USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 3 Bacteriology

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3.1 Bacteriology Introduction

The following chapter describes inspection procedures for bacterial pathogens of fish that may be required for a fish health inspection. The target bacterial species include *Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri*, *Renibacterium salmoninarum,* and *Piscirickettsia salmonis*. Chapter 2 Sampling describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

Presumptive identifications of *A. salmonicida (*subspecies *salmonicida and achromogenes)* (Section 2, 3.2 *Aeromonas salmonicida* (Furunculosis)*), E. ictaluri* (3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC))*,* and *Y. ruckeri* (3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)) are based on Gram staining properties, and characteristic biochemical reactions. Confirmatory identification consists of fluorescent antibody testing using fluorescein-conjugated, species-specific antibody (3.8.E "Fluorescent Antibody Test (FAT)"). Known isolates of *A. salmonicida, E. ictaluri,* and *Y. ruckeri* are purchased from ATCC and are used as positive controls. Single, unknown isolates may be used to test for all three of these organisms.

The presumptive identification of the Gram-positive bacterium *R. salmoninarum* (3.5 *Reibacterium salmoninarum* (Bacterial Kidney Disease, BKD)) is based upon serological methods. For purposes of initial screening and detection of the pathogen, the direct fluorescent antibody technique (FAT) on kidney smears and ovarian fluid samples is employed (3.8.E "Fluorescent Antibody Test (FAT)"). Documentation exists which indicates the possibility for false positive results caused by bacterial organisms which cross react with antibodies prepared against *R. salmoninarum* (Austin 1985; Bullock 1980; Brown et al. 1995). For this reason, it is important to follow steps described below to confirm that a positive FAT result is due to the presence of this pathogen. **Exception:** Anadromous salmonids regularly monitored for *R. salmoninarum* with ELISA or quantitative PCR techniques may be considered positive without additional testing by FAT.

Any FAT results which appear positive for *R. salmoninarum* should be confirmed by either culture of kidney tissue on selective kidney disease medium (SKDM-2) (3.5.B.1 "Bacterial Culture") or by testing the positive tissues with the polymerase chain reaction (PCR) technique (3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA").

The presumptive identification of the gram-negative, intracellular bacterium *P. salmonis* (3.6 *Piscirickettsia salmonis*) is based on isolation in tissue cell culture without antibiotics and/or detection in stained tissue impressions (3.6.A "Summary of Screening Tests"). Confirmatory testing is by serological methods or PCR (3.6.B "Confirmatory Tests").

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

3.2 *Aeromonas salmonicida* (Furunculosis)

Aeromonas salmonicida may be difficult to detect based on a variety of environmental, physiological, and host factors. At water temperatures between 14 to 24°C, fish can develop clinical disease within 4 to 12 days after bacterial exposure in the water supply. At temperatures below 13°C, chronic and latent infections are more likely to develop. This pathogen has been associated with disease in a variety of salmonid and non-salmonid species. It is generally accepted that any freshwater fish can carry the bacteria with or without showing signs of disease (Bullock et al. 1983; Thoesen 1994). The typical form of the bacterium (subspecies *salmonicida*) produces a soluble brown pigment on tryptic soy agar after two to three days of growth at 20°C. The less common and atypical strain of this pathogen (subspecies *achromogene*s) does not produce pigmentation under these incubation conditions. Both strains, however, have been associated with disease in a variety of fish species (Paterson et al. 1980).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples into TSA tubes or onto plates as described in 2.2 Sampling.

b. Incubate for 24 to 48 hours at 20 to 24°C (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are reported as negative for *A. salmonicida***.

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single, isolated colony to subculture onto fresh TSA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at 20 to 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies representing each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each isolated colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (3.A1 Laboratory Reference Flow Chart Appendix 1).

   i. Colony Pigmentation
   Typical strains of *A. salmonicida* have brown diffusible pigment after 2 to 3 days of incubation. Some strains may not be pigmented (subsp. *achromogene*s).

   ii. Gram Determination (3.8.A "Gram Reaction")

A. salmonicida is Gram-negative. Gram-positive isolates may be reported as negative for *A. salmonicida*.

iii. Presence of Cytochrome Oxidase (CO) (3.8.B "Cytochrome oxidase")
Most are CO positive. Rarely, CO negative strains of *A. salmonicida* subsp. *salmonicida* have been encountered (Chapman et al. 1991).

iv. Motility (3.8.C "Motility")
A. salmonicida is non-motile. Motile isolates may be reported as negative for *A. salmonicida*.

g. Perform biochemical testing on each isolate (3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Tube Method (3.8.D.1 "Tube Method")

1. Glucose fermentation (3.8.D.1.a "Glucose Fermentation") using OF basal medium containing glucose will produce an oxidative/fermentative (O/F) or a fermentative (F) result with gas (most strains produce gas, but some may be weak or variable in this production). **Any isolate with a result other than this may be reported as negative for *A. salmonicida*.**

2. Gelatinase (3.8.D.1.c "Gelatinase") and Indole (3.8.D.1.d "Indole Test") are considered together with the pigmentation of the isolate for interpretation of the results.

a. Brown diffusible pigmented isolates that are Gelatinase positive and Indole negative are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *salmonicida*.

b. Non-pigmented isolates that are Gelatinase negative and Indole positive are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *achromogens.*

3. **Isolates yielding any other combination of these results may be reported as negative for *A. salmonicida***.

4. Additional biochemical tests will differentiate *A. salmonicida salmonicida* from *A. salmonicida achromogens*.

a. Maltose fermentation (Section 2, 3.8.D.1.e "Carbohydrate Utilization") is determined using OF basal medium containing maltose, producing a fermentative F result. These tests may be done in a closed (i.e., over-layed) tube as is described for glucose (Section 2, 3.8.D.1.a "Glucose Fermentation") or by interpreting the reaction at the bottom of an open tube that contains sufficient medium to distinguish fermentation from oxidation (e.g., minimum of 5 mL in a 16 x 25 mm tube). *A salmonicida* sub-species *salmonicida* ferments maltose. *A. salmonicida* subspecies *achromogens* typically does not ferment maltose; however, maltose utilization has been documented for a few isolates.

b. Esculin hydrolysis (Section 2, 3.8D.1.h "Esculin Test") is determined on Bile Esculin Agar slants with a resulting positive hydrolysis by *A. salmonicida* sub-

species *salmonicida*, whereas strains of *A. salmonicida* sub-species *achromogens* are negative.

ii. Commercial Identification Systems (3.8.D.2 "Commercial Identification Systems")

1. Biolog (3.8.D.2.b "Biolog")

2. API
If isolates are tested with the commercial system API described in 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens.

h. When testing is complete, either cryopreserve isolates of interest or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

i. Positive control isolates of *Aeromonas salmonicida* can be obtained from the American Type Culture Collection (ATCC). The Internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:

i. *A. salmonicida* subspecies s*almonicida* – ATCC # 14174.

ii. *A. salmonicida* subspecies *achromogenes* – ATCC # 10801.

iii. A **cytochrome oxidase negative** isolate is also available – ATCC # 49385.

B. Confirmatory Test

1. **Fluorescent Antibody Test (FAT)** (3.8.E "Fluorescent Antibody Test (FAT)")
FAT is performed on at least one representative isolate from each lot inspected and found positive during screening. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.8.E.6 "Commercial Sources for Antibodies."

3.3 *Yersinia ruckeri* (Enteric Redmouth Disease, ERM)

Yersinia ruckeri affects fingerlings of potentially all salmonids. In salmonids, mortality increases dramatically following periods of stress due to environmental conditions and handling. Survivors of enteric redmouth disease outbreaks become carriers, after which the bacteria are shed from the intestinal tract in large numbers during a regular 36 to 40 day cycle. Severity of infection decreases at water temperatures below 10°C. *Yersinia ruckeri* has been isolated from the kidney of some non-salmonids as well. It is generally accepted that, although not ubiquitous in the environment, any freshwater fish can carry the bacteria with or without exhibiting signs of the disease (Bullock 1984; Thoesen 1994).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples onto tubes or plates as described in 2.2 Sampling.

b. Incubate for 24 to 48 hours at 20 to 24°C, (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *Y. ruckeri*.**

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single isolated colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at 20 to 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Gram Determination (3.8.A "Gram Reaction")
Yersinia ruckeri is Gram-negative. **Gram-positive isolates may be reported as negative for *Y. ruckeri*.**

ii. Presence of Cytochrome Oxidase (CO) (3.8.B "Cytochrome Oxidase")

Yersinia ruckeri is CO negative. **CO positive isolates may be reported negative for *Y. ruckeri***.

iii. Motility (3.8.C "Motility")
Yersinia ruckeri is motile. **Non-motile isolates may be reported as negative for *Y. ruckeri***.

g. Perform biochemical testing on each isolate 3.A1 Laboratory Reference Flow Chart Appendix 1)

i. Tube Method (3.8.D.1 "Tube Method")

1. Triple Sugar Iron (TSI) (3.8.D.1.b "Triple Sugar Iron (TSI)")
Yersinia ruckeri will yield an alkaline over acid (K/A) or alkaline over acid with gas (K/AG) result. **Any isolate with a result other than this may be reported as negative for *Y. ruckeri.***

2. Carbohydrate Utilization (3.8.D.1.e "Carbohydrate Utilization")

a. Arabinose cannot be utilized (fermented) by *Y. ruckeri*.

b. Rhamnose cannot be utilized (fermented) by *Y. ruckeri*.

c. Sucrose cannot be utilized (fermented) by *Y. ruckeri*.

i. Salicin cannot be utilized (fermented) by *Y. ruckeri*.

ii. **Isolates yielding positive results for any of these tests may reported as negative for *Y. ruckeri***.

3. Malonate Test (3.8.D.1.g "Malonate Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

4. Indole Test (3.8.D.1.d "Indole Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

5. Esculin Test (3.8.D.1.h "Esculin Test")
Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri*.

6. Decarboxylase Test (Lysine) (3.8.D.1.f "Decarboxylase Test (Lysine)")
Isolates yielding negative results for this test may be reported as negative for *Y. ruckeri.*

7. Sorbitol (3.8.D.1.e "Carbohydrate Utilization")

a. Isolates that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *Y. ruckeri*.

b. Sorbitol is used to differentiate between Type I and Type II *Y. ruckeri*.

i. Isolates yielding negative results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type I.

ii. Isolates yielding positive results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type II.

ii. Commercial Identification Systems (3.8.D.2 "Commercial Identification Systems")

1. Biolog (3.8.D.2.b "Biolog")

2. API
If isolates are tested with the commercial system API described in 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens."

3. When testing is complete, either cryopreserve isolates of interest or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

4. Positive control isolates of *Yersinia ruckeri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:

a. *Yersinia ruckeri* serovar Type I – ATCC # 29473.

b. *Yersinia ruckeri* Type II – ATCC # 29908.

B. Confirmatory Test

1. Fluorescent Antibody Test (FAT) (3.8.E "Fluorescent Antibody Test (FAT)")
FAT is performed on at least one representative isolate from each lot inspected and found positive during screening.

a. A separate antibody must be utilized for confirmation of either Type I or II strains.

b. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control.

c. A list of sources from which antibodies may be obtained is provided in 3.8.E.6 "Commercial Sources for Antibodies."

