regime:
 1. Preheat sample to 94°C for two minutes.
 2. Denaturing at 93°C for 30 seconds.
 3. Annealing at 60°C for 30 seconds.
 4. Extending at 72°C for one minute.
 5. Post dwell at 4 to 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.

- d. Visualization of PCR Product by Electrophoresis
See Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures.
 - i. 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”).
 - ii. 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.
 - iii. After electrophoresis, stain gel with ethidium bromide and visualize on an UV transilluminator.
 - iv. 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.**

3.5 *Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD) - 7

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.
- e. 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.