CHAPTER 14

Non-Lethal Methodology for Detection of Fish Pathogens

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I. Introduction

Fish health plays a key role in monitoring, evaluating and protecting the health of all aquatic animals within an ecosystem whether it relates to restoring depleted populations or the recovery of threatened and endangered (T&E) species. For this reason, fish species involved in special recovery projects and those of special management concern (T&E) have been targeted as a high priority for the Fish Health Program to address in the National Wild Fish Health Survey (Survey).

A. ENDANGERED SPECIES ACT

The Endangered Species Act of 1973 was enacted to protect and enhance the recovery of endangered (in danger of becoming extinct) and threatened (likely to become endangered) species. The Act prohibits harmful actions to any endangered or threatened plant or animal species. Increasing numbers of fish species are being listed under the Endangered Species Act (ESA). Biologists participating in the Survey will be responsible for the proper treatment of T&E species that are captured for disease sampling and/or other reasons, such as population monitoring.

B. PERMITTING

Permitting - The Secretary of the Interior, through the Regional Directors of the U.S. Fish and Wildlife Service, may issue permits for the taking and possession of T&E species, under certain circumstances.

Fish Health work, whether lethal or non-lethal to the fish collected, may be considered a harmful action which is governed by this permitting process. All personnel involved in collection of samples for the Survey should contact their regional ESA permitting office or recovery team to determine how the Act applies to the work they will be performing, should T&E species be encountered. Decisions on lethal or invasive sampling techniques discussed herein should be made with the participation of the collecting field biologists and the specific recovery team involved.

For more detail, see 50 CFR 17, Code of Regulations, 50 CFR 17.22-17.32 and the Endangered Species Act.

C. VALIDITY AND SENSITIVITY

Many concerns must be considered when evaluating results from non-lethal versus lethal fish health sampling protocols:

1. Sensitivity - in many cases detection of pathogens from blood serum is less sensitive than internal tissues. In other cases, detection of an organism may be enhanced by utilization of mucus or serum.

2. Validity - specific protocols for detection of fish pathogens using non-lethal sampling means are scarce, and many of those that are utilized have not been

validated. Clearly, more research must be accomplished to improve sensitivity and validity of non-lethal detection protocols available in the literature.

3. Statistical concerns - most of the stocks being considered for non-lethal fish health sampling are valuable and/or few in number, further reducing the sensitivity for detection of any particular pathogen which may be carried by members of that population.

II. Non-lethal Assay Methods to Employ

A. GENERAL CONSIDERATIONS:

1. Most of the tissues and materials listed in the table in Section E below can be processed and analyzed in the laboratory according to the procedures described within the chapters referenced in the chart. Several procedures are not described in detail within this manual. An attempt will be made, therefore, to detail them within this chapter of the manual.
2. The collection of blood and other tissue biopsies for use in PCR or RT-PCR assays should be done with consideration of the integrity of the DNA or RNA during collection, processing, and performing assays. Samples should be frozen on dry ice immediately after taken. In the case of samples to be assayed by RT-PCR, special commercially available buffers can be used, instead of immediate freezing, to help preserve the integrity of the sample RNA until the assay can be run (RNAlater® is a buffer available from Ambion, Inc. Telephone: 800-888-8804, cat# 7020).

B. CONSIDERATIONS FOR VIROLOGY:

1. Cell Lines: An impressive number of fish cell lines have been and continue to be developed and established by professionals in the fish health community. Many of these lines are catalogued in the American Type Culture Collection (ATCC). The ATCC database can be queried on the Internet for availability and purchasing information at http://www.atcc.org.

2. Additional cell lines, which may not be available at ATCC, are reviewed by Fryer and Lannan (1994).

NOTE: The detection of virus from water samples using an adsorption-elusion technique (as listed in the chart) is labor intensive, and therefore only the literary reference for this procedure is given (McAllister & Bebak, 1997).

C. CONSIDERATIONS FOR BACTERIOLOGY:

1. Culture on Selective Media - a brief list of several media, which are considered selective for culturable bacterial fish pathogens, is given in Section III of this chapter.