3.4 *Edwardsiella ictaluri* (Enteric Septicemia of Catfish, ESC)

The primary host species of this pathogen include all species of catfish and tilapia, as well as other warm water species. Both fingerlings and adults can be affected by the disease. In catfish, the bacterium is transmitted through the olfactory system to the brain, where the typical "hole-in-the-head" lesions can be observed during an *E. ictaluri* epizootic. Disease typically occurs at water temperatures between 20°C and 30°C. Experimental infections have been established in salmonids. It is generally accepted, that any warmwater species of fish can carry this pathogen with or without exhibiting signs of disease (Hawke et al 1981; Bullock and Herman 1985; Austin and Austin 1987; Thoesen 1994).

A. Summary of Screening Test

1. Bacterial Culture and Biochemical Analysis

a. Aseptically inoculate samples onto tubes or plates as described in 2.2 Sampling.

b. Incubate for 24 to 48 hours at 28 to 30°C. Alternatively, this organism can be grown at 20 to 24°C (Plumb et al. 1989). If no growth occurs at 24 and 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *E. ictaluri*.**

c. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

d. Incubate at the temperature used above for 24 hours to allow bacterial growth; all tests should be performed on 24 to 48 hour cultures.

e. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

f. Begin initial identification of pure strain bacterial cultures (3.A1 Laboratory Reference Flow Chart Appendix 1).

   i. Gram Determination (3.8.A "Gram Reaction")
   Edwardsiella ictaluri is Gram-negative. **Gram-positive isolates may be reported as negative for *E. ictaluri*.**

   ii. Presence of Cytochrome Oxidase (CO) (3.8.B "Cytochrome Oxidase")
   E. ictaluri is CO negative. **CO positive isolates may be reported negative for *E. ictaluri*.**

g. Perform biochemical testing on each isolate (3.A1 Laboratory Reference Flow Chart Appendix 1).

i. Tube Method (3.8.D.1 "Tube Method")

1. Triple Sugar Iron (TSI) (3.8.D.1.b "Triple Sugar Iron (TSI)")
E. ictaluri will yield an alkaline over acid (K/A) or alkaline over acid with gas (K/AG) result. **Any isolate with a result other than this may be reported as negative for *E. ictaluri*.**

2. Carbohydrate Utilization (3.8.D.1.e "Carbohydrate Utilization")
The following carbohydrates cannot be utilized (fermented) by *E. ictaluri*:

Arabinose
Rhamnose
Sucrose
Salicin

Isolates yielding positive results for any of these tests may be reported as negative for *E. ictaluri*.

3. Malonate Test (3.8.D.1.g "Malonate Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

4. Indole Test (3.8.D.1.d "Indole Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

5. Esculin Test (3.8.D.1.h "Esculin Test")
Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.

6. Decarboxylase Test (Lysine) (3.8.D.1.f "Decarboxylase Test (Lysine)")
Isolates yielding negative results for this test may be reported as negative for *E. ictaluri*.

7. Manitol Utilization Test (3.8.D.1.e "Carbohydrate Utilization")

a. **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri*.**

b. Isolates yielding negative results for this test and that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *E. ictaluri.*

ii. Commercial Identification Systems (3.8.D.2 "Commercial Identification Systems")

1. Biolog (3.8.D.2.b "Biolog")

2. API

If isolates are tested with the commercial system API described in 3.8.D.2.a "API-20E," it is recommended that the reference profiles be consulted in 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2."

3. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

4. Positive control isolates of *Edwardsiella ictaluri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org.

B. Summary of Confirmatory Test

1. **Fluorescent Antibody Test (FAT)** (3.8.E "Fluorescent Antibody Test (FAT)")
   FAT is performed on at least one representative isolate from each inspection. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.8.E.6 "Commercial Sources for Antibodies."

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 1987; Bullock and Herman 1988; Thoesen 1994).

A. Summary of Screening Test

1. **Fluorescent Antibody Test (FAT)** (3.8.E "Fluorescent Antibody Test (FAT)")

    a. Collect tissues as described in 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 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.

Note: Elliot 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 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 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 (2.2.E.2 "Collection of Kidney Cultures"), aseptically inoculate samples of tissues onto plates containing selective kidney disease media (SKDM-2) (3.7.A.3 "Selective Kidney Disease Medium-2 (SKDM-2)").

b. Incubate for 2 to 3 weeks at 15^{o}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. 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 µL ovarian fluid, into a 1.5 mL microcentrifuge tube. Tissue can be fresh or previously frozen.

2. Add 180 µL of lysozyme lysis buffer (3.7.G.1 "Lysozyme Lysis Buffer"). Incubate at 37°C for one hour, vortexing occasionally.

3. Add 25 µL of Proteinase K stock solution and 200 µL 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 µL 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 1minute 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 µL buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.

9. Repeat steps 7 and 8, using 500 µL 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 µL 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 µL. Discard spin column and store DNA solution at -20-70^{o}C until use.

12. Quantify the amount of DNA extracted with a spectrophotometer (see Chapter 6 Polymerase Chain Reaction (PCR)).

a. 25 to 50 mg of fish kidney tissue should produce between 100 and 300 ng DNA per µL using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.

b. 50 µL ovarian fluid produces a much lower amount of DNA per µL using this procedure. Dilute the template if DNA exceeds 300 ng/µL.

b. Initial Amplification of *R. salmoninarum* DNA

i. General QA/QC considerations must be considered before performing PCR (see Chapter 6 Polymerase Chain Reaction (PCR)).

ii. Procedures for initial round:

1. Using 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

2. Using 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 µL). 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 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 µL 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 µL 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 µL 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 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 Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process:

i. Using 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 µL).

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^{o}C for two minutes.

2. Denaturing at 93^{o}C for 30 seconds.

3. Annealing at 60^{o}C for 30 seconds.

4. Extending at 72^{o}C for one minute.

5. Post dwell at 4 to 16^{o}C for holding samples after cycling is complete.

Note: PCR Products can be refrigerated for one month or frozen at -70^{o}C for long-term storage.

d. Visualization of PCR Product by Electrophoresis
See 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 (6.3.C "Detection of Product").

ii. Using 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.**

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 (6.3.G "Visualize the DNA"). Photograph all gels and attach the photo to 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel. Attach to case history information**.**

3.6 *Piscirickettsia salmonis*

Piscirickettsia salmonis has primarily been reported as an emerging marine pathogen of salmonids (Bravo and Campos 1989; Fryer et al. 1990; Cvitanich et al. 1991; Brocklebank et al. 1992; Rodger and Drinan 1993; Grant et al. 1996; Cusack et al. 1997; Olsen et al. 1997), although there are reports of isolations in other species (Chen et al. 2000b) and in juvenile salmonids in freshwater (Bravo 1994; Gaggero et al. 1995). Additionally, there are an increasing number of reports on isolations of *P. salmonis*-like organisms from other fish species (Chern and Chao 1994; Chen et al. 1994; Chen et al. 2000a; Mauel et al. 2003). Clinical signs of disease and mortality have been reported in fish in marine net pens (Cvitanich et al. 1991; Branson and Diaz- Munoz 1991; Brocklebank et al. 1992; Olsen et al. 1997; Kent and Poppe 1998). The first evidence of disease may be the appearance of small white lesions or shallow hemorrhagic ulcers on the skin. Affected fish appear dark and lethargic. The major gross pathological changes are gill pallor, peritonitis, ascites, enlarged spleen, swollen gray kidney, and in some cases, a liver with large pale necrotic lesions.

A. Summary of Screening Tests

These techniques represent the best methods available for isolating and identifying *Piscirickettsia salmonis*, but they have not been demonstrated to be sensitive enough to detect a covert infection or a carrier state of infection. These methods are most effective in fish with clinical cases of infection.

1. **Culture of Obligate Intracellular Bacteria in Cell Culture** (Lannan and Fryer 1991)
   Piscirickettsia salmonis is a Gram-negative, non-motile, highly fastidious intracellular bacterial pathogen. *Piscirickettsia salmonis* does not replicate on bacteriological media, but instead it must be grown in cell culture, and therefore escapes detection by routine techniques used for bacterial isolation. Additionally, *in vitro*, this organism is sensitive to many antibiotics used in routine virus isolations, and will not grow even if inoculated onto suitable host cells if such compounds are included in the culture medium.

   a. Samples of kidney, spleen, liver and blood suitable for virology testing are aseptically collected from diseased fish during either overt or covert infections as described in 2.2.E.3 "Collection of Tissues for the Detection of Viral Agents." DUE TO SENSITIVITY OF *P. SALMONIS* TO ANTIBIOTICS *IN VITRO*, NONE SHOULD BE USED IN MEDIA DURING COLLECTION OF TISSUE OR THE CULTURE OF CELLS and because no antibiotics will be used, stringent aseptic techniques should be used to collect tissues. Musculature underlying skin lesions may also be a suitable tissue if collected aseptically by removing the contaminated outer skin layer.

   b. Tissue should be homogenized at 1/20 (w/v) in sterile antibiotic-free HBSS. Do not centrifuge. *Piscirickettsia salmonis* cells are bound in membranes and centrifugation will remove the bacteria from the supernatant. The 1:20 homogenate should then be further diluted 1/5 and 1/50 in antibiotic-free HBSS. These final two dilutions of the homogenate are the inocula for cell cultures, and are 1:100 and 1:1,000 dilutions of the original tissue.

c. The diluted homogenate can be inoculated directly (0.1 mL/culture per well of a 24-well plate) into the antibiotic-free culture medium overlaying the CHSE-214 cell monolayer (refer to 4.3 Cell Culture).

d. The cell cultures must be incubated at 15 to 18°C for 28 days and observed for the appearance of cytopathic effect (CPE). The piscirickettsial CPE consists of plaque-like clusters or rounded cells (Figure 3.1). With time, the CPE progresses until the entire cell sheet is destroyed. A drop of the suspect cell culture supernatant can be dried on a glass slide, fixed with methanol and stained with Giemsa stain (described below). Examine for presence of the bacteria.

e. If CPE does not occur (except in positive control) cultures should be incubated at 15 to 18°C for an additional 14 days.

f. If CPE is absent after the 42 day combined incubation period, samples are reported as negative and may be discarded using the proper decontamination procedures. If CPE associated with the bacteria occurs at any time during this assay, it is considered a PRESUMPTIVE positive and the identification of *P. salmonis* should be confirmed by the appropriate method.

