Standard Operating Procedure (SOP)

Title: Membrane-Filtration Fluorescent Antibody Test (MF-FAT) for the detection of *Renibacterium salmoninarum*

Number: **BACT-4**

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Approval:	Date:	Signature:
Bacteriology Supervisor:	January 10, 2012	William K. Purcell

Effective Date:	January 10, 2012
Document Replaced:	
Reason for Revision:	Addition of filtration step to PBS-triton
Area of Application:	

Purpose:

Detection of *Renibacterium salmoninarum* in fish fluids or broth culture

Sections:

I. References

II. Reagents

III. Filters

IV. Procedure

Disclaimer:

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I. References

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.M. 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.

II. Reagents

PBS pH 7.1 + 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
  - NaCl 8.5 g
  - $NaH_2PO_4 \cdot H_2O$ (monohydrate) 0.34 g
  - Na_2HPO_4 (anhydrous) 1.07 g
- Deionized H_2O to 1.0 L
- Mix with stir bar and confirm pH is 7.1
- Add thimerosal as preservative
  - Thimerosal 0.10 g

PBS pH 7.1 + Triton: Recipe for 1 liter of 1X phosphate buffered saline (PBS) pH 7.1 + thimerosal containing 0.5% (v/v) Triton X-100.

- Add 5.0 mL of Triton X-100 to 1 L of 1X PBS pH 7.1 + thimerosal
- Pass through a 0.2 µm filter

Trypsin Solution: Recipe for 1% (w/v) trypsin solution.

- Add measured ingredients to flask
  - Trypsin 1:250 powder 1.0 g
  - Deionized H_20 100 mL
- Mix trypsin with water at 4°C
- Filter through Whatman No. 1 filter paper
- Filter through a 0.2 µm syringe filter
- Dispense small aliquots and freeze at -20°C to -80°C
- Trypsin will retain activity for 1-2 months at -20°C; long-term storage at -80°C
- Thaw new aliquots of trypsin each day as needed; do not re-freeze

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)

- Dilute the 1 ml of antibody solution to 39 ml of PBS pH 7.1 + thimerosol 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 (re-filter if high background fluorescence is observed on slides)
- Diluted antibody is stable for at least one year at 4°C

Eriochrome Black T counterstain: Eriochrome black T varies in purity depending on vendor and lot. We purchase this product from Sigma[§§] (part # E 3277) which has ~65% dye content.

To make 1:2000 working concentration*:

- Add measured amounts to a bottle
  - Eriochrome Black T 0.25 grams
  - PBS pH 7.1 500 mL
- Filter through Whatman No.1 followed by Whatman No. 42 filter papers
- Store in a dark or foil covered bottle at room temperature or 4° C

*Our laboratory uses Eriochrome black T for MF-FAT at a dilution of 1:2,000, but this may vary depending on the lot of stain used. Check the stain quality on test filters. The prepared filters should be a definite purple color, but should not be so heavily counterstained that they are nearly black (or stain clogs the filter). If the counterstain appears too dark, dilute further with PBS pH 7.1 until desired color is obtained.

1X Voller PBS pH 7.4: 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
  - NaCl 8.0 g
  - KH_2PO_4 0.2 g
  - Na_2HPO_4 (anhydrous) 1.09 g
  - KCL 0.2 g
- Deionized H_20 to 1.0 L
- Stir to dissolve and confirm pH 7.4
- Add thimerosal as preservative
  - 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
  - Glycerol 90 mL
  - DABCO 2.5 g
- Dissolve the DABCO by heating the mixture gently in a water bath (~37°C)
- Add measured amount of PBS
  - 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

III. Filters

Filter holder: Whatman[§] pop-top filter holders (part #420100).

Polycarbonate filter: Whatman[§]13mm, 0.2 µm polycarbonate filter (part #110406)*.

*The polycarbonate filter should be shiny side up (toward the syringe) in the filter holder. This is the smoother surface and therefore the best surface for counting bacteria. The orientation of the filters in the package should be designated in the manufacturer's instructions. You can also hold the filter up to the light with a pair of sterile tweezers to identify the shiniest side. Once the correct side is located, write a letter or a word such as "up" on the edge of the filter before placing it in the holder. Write carefully with a ball point pen, as close to the edge of the filter as possible (being careful not to damage the filter). This will ensure that the correct side faces up when the filters is taken out of the holders and placed on slides.

Nylon membrane filter: A supporting 13mm, 5.0 µm nylon membrane filter (GE Magna Nylon Membrane Filters part # R50SP01300[§]).

*The polycarbonate filters are thin and frequently develop "ridges" identical to the ridges on the support screens of the filter holders. Placing a thicker nylon membrane filter between the polycarbonate filter and the support screen helps reduce the formation of ridges and therefore making the surface smoother for easier observation and counting of bacteria. The nylon filter should be placed on top of the polycarbonate filter or on the "downstream" side of the polycarbonate filter. The support filter also helps promote an even flow of substrate through the filters and adds strength to prevent tearing or breakage of the weaker polycarbonate membranes during filtration. The use of a support filter with a much larger pore size (e.g. 5µm) minimizes the effect the support filter has on the filtration rate. Make certain the filters are lined up and lying flat before assembling the rest of the holder. To prevent any leakage, the clamp ring should be firmly seated as far down the holder as possible.