2. Part III and IV of this Chapter describe, in detail, procedures for detecting bacteria from fish mucus and water samples.

D. CONSIDERATIONS FOR PARASITOLOGY:

Any material collected non-lethally can be examined microscopically for parasites. Tests using PCR can be conducted to detect Survey target parasites such as *Myxobolus cerebralis* and *Ceratomyxa shasta*. See manual chapters referenced in the chart below (Section E).

E. GENERAL NON-LETHAL SAMPLING CONSIDERATIONS:

Below is a chart which lists seven main forms of fish tissue and other material that can be collected non-lethally and examined for fish pathogens targeted for the Survey. Chapters in which protocols can be found are referenced.

Sample Material	Target Pathogens	Protocols	Chapter Reference
Coelomic Fluid	Viral pathogens	Cell culture/ PCR	Chapter 10 & 11
	R.salmoninarum	ELISA/ PCR M-FAT/PCR	Chapter 6 & 7 Chapter 14
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
Blood and Blood Serum*	Viral pathogens	Cell culture/ PCR	Chapter 10 & 11
	R. salmoninarum	ELISA/ PCR	Chapter 6 & 7
	Bacterial pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy/PCR	Chapter 8
Fecal /Intestinal Lavage	IPN	Cell culture/PCR	Chapter 10 & 11
	R. salmoninarum	KDM culture/PCR	Chapter 5
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites (*C. shasta*)	Microscopy/PCR	Chapter 8
Mucus*	IHN, IPN	Cell Culture/PCR	Chapter 10 & 11
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy	Chapter 8
External Lesions	Viral pathogens	Cell Culture/PCR	Chapter 10 & 11
	Bacterial Pathogens	BHIA culture/ serology	Chapter 5
	Parasites	Microscopy	Chapter 8
Water/Sediments**	IPN* (see Bibliography)	Adsorption-elusion	
	Bacterial Pathogens*	Filtration/culture/ serology	Chapter 5
	M. cerebralis	Microscopy/PCR	Chapter 8
Tissue Biopsy: Gill*, fin, opercula	Parasites (Including *M.cerebralis*)	Microscopy/PCR	Chapter 8

* with special considerations explained within this Chapter.
** although detection does not come directly from fish, examination of water and sediments can indicate the presence of a pathogen in a particular watershed.

III. Collection of Fish Blood for Pathogen Assays

Blood can serve as the ideal non-lethal tissue for detection of systemic infections. The preservation and storage methods for blood collection depend largely upon the target pathogen(s) of interest.

A. COLLECTION PROCEDURE:

1. It is most advantageous to use Vacutainer® collection tubes with accessory needles and holders, available through most scientific supply catalogs. Vacutainer® tubes come in a variety of capacities, but the 2 or 5 mL draw are the most appropriate for pathogen detection purposes. Vacutainer® tubes are also available with a variety of preservatives and anti-coagulants (EDTA, heparin, clot activator,), depending upon the intended use of the sample. Some assays are not compatible with these additives (these substances can act as inhibitors in PCR), so untreated tubes are available with no additive.
2. Blood should be collected only from fish large enough to withstand the procedure. Collection of blood from small fish can be lethal. No more than 1 mL of blood can be obtained from a 100g fish without lethal results.
3. Fish should be thoroughly immobilized by anesthetic prior to handling to avoid injury by the needle.
4. Place the anaesthetized fish on a non-slip surface, or have an assistant hold large fish so that the collector has access to the ventral peduncle surface.
5. Affix a properly sized needle onto the Vacutainer® holder, and carefully place the stopper end of the Vacutainer tube down into the holder, but do not puncture the rubber seal. **It is necessary to maintain vacuum inside the tube in order to obtain blood. If the vacuum is broken, a new tube must be used.**
6. Blood is removed from the caudal vein located ventral to the spinal column. Insert the needle into the side or ventral surface of the caudal peduncle and approach the area with the needle. When the needle is near the vein, push on the end of the Vacutainer so that the rubber stopper is punctured and blood will flow into the tube. It may be necessary to move the needle tip slightly to locate the vein. Once located, the blood will flow freely into the tube. When enough volume is obtained, remove the needle from the fish and remove the tube from the holder. It is necessary to properly preserve the blood sample by freezing or distribution into appropriate buffers before the blood clots.