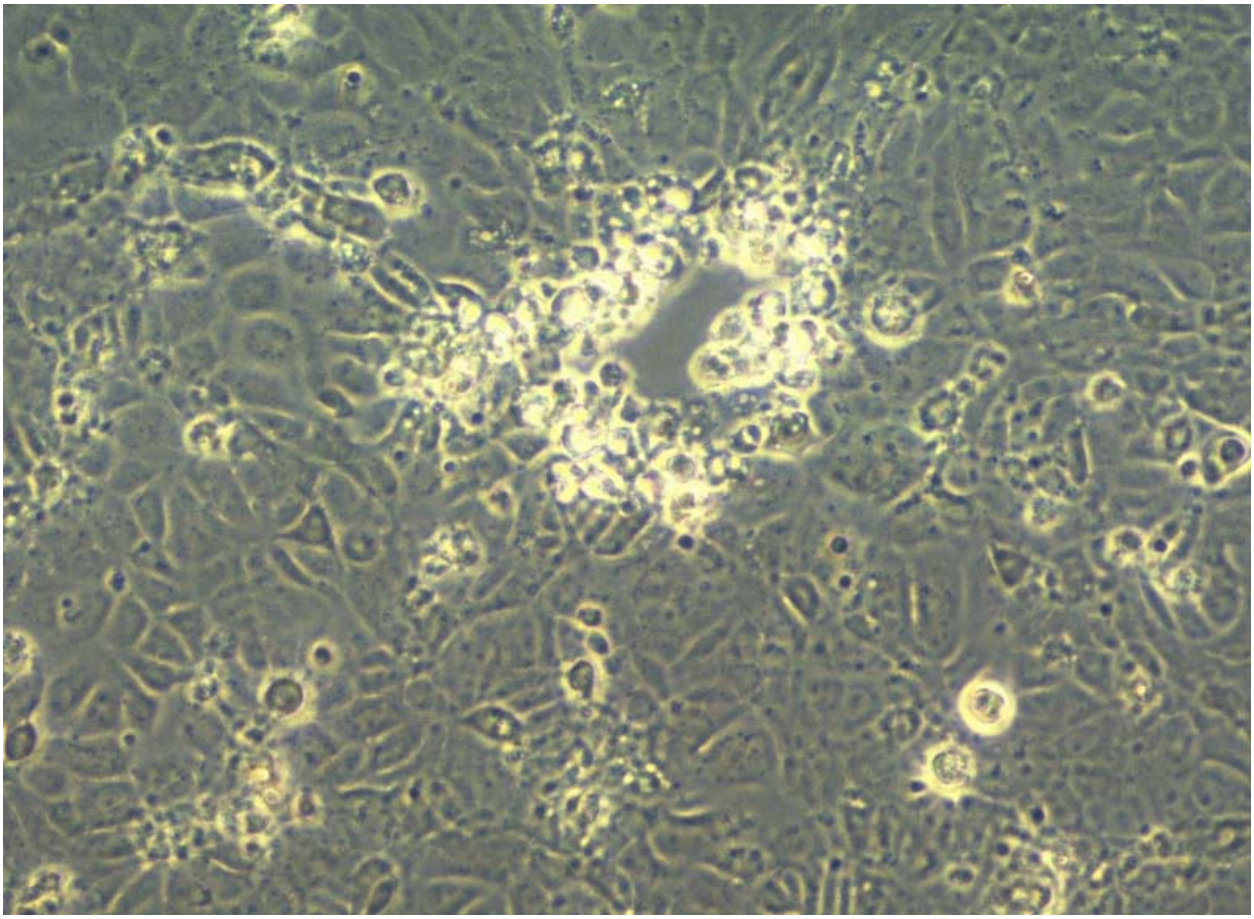


Figure 3.1. Cytopathic effect typical of *Piscirickettsia salmonis* on a monolayer of CHSE-214 cells. (Photo courtesy of Marcia House, NWIFC.)

2. Tissue Impressions of Kidney Stained with Giemsa Stain

Piscirickettsia salmonis can also be diagnosed based on clinical signs and detection in Giemsa stained tissue impressions, or sections (refer to **Note** below). It is pleomorphic, ranging from 0.5 to 1.5 μm in diameter and frequently is seen in pairs (Figure 3.2). Positive identification is confirmed by IFAT using *P. salmonis*-specific antibodies (3.8.E.3 "Indirect FAT (IFAT) Staining").

a. Preparations of tissue culture supernatant, smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.

b. Immerse slides in working solution of Giemsa stain for 30 minutes. Stock solution: 0.4 w/v in buffered methanol solution, pH 6.9 (commercially available). Working solution: diluted 1:10 in phosphate buffer pH 6.0 (0.074M NaH_2PO_4, 0.009M Na_2HPO_4).

c. Destain with tap water.

d. Observe slides under oil immersion. Examine a minimum of 50 fields. Tissue smears from infected organs show darkly stained pleomorphic organisms occurring in coccoid or ring forms, frequently in pairs, with a diameter of 0.5-1.5 μm (Figure 3.2). Visualization of organisms in smears is considered a PRESUMPTIVE positive result and the identification of *P. salmonis* should be confirmed by the appropriate confirmatory method.

Note: Every effort should be made to culture *P. salmonis* because it is the most sensitive screening method.

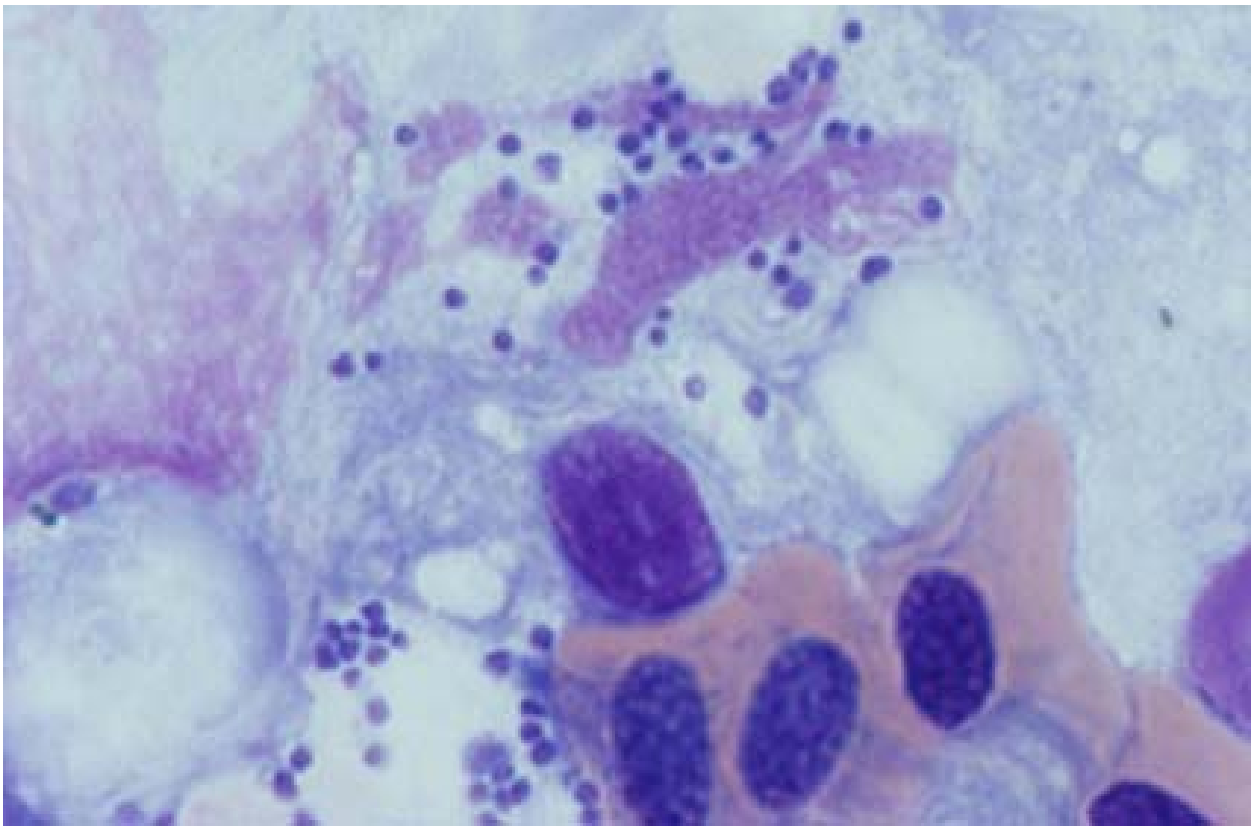


Figure 3.2. Giemsa stained liver impression from *Piscirickettsia salmonis* infected coho salmon (1000X magnification). Photo courtesy of Marcia House, NWIFC.

B. Confirmatory Tests

1. Serological Methods

The positive identity of *P. salmonis* isolated in cell culture or observed in Giemsa stained smears may be determined by serological methods, including IFAT or immunocytochemistry of histological sections.

a. Indirect Fluorescent Antibody Test (IFAT) (refer to 3.8.E.3 "Indirect FAT (IFAT) Staining") (Lannan et al. 1991)

i. Smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.

ii. Tissues smears to be examined by IFAT must be freshly prepared or stored at -20°C.

iii. The sample is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with fluorescein isothiocyanate (FITC).

iv. Following washing, apply glycerol-based mounting media and coverslip, then examine with a microscope equipped with epi-fluorescence.

b. Immunohistochemistry of Tissue Sections (Alday-Sanz et al. 1994)\

i. Sections (5 μm) of formalin fixed, paraffin-embedded tissues are deparaffinized and treated to eliminate endogenous peroxidase activity.

ii. The tissue is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with horse-raddish peroxidase.

iii. Following washing, the tissue is exposed to a chromagen (hydrogen peroxide and 3,3-diaminobenzidine), counterstained with hematoxylin, dehydrated and prepared for examination under a light microscope.

2. Polymerase Chain Reaction (PCR)

A nested polymerase chain reaction (PCR) has been developed to detect genomic DNA of *P. salmonis* using general bacterial 16S rDNA primers in the first amplification and *P. salmonis*-specific primers in a second reaction. A direct (single amplification) PCR can be run using the *P. salmonis* specific primers.

a. Preparation of Infected Cell Culture Supernatant or Tissue
Use of a commercially available spin column to purify DNA from cell culture supernatant or tissue is recommended for PCR sample preparation. In addition to following the manufacturer's instructions on use of the columns, initial digestion of the sample in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 4 mg/mL lysozyme) at 37°C for 30 minutes is suggested (refer to 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA").

i. The cell culture supernatant preparation:

1. Triturate the overlying cell culture media to disrupt the suspect cell culture, as if you were preparing a re-inoculation in a virology assay.

2. Transfer 0.2 mL of the suspension to a clean sterile 1.5 mL microcentrifuge tube and centrifuge at 4°C.

3. Discard the supernatant and treat the pellet as a tissue sample in the extraction procedure.

b. Nested Polymerase Chain Reaction (Mauel et al. 1996)

i. Initial amplification using eubacterial primers:

1. General QA/QC considerations must be reviewed before performing PCR (see Chapter 6 Polymerase Chain Reaction (PCR) for more specific QA/QC considerations for PCR).

2. Procedures for the initial round:

a. Using 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

b. Using 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record the 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 (45 µL). Add four to the number of samples so that there is enough to run controls.

c. Under a UV cabinet, add PCR reagents except for sample DNA to the MM tube in the order listed in 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis,* adding water first and Taq last. Keep all reagents cold during mixing, and return them to the freezer immediately after use. Do not expose enzymes, primers, or dNTP's to UV light.

i. Water to make a 45 µL total volume per reaction.

ii. 1X PCR buffer (10 mM Tris HCl, pH 9, 50 mM KCl, and 0.1% Trition X100).

iii. 1.5 mM $MgCl_2$.

iv. dNTP mix (0.2 mM).

v. Primers (1 mM each)

1) EubA 5'-AAG-GAG-GTG-ATC-CAN-CCR-CA-3'

2) EubB 5'-AGA-GTT-TGA-TCM-TGG-CTC-AG-3'

vi. Taq polymerase (2.5 units per reaction).

d. Place 45 µL of MM into each 0.2 mL PCR tube and close the caps tightly. Move PCR tubes to sample loading area.

e. In sample loading area, load 5 µL of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.

f. Load the sample tubes into the thermocycler wells.

g. The thermocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 50°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15°C after cycling is complete.

ii. "Nested" PCR-secondary amplification using *P. salmonis* specific primers.

