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 Section 2, 3.7.D "Reagents.")

a. Prepare a bacterial smear from a pure culture.

i. Put a drop of saline, distilled water, PBS (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 Section 2, 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 (Section 2, 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 Section 2, 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 (Section 2, 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.

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

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

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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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*

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 (Section 2, 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 (Section 2, 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 (Section 2, 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 Section 2, 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 Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 2.2.E.2.f).

i. After pooled ovarian fluid samples are processed and appropriate amount of supernatant removed for virology assays (Section 2, 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°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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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 (Section 2, 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.