B. WHOLE BLOOD SAMPLES FOR MICROSCOPIC EXAMINATION

1. Blood is collected in anti-clotting agent (heparin) and smears are prepared according to individual assay protocols (wet mount for immediate observation or dried and fixed for hematological staining).

C. WHOLE BLOOD SAMPLES FOR VIROLOGY AND BACTERIOLOGY

1. Blood is collected without a preservative, and immediately diluted 1:10 with buffered saline.
2. Store samples on ice until processing.
3. Samples can be homogenized by stomacher, or by repeated expulsion with a small gauge needle and syringe.

4. The samples are further diluted and inoculated according to standard protocols for the detection of the target pathogen.

D. WHOLE BLOOD SAMPLES FOR RT-PCR AND PCR
1. RNA viruses – samples must be collected in tubes with no additives.
2. Immediately after collection, a small amount of blood is frozen on dry ice, or placed in a preservative buffer solution for RNA (RNAlater®, Ambion, Inc.).
3. PCR: DNA or RNA are extracted according to tissue protocols for PCR and RT-PCR.

E. SERUM SAMPLES

Whole blood is allowed to clot or settle, and the serum is carefully aspirated from the remainder of the clot.

1. In case blood cells remain suspended in the serum, the sample can be centrifuged for 10 minutes at 3,000 rpm. Serum can be stored for 1 week at 4°C, or for one year at -20°C. The addition of thimersal (1:10,000) will preserve the samples for extended storage, as long as this additive does not interfere with intended assays.
2. Serum can be used in a variety of assays including immunological antigen and antibody detection.

IV. Non-lethal Detection of Infectious Salmon Anemia Virus in Blood

Infectious salmon anemia virus can be detected from whole fish blood using both tissue culture and reverse transcriptase polymerase chain reaction technology (RT-PCR), as reported by Giray et al. 2003. Refer to Section III for blood collection procedures. Blood is immediately diluted in physiological saline or PBS, processed, and inoculated onto plates containing both SHK-1 and ASK cell lines (Chapters 10 & 11). Any CPE detected by 28 days can be confirmed as ISAv by RT-PCR (Chapter 12).

Blood has been determined to be a suitable material for direct use in RT-PCR for detecting RNA from ISAv as well, and is already used for screening of wild anadromous salmonids during upstream migration. The following is a detailed protocol for RNA extraction and RT-PCR on whole blood preserved in RNAlater® (section III):

A. **RNA EXTRACTION**

Using QIAGEN® RNeasy Kit with optional vacuum manifold:

1. Materials and Reagents:

RNeasy Mini Kit if Tissue is used (QIAGEN # 74104)
QIAGEN Viral RNA Kit (QIAGEN # 52904)
Qia-shredder spin columns (QIAGEN # 79654)
QiaVac-24 vacuum manifold -Optional (QIAGEN # 19403)
Vacuum Pump- Optional
Microcentrifuge
RNALater® (Ambion, Inc. # 7020).

QIAGEN Viral RNA Mini Kit if Cell culture or serum is used (QIAGEN #52904)
Ethanol (Absolute 97-100%)
Ethanol (70%)
β-Mercaptoethanol (14.5 M)
Pipettor (100-200 µL and 0.5mL)
Aerosol Barrier Tips
1.5mL centrifuge tubes
Micro centrifuge
heat block(s) (70°C and 95°C)
latex or nitrile gloves

2. General QA/QC - **Wear and change gloves often**. This prevents spread or contamination of sample RNA/DNA and polymerase naturally occurring on the skin. **Review Chapter12 for important QA/QC considerations before proceeding with this protocol.**

3. Assay Preparation:

    a. Mix Beta-mercaptoethanol (ME) into Buffer RTL before starting (10 µL ME to 1 mL RLT).
    b. Ensure ethanol is added to Buffer RPE.
    c. Prepare 70% ethanol solution for step 4.
    d. Label QIAshredder and collection tubes.
    e. Set up QuaVac-24 unit with VacValves and/or VacConnectors, and place labeled spin columns into VacConnectors so they are ready for lysates.
    f. Label 1.5 mL mc tubes for lysing samples (make sure that a positive and negative controls are included).
    g. Fish blood should be stored refrigerated or frozen in RNAlater.