1. Materials, methods, and general QA/QC considerations of this subchapter and Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process.

2. Procedures for the second round:

a. Using 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, 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 (48 µL).

b. Add PCR reagents except the first round amplified DNA to the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the initial with the exception of the primers, which are:

i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'

ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'

c. Place 48 µL of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.

d. Load 2 µL of amplified sample DNA into the appropriate PCR tubes.

e. Load the tubes into the thermocycler wells.

f. Thermocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 65°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15°C after cycling is complete.

c. Direct (single round) polymerase chain reaction.

i. Materials, methods and general QA/QC considerations of this subchapter and Chapter 6 Polymerase Chain Reaction (PCR) also apply to the direct PCR process.

1. Using 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, 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 (45 µL). Add four to the number of samples so that there is enough to run controls.

a. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the nested with the exceptions of the volume of water and primers. The primers are:

i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'

ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'

b. Place 45 µL of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.

c. Load 5 µL of sample DNA into the appropriate PCR tubes.

d. Load the tubes into the thermocycler wells.

e. The therrmocycler should be programmed for the following regime:

i. Denature the mixture at 94°C for two minutes.

ii. Amplify by 35 cycles of:

1) Denaturing at 94°C for one minute.

2) Annealing at 65°C for two minutes.

3) Extending at 72°C for three minutes.

4) Post-dwell at 4 to 15 °C after cycling is complete.

d. Visualization of PCR product by electrophoresis (see Chapter 6 Polymerase Chain Reaction (PCR) for general procedures).

i. Visualization of amplified products resulting from PCR for detection of *P. salmonis* DNA is best accomplished after electrophoresis on a 2% agarose gel (6.3C "Detection of Product").

ii. Using 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel, 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 (EubA/ EubB) and second (PS2S/ PS2AS) round PCR assays (first round (EubA/ EubB) band = 1540 bp, nested and direct PCR (PS2S/ PS2AS) = 476 bp). **Bands occurring at the PS2S/ PS2AS product locations are confirmatory of *P. salmonis* and are reported as POSITIVE.**

2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates that PCR should be re-run on samples from the extracted DNA tube.

v. Document the electrophoresis results (6.3.G "Visualize the DNA"). Photograph all gels and attach the photo to 3.A3.G Photodocumentation of the PCR Product Gel. Attach to case history information.

Note: Other PCR assays have been developed to detect *P. salmonis* (Marshall et al. 1998). These primer sequences and reaction conditions would also be suitable for confirmation of the presence of *P. salmonis*. Furthermore, additional diagnostic tests, such as transmission electron microscopy (TEM) and enzyme-linked immunosorbent assay (ELISA) (Aguayo 2001) may also aid in the detection of *P. salmonis*.

3.7 Reagents, Media, and Media Preparation

A. Growth Media

Most bacteriological culture medias are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Recipes for those media which are not available in a commercial preparation are included below.

1. **Brain Heart Infusion Agar (BHIA)** (Difco 1998)
   A basic agar for most bacterial cultures.

2. **Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB)** (Difco 1998)
   A basic media for most bacterial cultures.

3. **Selective Kidney Disease Medium-2 (SKDM-2)** (Austin et al. 1983)
   A media used for selective isolation of *Renibacterium salmoninarum*.

Peptone	10g
Yeast extract	0.5g
L-Cysteine HCL	1g
Agar	15g
Distilled water	874mL

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121^{o}C. Cool to ~ 50^{o}C and add:

Fetal Bovine Serum	100.0 mL

The following antibiotics are added to reduce overgrowth from other bacterial organisms):

4.0 mL Cycloheximide (1.2 g Cyclohexamide in 96 mL dH_2O)
1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH_2O)
2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of dH_2O)
1.0 mL Oxolinic acid (0.06 g Oxolinic acid in 24 mL of 5% NaOH)

B. Media to Identify Growth and Biochemical Characteristics

Most of these media are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Unless otherwise specified, these media can also be prepared from basic laboratory ingredients according to recipes found in the following references: MacFaddin 1980, Difco 1998, and MacFaddin 2000.

1. **Motility Test Medium**
   A semi-solid media used as a tube test to detect the ability of a microorganism to exhibit motility. Several types are commercially available, including MIO (motility, indole, ornithine) which allows for the detection of motility, and the reaction of two biochemical tests in the same tube.

2. **Trytic Soy Broth (TSB)**
   A nutrient broth media used to determine motility of a microorganism with the hanging drop method.

3. **Triple Sugar Iron Agar (TSI)**
   A commercially prepared dehydrated media used to evaluate the utilization of glucose and two additional carbohydrates, as well as the production of hydrogen sulfide.

4. **Oxidation/Fermentation (OF) Medium**
   A basal media for carbohydrate utilization tests, available in a commercially prepared dehydrated powder. The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

   a. To prepare final medium aseptically add 10 mL of a filter-sterilized (0.45 μm) 10% carbohydrate solution to autoclaved and cooled (50°C) media resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in a 0.5% final concentration (see below). Only one carbohydrate is added to the basal medium for each test to be run.

      10% Arabinose (1 g Arabinose to 10 mL in dH_2O)
      10% Rhamnose (1 g Rhamnose to 10 mL in dH_2O)
      10% Sucrose (1 g Sucrose to 10 mL in dH_2O)
      10% Sorbitol (1 g Sorbitol to 10 mL in dH_2O)*
      10% Maltose (1 g Maltose to 10 mL in dH_2O)
      10% Glucose (1 g Glucose to 10 mL in dH_2O)
      5% Salicin (0.5 g Salicin to 10 mL in dH_2O)

   b. Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2 to 8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

      *A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle (1985).

5. **Nutrient Gelatin**
   A dehydrated medium for determining the presence of Gelatinase.

6. **Tryptone Broth**
   For use with the indole test.

Tryptone	10 g
Distilled water	1000 mL

   Heat gently to dissolve. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Store at 2 to 8°C.

7. **Decarboxylase Medium Base**
   A basal media for use in Lysine test. The basal media, without addition of lysine, serves as the

control.

To make L-Lysine media add 5 g L-Lysine to 1 liter of prepare basal decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 $\pm$ 0.2 at 25°C. Store at 2 to 8°C.

8. **Malonate Broth**
   A media used for the malonate test.

9. **Bile Esculin Agar**
   A commercially prepared, dehydrated medium used to determine a bacterium's ability to hydrolyze esculin into glucose and esculetin.

C. Media Preparation

1. Plate Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

b. Cover beaker with foil or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121^{o}C for 15 minutes at 15 pounds pressure (consult with the manual provided by the autoclave manufacturer for adjustment of time when large volumes of media are being sterilized).

c. Cool media to 50°C.

d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date, and initials. When media is needed, boil, microwave, or use a water bath to completely melt the agar. Cool to 50°C.

e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Mix any added ingredients into the media at this temperature.

f. Label the bottom of each plate with medium type and date prepared.

g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per 100 X 15mm petri dish. Replace lids as soon as the plate is poured.

h. Invert plates when the media has cooled completely (~ 30 to 60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

i. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

j. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with the date of preparation.

2. Tube Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise, compensation for temperature needs to be made.

c. Arrange test tubes in racks. Disposable, autoclavable, screw cap tubes can be used for all tube media.

d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette, prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 mL media in 16 X 125 mm or 20 X 125 mm tubes. Close caps loosely.

e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

f. Loosely place screw caps on tubes. Do not tighten caps. It is necessary to allow pressure to release from tubes while heating in the autoclave.

g. Follow manufacturer's recommendation for autoclave time and temperature.

h. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA). Then tighten caps.

i. Cool completely to room temperature in the slanted position.

j. Label the tubes or the tube rack with type of medium and date made.

k. Store at 2 to 8°C, following manufacturer's recommendation for period of long-term storage.

D. Reagents

Most of these reagents are commercially available pre-made. These commercial products are entirely acceptable and should be stored according to the manufacturer's recommendations. The formulations provided below were obtained from the references cited.

1. Gram Stain Reagents

These stains can be ordered as a complete kit or can be prepared as follows:

a. Crystal Violet

Crystal violet (90% dye content)	20.0 g
Ethanol (95%)	200 mL
Ammonium oxalate	8.0 g
dH_20	800 mL

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

b. Gram's Iodine

Iodine crystals	1.0 g
Potassium iodide	2.0 g
dH_20	300 mL

c. De-Colorizer

Acetone	40 mL
Ethanol (95%)	60 mL

d. Safranin

Safranin O	2.5 g
95% ethanol	100 mL
dH_20	900 mL

Filter safranin solution before use.

2. FAT Mounting Fluid (pH 9.0)

Glycerol	90.0 mL
DABCO*	2.5 g
PBS	10.0 mL

Suspend the DABCO in glycerol over low heat. Then add 1X PBS (see below). Adjust pH to 8.6 to 9.0 with 1N hydrochloric acid or 0.1N sodium hydroxide. The pH of the mounting media is important, as an acid pH will quench fluorescence. Check the pH frequently. Store at room temperature.

* Optional ingredient - DABCO is 1,4-diazabicyclo-(2,2,2)-octane. Its addition to mounting fluid can reduce quenching of fluorescence.

3. Phosphate-Buffered Saline for FAT (PBS), pH 7.1

a. 1x concentration (0.15 M NaCl, 0.01 M phosphate; makes 1 L)

NaCl	8.50 g
Na_2HPO_4 (anhydrous)	1.07 g
$NaH_2PO_4 \cdot H_2O$ (monohydrate)	0.34 g
DH_2O	to 1 L

Adjust pH to 7.1 with 1N hydrochloric acid or 0.1N sodium hydroxide.

b. 5x concentration (makes 10 L of 1x PBS)

NaCl	85.00 g
Na_2HPO_4 (anhydrous)	10.70 g
$NaH_2PO_4 \cdot H_2O$ (monohydrate)	3.45 g
DH_2O	to 2 L

Adjust pH to 7.1 with 1N hydrochloric acid or 0.1N sodium hydroxide.

4. Kovac's Indole Reagent

Isoamyl alcohol	30 mL
p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric acid (HCl)	10mL

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2 to 8°C in amber dropper bottle.

5. Counter Stains

a. Rhodamine
Rehydrate the rhodamine to 1 mg/mL in distilled water. If the rhodamine does not completely dissolve, add a small drop of 0.1 M sodium hydroxide. Store at 2 to 8°C.

b. Evans Blue
Prepare 0.1% stock solution with distilled water and decontaminate with 0.45 µm membrane filtration. Store at room temperature. Prepare a 0.01% working dilution with sterile PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") (Cvitanich 1994).

c. Eriochrome Black T
Prepare a solution at 1:60 (w/v) in PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1"), and filter through Whatman #1 and Whatman # 42 filter papers before initial use (Elliott & McKibben 1997).

