

Standard Operating Procedure (SOP)

Title: Direct Fluorescent Antibody Test (DFAT) for the detection of *Renibacterium salmoninarum* in tissues

Number: BACT-3

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Approval:	Date:
Bacteriology Supervisor:	December 15, 2011

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Reason for Revision:	Correction to rinse procedure
Area of Application:	

Purpose: Detection of *Renibacterium salmoninarum* cells in fish tissues or culture

Sections:

- I. Reference
- II. Reagents
- III. Procedure

Disclaimer:

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I. Reference

- Bullock, G.L., B.R. Griffin, and H.M. Stuckey. (1980) Detection of *Corynebacterium salmoninarum* by direct fluorescent antibody test. Canadian Journal of Fisheries and Aquatic Sciences. 37:719-721.
- Pascho, R.J., D.G. Elliott, and J.M. Streufert. 1991. Brood stock segregation of spring chinook salmon *Oncorhynchus tshawytscha* by use of the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody technique (FAT) affects the prevalence and levels of *Renibacterium salmoninarum* infection in progeny. Diseases of Aquatic Organisms 12:25-40.

II. Reagents

PBS pH 7.1 plus thimerosal: Recipe for 1 liter of 1X phosphate buffered saline (PBS) pH 7.1 containing thimerosal as preservative.

- Add measured amounts into a 2 L bottle
 - o NaCl 8.5 g
 - o NaH₂PO₄ · H₂O (monohydrate) 0.34 g
 - o Na₂HPO₄ (anhydrous) 1.07 g
- Deionized H₂O to 1.0 L
- Mix with stir bar and confirm pH is 7.1
- Add thimerosal as preservative
 - o Thimerosal 0.1 g

FITC conjugated antibody: *Fluorescein*-labeled affinity purified antibody to *Renibacterium salmoninarum* produced in goat.

- Purchase commercially from KPL[§] (Part #02-96-91)
- Rehydrate following manufacturer's instructions (1 mL of reagent quality water)
- Add the 1 ml of antibody solution to 39 ml of PBS pH7.1 containing thimerosal for 1:40 working dilution
- Filter through a 0.2 µm syringe filter into a dark colored (or foil covered) bottle and store at 4°C
- Label bottle with dilution and date (will keep for at least a year)
- Re-filter if high background fluorescence is observed in slides

Eriochrome Black T counterstain: Eriochrome black T varies in purity depending on vendor and lot. We purchase this product from Sigma^{§§} (part # E-2377) which has ~65% dye content.

To make 1:60 concentration:

- Add measured amounts to a bottle
 - o Eriochrome Black T 12 grams
 - o Deionized H₂O 720 ml
- Store in dark or foil covered bottle at 4°C
- Shake before use

1X Voller PBS pH 7.4 with thimerosal: Recipe for 1X Voller phosphate buffered saline (PBS) pH 7.4 with thimerosal for diluting the Glycerol-DABCO mounting medium below.

- Add measured amounts into a 2 L bottle
 - o NaCl 8.0 g
 - o KH₂PO₄ 0.2 g
 - o Na₂HPO₄ (anhydrous) 1.09 g
 - o KCL 0.2 g
 - o Deionized H₂O to 1.0 liter
- Stir to dissolve and confirm pH 7.4
- Add thimerosal as a preservative
 - o Thimerosal 0.1 g

Glycerol-DABCO mounting medium: Glycerol-1,4-diazabicyclo-(2,2,2)-octane (DABCO) mounting medium pH 8.6-9.0.

- Add measured amounts of DABCO to the glycerol
 - o Glycerol 90 ml
 - o DABCO 2.5 g
- Dissolve the DABCO by heating the DABCO-glycerol mixture gently in a water bath (~ 37°C)
- Add measured amount of 1X Voller PBS with thimerosal
 - o 1X PBS, pH 7.4 10 ml
- Adjust the pH to 8.6-9.0 by adding 0.1 N HCl or 0.1 N NaOH as necessary.
- Store at room temperature in a dark or foil covered bottle

[§]Vendors:

Kirkegaard and Perry Laboratories Inc. (KPL); 1-800.638.3167; www.kpl.com
Sigma-Aldrich 1-800-325-3010; www.sigmaaldrich.com

III. Procedure

1. **Create slides.** An 8 mm non-coated dual-welled slide is typically used. Acetone-clean the slide if necessary.
 - a. **Kidney tissue: Homogenize tissue and use a moistened (PBS) sterile swab to smear kidney onto slide.**
 - i. **Let tissue dry and heat fix by flaming the underside briefly so that it is warm (not hot) to touch.**
 - ii. **Acetone fix by soaking slide in acetone bath for 5 min.** Change acetone frequently to avoid debris buildup
 - b. **Bacterial culture (colony): Place a drop of deionized water in a slide well then use an inoculating loop to transfer a bit of a colony into the water.**
 - i. **Let dry and heat fix by flaming the underside briefly.**
2. **Add 50µl/well of the FITC conjugated antibody diluted 1:40 in PBS pH 7.1.** Gently tap the sides of the slide to ensure antibody completely covers the well.
3. **Place slides in a humidified chamber and incubate at room temperature for 1 hour in the dark.**
4. **Rinse and counterstain slides.** Use of a staining rack is recommended, but the slides should be protected from direct light during the rinsing and counterstaining procedures.
 - a. **Kidney tissue:**
 - i. **Rinse slides in PBS pH 7.1 plus thimerosal.** Quickly rinse slides twice by carefully flooding with PBS and then pouring off by tilting slides. Rinse again twice more but leave PBS on slides for 3 min per rinse before pouring off.
 - ii. **Counterstain slides using a 1:60 (w/v) solution of Eriochrome Black T by flooding slides for 10 seconds.**
 - iii. **Rinse in PBS pH 7.1 plus thimerosal.** Follow instructions outlined in step i.
 - iv. **Perform final rinse by flooding slides with deionized H₂O to remove all PBS.**
 - b. **Bacterial culture (colony):**
 - i. **Rinse twice in deionized water.** Flood slide with water and then pour off.
5. **Allow slides to air dry completely.**

6. **Mount a cover glass using a small drop of DABCO-glycerol medium on each well.**
7. **Examine slides by epifluorescence microscopy using 100x oil immersion objective (1000x total magnification).** Count the number of bacteria in 100 fields (50 fields per well). Scan the wells using an across down across pattern to ensure that no field is counted twice.
 - a. If slides cannot be read immediately, then store overnight at 4°C.