4. Procedure (adapted from RNeasy Handbook):

    a. Pippette 30 µL blood into a 1.5 mL mc tube.
    b. Add 600 µL Buffer RTL, and vortex for 1 minute.
    c. Transfer lysate to a QIAshredder spin column.
    d. Homogenize the tissue by centrifuging the QIAshredder for 2 minutes at **maximum speed. Discard the QIAshredder spin column and retain** the filtered lysate.
    e. Either place a cap on the collection tube containing the lysate, or transfer to a new 1.5 mL microcentrifuge tube.
    f. Add 600 µL of 70% ethanol to the cleared lysate, and mix well by pipetting. A precipitate may form, but should not affect the outcome of the procedure.
    g. Apply 700 µL of the sample at a time (including any precipitate) to an RNeasy spin column that has been placed on the QiaVac 24 manifold. Vacuum until all lysate has passed through filter.
    h. Apply the remainder of the sample to the spin column and vacuum filter again.
    i. Pipette 700 µL Buffer RW1 into the spin column, and vacuum filter.
    j. Be sure ethanol is added to Buffer RPE, then pipette 500 µL Buffer RPE to spin

column and apply vacuum.

k. Add another 500 µL Buffer RPE to the spin column and vacuum.
l. Place spin column in a clean collection tube. Centrifuge for 3 minutes at maximum speed (14,000 X g). This step ensures drying of the filter.
m. Transfer spin column to an RNase free 1.5 mL microcentrifuge tube and pipette 50-100 µL RNase-free water directly into the spin column. Centrifuge for 1 min at 8000Xg to elute. Repeat elution with fresh water (50-100 µL) into the same collection tube. Measure and dilute RNA to 10 to 100 ng/µL.

B. **RT-PCR PROCEDURES (ISAV)** (adapted from Keleher et al. 2003)**:**

1. Materials and Reagents:
   a. Superscript One-Step RT-PCR System (GIBCO #10928-018)
   b. Rnasin RNase Inhibitor (Promega)
   c. Upstream primer for ISAv 1D: 5' GGC TAT CTA CCA TGA ACG AAT C
   d. Downstream primer for ISAv 2: 5' TAG GGG CAT ACA TCT GCA TC
   e. Molecular Grade (RNase free) d-H_2O
   f. 0.5 and 1.5 mL microcentrifuge tubes (RNase/DNase Free)
   g. Thermal cycler
   h. 0.5-25µL and 20-200µL pipetters: (positive displacement with matching lunger/tips and/or regular with aerosol barrier tips)
   i. gloves (latex or nitrile)
   j. Bench top UV cabinet.

2. Procedures:
   a. Master Mix Preparation using One-Step RT-PCR Systems (Invitrogen): Add water first and RT/Taq mix last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use. Refer to Chapter 12 for general procedures and considerations when performing PCR. The following are final concentrations of each component to be added into each reaction tube:

Component	Final Concentration
2X Reaction Mix	1X
Sense primer	50 pMole
Antisense primer	50 pMole
RT/TAQ Mix(Gibco)	1 µL
Rnasin(Promega)	10 units
RNase free Water	added to above to 49 µL total

   b. Place 49µL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes with MM to sample loading area. Add 1 µL template RNA.

   c. Thermocycler should be programmed for the following regime:

Reverse Transcription:	42°C 15 min.
Pre-dwell	94°C 5 min.
30 Cycles of:	94°C 45 sec.

59°C 45 sec
72°C 1 min.45 sec.

Post dwell at 72°C for 7 min.
Hold to 4°C chill at end of program.

d. Retain amplified product for electrophoresis as described in Chapter 12. The resulting cDNA product will produce a 493 base pair band after electrophoresis in a 2% agarose gel, indicating a positive finding for ISAv.