6. FITC Conjugated Rabbit Anti-X /Rhodamine Counter Stain

Rhodamine stock	10.0 µL
FITC conjugated antibodies	10.0 µL
Phosphate buffered saline	480.0 µL

Store at 4°C.

Note: Evans Blue or Eriochrome Black T can also be used as counterstain in FAT in a separate step during the staining process, but neither can be added directly to the conjugate solution.

E. Cytochrome Oxidase Spot Test

Individual test strips can be purchased from several suppliers (Catalogue # 38-191, Remel, Tel. 800-255-6730).

F. Determination of Antiserum and Conjugate Working Dilutions for FAT

Commercially prepared anti-sera and conjugates should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 mL can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome on positive control FAT preparations. Generally, the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases, each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or

testing dilutions on either side of the recommended concentration. The following example shows how to determine the correct working dilution of FAT conjugate where the manufacturer recommends a working dilution of 1:40.

1. Using the stock solution, dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended by antibody manufacturer.

2. Rhodamine counter stain is added directly to the optimum antiserum working dilution at a 1:50 (alternatively, Evans Blue may be used to counterstain FAT stained slides – do not add Evans Blue to the conjugate directly).

3. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way, the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

4. Prepared conjugate solutions should be filtered through 0.2 to 0.4 micron filter prior to use and storage. Store frozen in small aliquots. Do not thaw and re-freeze antibodies repeatedly.

G. PCR Reagents

The following are formulations for extraction reagents used specifically in the protocols described in 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA."

1. Lysozyme Lysis Buffer

100 mL (this formulation is used for lysis of gram positive bacteria with Qiagen extraction kits).

Lysozyme	2 g
Tris HCl Stock	2 mL
EDTA stock	2 mL
Triton	1.2 mL

Bring these components to 100 mL with sterile distilled water (molecular grade).

2. Tris HCl Stock Solutions

100 mL at 1M pH 8.0 (for use in lysozyme buffer).

Trizma base	5.7 g
Tris HCl	8.9 g
dH2O	85.4 mL

3. EDTA Stock Solution

100 mL at 0.1 M (for use in lysozyme buffer).

EDTA	3.72 g

Bring to 100 mL with sterile distilled water (molecular grade).

3.8 Bacterial Identification Techniques

Unless otherwise specified, all of the materials and techniques described in 3.8 Bacterial Identification Techniques are described in detail in MacFaddin's (2000) and or the 11th Edition Difco Manual (1998). Each of the tests listed are provided with a set of control bacterial species available from ATCC, which will provide quality control for each biochemical test. It is not necessary, however, to set up control isolates for every test run in these protocols. It is strongly suggested that newly prepared batches of media and reagents be tested using the control bacterial isolates listed for each.

A. Gram Reaction

Gram staining detects a fundamental difference in the cell wall composition of bacteria.

1. **Gram Stain**
   (Kits are available commercially, or formulas for reagents are listed in 3.7.D "Reagents.")

   a. Prepare a bacterial smear from a pure culture.

      i. Put a drop of saline, distilled water, PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1"), or formalin saline (0.4% formalin, 0.85% NaCl) on a clean glass slide.

      ii. Using a sterile loop or needle, touch an isolated colony and mix in the water drop.

      iii. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).

      iv. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.

      v. Allow to cool.

   b. Flood the slide with crystal violet (3.7.D.1.a "Crystal Violet"), and allow to remain on the slide for 60 seconds.

   c. Wash off the crystal violet with running tap water.

   d. Flood the slide with Gram's iodine (3.7.D.1.b "Gram's Iodine"), and allow to remain on the slide for 60 seconds.

   e. Wash off with running tap water.

   f. Decolorize with decolorizer solution (3.7.D.1.c "De-Colorizer") until the solvent flows colorless from the slide (approximate 5 to 10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading. Too little decolorization can result in a false positive result.

g. Rinse immediately with running tap water.

h. Counter stain with safranin (3.7.D.1.d "Safranin") for 60 seconds.

i. Rinse with tap water and allow to air dry.

j. Results

   i. Gram-Negative
   Cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counter stained with safranin.

   ii. Gram-Positive
   Cells retain the crystal violet and remain purple to dark blue.

k. Quality Control
   Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2 to 8°C for long term use).

   i. Positive
   Staphylococcus sp. (ATCC – any isolate)

   ii. Negative
   Yersinia ruckeri

   iii. Commercially prepared Gram stain control slides are available (Fisher Scientific, #08-801).

2. 3% Potassium Hydroxide
Alternative test for Gram reaction.

a. Add a heavy inoculum of a pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mL distilled water) on a clean glass slide.

b. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

c. Results

   i. Gram-Positive
   Bacteria will not appear to change the viscosity of the KOH solution.

   ii. Gram-Negative
   Bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency.

d. Quality Control

Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2 to 8°C for long term use).

i. Gram-Positive
Staphylococcus sp.

ii. Gram-Negative
Yersinia ruckeri

B. Cytochrome Oxidase

See 3.7.E "Cytochrome Oxidase Spot Test." This test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of a pure 18 to 24 hour old bacterial culture to the surface of the test strip impregnated with reagent.

2. Results

a. Positive
Purple color within 5 to 10 seconds (reactions that occur after 10 seconds are negative).

b. Negative
No purple color.

3. Quality Control

a. Positive
Pseudomonas aeruginosa (ATCC 10145)

b. Negative
Yersinia ruckeri

c. Observe expiration dates of reagent strips.

C. Motility

This test determines if a bacterial isolate is motile by means of flagella.

1. Hanging Drop Method

a. Inoculate a tryptic soy agar (TSA) slant or tryptic soy broth (TSB (3.7.A.2 "Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) (Difco 1998)")) with the isolate. Note: Use suitable medium for those organisms that do not grow on TSA or in TSB (e.g., yellow-pigmented organisms).

b. Incubate at room temperature until growth is obtained, usually 24 hours.

c. For isolates grown on agar, place a drop of sterile distilled water or PBS onto the center of a clean cover slip. Inoculate the center drop with pure strain culture using a sterile loop. For isolates grown in broth, use a sterile loop or sterile dropper and place a drop in the center of a clean clover slip.

d. Carefully invert the cover slip and place over the concave portion of a hanging drop slide.

e. Observe for motility at 400X magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility.

f. Record results as motile or non-motile. Note: If the hanging drop slide was prepared from an isolate grown on agar and the bacterium appears to be non-motile, an additional hanging drop test using TSB (or other suitable broth medium) or semi-solid medium method must be done to confirm true non-motility.

2. Semi-Solid Medium Method
Refer to 3.7.B.1 "Motility Test Medium."

a. Stab the semi-solid medium with a small amount of inoculum.

b. Incubate overnight at room temperature.

c. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

d. Confirmation of results using the hanging drop method is recommended.

3. Quality Control

a. Positive
Escherichia coli (ATCC 25922)

b. Negative
Aeromonas salmonicida

D. Biochemical Testing

1. Tube Method

a. Glucose Fermentation
An OF basal medium (3.7.B.4 "Oxidation/Fermentation (OF) Medium") is used to test the fermentation of glucose by bacterial isolates.

i. With a sterile needle, inoculate two tubes of OF-glucose by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

1. Fermentation Test
One tube is over-layed with sterile mineral oil or paraffin. Sterile petroleum jelly (heated to melting) should be used for more accurate observation of gas production.

2. Oxidation Test
The second tube is not overlayed.

ii. Incubate at 20 to 24^{o}C and read after 18 to 24 hours.

	Fermentation Test Tube	**Oxidation Test Tube**
Fermentative	A or AG	A or AG
Oxidative	N	A or AG
Non-reactive	N	N

iii. Results
A = Acid (yellow)
AG = Acid + Gas
N = No change or Alkaline (green or blue-green).

iv. Quality Control

1. Fermentative
Aeromonas species

2. Oxidative
Pseudomonas fluorescens

b. Triple Sugar Iron (TSI)
TSI agar (3.7.B.3 "Triple Sugar Iron Agar (TSI)") contains the three sugars in varying concentrations: glucose (1X), which is a simple monosaccharide, and lactose and sucrose (10X each), both of which are disaccharides. It also contains the pH indicator phenol red. All organisms that utilize glucose will yield an initial acidic reaction throughout the tube (yellow - see below) regardless of whether they utilize sucrose or lactose. Reversion will not occur until all the glucose in the slant portion is completely utilized. At this point, the bacterium will utilize either one or both of the disaccharides, continuing the production of acids in the media (the slant remains yellow). If, after all the glucose in the slant is used and the bacterium cannot utilize either lactose or sucrose, the bacterium is forced to revert to protein (peptone) present in the agar. In this case, nitrogenous bi-products are produced and the pH in the media rises until the pH indicator shows a reversion from yellow (acid) to red (alkaline). Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

i. With a sterile needle, inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

ii. Incubate at 20 to 24°C. Read after 18 to 24 hours.

iii. Results

A = Acid
K = Alkaline
H_2S= Hydrogen sulfide produced

Slant / Butt	Color	Interpretation
K / N or K / A	Red / Orange	Only Peptone Utilized
	Red/ Yellow	Only Glucose Fermented
A /A	Yellow /Yellow	Glucose plus Lactose and/or Sucrose Fermented
Gas	Splitting or Bubbles	Gas Production
H_2S	Black Butt	Hydrogen Sulfide Produced

N = No change

iv. Quality Control

1. K/A
Shigella flexineri (ATCC 12022)

2. A/AG
Escherichia coli (ATCC 25922)

c. Gelatinase
A test to determine bacterial production of gelatinase enzymes that liquefy gelatin.

i. Inoculate by stabbing ½ to 1 inch deep into the nutrient gelatin media (3.7.B.5 "Nutrient Gelatin") with a heavy inoculum from an 18 to 24 hour pure culture.

ii. Incubate 18 to 24 hours at 20°C.

iii. Results

1. Positive
Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

2. Negative
No liquefaction occurs in media.

iv. Quality Control

1. Positive
Proteus vulgaris (ATCC 8427)

2. Negative
Escherichia coli (ATCC 25922)

v. Precautions

1. The liquid will generally appear turbid due to bacterial growth.

2. Nutrient gelatin softens at temperatures above 20°C. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35°C, should be refrigerated prior to recording results.

d. Indole Test
A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole), and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.

i. Inoculate tryptone broth (3.7.B.6 "Tryptone Broth") with a light inoculum from an 18 to 24 hour pure culture.

ii. Incubate 24 to 48 hours at 20°C

iii. At the end of 24 hours incubation do the following:

1. Aseptically remove 2 mL of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.

2. Add about 5 drops of Kovac's reagent (3.7.D.4 "Kovac's Indole Reagent") to one of the tubes and agitate tube.

3. If a positive reaction is observed, the test is complete.

4. If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

iv. Results

1. Positive
Within 1 to 2 minutes, a cherry red ring will form at the surface of the media.

2. Negative
No color formation is observed at the surface; the color remains that of the reagent – yellow.

3. Variable
An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

v. Quality Control

1. Positive
Escherichia coli (ATCC 25922)

2. Negative
Pseudomonas aerugenosa (ATCC 27853)

vi. Precautions

1. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

2. Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.

3. Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!

4. Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.