[§]Vendors:

- Kirkegaard and Perry Laboratories Inc. (KPL); 1-800.638.3167; www.kpl.com
- Sigma-Aldrich; 1-800-325-3010; www.sigmaaldrich.com
- Whatman: www.whatman.com; available through a variety of vendors
- GE Osmonics; www.geosmolabstore.com; available through a variety of vendors

IV. Procedure

1. **Assemble filter holders and place filters inside.** Label each of the holders to keep track of the sample number (e.g. with a small piece of lab tape).

2. **Process samples**
   a. **<u>Ovarian Fluid</u>: Thaw and vortex mix 0.5 ml of selected ovarian fluid sample**. Ovarian fluid samples should be stored frozen (-20° to -80°C) until processing. Always include a negative and positive control:
   – *<u>Negative control</u>: l ml PBS-Triton + 0.5 ml trypsin*
   – *<u>Positive control</u> : 0.1 ml known positive sample or R. salmoninarum-spiked sample + 0.9 ml PBS-Triton + 0.5 ml trypsin*
      i. **Combine ovarian fluid with 0.5 mL of PBS pH 7.1 + Triton and 0.5 ml of trypsin solution.** Vortex mix for 20-30 seconds.
      ii. **Heat mixtures in 50°C water bath for 10 min.**
      iii. **Triturate the sample three times using a 3 ml syringe fitted with 22 gauge x 1-1/2" needle.**
      iv. **Pull the entire sample into the syringe and remove the needle.**

   b. **<u>Broth Culture</u>**: **Centrifuge cells at 5000 x *g* for 20 min at 4°C.** See BACT-1 SOP for *R. salmoninarum* media recipes.
      i. **Re-suspend pellet in diluent of choice**. e.g. 1X PBS-peptone (see BACT-1 SOP for recipe). Triturate repeatedly with a pipet to break up *R. salmoninarum* clumps.
      ii. **Make serial 10-fold dilutions in diluent.**
      iii. **Subject several dilutions to MFAT analysis.** We typically analyze the 10^{-4}, 10^{-5}, and 10^{-6} dilution but this will depend on starting concentration of culture.
      iv. **Pull 1 mL of diluted bacterium into a syringe**.

3. **Attach the syringe containing the sample to the prepared holders containing filters and force samples through the filter with the syringe.** A slow steady pressure on the syringe handle is best for especially viscous samples, pushing too fast can cause the syringe to separate from the filter holder resulting in loss of sample.

4. **Rinse the filter with 3 mL of PBS pH 7.1 +Triton**. Force buffer through the filter using the syringe.

5. **Remove syringe and discard, leave the filter in the holder, and pipette 100 µl of FITC-conjugated antibody onto the filter.** Use a separate tip for each filter and be careful not to puncture the filter when applying antibody. Tilt the filter holder to ensure that the entire surface gets covered by antibody.

6. **Incubate filter upright in a dark humid chamber for 1 hour at room temperature.**

7. **Rinse filter again with 3 mL of PBS pH 7.1 + Triton by forcing the PBS through the filter with a new syringe**.

8. **Counterstain with 1 mL of diluted Eriochrome Black T by forcing it through the filter with the syringe.** Typical dilution of Eriochrome Black T is 1:2000 but see notes regarding this reagent in section II.

9. **Remove the polycarbonate filter from the holder.** Disassemble the holder and separate the polycarbonate filter from the support filter. Discard support filter.

10. **Place filter on a microscope slide and air dry completely**. Transfer label from holder to microscope slide. Make sure to place the filter with the correct side facing up.

11. **Place one drop of the DABCO-glycerol mounting medium onto the center of the filter, then mount a cover glass.**

12. **Examine the slides with a microscope equipped for FITC epifluorescence.** Read 150 fields per filter, or if you reach 200 bacteria in fewer than 150 fields, then stop and record number of bacteria counted in total number of fields examined. If there are obviously too many bacteria to count then record as TNTC.

13. **Filter counts of the number of *R. salmoninarum* in a given number of fields can be converted to cells/ ml of the original ovarian fluid sample according to the formula:**

$$\text{cells/ml}^{*} = \frac{(\text{conversion factor}^{\S})\ \times\ (\text{dilution factor})\ \times\ (\text{total number of cells counted})}{\text{total number of fields examined}}$$

*To calculate the theoretical sensitivity of the technique for the desired number of fields to be examined, enter the value '1' for the 'the total number of cells counted'.

§The conversion factor is the filtering surface area divided by the area of a single field at the magnifications used. We have determined that the filtering surface area using the Whatman filter holders and polycarbonate filters described above is 78.5398 mm^2. To calculate the conversion factor you will need to know the field surface area for your specific microscope at 1000X magnification. Use a stage micrometer divided into 0.01or 0.02 mm increments to measure the diameter of a field at 1000X. Then calculate the surface using the area of a circle formula (πr^2).

Example:
If the measured field diameter is 0.17 mm, then the radius is 0.085 mm.
The surface area = 3.1416 x 0.085^2 = 0.0227mm^2
The conversion factor for this microscope will be 78.5398 ÷ 0.0227 = 3460.

If you are using a different filter holder, then the filtering surface area should be calculated. This can be accomplished by filtering 1 ml of the Eriochrome Black-T solution through the chosen setup and measuring the diameter of the colored area on the filter. Calculate the surface area used in filtration with the area of a circle formula (πr^2).