V. Non-lethal Detection of Bacterial Pathogens in Mucus of Fish

The bacterial fish pathogen Aeromonas salmonicida can be readily isolated from the mucus of salmonids using the following techniques. Some success in isolating Yersinia ruckeri and Flavobacterium columnare has also been reported by field personnel. Bacteria can be detected using simple swab/streaks onto agar media, or they can be quantified through serial dilution in PBS. Quantification can help reflect the level of systemic infection in some fish. The following methodologies have been adapted from Cipriano et al(1992).

A. NON-QUANTITATIVE METHOD:

1. Mucus is scraped gently from the lateral surface of the fish with a 10µL inoculating loop, and streaked directly onto the agar media of choice, according to the target pathogen(s) for detection.

2. Alternatively, commercially prepared transport swabs (see source list) can be used to collect mucus specimen, for later streaking onto agar media. Follow manufacturer's instructions on use of the swabs. Be sure to store transport swab samples cold and streak the sample onto appropriate media within 24 hours of specimen collection.

B. DILUTION PLATE COUNTS FROM NON-LETHAL MUCUS SAMPLES:

Small samples of mucus can be weighed and diluted in PBS, then plated on an appropriate agar medium. After incubation, colonies of target bacterial pathogens can be quantified and reported as colony forming units per gram of mucus (cfu/gm).

1. With a sterile scalpel or bladed instrument, gently collect a small amount of mucus from the lateral surface of the fish.
   a. Place into a pre-weighed sterile culture tube and keep sample cold until processing can be accomplished.
   b. Determine the weight of the sample by subtracting the tube weight from the gross weight after sample is collected.

2. Make a 1:10 dilution according to tissue weight with phosphate buffered saline(PBS).

Homogenize tissue with rigorous pipetting motions.

3. Make serial log $_{10}$ dilutions of the 1:10 dilution in PBS: Fill micro titer plates with 90µL PBS per well (4 wells per sample will be needed), or any other vessel with appropriate volume to accomplish 10 fold dilutions.

4. Add 10µL of the 1:10 dilution tube to the first micro titer well

5. Make 10 fold dilutions by taking 10µL from the first well and placing it in the second.
   a. Change pipet tips in between dilutions.
   b. Do the same from the second to third to fourth wells or tubes of PBS.

6. Prepare media plates with appropriate labels. It is helpful to space the numbers 1 through 5 around the edge of the plate and in the middle to aid in placement and tracking of each dilution.

7. Plate all dilutions, using the same tip but working from the fourth dilution backwards to the higher dilutions. 10µL drops are most readily absorbed by the agar, but larger volumes can also be plated and spread onto individual media plates.

8. Keep plates upright until the fluid of every drop has been absorbed. Then turn t Them over and incubate appropriately according to optimal conditions required by the target pathogen.

9. Quantify and isolate bacterial colonies produced from samples:
   a. Drops will vary in bacterial load depending upon the sample.
   b. Pick a spot that contains a countable number of colonies.
   c. Pick representative isolates of each colony type observed for characterization and identification.
   d. Count each colony type and record on media tubes as dilution number times number of colonies counted (example: 1 X 16).

10. Calculate colony forming units per gram mucus (cfu/gm):
    a. cfu/gm = colonies counted X dilution factor
    b. example: 30 colonies counted in 10^{-2} dilution =
       30 X 10^2 per 0.01 gm (10^{-2} contains 0.01gm of mucus)
       or 30 X 10^4 per 1.00 gm (decimal moved)
       3 X 10^5 cfu divided by original sample weight = cfu/gm tissue

Quantification of bacteria on the surface of fish can give a good indication of the level of internal infection that may exist. For instance, on salmonids, when *A.salmonicida* exceeds 10^3 cfu/gm mucus, there is a strong possibility that the fish has a systemic infection that could be lethal to the fish. This work has been done under normal fish culture circumstances, however, and adjustments to determine lethal versus carrier infections in wild fish may be necessary.

C. MATERIALS AND SOURCES

BACTI-SWAB™ Modified Stuart's Transport Medium
available from Remel (800-255-6730).