5. The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

e. Carbohydrate Utilization (MacFaddin 1980)
The following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.

i. Inoculate carbohydrate tube (3.7.B.4 "Oxidation/Fermentation (OF) Medium") with growth from an 18 to 24 hour pure culture.

ii. Incubate with loosened cap 18 to 24 hours at 20^{o}C. A prolonged incubation of up to four days may be necessary for some negative results.

iii. Results

1. Positive
Acid is produced from fermentation, which turns media yellow.

2. Negative
No fermentation of carbohydrate, media remains green.

3. Aerogenic
Gas bubbles are present within the media.

iv. Quality Control

Carbohydrate	Positive Control Isolate	Negative Control Isolate
Arabinose	*Escherichia coli* (ATCC 25922)	*Yersinia ruckeri*
Sorbitol	*Escherichia coli* (ATCC 25922)	*Y. ruckeri* Type I
Rhamnose	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*
Salicin	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*
Maltose	*Yersinia ruckeri*	*Pseudomonas fluorescens*

v. Precautions

1. Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.

2. Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

f. Decarboxylase Test (Lysine)
A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

i. For each isolate to be tested, it is necessary to inoculate a decarboxylase control tube and lysine test tube (3.7.B.7 "Decarboxylase Medium Base"). Use light inoculum from 18 to 24 hour pure culture.

ii. Add 1 to 2 mL oil overlay to each tube.

iii. Incubate 24 hours at 20^{o}C. A prolonged incubation of up to four days may be necessary.

iv. Results

Test Result	Lysine Tube	Control Tube
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented)	Yellow
Negative	Purple (glucose not fermented, decarboxylase not produced	Purple (glucose not fermented)

v. Quality Control

1. Positive
Yersinia ruckeri

2. Negative
Enterobacter cloacae (ATCC 13047)

vi. Precautions

1. At the end of incubation, the lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.

2. An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.
3. Do not interpret tests prior to 18 to 24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

g. Malonate Test
A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.

i. Inoculate malonate media (3.7.B.8 "Malonate Broth") with a light inoculum from an 18 to 24 hour pure culture.

ii. Incubate 24 to 48 hours at 20^{o}C.

iii. Results

1. Positive
Light blue to deep blue color throughout the media.

2. Negative
Color remains the same as un-inoculated tube - green.

iv. Quality Control

1. Positive
Enterobacter aerogenes (ATCC 13048)

2. Negative
Yersinia ruckeri

v. Precautions
The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

h. Esculin Test
To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).

i. Inoculate the surface of the bile esculin slant (3.7.B.9 "Bile Esculin Agar") with inoculum from an 18 to 24 hour old pure culture.

ii. Incubate 20°C for 24 to 48 hours.

iii. Results. Note: Bile Esculin Agar was originally designed for isolation and presumptive characterization of Group D Streptococcus spp. As the bile salts inhibit nearly all other Gram-positive bacteria. The presence of bile in the medium does not affect differentiation of esculin by A. salmonicida; this medium is used because it is commercially available and easily prepared.

1. Positive
Dark brown to black color diffuses into the medium beginning at the site of inoculation. Often, the entire slant and butt become colored.

2. Negative

Bacterial growth is present on the slant, but the color of the medium remains similar to un-inoculated.

iv. Quality Control

1. Positive
Enterobacter aerogenes (ATCC 13048)

2. Negative
Yersinia ruckeri

v. Precautions
False positives may occur with hydrogen sulfide producing organisms, such as S*hewanella putrefaciens.* Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

2. Commercial Identification Systems

Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. Therefore, it may be necessary to refer to the charts in 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2 for identification when API strips are employed.

a. API 20E™
The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a microtube system designed for the performance of 23 standard biochemical tests from isolated colonies on plating medium. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179). Reference charts of API profiles for *A. salmonicida* and *Y. ruckeri* are listed in 3.A2 Profiles Obtained with API-20E for Known Fish Pathogens Appendix 2.

b. Biolog
MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

E. Fluorescent Antibody Test (FAT)

The Fluorescent Antibody Test (FAT) is one serological method for corroboration testing of bacterial isolates. FAT can be performed either with a direct antibody staining (DFAT) or indirect (IFAT) technique, depending upon the availability of pathogen-specific FITC-conjugated or unconjugated antibody preparations from the manufacturers listed below (3.8.E.6 "Commercial Sources for Antibodies"). There are three basic steps for FAT: preparing and fixing bacterial samples; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the Slides

a. Pure Bacterial Cultures
(Confirmatory testing of pure isolates of *A. salmonicida*, *Y. ruckeri,* or *E. ictaluri.*)

i. Pure isolates of bacteria are diluted in sterile PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") and applied to two replicate wells of an FAT slide.

ii. Allow air-drying completely or heat fix.

b. Kidney
(For presumptive detection of *R. salmoninarum*) – prepare kidney smear on a non-coated or acetone-cleaned glass slide.

i. Homogenize a piece of posterior kidney tissue and create a thin smear on the surface of a slide.

ii. Allow to air dry completely or heat fix.

c. Ovarian Fluid Pellet Smear
(DFAT-for presumptive detection of *R. salmoninarum*) – ovarian fluid is collected (2.2.E.2.f).

i. After pooled ovarian fluid samples are processed and appropriate amount of supernatant removed for virology assays (4.4.C "Processing of Coelomic (Ovarian) Fluid Samples"), the pellet is re-suspended in the remaining supernatant by thorough vortexing or repeat pipetting.

ii. Transfer two 1.5 mL aliquots from each pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL micro-centrifuge tubes (see **Note**). Freeze the remainder of the sample at -20^{o}C for PCR confirmation.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes.

Note: Elliot 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 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.

iv. The pellet is carefully re-suspended and a thin smear prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

v. Allow to air dry completely or heat fix.

d. After the tissue is completely air dried or heat fixed, slides are fixed in acetone for five minutes. Other fixing solutions are suitable as long as a component of the solution contains a lipid-dissolving reagent such as acetone or xylene, which helps remove lipids and improve the overall fluorescence quality and intensity.

2. Direct FAT (DFAT) Staining (Thoesen 1994)

Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).

a. Positive and Negative Controls
Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in confirmation testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate (3.7.D.6 “FITC Conjugated Rabbit Anti-X/Rhodamine Couter Stain”) on each sample slide and control slides.

c. Incubate for 30 to 60 minutes at room temperature, according to manufacturer’s recommendation.

d. Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (3.7.D.3 “Phosphate-Buffered Saline for FAT (PBS), pH 7.1”).

e. If rhodamine counterstain has not been incorporated in the FAT stain, apply the counterstain of choice (3.7.D.5 “Counter Stains”) at this point for 1-2 minutes.

f. Final rinse/soak in PBS for 5 to 10 minutes. Air-dry completely.

g. Add a small drop of FA mounting fluid, pH 9 (3.7.D.2 “FAT Mounting Fluid (pH 9.0)”) to each slide, being careful not to touch the dropper to the slide to prevent the possibility of cross contamination.

h. Place a 24 x 50 mm cover slip over the slide using care not to trap air bubbles.

i. Spread the mounting fluid by gently pressing the cover slip with the blunt end of a pen or lab marker.

j. Add one drop of immersion oil to the cover slip and examine at 1000X using the epifluorescent filter.

3. Indirect FAT (IFAT) Staining (Thoesen, 1994)

IFAT is a double layered antibody technique, where the first layer consists of unconjugated, purified immunoglobulin (IgG) or antibody prepared in one animal species (e.g. rabbit) against the target antigen. The second antibody applied is a FITC-conjugated antibody prepared in a second animal species (e.g. goat), and specific for IgG of the first animal species (e.g. goat anti-rabbit IgG). Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).

a. Positive and Negative Controls
Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

b. Place slides in dark, humidified chamber, and place one drop of unconjugated, pathogen specific antibody on each sample slide and control slides.

c. Incubate at room temperature for 30 to 60 minutes, according to manufacturer's recommendations.

d. Gently rinse slides with PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1").

e. Place a drop of FITC-conjugated second antibody on slides and incubate at room temperature in dark chamber according to manufacturer's recommendation.

f. Rinse briefly with PBS. If rhodamine counterstain has not been incorporated into the FAT stain, apply the counterstain of choice (3.7.D.5 "Counter Stains") at this point for 1 to 2 minutes.

g. Rinse and wash in PBS for 5 to 10 minutes.

h. Air dry completely and apply a small drop of FA mounting fluid, pH 9 (3.7.D.2 "FAT Mounting Fluid (pH 9.0)"). Apply a cover slip to mounting fluid.

i. Add one drop of immersion oil to the coverslip and examine at 1000X using the epifluorescent filter.

4. Reading Results

a. Slides are read at 1000X on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence

microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells. Positive fluorescence appears "apple green" in color.

i. Pure Culture Bacterial Confirmatory Testing
Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control. Negative isolates will not fluoresce.

ii. *R. salmoninarum* Screening
Examine at least 50 fields. Positive bacterial cells are 1.0 X 0.5 μm, which fluoresce. Negative smears will not fluoresce.

iii. *P. rickettsia* Screening
Examine at least 50 fields. Positive bacterial cells are 0.5 to 1.5 μm in diameter, pleomorphic, occur in coccoid or ring forms which fluoresce. Observation of fluorescing cells within host tissue cells provides strong evidence of *P. salmonis* infection. Negative smears will not fluoresce.

5. Hints for Good Results

a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (3.7.F "Determination of Antiserum and Conjugate Working Diutions for FAT").

b. Filter all conjugated antibody reagents (.45 μm filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.

c. Prepare thin smears; thick smears will not fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

d. Evenly distribute the kidney material in PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") or use a very light inoculum of pure bacterial culture for each well (excess bacteria will stain poorly).

e. Heat-fix slides prior to fixing in acetone. If there is not possible to heat-fix the slides they can be air-dried and sent to the lab without fixation. Fixed slides should be stored refrigerated until stained.

f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process. Fixing solutions should be completely changed, at a minimum, between separate inspection cases and/or when positive results occur.

g. Use anhydrous acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

6. Commercial Sources for Antibodies

a. Kirkegaard and Perry Laboratories, Inc.
2 Cessna Court, Gaithersburg, MD 20879-4145 USA
Phone: 800/638-3167, 301/948-7755
Web Site: http://www.kpl.com

Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.

b. Microtek International, LTD (Bayotek)
6761 Kirkpatrick Crescent, Saanichton, B.C., CA
Phone: 250-652-4482
Web Site: http://www.microtek-intl.com

Antibodies available: Polyclonal antibodies available for Indirect FAT for he following bacterial pathogens: *Renibacterium salmoninarum, Piscirickettsia salmonis, Aeromonas salmonicida,* and *Yersinia ruckeri* serotypes 1 and 2.

3.9 Glossary

Acute - a disease having rapid onset, severe symptoms and a relatively short course – not chronic.

Anneal - the attachment of oligonucleotide primers to a specific site on a single stranded DNA segment.

Aseptic - the nature of preventing contamination of foreign microorganisms.

Basal media - a media formulation to which additional components may be added for a particular test.

Carbohydrate - a particular sugar used in a test.

Chronic - describes the course of a disease which is long and drawn out – not acute.

Commercially prepared - a component or test, which is available from a commercial source for purchase.