V. Procedures for the Detection of Bacteria in Filtered Water Samples

Water can be examined for a variety of bacterial species, including those that can serve as pathogens to fish. Water can be sampled directly from streams, ideally in areas where more fish are congregated, such as below pools, logs, and other habitat fish use for shelter. Sampling at effluents of fish culture facilities can also provide information on cycles of pathogens being shed into the environment. Bacteria can be enumerated on the media plates and reported as colony forming units (cfu) per milliliter (mL). The following methodologies have been adapted from Ford (1994).

A. METHODS:

1. Collect water sample in sterile 200 mL container.
   a. Take care not to contaminate sample with hands
   b. Keep sample cold until filtering and plating can be accomplished

2. Wipe filter unit with alcohol and carefully load with .45µm pore filter paper (grid side up). Take care not to contaminate filter by touching - use forceps that have soaked in alcohol.

3. Filter several dilutions of sample with enough sterile distilled water to make a 100 mL total volume.
   a. Start with the most dilute volume of sample. For example: if plating 1,10 and 100 mL of water, fill filter unit with 99mL d-H_2O and transfer 1mL sample. The next dilution (10 mL sample in 90 mL d-H_2O) can be done without disinfecting filter unit. However, wipe the filter unit with alcohol between different water samples.
   b. The number and volume of dilutions should be adjusted with the quality of water samples: murky water will contain a lot of bacteria, and therefore, the smallest filtered volume may have to be 0.5 or 0.1 mL
   c. Always dilute with sterile distilled water to bring total filtered volume to 100 mL for consistency and even distribution of bacteria.

4. After filtering, plate samples onto appropriate media:
   a. With disinfected forceps, gently grasp each filter paper from unit and place grid side down onto agar media surface, removing any air bubbles with the forceps.
   b. The filter need remain for only a few minutes before it can be removed and discarded with clean forceps.

5. Incubate plates for appropriate time and temperature depending on target organisms.

6. Perform bacterial counts and isolations directly from plates. Frequency plots and histograms of the major bacterial genera can be plotted as well as the number of cfu/mL of target pathogen in a particular sample.

B. MATERIALS AND SOURCES:

Sample Containers (Sterile)240 mL (Thomas Scientific/6186-M40)
Nalgene Filter Apparatus (Thomas Sci./4618-N60)
Nalgene Filter Apparatus (Thomas Sci/ 4618-N62)
Filters (Sterile), 47mm, 0.45Φm pore (Thomas Sci./4626-J20)
Filter Pump (aspirator type - VWR/ 28610-008) or a vacuum pump
1 mL Pipets (Sterile-Fisher Sci./13-678-11A)
Coomassie Brilliant Blue (R250-Sigma/B-0149)

Other equipment needed: Forceps
Alcohol (70% isopropyl)
Sterile Distilled Water
Agar Plates (depending on target pathogens)

C. MEDIA SUGGESTIONS:
The following are media and components which select for, or enhance isolation of the given target pathogen. A literary reference is provided for each.

1. ***Aeromonas salmonicida*:**

Tryptic soy agar (TSA)-commercial media - follow preparation instructions.

Coomassie Brilliant Blue agar (CBBA) - (Cipriano & Bertolini, 1988).

TSA	40 g
Coomassie brilliant blue R-250(CBB)	0.1 g
d-H_2O	1.0 L

Autoclave for 15 min at 15 psi (121^{o}C) and pour into petri plates. *A. salmonicida* colonies will appear as dark blue, friable colonies after 48 hours at 20^{o}C.

2. ***Flavobacterium psychrophilum, F. columnare:***

Tryptone Yeast Extract Supplemented (TYES) - (Holt & Amandi, 1989)

Tryptone	4.0 g
Yeast Extract	0.4 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.5 g

Agar	10.0 g
d-H_2O	1.0 L

Dissolve ingredients and adjust pH to 7.2. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

Tryptone Yeast Gelatin (TYG) - (Bullock, et al, 1986)

Tryptone	2.0 g
Yeast Extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
d-H_2O	1.0 L

Dissolve ingredients and adjust pH to 7.0. Heat to boiling for 1 minute. Autoclave for 15 min at 15 psi (121°C) and pour into petri plates.

3. Yersinia ruckeri:

TSA, BHIA - both commercially prepared

Shotts-Waltman (SW) -(Waltman&Shotts, 1984)

Sodium Chloride	5.0 g
Tryptone	2.0 g
Yeast Extract	2.0 g
Tween 80	10 mL
$CaCl_2 \bullet 2H_2O$	0.1 g
Bromthymol Blue	0.003 g
d-H_2O	950 mL
pH to 7.4 and add:	
Agar	15 g

Heat to boiling. Autoclave for 15 min at 15 psi (121°C). Add 10 mL of 0.5g/mL sucrose solution which has been filter sterilized. Refrigerate poured plates until use.

Y. ruckeri will produce a green colony with a zone of hydrolysis (precipitation of calcium oleate from Tween 80). Always confirm colony identity with biochemical characterization of isolates. (Type II will not hydrolyze Tween 80).

4. *Renibacterium salmoninarum:*

Kidney Disease Medium (KDM2) - (Evelyn, 1977)

Peptone	10.0 g

Yeast Extract	0.5 g
L-Cysteine HCl	1.0 g
Distilled water	1000 mL
Adjust pH to 6.5	
Agar	15.0 g

Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

FBS	200.0 mL

The following volumes of antibiotics can also be added to the KDM2 (SKDM) to reduce overgrowth from other bacterial organisms (Austin, et al. 1983).

Cyclohexamide	4.0 mL (see below)
D-cycloserine	1.0 mL
Polymyxin B-sulfate	2.0 mL
Oxolinic Acid	1.0 mL

Prepare the above antibiotics following these formulas:

Antibiotic Solution Preparation:

	grams	mL
Cyclohexamide	1.2	96 d-H_2O
D-Cycloserine	0.3	24 d-H_2O
Polymyxin B-sulfate	0.3	24 d-H_2O
Oxolinic Acid	0.06	24(5% NaOH)

Researchers found that variable lots of peptone could adversely affect the ability to successfully culture *R.salmoninarum* using these media. Evelyn et al. (1990) reported on the use of a metabolite solution from KDM broth containing *R.salmoninarum* (autoclaved or filter sterilized using 2 µm pore size) and added to the KDM media at 2% (v/v). The addition of metabolite solution to the media has shown to improve success in culturing this organism, and seems to negate the adverse effects of poor peptone lots used in the media.

5. *Edwardsiella ictaluri*

S-W *E. ictaluri* Selective Media - (Shotts & Waltman, 1990)

Tryptone	10 g
Yeast Extract	10 g
Phenylalanine	1.25 g
Ferric ammonium chloride	1.2 g

Bromthymol blue	0.003 g
Bile salts	1.0 g
Agar	15.0 g
Distilled water	980 mL

Dissolve ingredients by boiling, then cool to 50°C and adjust pH to 7.0.
Autoclave for 15 min at 15 psi (121°C).
Cool to 50°C again and add mannitol (filter sterilized) to 0.35% (v/v) and colistin sulphate to 10 ug/mL.

Interpretation:
Proteus species will produce brown colonies (caused by phenylalanine and ferric ammonium chloride).
Serratia and *Aeromonas* will ferment mannitol producing yellow colonies.
Edwardsiella ictaluri will produce translucent, colorless colonies.

VI. Method for Non-Lethal Gill Biopsy

Gill filaments can be removed from fish while under anesthesia with little injury to the fish. The tissue can be examined directly under microscopy for parasites; preserved for histology; or frozen for examination using other diagnostic methods, such as PCR for *Myxobolus cerebralis*. The following protocol has been adapted from methods used for collection of gill tissues for gill Na^+, K^+-ATPase activity measurements in salmonids (McCormick, Personal communication).

A. METHODS:

1. While fish is under anesthesia, place on a moistened chamois cloth to minimize scale loss and damage. A right handed individual should place the fish on its right side so that the head is to the left and tail to the right.

2. Gently pull back the operculum with rounded forceps. A cartilaginous septum (present in some species such as salmonids, but not in others) holds filaments together for one-half their length.

3. Using a fine pointed scissors, remove 4-6 filaments just above the septum from a fish weighing between 20 and 80 grams (remove more or less tissue for larger and smaller fish, respectively).
   a. With the operculum reflected, isolate several filaments with the open blades of the scissors (see Figure 1).
   b. Turn scissors so that they are perpendicular to the filaments and cut in a single quick motion.
   c. To retain the filaments on the scissor blades, turn the scissors slightly as you finish cutting.

   Take care not to crush sample or remaining filaments. If there is any movement from the fish, be sure to retract forceps and scissors quickly to avoid injuries.

Return fish to fresh water immediately.

4. Transfer filament samples to appropriate containers for storage until assays cane be performed.

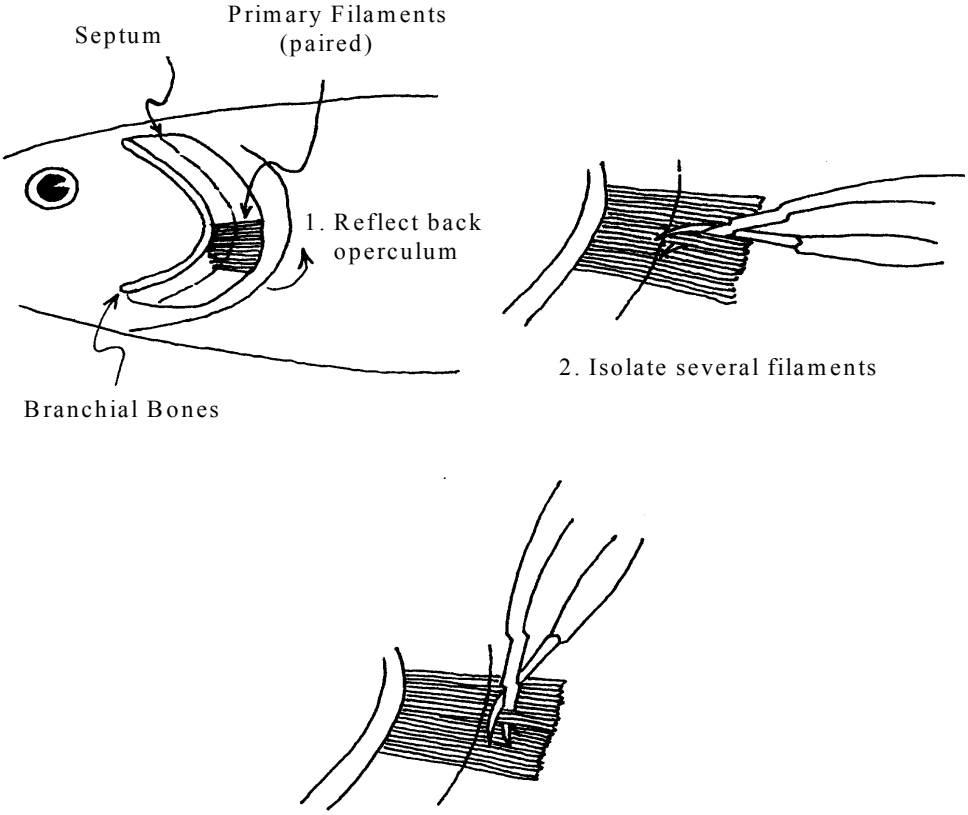
B. RECOVERY OF FISH SUBJECT TO GILL BIOPSY

Upon return to the water, the fish may bleed slightly for up to one minute. Excessive bleeding, beyond one minute is usually associated with cutting to deeply into the filaments (i.e. below the septum). Even excessive bleeding does not usually result in mortality.

C. MATERIALS

Chamois Cloth
Rounded forceps
Fine point scissors
(Vannas eye scissors - 7mm curved blade, Sicoa-phone 201-941-6500, Cat.# OM-1401)
Collection vials
Fish anesthetic

Figure 1 – Non-Lethal Gill Biopsy



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