Confirmatory identification - identification of a pathogen through the completion of both initial screening techniques and another confirmatory test as described in this document, the results of which concur with each other for positive identification of a particular organism.

Counterstain - a stain used in FAT to achieve a dark background color of tissues and materials stained, aiding in the observation of fluorescing bacterial cells.

Decolorize - the application of a solution to stained material to remove excessive stain.

Denature - the enzymatic/temperature dependent activity which converts double stranded DNA to single stranded DNA.

DNA extraction - the process of obtaining pure, double stranded DNA from sample tissues and materials.

Elution - the washing of DNA from a spin column filter membrane.

Extension - the synthesis of a new, copied segment of DNA following denaturing and annealing processes involved with PCR.

Fermentation - bacterial utilization of a compound in the absence of oxygen.

Filtrate - the resultant liquid obtained from filtration.

FITC - fluorescein isothiocyanate, a reagent, which is used as an antibody label for the fluorescent antibody test.

FITC-conjugated (antibody) - describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

Lysate - the product solution of tissue lysis.

Nested-PCR - a second PCR is performed, targeting a sub-segment of DNA produced in the first round PCR specific for a particular organism.

Oxidation - bacterial utilization of a compound in the presence of oxygen.

Pellet - the product of sedimentation of solid materials from a liquid resulting from centrifugation.

Phenotypic characterization - the detectable expression of a bacterial isolate to environmental conditions, biochemical testing and morphological observations recorded for the purpose of differentiation and determination of the species of bacteria.

Polymerase chain reaction (PCR) - a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

Positive control isolates - bacterial isolates may be obtained from the American Type Culture Collection (ATCC: http://www.atcc.org).

Post-dwell - the period of time after cycles are completed in PCR.

Presumptive identifications - identification of a pathogen through the completion of initial screening tests as described in this document, where no other test has been performed to confirm the positive identification of the organism.

Primary culture - bacterial cultures achieved from media inoculated directly from fish tissues.

Primer - oligonucleotides that, in the presence of DNA and excess dNTP's, hybridize specifically to a target sequence and "prime" new DNA synthesis.

Pure bacterial culture - a culture of bacteria originating from an isolated colony.

Quality control - taking steps to assure that testing and results are accurate and reliable.

Selective media - a medium containing ingredients, which may either exclude growth of some microorganisms, select for growth of a particular species of microorganism, or both.

Serological methods - detection methods, which employ the use of an antibody against the target organism.

Spin column - a small filter unit provided by many commercial DNA extraction kits used to bind, wash, and elute DNA from tissue lysates.

Stock suspension - suspension of a compound, which must be further diluted before direct application in a particular test.

Sub-acute - a course of disease which exhibits some acute and some chronic symptoms.

Subculture - the transfer of an established bacterial culture to an uninoculated medium.

Supernatant - the surface fluid resulting from centrifugation of a liquid.

Working suspension (dilution) - suspension of a compound at the correct dilution for direct application in a particular test.

Yellow-pigmented bacteria - bacterial isolates which exhibit a yellow pigmentation on colony formations (e.g. *Flavobacterium* species).

3.10 References

Aguayo, J., A. Miquel, N. Aranki, A. Jamett, P. D. T. Valenzuela, and L. O. Burzio. Detection of *Piscirickettsia salmonis* in fish tissues by an enzyme-linked immunosorbent assay using specific monoclonal antibodies. Diseases of Aquatic Organisms 49:33-38.

Alday-Sanz, V., H. Rodger, T. Turnbull, A. Adams, and R. H. Richards. 1994. An immunohistochemical diagnostic test for rickettsial disease. Journal of Fish Diseases 17:189-191.

Austin, B., D. Bucke, S. Feist, and J. Rayment. 1985. A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* with a 'coryneform' organism. Bulletin of European Association of Fish Pathologists 5:8-9.

Austin, B., and D. A. Austin. 1987. Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Ellis Horword LTD, West Sussex, England.

Austin, B., T. M. Embley, and M. Goodfellow. 1983. Selective isolation of *Renibacterium salmoninarum*. FEMS Microbios Letters 17:111-114.

Branson, E. J., and N. Nieto Diaz-Munoz. 1991. Description of a new disease condition occurring in farmed coho salmon, *Oncorhynchus kisutch* (Walbaum), in South America. Journal of Fish Diseases 14:147-156.

Bravo, S. 1994. Piscirickettsiosis in freshwater. Bulletin of European Association of Fish Pathologists 14(4):137-138.

Bravo, S., and M. Campos. 1989. Coho salmon syndrome in Chile. AFS/FHS Newsletter 17(3):3.

Brocklebank, J. R., D. J. Speare, R. D. Armstrong, and T. Evelyn. 1992. Septicemia suspected to be caused by a rickettsia-like agent in farmed Atlantic salmon. Canadian Veterinary Journal 33:407-408.

Brown, L. L., T. P. T. Evelyn, G. K. Iwama, W. S. Nelson, and R. P. Levine. 1995. Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene of *R. salmoninarum* as detected by the polymerase chain reaction (PCR). Diseases of Aquatic Organisms 21:227-231.

Bullock, G. L., B. R. Griffin, and H. M. Stuckey. 1980. Detection of *Corynebacterium salmonis* by direct fluorescent antibody test. Canadian Journal of Fisheries and Aquatic Sciences 37:719-721.

Bullock, G. L., R. C. Cipriano, and S. F. Snieszko. 1983. Furunculosis and other diseases caused by *Aeromonas salmonicida*. Fish Disease Leaflet 66, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D. C. 20240.

Bullock, G. L. 1984. Enteric redmouth disease of salmonids. Fish Disease Leaflet 67, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D. C. 20240.

Bullock, G. L., and R. L. Herman. 1985. *Edwardsiella* infections of fishes. Fish Disease Leaflet 71, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D. C. 20240.

Bullock, G. L., and R. L. Herman. 1988. Bacterial kidney disease of salmonid fishes caused by *Renibacterium salmoninarum.* Fish Disease Leaflet 78, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D. C. 20240.

Chapman, P. F., R. C. Cipriano, and J. D. Teska. 1991. Isolation and phenotypic characterization of an oxidase-negative *Aeromonas salmonicida* causing furunculosis in coho salmon (*Onchorhyncus kisutch*). Journal of Wildlife Diseases. Cvitanich, J. D 27:61-67.

Chase, D. M., and R. J. Pascho. 1998. Development of a nested polymerase reaction for amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Diseases of Aquatic Organisms 34:223-229.

Chen, S. C., M. C. Tung, S. P. Chen, J. F. Tsai, P. C. Wang, R. S. Chen, S. C. Lin, and A. Adams. 1994. Systemic ganulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochronuis niloticus* (L.), from southern Taiwan. Journal of Fish Diseases 17:591-599.

Chen, S. C., P. C. Wang, M. C. Tung, K. D. Thompson, and A. Adams. 2002a. A *Piscirickettsia salmonis*-like organism in grouper, *Epinephelus melanostigma* in Taiwan. Journal of Fish Diseases 23:415-418.

Chen, M. F., S. Yun, G. D. Marty, T. S. McDowell, M. L. House, J. A. Apperson, T. A. Guenther, K. D. Arkush, and R. P. Hedrick. 2000b. A *Piscirickettsia salmonis*-like bacteriurm associated with mortality of white seabass *Atractoscion nobilis*. Diseases of Aquatic Organisms 43:117-126.

Chern, R. S., and C. B. Chao. 1994. Outbreaks of a disease caused by a rickettsia-like organism in cultured tilapias in Taiwan. Fish Pathology 29(2):61-71.

Cipriano, R. C., and J. B. Pyle. 1985. Development of a culture medium for determination of sorbitol utilization among strains of *Yersinia ruckeri*. Microbios Letters 28:79-82.

Cipriano, R. C., C. E. Starliper, and J. H. Schachte. 1985. Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. Journal of Wildlife Diseases 21(2):144-148.

Cusack, R., D. Groman, and S. Jones. 1997. The first reported rickettsial infections of Atlantic salmon in Eastern North America. (Abstract) European Association of Fish Pathologists, VIIIth International Conference. "Diseases of Fish and Shellfish," Sept. 14-19, 1997, Edinburgh, Scotland.

Cvitanich, J. D., N. O. Garate, and C. E. Smith. 1991. The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. Journal of Fish Diseases 14:121-145.

Cvitanch, J. D. 1994. Improvements in the direct fluorescent antibody technique for the detection, identification, and quantification of *Renibacterium salmoninarum* in salmonid kidney smears. Journal of Aquatic Animal Health 6(1):1-12.

Difco Manual, 11th Ed. 1998. Difco Laboratories, Division of Becton Dickinson & Co., Sparks, Md. Available on the Internet at the following website: http://www.bd.com/industrial/difco/DifcoManual.pdf

Elliott, D. G., and T. Y. Barila. 1987. Membrane filtration-fluorescent antibody staining procedure for detecting and quantifying *Renibacterium salmoninarum* in coelomic fluid of chinook salmon (*Oncorhynchus tshawytscha*). Canadian Journal of Fisheries and Aquatic Sciences 44:206-210.

Elliott, D. G., and C. L. McKibben. 1997. Comparison of two fluorescent antibody techniques (FATs) for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid of spawning chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 30:37-43.

Fryer, J. L., C. N. Lannan, L. H. Garcès, J. J. Larenas, and P. A. Smith. 1990. Isolation of a rickettsiales-like organism from disease coho *Oncorhynchus kisutch* in Chile. Fish Pathology 25(2):107-114.

Gaggero, A., H. Castro, and A. M. Sandino. 1995. First isolation of *Piscirickettsia salmonis* from coho salmon, *Onchorhynchus kisutch* (Walbaum), and rainbow trout, *Onchorhynchus mykiss* (Walbaum), during the freshwater stage of their life cycle. Journal of Fish Diseases 18:277-279.

Grant, A. N., A. G. Brown, D. I. Cox, T. H. Birkbeck, and A. A. Griffen. 1996. Rickettsia-like organism in farmed salmon. Letter in Vet Rec 138(17): 423.

Hawke, J. P., et al. 1981. *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish. International Journal of Systematic and Evolutionary Bacteriology 31:396-400.

Kent, M. L., and T. T. Poppe (eds). 1998. Diseases of seawater netpen-reared salmonid fishes. Canadian Special Publication of Fisheries and Aquatic Sciences 116. Dept. Fisheries and Oceans, Nanaimo, B. C.

Lannan, C. N., S. A. Ewing, and J. L. Fryer. 1991. A fluorescent antibody test for detection of the rickettsia causing disease in Chilean salmonids. Journal of Aquatic Animal Health 3:229-234.

Lannan, C. N., and J. L. Fryer. 1991. Recommended methods for inspection of fish for the salmonid rickettsia. Bulletin of European Association of Fish Pathologists 11:135-136.

MacFaddin, J. F. 2000. Biochemical Tests for Identification of Medical Bacteria, Third Ed. Lippincott Williams & Wilkins, 227 East Washington Square, Philadelphia, PA 19106.

Marshall, S., S. Heath, V. Henriquez, and C. Orrego. 1998. Minimally invasive detection of *Piscirickettsia salmonis* in cultivated salmonids via the PCR. Applied Environmental Microbiology 64(8):3066-3069.

Mauel, M. J., S. J. Giovannoni, and J. L. Fryer. 1996. Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. Diseases of Aquatic Organisms 26:189-195.

Mauel, M. J., D. L. Miller, K. Frazier, A. D. Liggett, L. Styer, D. Montgomery-Brock, and J. Brock. 2003. Characterization of a piscirickettsiosis-like disease in Hawaiian tilapia. Diseases of Aquatic Organisms 53:249-255.

Olsen, A. B., H. P. Melby, L. Speilberg, O. Evensen, and T. Hastein. 1997. *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway-epidemiological, pathological, and microbiological findings. Diseases of Aquatic Organisms 31(1):35-48.

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 Veterinary Diagnostic Investigation 10:60-66.

Pascho, R. J., D. G. Elliott, R. W. Mallett, and D. Mulcahy. 1987. Comparison of five techniques for the detection of *Renibacterium salmoninarum* in coho salmon. Transactions of the American Fisheries Society 116(6):882-890.

Paterson, W. D. et al. 1980. Relationships between selected strains of typical and atypical *Aeromonas salmonicida, A. hydrophila*, and *Haemophilus piscium*. Canadian Journal of Microbiology 26:588-598.

Plumb, J. A., and S. Vinitnantharat. 1989. Biochemical, biophysical, and serological homogeneity of *Edwardsiella ictaluri.* Journal of Aquatic Animal Health 151-56.

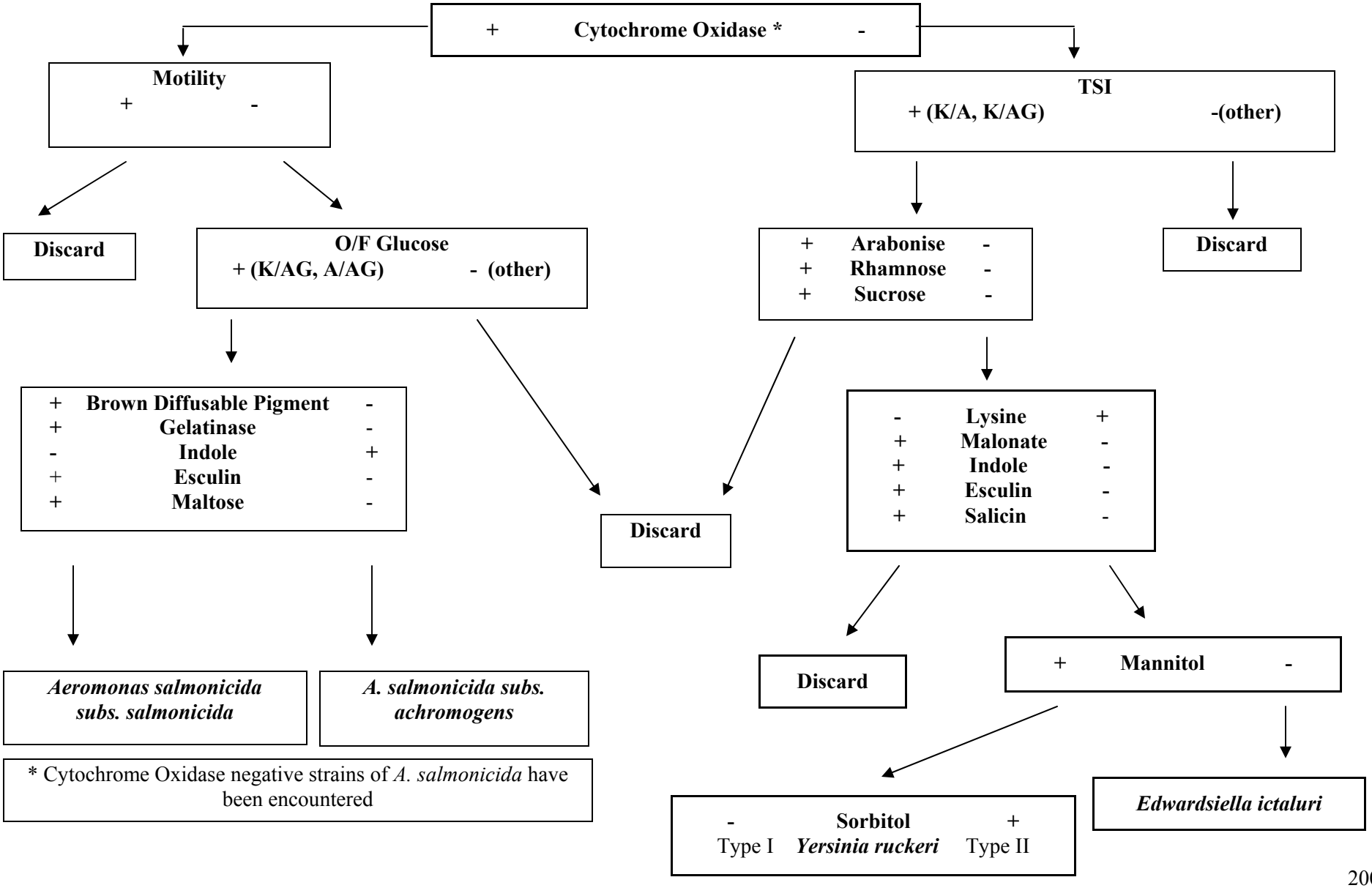
Plumb, J. A., and C. Shoemaker. 1995. Effects of temperature and salt concentration on latent *Edwardsiella ictaluri* infections in channel catfish. Diseases of Aquatic Organisms 21:171-175.

Rodger, H. D., and E. M. Drinan. 1993. Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L., in Ireland. Journal of Fish Diseases 16:361-369.

Sanders, J. E., and J. L. Fryer. 1980. *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease in salmonid fishes. International Journal of Systematic and Evolutionary Bacteriology 30: 496-502.

Thoesen, J. C., editor. 1994. Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th edition, Version 1. Fish Health Section, American Fisheries Society.

3.A1 Laboratory Reference Flow Chart for Identification of Gram-Negative Bacterial Pathogens Which Grow on TSA or BHIA and are Targeted for Detection to Complete Fish Health Inspection Requirements



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3.A2 Profiles Obtained with API-20E for Known Fish Pathogens

The following table represents API20E profiles for ***Yersinia ruckeri*** when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35 to 37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database (National Wild Fish Health Survey Lab Procedures Manual, USFWS).

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1. *Serratia marcescens* 2. *Serratia liquefaciens* 3. *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1. *Serratia marcescens* 2. *Serratia liquefaciens* 3. *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1. *Escherichia coli* 2. *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	"Acceptable ID" 1. *Hafnia alvei*
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	"Very good ID" 1. *Yersinia ruckeri*
Coho, Quilcene R., WA (11/88)	**5104000**	"Very good ID" 1. *Yersinia ruckeri*
Unknown source	**5100100**	"Excellent ID" 1. *Yersinia ruckeri*
Unknown source	**4105100**	1. *Hafnia alvei*
Unknown source	**4104100**	1. *Yersinia ruckeri* 2. *Salmonella gallinarum*
Unknown source	**4104000**	"Acceptable ID" 1. *Yersinia ruckeri*
Unknown source	**0104100**	"Acceptable ID" 1. *Yersinia ruckeri*

* *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22 to 25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-27.

The following represents API20E profiles for ***Aeromonas salmonicida*** isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**Computer/Manual ID**
spp not identified, Makah NFH, WA (8/88)	**0006104**	"Acceptable ID" 1. *Pseudomonas pseudomaleae*
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	0006104	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	0006104	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	0006104	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	1. *Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	"Good to Excellent ID" 1. *Aeromonas salmonicida*

**Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22 to 25° C).

Profiles provided in the API Manual are based on positive reactions for some or all of these first three biochemical tests, therefore the first digit of the "acceptable" profiles for *A.salmonicida* include the values 2,4, or 6. More often, a zero value is obtained after 24 to 48 hours incubation at room temperature. Longer incubation periods are required for these tests.

References

Romalde, J. L., and A. E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. Bulletin of European Association of Fish Pathologists 11(4), 147.

Kent, M. L. 1982. Characteristics and identification of Pastuerella and Vibrio species pathogenic to fishes using API-20E (Analytab Products) multitube test strips. Canadian Journal of Fisheries and Aquatic Sciences 39:1725-1729.

Toranzo, A. E., Y.Santos, T. P. Nieto, and J. L. Barja. 1986. Evaluation of different assay systems for identification of environmental Aeromonas strains. Applied Environmental Micorbiology 51:652-656.

3.A3.A Worksheet A – PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue Type_____

Tissue Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

3.A3.B Worksheet B – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 40 µL		30.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		20 pMole	20 pMole/µL	1	
(-)Primer		20 pMole	20 pMole/µL	1	
TAQ		2 units/Rx	5U/µL	0.4	
DNA±		-	-	10 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

±Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *R. salmoninarum* 1st Round Amplification

P3 (round 1 forward)	5'-A GCT TCG CAA GGT GAA GGG-3'
M21 (round 1 reverse)	5'-GC AAC AGG TTT ATT TGC CGG G-3'

3.A3.C Worksheet C – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 49 µL		39.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.4 mM	10 mM	1	
(+)Primer		20 pMole	20 pMole/µL	1	
(-)Primer		20 pMole	20 pMole/µL	1	
TAQ		2 units/Rx	5U/µL	0.4	
DNA$^{\pm}$ (round 1)		-	-	1 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *R. salmoninarum* 2nd (Nested) Round Amplification

P4 (round 2 forward)	5'-AT TCT TCC ACT TCA ACA GTA CAA GG-3'
M38 (round 2 reverse)	5'-C ATT ATC GTT ACA CCC GAA ACC-3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)
Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 45 µL		35.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
DNA$^{\pm}$		-	-	5 µL	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

$^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 1st Round Amplification

EubA (round 1 forward)	5'- AAG-GAG-GTG-ATC-CAN-CCR-CA -3'
EubB (round 1 reverse)	5'- AGA-GTT-TGA-TCM-TGG-CTC-AG -3'

3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 48 µL		38.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
DNA± (round 1)		-	-	2 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. ±Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 2nd (Nested) Round Amplification

PS2S (223F) (round 2 forward)	5'- CTA-GGA-GAT-GAG- CCC-GCG-TTG -3'
PS2AS (690R) (round 2 reverse)	5'- GCT-ACA-CCT-GAA-ATT-CCA-CTT -3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.F Worksheet F – Direct Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		Add to total 45 µL		35.0	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5 mM	50 mM	1.5	
dNTP's		0.2 mM	10 mM	1	
(+)Primer		1 mM	1 mM	1	
(-)Primer		1 mM	1 mM	1	
TAQ		2.5 units/Rx	5U/µL	0.5	
DNA$^{\pm}$		-	-	5 µL	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent mixing (MM) area.

Primer Sets for *P. salmonis* 2nd Direct Amplification

PS2S (223F) (round 2 forward)	5'- CTA-GGA-GAT-GAG- CCC-GCG-TTG -3'
PS2AS (690R) (round 2 reverse)	5'- GCT-ACA-CCT-GAA-ATT-CCA-CTT -3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel

Case Number _____ Date _____

Samples _____

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

AFFIX PHOTODOCUMENTATION

Notes: