FISH PATHOLOGY SECTION

LABORATORY MANUAL

Edited by

Theodore R. Meyers, Ph.D.

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PREFACE

There are many published sources for laboratory procedures used in the diagnosis of finfish diseases and less so with shellfish. This laboratory manual is not intended to be comprehensive in its treatment of this large subject area. Many of the finfish diseases found elsewhere in the United States and the world have not been found in the state of Alaska. Consequently, this manual addresses only those agents known to occur in Alaska (*Myxobolus cerebralis* has yet to be confirmed) while still providing a general scheme of approach to the disciplines of virology, bacteriology, histology, etc., to allow detection of potentially new or exotic agents as well. The procedures herein follow the AFS Fish Health Section Bluebook standards for the detection of fish pathogens where appropriate and in several instances protocols cited from other investigators in the published literature have been included as well. However, the real purpose of this manual is to provide a working document of very detailed information for ADFG pathology staff and clients regarding the daily routine in which we conduct finfish and shellfish diagnostics. As with most such manuals, this one will be continually updated as new and other procedures become necessary in our everyday use.

NOTE: Mention of brand names or trademarks in the text of this manual is not an endorsement of any such product by ADF&G but rather serves as a descriptive model for the reader.

EDITOR

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CHAPTER 1

Sample Collection and Submission

Theodore R. Meyers, Norman Starkey, Sally Short, and Karen Lipson

I. Finfish Diagnostics

Diagnostic procedures used for detection of fish disease agents will be according to the *American Fisheries Society Fish Health Section Bluebook* (AFS-FHS.2005. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2005 edition. AFS-FHS, Bethesda, Maryland). The prioritization of the basic user diagnostic needs are as follows, in descending order of importance:

- Disease outbreaks or finfish/shellfish mortality
- Broodstock screening for Family Tracking of bacterial kidney disease (BKD) (finfish)
- Broodstock screening for shellfish (oyster) certification and importation of *Crassostrea gigas* spat
- Screening of broodstock or resident animals to establish a disease history, generally to satisfy a Fish Transport Permit (FTP) for finfish and shellfish
- Required pre-release inspection of apparently healthy fish or shellfish

The major purpose of this section is to clarify to laboratory staff and user groups the proper sampling procedures to be carried out by clients when finfish or shellfish disease problems arise. This is an absolute necessity to insure that samples received by the pathology labs are adequate for allowing a definitive disease diagnosis.

- Disease Recognition and Action – Whenever abnormal behavior patterns, external abnormalities, or high mortality occur at a hatchery, an immediate response from the hatchery staff in charge is imperative. Assistance should be requested from the Fish Pathology Section (FPS) of ADF&G whenever mortality appears excessive and is not related to known handling or mechanical malfunction of the physical plant. An epizootic is occurring when mortality reaches 1.5% per day. This requires immediate attention. A total commitment of the facility staff and appropriate personnel is needed to save the remaining fish.

  Mortality less than 1.5% down to 0.5% indicates that a fish health problem is present and the FPS should be notified for consultation.

Mortality of less than 0.5% per day but greater than 0.3% should be investigated. Hatchery personnel should attempt to remedy the situation by modifications of environment or feeding and notify the FPS.

The percentages given above are for total mortality. It is no less a matter of concern, however, if one lot of fish or shellfish is dying at 1.5% per day while the others remain healthy. The sick animals should be isolated as much as possible to prevent transmission of the disease to other lots.

In order to reduce the spread of disease, dead fish and shellfish should be incinerated or soaked in a solution of 200 ppm of chlorine or iodine (active ingredient) for 12 hours before disposal.

- Sample Collection and Shipment – Prior to collecting any samples, the FPS must be contacted to discuss whether samples are necessary, and if so, the appropriate type of sample and numbers of fish or shellfish needed. Advance notice of sample submission by at least one week is preferred. Obviously, serious disease outbreaks will merit an exception. If advance notice is not given, samples may not be processed if other samples have priority or if appropriate lab personnel are not alerted and therefore unavailable to process the samples. The following instructions are general guidelines but some samples need special treatment and the pathology personnel will provide details. Samples that are not in an adequate condition (either substandard or improperly packaged) upon arrival may not be processed. All proposals for sampling (Southeast Region, Southcentral Region and AYK-Westward Regions) should be cleared through pathology staff by contacting the appropriate lab personnel.

- Preparing Samples – Different procedures are followed in sampling for bacteriological, virological, parasitological, ELISA, FAT or histological analyses. Further details regarding the procedures below will be provided to hatchery personnel upon initial contact with the FPS.

- In clinical cases of disease ($\geq$ 0.5% mortality/day) 5-10 moribund fish or shellfish are generally a sufficient sample size to make a diagnosis. In situations where no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be necessary. However, depending upon individual circumstances, sample sizes may vary between 5 and 60. Samples from each affected lot, incubator, or rearing container do not have to be examined when clinical signs of disease are similar among the groups. Consult with the FPS for specific sampling requirements in each situation.

II. Finfish Bacteriology

Small fish must be received either alive or freshly dead (within 1-2 hours) on blue ice in a cooler. Fish must not be frozen. Bagged fish should not be in direct contact with blue ice or they will freeze.

Live fish are preferred for diagnostic samples. At least 5-10 moribund fish should be placed in one or more large leak-proof plastic bags containing hatchery water. Seal the bags so space for air remains and leakage will not occur. Label bags with fish status (moribund or healthy), incubator or raceway number, stock and species and enclose a Sample Submission Form (see page 1-8) with each shipment. If oxygen is available, add to bags before sealing.

Addition of an oxygen tablet to each bag is recommended, particularly for samples that must be shipped.

III. Virology (also see Chapter 5 regarding sample collection)

A. Clinical Disease: In clinical disease outbreaks of suspected IHNV in sockeye salmon, 10 moribund or freshly dead fish are sufficient to isolate the virus for a confirmed diagnosis. In other salmonid species, 60 moribund fish may be required to establish an etiology. For alevins, fry, and fingerlings, whole fish should be sent by following instructions given above under finfish bacteriology.

B. Broodstock and Disease History Examination: For establishing a disease history in adult fish or in broodstock screening, 60 samples from adult fish will be required. Samples of choice are from spawning or postspawning female fish consisting of ovarian fluids collected from each fish and shipped in separate disposable centrifuge tubes with snap caps. When required, samples from spawning or postspawning males should consist of 0.5 g each anterior and posterior portions of kidney and whole spleen from each fish, aseptically removed and pooled in individual sealed 2 ounce white-labeled plastic Whirl-Pak® bags. Tissues from more than one fish should not be combined in one bag. All tissues and fluids for virus assays should be shipped to the FPS on blue ice (4°C) but never frozen. Freezing at low temperatures and subsequent thawing can inactivate IHNV or other viruses, producing lower titers, which in some samples may be too low to detect routinely. Virus samples on blue ice should be sent to the FPS lab as soon as possible within 72 hours of collection.

These sampling procedures are applicable to assays for other finfish viruses should the need arise.

1. Ovarian fluids for virology testing: Obtain instructions from the lab staff regarding whether you should take ovarian fluids from ripe fish used in the egg take or from postspawning fish. Disinfect the external ventral surface and wipe dry with paper towels.

For postspawners, partially strip a single fish's ovarian fluids into a paper cup (recommend 4 oz pleated cups but paper drink cups can be used), avoiding the extrusion of blood, fecal material or nematode worms if present. For ripe fish, you may either extrude a small amount of fluid prior to taking eggs or pour fluid off the eggs. Two ml of fluid are adequate for ripe fish, but 3-5 ml should be obtained if sampling postspawners in case there is a need to filter the samples.

Crimp edges of the cup to form a spout and pour fluid into a 10 ml centrifuge tube with cap, "straining out" any eggs. Avoid contaminating the rim with your hands. Discard the cup after each fish. Do not provide more than 5 ml of ovarian fluid.

Cap the tube tightly making sure that the cap is properly seated. Place tubes in a rack in a plastic Ziploc® bag labeled with stock of fish and species, sampling location, date, fish life stage, and number of samples. Place upright in a cooler with cold packs. Do not freeze. If samples cannot be shipped within 72 hours they may be kept on ice up to 6 days if not grossly contaminated. Otherwise they must be frozen.

2. Tissue samples from males for virology testing: Disinfect the external ventral surface and either rinse with clean, pathogen-free water or wipe dry with paper towels.

   Carefully cut open fish, taking care not to cut the gastrointestinal tract which would contaminate tissues with bacterial flora.

   Aseptically remove the spleen and anterior and posterior portions of the kidney, each about 0.5 g in size (size of thumbnail) and place into a single 2 oz white-labeled plastic Whirl-Pak® bag using a spoon, knife or forceps.

   Seal each bag and keep cool (4°C).

   Between the sampling of each fish, clean dissecting utensils with 3% iodine and dry with a clean paper towel. Organic matter will affect the working ability of the disinfectant, so any tissue should be wiped off utensils with a separate paper towel prior to disinfection. Wooden tongue depressors, discarded between samples, eliminate the need for disinfection. Disinfect hands between the sampling of each fish. Rubber gloves should be worn to reduce human contact with disinfectant.

   When sampling is done, place all sealed sample bags in a large plastic bag. Label the bag with the number of samples, stock of fish and species, sample location, fish life stage and date. Place in a cooler on ice or a cold pack and transport within 3 days. Do not freeze. Make sure you include a Sample Submission Form with each shipment.

IV. Fluorescent Antibody Test (FAT).

The following procedures are for *Renibacterium salmoninarum* (bacterial kidney disease, BKD), *Yersinia ruckeri* (enteric redmouth, ERM), and *Aeromonas salmonicida* (furunculosis). In disease outbreaks involving small fish, 5-10 moribund or freshly dead fish per affected lot(s) shipped in plastic bags on ice (not frozen) are sufficient for a disease diagnosis. A presumptive diagnosis of *A. salmonicida* may not be confirmed by bacteriologic culture if samples are frozen. Sampling is according to that described earlier under bacteriology. An additional sample of 60 randomly selected normal appearing fish from the same lot(s) may be required at a later date to determine the prevalence of sub-clinical disease within a given group of fish before release is approved.

In situations where a disease history and/or broodstock screening is desired, a minimum sample size of 60 fish will be required. Family tracking for BKD will require screening of all parent fish involved in the egg take. Whole fish should be sent when sampling alevins, fry and fingerlings. In situations where large fish are to be examined, only kidney tissues are required. Sampling procedures are identical to those described for virology sampling of male kidney tissues.

Although fresh-on-ice samples are necessary for successful isolation of certain disease agents, freezing is the least desirable, but may be a necessary alternative if there will be excessive delay in getting the samples to the FPS.

In situations where it is more practical for field personnel to prepare the slides for FAT rather than mail tissues, the appropriate materials will be provided by the FPS. Briefly, after collection of kidney tissues the procedure requires:

A. Homogenization of the kidney sample from each fish by kneading within the plastic sample bag.

B. A sterile wooden applicator stick is touched to an individual homogenized kidney sample and then mixed with a drop of phosphate buffered saline (PBS) deposited in a single numbered well on a multiple well slide.

C. The samples are allowed to air dry at room temperature and the slides may be mailed to the FPS in slide boxes.

Each kidney sample requires a separate applicator stick and well. Slides are prepared in multiples for parallel testing if fish are to be screened for BKD, *A. salmonicida* and *Y. ruckeri* (There are 2 serotypes that require duplicate slides). Homogenization of the kidney is important to break open BKD pustules and distribute the causative organism or any other target bacteria for easier detection. It is also important to not make kidney smears too thick within the depressions, which makes interpretation difficult. Also, such smears may wash off the slide during processing.

V. ELISA Sampling of Kidneys for the BKD Agent (see ELISA Chapter 9)

VI. Parasitology and General Necropsy

The same sampling procedures as in Bacteriology apply here. Live fish are preferred to frozen or preserved fish. This is especially true for detection of external protozoan parasites and general gross tissue lesions, which are usually lost during freezing. Fish may be fixed in 10% buffered formalin if live fish are not available. Fish longer than 6 cm should be opened along the abdomen to ensure adequate formalin fixation of all tissues.

The FPS will not routinely process large numbers of fish ($\geq$ 20) for purposes of establishing parasite (helminth) prevalences.

VII. Histology

Histological samples should be fixed in Bouin's solution or 10% buffered formalin. Fix live fish after anesthetized. Use 5-10 moribund fish. Dead fish are unsuitable for histology. The volume of fixative must be 10 times the volume of tissue. For fish longer than 6 cm, the abdomen should be opened and the intestine detached at the anus so that the internal organ mass may be pulled out slightly. For large fish, only specified organs should be sent in fixative. Call the FPS for specific instructions prior to fixing. Further details regarding necropsy and histologic sampling for both finfish and shellfish are provided in the histology chapter (Chapter 6) of this manual.

Sample sizes for shellfish are: 30 live adult animals for a disease history; 60 live adults, 200 live spat and 1,000 live larvae for certification to import seed from an out-of-state Pacific oyster stock; maximum of 5-10 live or fixed animals for diagnostic purposes.

VIII. Sample Shipment Instructions (for all samples)

A. Pack samples in a small ice chest made of plastic or sturdy styrofoam which will not be damaged in transit. Ice chests (other than styrofoam) will be returned to the sender.

B. Add pre-packaged ice substitutes. To prevent freezing, separate the samples from the ice with newspaper or other insulative material.

C. Place completed Sample Submission Form(s) (page 1-8) (forms available from FPS) for each stock sampled within a waterproof plastic bag and enclose in ice chest.

NOTE: Clients should always include a Sample Submission Form (page 1-7) with a submitted sample which provides the species, brood year, clinical signs, time of onset, sample date, number of samples submitted and other very important information. Clients should not assume a previous telephone call will take the place of submitting this written information. In the future, samples submitted without this paperwork may be refused or at least delayed in processing. When in doubt about anything, clients should consult a pathology staff member and not forget the paperwork.

D. Close, seal, and label the ice chest with appropriate instruction for the type of sample enclosed (i.e., "Live Fish – Do Not Freeze" for live samples, "Keep Frozen" for ELISA, "Refrigerate but do not freeze" for virology . . .) Label with mailing address, telephone number and the name of the person contacted in the lab. The mailing addresses for the pathology labs are:

Fish Pathology Lab
ADF&G, CF Division
333 Raspberry Road
Anchorage, AK 99518-1599

Juneau Fish Pathology Lab
ADF&G, CF Division
3333 Old Glacier Highway
PO BOX 115526
Juneau, AK 99811 -5526

E. Ship via express air or air freight (if you know it will not get bumped off the flight) as soon as possible. Instruct the airline to refrigerate the sample upon its arrival in Anchorage or Juneau. If sent early in the week, fewer air freight and delivery problems are encountered. Avoid shipping on Fridays.

F. Unless special circumstances dictate otherwise, clients are expected to ship samples such that they may be received by the labs and therefore, processed during regular working hours.

NOTE: The cost of shipping samples to the pathology labs is the direct responsibility of the user unless the samples are submitted as a courtesy request by lab personnel.

G. Phone the Fish Pathology Lab to notify that the sample is in route. Please provide the flight number, air waybill number, and expected time of arrival. Subsequently check to see if it has arrived. It is the responsibility of the sender to ensure that the sample arrives at the laboratory in satisfactory condition.

Fish Pathology Laboratory
Sample Submission Form

Date received at laboratory_____

Date collected: _____

Facility/contact person & address:_____

_____

Lot (brood year/stock/species):_____

Life stage:_____

Sex if applicable:_____

Date outbreak noticed:_____

Problem history:_____

_____

_____

_____

_____

_____

_____

_____

_____

Recent medications: _____

Are these samples an FTP requirement? YES____ NO___

If yes, what is the FTP number? _____

CHAPTER 2

Materials List for Sample Submission

Norman Starkey

The following list of vendors and materials has been compiled through trial and error over time. Equipment in place at the pathology laboratories requires the exact materials listed. The use of different styles of tubes and bags make processing samples difficult and excessively time consuming.

Ovarian Fluid Samples - Juneau Laboratory

10-ml polypropylene opaque tube (Sarstedt)	Cat. #57.519	1,000 for $79.90
Caps for above	Cat. #65.793	1,000 for $27.30
Styrofoam racks	Cat. #95.064.250	10/pk for $38.00

Ovarian Fluid Samples - Anchorage Laboratory

15-ml centrifuge tubes with caps VWR	Cat. # 21008-103	$155/case of 500

The 15-ml VWR tubes are preferred, although the 10-ml opaque tubes can also be used.

MATERIALS LIST FOR HATCHERY MANAGERS

Additional Materials (Approximate prices)

➔ 10 ml conical centrifuge tubes / Sarstedt Inc. 57.519	$79.90/1000
➔ caps for centrifuge tubes / Sarstedt Inc. 65.793	$24.22/1000
Disposable racks for centrifuge tubes / VWR. 12000-802	$28.51/10 pk
➔ Whirl-Pak® 2 oz. bags (with white strip) (500) / VWR 11216-772	$62.45/500
Applicator sticks / VWR 10805-018	$12.89/1000
Wooden tongue depressors/ VWR 62505-007	$15.76/500
Gram stain (4 x 250 set) VWR 90000-708	$44.08
Slides (glass one-end frosted) / VWR 48311-600 cost/box = ½ gross	$43.870
Cover-slips (22 x 22 mm) / VWR No. 1 48366-067 1 oz. will cover 1 box of slides	$16.42/oz
Filters, disposable 25-mm syringe filters, sterile VWR 28145- 481 0.45 µm (cellulose acetate)	$97.56 /50
Hema 3 Differential Staining Set / Fisher 22-122911 (15-sec. staining procedure) 3 X 500 ml	$132.04 /set
Pasteur pipets (disposable) 5 ¾" / VWR 14672-410	$118.79 /1000
Rubber bulb (for use with Pasteur pipet) / VWR 56310-240 12/pkg	$4.70/12 pkg
FAT slides (Erie Scientific 10-2255) can be obtained either from the Anchorage or Juneau Pathology Labs.	
Tryptic Soy Agar pre-poured plates VWR 29452-106	$8.70/10 plates
Disposable petri dishes (The pathology labs will provide these if needed)	
Sterile syringes with Luer-Lok tips 10 cc / VWR BD309604	$18.70 /100

➔ **NOTE**: Substitutions for the Whirl-Pak® 2 oz. Bags and the centrifuge tubes are not acceptable.

VENDOR NAMES AND ADDRESSES

Fisher Scientific
4500 Turnberry Dr.
Hanover Park, IL 60133
(800) 766-7000

Sarstedt, Inc.
P.O. Box 468
Newton, NC 28658
(800) 257-5101

VWR Scientific
355 Treck Dr.
Seattle, WA 98188-7603
(800) 932-5000

CHAPTER 3

Standard Necropsy Procedures for Finfish

Theodore R. Meyers

After assigning an accession number to the sample, the case data information received with the sample should be used to fill out the laboratory worksheet that will accompany the case to completion. Live fish should be examined for behavioral abnormalities (spiral swimming, flashing, flared gill opercula, prostration, etc.) then anesthetized to avoid tissue artifacts caused by alternate methods of euthanasia such as pithing or a blow to the head. Some external abnormalities (whitened or eroded fin tips, cloudy cornea, body discoloration, excessive mucus) are best observed while the fish is submerged in water. In many cases postmortem change in fish received dead will prevent this latter opportunity.

I. General Necropsy Procedure

A. Necropsy subjects should first be examined for external abnormalities or lesions that could include: pinheadedness or otherwise poor body condition; exophthalmia; cloudy cornea or lens opacity; hemorrhaging within the anterior chamber of the eyes, fins, body surface or body orifices (anus, nares, mouth, gill chamber), frayed or missing fins; gas bubbles within the fin rays or connective tissues of the eyes; ulcerations, abscesses, abrasions; body discolorations; excessive mucus; trailing fecal casts or rectal prolapse; external foreign bodies such as fungus, metazoan or protozoal parasites, cysts or tissue growths; potbelly or other protrusion or body malformations (spinal deformities, cranial swelling, shortened opercula, pugheadedness, microeye).

B. External lesions such as ulcerations or abrasions should be struck onto TSA. Use of TSA with 1.5% NaCl may be necessary depending upon case information and whether fish are in saltwater and a halophilic bacterial pathogen is suspected.

C. A peripheral blood smear should be made by excising the caudal peduncle (for small fish) and allowing a drop of blood to be deposited near the frosted end of a clean glass slide. The blood is smeared <u>before clotting</u> with a second glass slide by touching the drop with the slide at a 45^{o} angle to the first slide and pushing the angled slide to the end of the first slide. Capillarity draws the smear across the first slide and the smaller the angle the thicker the smear (Fig. 1, p. 3-6). Stain the smears in a differential 3-part stain (such as Hema 3) (see staining procedures in this section) and observe on the microscope at 1000X for bacterial rods, erythrocytic inclusion bodies (EIB) and viral erythrocytic (VEN) cytoplasmic inclusions, necrobiotic bodies (IHNV) and erythroblastosis or other blood abnormalities in cell composition and morphology. Larger fish may be bled by caudal vein puncture into a heparinized syringe or Vacutainer® and blood expressed onto a slide for subsequent smearing. For blood collection, the needle should be inserted at the location just below the lateral line that intersects with the rear margin of the anal fin. The needle should be inserted until just penetrating the vertebra (hemal canal) as indicated by slight resistance. Blood will automatically begin to flow

when the Vacutainer® is punctured by the needle base or when the plunger of the syringe is pulled back.

D. Fish should be placed on their right sides for performance of the remaining necropsy procedures. Skin scrapes of normal and lesion areas mounted with a drop of PBS and coverslip on a glass slide should be made by using either the edge of the coverslip as the scraping instrument, or a scalpel. Bacteria or fungus from lesion areas or protozoal parasites such as *Ichthyobodo* and *Trichodina* are common organisms to look for beginning at 40x and then at 200X on a compound microscope using the aperture diaphragm.

E. Wet mounts of gill filaments are made by using a small pair of surgical scissors to remove a portion of one gill arch. Gill filaments should be slightly teased apart for good viewing of filament and lamellar profiles and mounted in PBS with or without a coverslip. These should be examined immediately since branchial epithelium rapidly deteriorates causing postmortem artifact. Look for gas bubbles in the capillaries, telangiectasia, hyperplasia, external parasites (bacterial, protozoal, fungal, metazoan), or other foreign bodies. Should bacteria be observed or suspected the coverslip may be removed and used to mince the gill tissue. This is allowed to air dry for later Gram staining. After staining, the gill tissue is removed with forceps for viewing of the stained slide for bacteria by oil immersion.

F. Disinfect the outer surface of the fish by flooding with 70% ethanol. Disinfect a pair of scissors, forceps and scalpel by immersion in 100% ethanol and passing the instruments through a Bunsen flame allowing the alcohol to ignite and burn off. Repeat one or two more times. Wipe instruments clean of any organic matter beforehand for effective disinfection.

G. The abdominal cavity is entered by pulling the pectoral fin with sterile forceps while cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors. The cut is continued dorsally to just below the lateral line where resistance is encountered. Start again at the base of the pectoral fin and continue the incision towards the posterior of the fish along the ventral abdominal wall to the vent. Stay slightly above the intestinal tract when making the incision so that it is not punctured, thereby contaminating the tissues. At the vent continue dorsally to just below the lateral line and continue cutting anteriorly to connect with the first incision. Remove the flap of abdominal tissue, thus exposing the internal viscera and cavity. When done correctly on a moribund specimen the air bladder should remain inflated and the GI tract completely intact. Instruments may need wiping of organic material and flaming repeatedly during this procedure.

H. Visually examine viscera (heart, liver and gall bladder, kidney, pancreas, adipose tissue, spleen, air bladder, pyloric caeca and entire GI tract) for abnormalities such as: discoloration or mottled appearance; enlargement (hypertrophy); hemorrhage or erythema; abscesses or cysts; fluid in the abdominal cavity (ascites causing potbelly); foreign bodies such as fungus, metazoan parasites or tissue growths, etc.

I. If bacteriologic samples are to be taken they should be struck onto TSA from the kidney and/or from visceral lesions before other samples are taken to avoid bacterial contamination (see bacteriology Chapter 4 for procedures). If *Phoma* or other fungus is

suspected, samples from the suspect lesion or air bladder should be struck onto PA and either OA or CMA.

J. Tissues to be taken for viral assay of larger fish (kidney/spleen pool) should also be placed into sterile tissue culture fluid for refrigeration and homogenization at a later time. Fry are generally processed whole for virology (see processing and storage details in virology Chapter 5).

K. Kidney smears for FAT detection of the BKD agent should be taken at this step. Generally, bacterial problems due to Gram-negative bacteria such as furunculosis and ERM agents can be detected more efficiently by isolation on prepared media. Kidney tissues for ELISA are generally not taken from a diagnostic case since clinical disease caused by the BKD agent can easily be detected using FAT or Gram stain when lesions are apparent.

L. If the spleen has not been completely removed for virus assay, a spleen squash can be made by placing a cut section of the tissue with a drop of PBS on a glass slide and covering with a coverslip. Whole spleen squashes will be necessary when small fish are examined. Look for the presence of motile or non-motile bacterial rods and fungal hyphae. The coverslip may be removed and the squash Gram stained for confirmation of bacteria as described for gill tissues.

M. A squash of a small section of the lower intestine (rectum) should also be made on a glass slide using PBS and a coverslip. Look for presence or absence of food and *Hexamita* or amoebae. Bacteria should obviously be abundant as part of the normal gut flora. Also look for fungal hyphae within the gut wall or lumen.

N. A squash of lesion material from a visceral organ or organs may be warranted if present and if its cause is not readily discernible. Gram stains and/or a differential stain of this material may also be warranted (see staining procedures in this section). An example would be stained impression smears of kidney tissue to examine for possible BKD, PKD or *Nucleospora salmonis.*

O. If the cause of mortality or morbidity is in question as to whether or not the above procedures will provide an answer, histology samples should be taken as a backup measure, but only if moribund fish are available. Fish that have been dead for several hours or longer are generally not suitable for histology due to postmortem tissue autolysis. If fry are involved, whole fish may be dropped into Bouin's fixative or 10% buffered formalin. Fingerlings should have the abdomens opened with scissors for better fixative penetration (see the histology section for more detail regarding fixation of tissues).

P. If clinical signs suggest a central nervous system disorder the top of the cranial cavity should be opened and the brain included in bacteriologic sampling using TSA and cytophaga or TYES agar. Heads from additional affected fish should be severed behind the gill opercula and placed into fixative for later histological sectioning of the brain.

Q. During necropsy, occasional serial sectioning of skeletal muscle using a razor blade may be necessary should a lesion within that tissue be suspected. Examples would include abscesses, hematomas, neoplasms or encysted parasites causing a protrusion of the

musculature. Depending upon the nature of the lesion, bacteriological sampling, Gram staining or fixation for histology may be necessary.

This necropsy procedure should include at least 5-10 moribund or otherwise affected fish. Control or healthy fish may be requested for comparison of whether abnormalities perceived are real or not. The number of control fish processed will depend upon the particular case and may range from 10 to none.

Necropsies are best performed as a 2-3 person team effort in which a microbiologist and/or technician can make gross external and internal observations and the bacteriologic and tissue preparations. The pathologist in charge can devote his or her time to interpreting the sample preparations on the microscope. In this approach a case can be processed in a minimum amount of time and provides further pathology experience to the support staff.

In summary, a standard necropsy should include:

- Accession number, case data information, laboratory worksheet
- External and internal gross observations which could include brain and serial sectioning of skeletal muscle.
- Wet mounts or squashes of:
  - ◊ gills
  - ◊ skin
  - ◊ spleen
  - ◊ lower gut
  - ◊ lesions (if any)
- Peripheral blood smear – differential stain
- Bacteriology/mycology - TSA/PA from kidney and lesions (if any); Gram stains
- Virology sample (only necessary in some cases)
- Kidney smear for FAT
- Tissues for histology (only necessary in some cases)

NOTE: For Standard Laboratory Shellfish Necropsy Procedures see histology section for details by species.

II. Staining Procedures

A. Gram stain

1. Make smears of blood, fluid, tissue or bacterial colony from an agar plate, as described in previous sections and air dry. (Heat fix smears but do not heat excessively, i.e., slide should not be too hot to touch.)

2. Place slide on a rack over a sink.

3. Cover smear with crystal violet dye and let stand for 1 minute.

4. Rinse briefly in tap water and drain. Flood smear with Gram's iodine, pour off and flood again. Let stand for 1 minute.

5. Wash briefly in tap water and drain. Rinse with decolorizer (95% ethanol or ethanol-acetone mixture from a pipet) until no more purple dye comes off the slide (usually 2-5 seconds). Excessive decolorization may give a false Gram-negative reaction.

6. Wash briefly with tap water and drain. Flood smear with safranin stain and let stand 1 minute.

7. Wash briefly with tap water, drain, and air dry.

8. Examine the slide using the oil immersion lens (100x)

9. Gram-negative bacteria will be pink to red; Gram-positive bacteria will be purple.

B. Differential stain (such as Hema-3)

1. Make smears of blood, fluids, or tissues.

2. Dip 5 times in fixative solution 1, one second each time, and drain.

3. Dip 5 times in stain solution 2, one second each time, and drain.

4. Dip 5 times in stain solution 3, one second each time, and drain.

5. Rinse in tap water and drain.

6. Air dry and examine using 10x, 40x, or 100x.

NOTE: Stain solution 3 can become weakened with age or use. Check stain intensity on slides periodically. Slides may be re-stained with fresh solution 3 if necessary. Periodically pass solutions 2 and 3 through separate 0.45-µm filters to remove precipitates and contaminating bacteria.

Figure 1. Demonstration of how to make a thin blood smear.

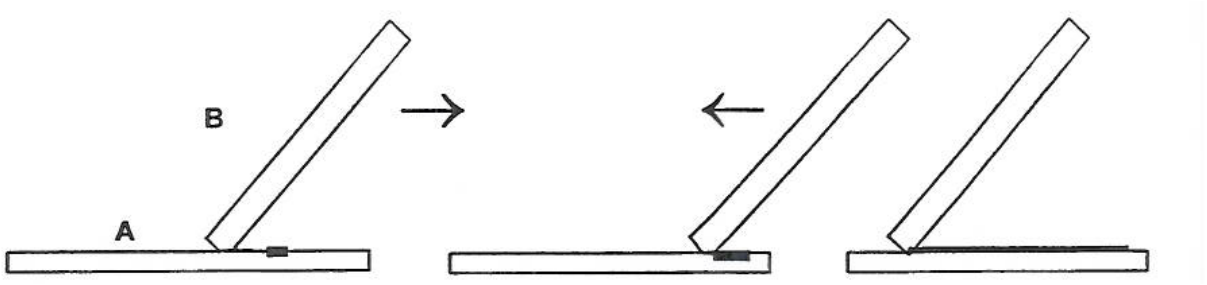
a. On slide “A” express a drop of blood or hemolymph about one-half inch from the end.

b. The edge of a second slide “B” is placed on the surface of slide “A” at about a 45° angle and is moved to the right until contact with the drop of blood.

c. Contact with the blood will cause the drop to spread along the edge of slide “B” due to capillarity. Slide “B” is then pushed to the left, being careful to keep the edge pressed uniformly against the surface of slide “A”.

d. The size of the drop of blood and acuteness of the angle formed between the slides will determine the thickness of the film. A more acute angle results in a thicker film.

e. The smear is allowed to air dry for transport in a slide box and later staining.



Preparation of thin blood smear

CHAPTER 4

Bacteriology

Tamara Burton, Jill Follett, Sally Short,
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I. Media Preparation

A. Plate Media

1. Prepare media in stainless steel beakers or clean glassware according to written instructions. Check pH and adjust if necessary. Media must be heated to boiling to dissolve agar before dispensing into bottles.
2. Pour into suitable screw-capped glassware.
3. Autoclave for 15 minutes at 15 pounds pressure or according to instructions of manufacturer. Be sure to leave lids loose.
4. Cool to approximately 50°C in a water bath before pouring.
5. Alternatively, cool media to room temperature and store in refrigerator. Label with type, date and initials. When media is needed, boil or microwave stored media bottles to melt agar and cool to 50°C before pouring.
6. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface.
7. Label the bottom of each plate with medium type and date prepared.
8. Remove bottle cap and pour plates lifting each petri dish lid as you go. Replace lids as soon as the plate is poured. Do not allow medium to run down the outside of the bottle. Hold the bottle as horizontal as possible while pouring.
9. Immediately rinse medium bottle and cap with hot water to remove agar and clean up any spilled agar.
10. Allow plates to sit overnight at room temperature or for a few hours at 37°C to reduce excess moisture. Store plates upside down in the refrigerator in a tightly sealed plastic bag.
11. Always perform sterility check on media batch and record results.

B. Tube media

1. Prepare media in stainless steel beakers or clean glassware according to written instructions.
2. If the medium contains agar, boil to dissolve the agar.
3. Media with indicators must be pH adjusted. This is best done when each medium is at room temperature, otherwise a compensation for temperature needs to be made.
4. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.
5. Use an automatic pipettor or pipet-aid to dispense the medium.
6. Immediately after use, rinse the pipettor with hot tap water followed by distilled water to remove media.
7. Cap tubes loosely.

8. Autoclave as directed.
9. If making slants, put tubes in slant racks after autoclaving and tighten caps when cooled to room temperature.
10. Label the tubes or the tube rack with type of medium and date made.
11. Store at 2-8°C.
12. Always perform sterility check on media batch and record results.

II. Media Formulations

Arginine dihydrolase broth
Suspend 10.5 g dehydrated medium (decarboxylase broth, Difco #0890) in 1 L distilled water and heat to dissolve completely. Add 10 g of L-arginine HCl (Difco #0583) and mix the solution. Dispense the medium in 5 ml/tube and autoclave for 15 minutes at 15 pounds pressure (121°C). Final pH = 6.0 at 25°C. Store at 2-8°C.

Bacterial freeze media
Prepare a 200 ml solution of Tryptic soy broth by adding 6 gm media in 200 ml water. Stir until dissolved. Add 50 ml glycerol. Mix and filter sterilize into 250 ml container. Keep refrigerated until used.

Bile esculin agar
Suspend 64 g dehydrated medium (Difco #0879) in 1 L distilled water and heat to boiling to dissolve completely. Dispense 7 ml medium into tubes and autoclave for 15 minutes at 15 pounds pressure. Cool medium in a slanted position. Store tubed medium at 2-8°C. Final pH = 6.6 $\pm$ 0.2 at 25°C.

2,3-Butanediol
Prepare the following basal medium:

nutrient broth	8.0 g
dibasic potassium phosphate	5.0 g
dH_2O	1 L

Distribute medium 4.5 ml/tube; autoclave at 15 psi for 15 minutes. Aseptically add 0.5 ml of a sterile 10% glucose solution (1% final glucose concentration).

Citrate agar (Simmon's)
Suspend 24.2 g dehydrated medium (Difco #0091) in 1 L distilled water and heat to boiling to dissolve completely. Dispense 7 ml/ tube. Autoclave for 15 minutes at 15 pounds pressure and cool in a slanted position. Store at 2-8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

Cornmeal agar
Suspend 17.0 g dehydrated medium (Difco #0091) in 1 L distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C. Final pH = 6.0 $\pm$ 0.2 at 25°C.

Cytophaga agar

tryptone	0.5 g
yeast extract	0.5 g
sodium acetate	0.2 g
beef extract	0.2 g
agar	11.0 g
dH_20	1 L

Suspend the above ingredients in distilled water and heat to dissolve. Adjust pH to 7.2-7.4 and autoclave 15 minutes at 15 pounds pressure. Store at 2-8°C. Add 5% sterile fetal bovine serum just before pouring plates. For marine organisms, replace 1 L water with 500 ml water and 500 ml instant ocean or sea water. Label media salt water cytophaga agar (SWCA).

Modified cytophaga agar (from Rich Holt, Oregon Department of Fish and Wildlife, April 1996)

I. Agar

tryptone	4.0 g
yeast extract	0.4 g
$MgSO_4$ - 7 H_2O	0.5g
$CaCl_2$ - 2 H_2O	0.2 g
agar	10.0 g
dH_2O	1 L

II. Skim milk stock

skim milk powder	40.0 g
dH_2O	200 ml

Add distilled water to the skim milk powder to bring to a volume of 200 ml and dispense into 5-10 ml capped tubes. Autoclave for barely 15 minutes.

Add about 1 ml of the sterile skim milk stock (warmed to room temperature) to about 100 ml of the modified cytophaga agar medium above, just before pouring. The amount of skim milk stock added should be just enough to make the agar medium slightly turbid.

NOTE: Isolation of marine organisms requires the addition of artificial sea salts at concentrations of 1.5 to 2%.

KDM-2 agar

For isolation of *Renibacterium salmoninarum* (Rs). Selective kidney disease medium (SKDM) may also be used and contains antibiotics to inhibit other organisms.

Basal medium

peptone	10.0 g
yeast extract	0.5 g
cysteine HCl	1.0 g
dH_2O	800 ml
agar	15.0 g

Final pH = 6.5

Dissolve first three ingredients in dH_2O and adjust to pH 6.5 with NaOH. Add agar and bring to boiling. Autoclave at 15 pounds pressure 15 minutes. This medium can be stored in the dark for up to 3 months.

NOTE: The ability of peptone to support growth of the Rs organism can vary with the manufacturer and within different product lots. To circumvent this problem see page 4-15 for isolation and maintenance of the Rs organism.

Completed medium

1. Cool melted basal medium to 45°C.
2. Add 20 ml sterile calf serum or bovine serum (warmed to 45°C) per 100 ml basal medium.
3. Pour into petri dishes or 25-cm^2 tissue culture flasks. Petri dishes should be taped around the edges to prevent dehydration during prolonged incubation.
4. For best results use within 1-2 weeks of preparation. The medium will begin to form a white precipitate with age.
5. Incubate at 15°C. It takes several weeks for growth.
6. Shelf life of this medium is 3 months. Store at 2-8°C.

KDM-2 Broth growth medium

For growing up cultures of *Renibacterium salmoninarum* (Rs).

Peptone	10.0 g
Yeast Extract	0.5 g
Cysteine-HCl	0.5 g
dH_2O	900 ml

Adjust pH to 6.5 then add 15 ml of nurse medium (filter sterilized spent KDM_2 broth that has supported growth of Rs cells) if you have it. See section on isolation and maintenance of *R. salmoninarum* for further details.

Autoclave, then temper sterile media in 50ºC waterbath and add 100 ml fetal bovine serum for final broth.

KDM-2 Freeze medium

For freezing and storing *Rs* isolates.

Add 2% gelatin to KDM-2 broth above used as the freeze medium.

Peptone-saline used for resuspending bacteria cultured in broth (see isolation and maintenance section for procedure).

Peptone	1.0 g
NaCl	8.5 g

Bring to 1 liter with dH_2O and autoclave.

Lysine decarboxylase broth
Suspend 14 g of the dehydrated medium (Difco #0215) in 1 L distilled water and heat to boiling. Dispense 5 ml/tube and autoclave at 15 pounds pressure for 15 minutes. Final pH = 6.8 at 25°C. Store at 2-8°C.

MacConkey agar
Suspend 50 g of the dehydrated medium (Difco #0075) in 1 L distilled water and heat to boiling. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes. Pour into petri plates. Final pH = 7.1 ± 0.2. Store at 2-8°C.

Motility test medium
Suspend 20 g of dehydrated medium (Difco #0105) in 1 L distilled water and heat to boiling. Dispense 5 ml/tube and autoclave at 15 pounds pressure for 15 minutes. Cool medium in an upright position in a cold water bath. Final pH = 7.2 ± 0.2 at 25°C. Store at 2-8°C.

MRVP broth-(Methyl Red/Voges-Proskauer)
Suspend 17 g of dehydrated medium (Difco #0016) in 1 L distilled water. Distribute 5 ml/tube and autoclave for 15 minutes at 15 pounds pressure. Final pH = 6.9 at 25°C. Store at 2-8°C.

Mueller-Hinton agar
Suspend 38 g of dehydrated medium (Difco #0252) into 1 L distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure (121°C). Aseptically dispense into sterile petri dishes. Store at 2-8°C. Final pH = 7.3 ± 0.1 at 25°C.

Nutrient broth
Dissolve 8 g of the dehydrated medium (Difco #0003) in 1 L of distilled water. Dispense 5-7 ml/tube and autoclave for 15 minutes at 15 pounds pressure. Final pH = 6.8 at 25°C. Store at 2-8°C.

Oatmeal agar
Suspend 72.5 g dehydrated medium (Difco #0091) in 1 L distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C.

Oxidation/Fermentation medium
Suspend 9.4 g of dehydrated medium (Difco #0688) in 1 L of distilled water and heat to boiling. Distribute in 100-ml amounts, autoclave for 15 minutes at 15 pounds pressure (121°C). To prepare final medium aseptically add 10 ml of a filter-sterilized (0.45µm) 10% glucose solution. Mix flask thoroughly and aseptically dispense 5.0 ml into sterile tubes. Store at 2-8°C. Final pH = 6.8 ± 0.2 at 25°C.

Potato dextrose agar (PA)
Suspend 39 g of the dehydrated medium (Difco #0013) in 1 L of distilled water. Bring the solution to a boil to completely dissolve. Autoclave for 15 minutes at 15 pounds pressure. Cool medium to 45°C. Pour into sterile petri dishes immediately. Final pH = 3.5 ± 0.2 at 25°C. Store at 2-8°C. To reduce bacterial growth, add 1.6 ml of sterile 1:10 dilution of tartaric acid per 100 ml of medium. After addition of acid do not heat medium.

Sabouraud dextrose agar

Suspend 65 g of dehydrated medium (Difco #0109) in 1 L distilled water. Heat to boiling to dissolve and dispense 7.0 ml into screw cap tubes. Autoclave for 15 minutes at 15 pounds pressure and cool in slanted position. Store at 2-8°C. Final pH = 5.6 ±0.2 at 25°C.

Selective kidney disease agar (SKDM)

tryptone (1%)	10.0 g
yeast extract (0.05%)	0.5 g
cyclohexamide (0.005%)	0.05 g
agar (1%)	10.0 g
L-cysteine HCl (0.1%)	1.0 g

Suspend the above ingredients in 900 ml of distilled water and heat until dissolved. Adjust the pH to 6.8 with NaOH. Autoclave for 10 minutes at 15 pounds pressure. After sterilization, cool to 45°C and add 10% sterile calf serum . Finally, add filter-sterilized (0.45 µm) antimicrobial solutions for a final concentration of:

D-cycloserine (0.00125% w/v), polymyxin B sulfate (0.0025% w/v), and oxolinic acid (0.00025% w/v). The plates should be stored at 2-8°C in the dark. The antibiotics significantly enhance the isolation capacity of the KDM_2 medium (Gudmundsdottir et al. 1991).

Triple sugar iron agar (TSI)

Suspend 65 g of the dehydrated medium (Difco #0265) in 1 L of distilled water and heat to boiling. Dispense 7 ml/tube. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool in slanted position. Store at 2-8°C. Final pH = 7.4 at 25°C.

Tryptic soy agar (TSA)

Suspend 40 g of dehydrated medium (Difco #0369) in 1 L distilled water and heat to boiling to dissolve completely. Autoclave at 15 pounds pressure for 15 minutes. Pour into petri plates. Store at 2-8°C. Final pH = 7.3 ±0.2 at 25°C. If a marine bacteria is suspected, 1.5% NaCl can be added to the media formulation.

Tryptone-Yeast agar

tryptone	5.0 g
yeast extract	2.5 g
agar	15.0 g
dH_2O	1 L

Mix ingredients and heat until dissolved. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes. Pour into petri plates. Store at 2-8°C.

TYES agar

tryptone	5.0 g
yeast extract	0.4 g
$MgSO_4$ $7H_2O$	0.5 g
$CaCl_2$ $2H_2O$	0.2 g
agar	15.0 g
dH_2O	1 L
Fetal bovine serum	50 ml

Mix ingredients and heat until dissolved completely. Autoclave at 15 pounds pressure for 15 minutes. Cool to 45°C and aseptically add warmed FBS and pour into petri plates. Store at 2-8°C. Final pH = 7.1 ±0.2. If a marine *Flexibacteria* or *Flavobacterium* is suspected, 1.5% NaCl can be added to media formulation.

Congo red dye may be added (100 ug/ml) to TYES agar to examine for growth inhibition of *Flavobacterium psychrophilum*. Alternatively, congo red discs may be prepared by dissolving 100 ug/ml of congo red in sterile distilled water followed by standard autoclaving for 15 minutes. Place sterile blank discs (BD-Difco, BD-Taxo) in the congo red solution overnight, remove and let dry at 37°C. Store at 2-8 °C in a screw capped bottle. Use on TYES agar plates to test for a zone of inhibition to identify isolates of *Flavobacterium psychrophilum*.

Yeast Extract agar

yeast extract	10.0 g
dextrose	10.0 g
agar	15.0 g
dH_2O	1 L

Heat water, dextrose, and agar. Add yeast extract and continue to heat until dissolved. Dispense into bottles and autoclave at 15 pounds pressure for 15 minutes. Final pH = 6.5. Store at 2-8°C.

III. Stains and Reagents

2,3-Butanediol reagents

- 2.3% aqueous Periodic Acid: stored in brown, glass-stoppered bottle
- Piperazine solution:
  - Dissolve: 25.0 g of Piperazine hexahydrate in 99 ml dH_2O
  - Then add: 1.3 ml 87% formic acid
- 4% aqueous sodium ferricyanide (or sodium nitroprusside) solution (prepare fresh with each use) e.g. 0.1 gm/2.5 ml water = 4%

Gram stain reagents

These stains can be ordered as a complete kit from VWR (#9000-708) or can be reconstituted as follows:

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.

Gram's iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH_20	300 ml

Decolorizer

acetone	40 ml
ethanol (95%)	60 ml

Safranin

safranin O	2.5 g
95% ethanol	100 ml
dH_20	900 ml

Filter safranin solution before use.

Methyl red reagent

Dissolve 0.1 g of methyl red in 300 ml of 95% ethanol.
Add 200 ml of distilled water to the alcohol-indicator mixture.
Store reagent at 4°C.

Oxidase reagent

Tetramethyl-P-Phenylenediamine Dihydrochloride	1.0 g
dH_20	100 ml

Purchased BD-TAXO discs or Spot-Test applicators (BD-DIFCO) may also be used.

O/129 Discs

0.1% solution of pteridine dissolved in acetone.

or

0.1% solution of pteridine phosphate dissolved in sterile distilled water.

Autoclave at 15 pounds pressure for 15 minutes. Place sterile blank discs (BD-Difco, DD-Taxo Concentration Discs 1/4") in pteridine or pteridine phosphate solution, remove and let dry at 37°C. Store at 2-8°C in a screw-cap bottle. Use on agar plates (usually Mueller-Hinton or TSA) to test the sensitivity of cultures to this vibrio-static agent.

Potassium hydroxide (KOH) 40%

Weigh out 40 g of KOH.
Dissolve in less than 100 ml of distilled water in a beaker placed in a circulating cold water bath.
Bring the solution to 100 ml with distilled water.
Store in a polyethylene or glass reagent bottle.

NOTE: KOH solutions are extremely caustic.

Voges-Proskauer reagent

Dissolve 5.0 g of alpha-naphthol in less than 100 ml of absolute ethanol (100%).
Bring solution to 100 ml with absolute ethanol.
Prepared reagent also available.

IV. Test Descriptions

A. Antibiotic sensitivities

This test determines the sensitivity or resistance of a bacterial isolate to specific antibiotics. Filter paper discs, each saturated with a different antibiotic, are evenly spaced on an agar surface inoculated with a lawn of the bacterial isolate to be tested. The antibiotics diffuse into the surrounding medium, and create a decreasing gradient of antibiotic concentration. If sensitive, a zone of inhibition or clear zone will be present around the antibiotic disc. The following antibiotics are usually tested: novobiocin, 0/129, penicillin G, erythromycin, oxytetracycline, Romet 30 and Florfenicol.

1. Suspend cells from 5 colonies of a pure bacterial culture in sterile saline or nutrient broth to obtain a turbidity equivalent to a 0.5 McFarland standard. Alternatively, use a purchased inoculation system (such as BBL Prompt) to prepare a standardized suspension of bacteria for making a test lawn according to product directions.
2. Streak a Mueller-Hinton agar plate with a sterile cotton swab soaked with the bacterial suspension. Swab the plate in three separate planes.
3. Aseptically place antibiotic discs to be tested onto a freshly inoculated plate. Press onto agar surface lightly.
4. Invert the plates and incubate at 20°C for 24-48 hours. Observe and record results by measuring the diameters of the zone of inhibition around each disc.

RESULTS: Sensitive: A significant zone of inhibition around the disc.
Resistant: Bacterial growth adjacent to disc or insignificant zone of inhibition.

Zone Diameter Standards

Antimicrobial	Disc content	Zone Diameter (mm) Resistant	Intermediate	Sensitive
Erythromycin[1]	15 µg	No zone	<15	≥15
Florfenicol[2]	30 ug	No zone	<15	≥20
Novobiocin[2]	30 µg	No zone	<10	≥10
Oxolinic Acid[1]	2 µg	No zone	<15	≥15
Oxytetracycline[1]	30 µg	No zone	<15	≥15
Penicillin G[3]	10 units	≤11	12-21	≥22
Romet 30[1]	25 µg	No zone	<15	≥15
0/129[2]	0.1%(W/V)	No zone	<7	≥7

[1] From the Model Comprehensive Fish Health Protection Program. Pacific Northwest Fish Health Protection Committee, September 1989.
[2] From CF pathology case materials.
[3] From "Performance Standards for Antimicrobial Disc Susceptibility Tests," NCCLS 1981.

Known sensitive and resistant control organisms should be tested using the same antibiotic discs. Antibiotic discs should be stored in a freezer. Replacement after exceeding the labeled expiration dates should depend on efficacy testing with control organisms.

B. Arginine dihydrolase

This test is used to differentiate organisms by their ability to dihydrolize the amino acid arginine into ornithine, ammonia and CO_2. If the dihydrolase enzyme is present, its action on arginine produces an alkaline reaction resulting in the intensification of the

bromcresol purple indicator. If the enzyme is not present, the organism ferments the glucose present in the medium causing an acid pH and a color change to yellow (negative).

1. Boil tube to create an anaerobic environment by expelling oxygen. Allow to cool.
2. Stab to the bottom of the tube with the inoculum, overlay with 4-5 mm sterile mineral oil.
3. Incubate: 20 °C for 24-48 hours.

RESULTS: Positive: Purple (no change)
Negative: Yellow

QUALITY CONTROL:
Positive: *Listonella anguillarum or Aeromonas salmonicida*
Negative: *Serratia liquefaciens*

C. Bile esculin

Esculin hydrolysis reacts with iron salt in the medium and the resultant Fe^{3+} complex colors the agar black.

1. Aseptically streak slant with a heavy inoculum from a pure culture (24-48 hours old).
2. Incubate at 20° C
3. Check slant cultures at 24 and 48 hour intervals.

RESULTS: Positive: Blackening of the medium
Negative: No blackening of the medium

NOTE: Growth may occur but this alone is not a positive reaction.

QUALITY CONTROL:
Positive: *Serratia liquefaciens*
Negative: *Yersinia ruckeri* Types 1 and 2

D. 2,3-Butanediol

Production of 2,3-Butanediol will confirm the presence of *Aeromonas hydrophila.*

1. Inoculate basal medium containing glucose, incubate (20°C) until fairly turbid (24-48 hours).
2. Add to each tube 1.0 ml 2.3% periodic acid; shake tube and let stand for 15 minutes.
3. Add: 1.5 ml of piperazine solution
   0.5 ml of 4% sodium ferricyanide or sodium nitroprusside (prepared daily).

RESULTS: Positive: distinct blue within 2 minutes
Negative: pale green

QUALITY CONTROL:
Positive: *Aeromonas hydrophila*
Negative: *Aeromonas salmonicida*

E. Citrate utilization-(Simmon's)

Utilization of citrate as a sole carbon source results in the breakdown of the citrate and ammonium salts in the medium leading to an alkaline pH with a color change from green to blue.

1. Using a loop, lightly inoculate a Simmon's citrate agar slant from a pure culture of the suspect organism.
2. Keeping the lid very loose, incubate overnight at 20°C for 24 to 48 hours. Longer incubation may be necessary.

RESULTS: Positive: Bacterial growth with slant color changing to navy blue
Negative: Sparse or no growth with medium remaining original green

QUALITY CONTROL: Positive: *Pseudomonas fluorescens*
Negative: *Aeromonas salmonicida*

F. Congo red TYES agar

Suspected isolates of *Flavobacterium psychrophilum* are inoculated onto a divided plate – one side with TYES and the other side with congo red TYES and examined for growth inhibition.

RESULTS: Positive: No bacterial growth on congo red TYES
Negative: Growth on congo red TYES

QUALITY CONTROL: Positive: *Flavobacterium psychrophilum*
Negative: *Flavobacterium sp.*

G. Congo red disc sensitivity

This test is an alternative to the congo red TYES for identification of *Flavobacterium psychrophilum*.

1. Suspend bacterial isolate in sterile saline with an optical desnsity equivalent to a 0.5 McFarland standard.
2. Streak suspension on TYES plate in three planes with sterile cotton swab.
3. Aseptically place congo red disc in center of inoculated plate.
4. Incubate at 20°C for 72 hrs or longer until good bacterial growth.

RESULTS: Positive: Zone of inhibition around disc
Negative: Growth adjacent to disc margin

QUALITY CONTROL: Positive: *Flavobacterium psychrophilum*
Negative: *Flavobacterium sp.*

H. Cytophaga agar

This agar is best for isolating flavobacteria and should be used when cold water disease is suspected. Differences in cell and colony morphology aid in distinguishing the numerous bacteria that grow on this partially selective agar. Streak for isolation on two cytophaga agar plates with inoculum from kidney, spleen, brain, gill tissues or external lesion material. Incubate one plate at 20°C and the other at 25°C for 3-5 days.

RESULTS:

Flavobacterium psychrophilum No growth at 25°C. Growth of bright yellow colonies at 20°C with convex center, spreading periphery, and fried-egg appearance.

Flavobacterium columnare Light or no growth at 20°C. Greater growth of yellow convoluted centered colonies with rhizoid edges at 25°C.

Tenacibaculum (marine) Orange or yellow colonies with uneven edges.

Flavobacterium sp. Growth range 10-25°C. With best growth at 18°C. Light yellow round colonies, transparent and smooth.

Modified cytophaga agar

This agar has been successful in isolating fastidious cold water disease organisms that have not grown on conventional cytophaga agar and is useful in differentiating from faster growing organisms. Plates should be struck with kidney, brain or lesion material and incubated at 19-20°C.

RESULTS:

Flavobacterium sp. Clear zones due to proteolysis of milk.
Pseudomonas sp. Green discoloration of the medium.

I. Gram stain (also see Chapter 3 page 3-4)

The response of bacteria to the Gram stain is an important characteristic in bacterial classification. It detects a fundamental difference in the cell wall composition of bacteria.

1. Prepare a bacterial smear from a pure culture.
   a. Put a drop of sterile saline or distilled water on a clean glass slide.
   b. Using a sterile loop/needle touch an isolated colony and mix in drop.
   c. Mix until just slightly turbid.
   d. Let air dry and heat fix. Do not heat excessively; slide should not be too hot to touch.
2. Flood the slide with crystal violet stain and allow to remain on the slide for 60 seconds.
3. Wash off crystal violet with running tap water.
4. Flood slide with Gram's iodine, pour off and flood again and allow to stand for 60 seconds.
5. Rinse off with running tap water.
6. Decolorize with 50% alcohol-50% acetone solution until the solvent flows colorless from the slide. Excessive decolorization should be avoided since it may result in an incorrect reading.
7. Rinse immediately with running tap water.
8. Counterstain with Safranin O for 60 seconds.
9. Rinse with tap water and allow to air dry.

RESULTS: Gram-negative: cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counterstained with safranin.
Gram-positive: cells retain the crystal violet and remain purple to dark blue.

QUALITY CONTROL: Positive: *Renibacterium salmoninarum*
Negative: *Aeromonas* sp.

J. Indole production—Kovac's method

This test determines the ability to split indole from the tryptophan molecule.

1. Inoculate the test organism into tryptone broth, nutrient broth or a basal medium containing tryptophan.
2. Incubate at 20 °C for 24-48 hours, preferably 48 hours.
3. Add 5 drops of Kovac's reagent (VWR EM1.11350.0001) to the incubated tube.
4. If culture is negative for indole production, test again after 48 hours.

RESULTS: Positive: A bright red color in the reagent layer indicates the presence of indole.
Negative: No color change.

QUALITY CONTROL: Positive: *Aeromonas hydrophila*
Negative: *Yersinia ruckeri*

K. Lysine decarboxylase

This test differentiates organisms by their ability to decarboxylate the amino acid lysine using the lysine decarboxylase enzyme system. Decarboxylation of lysine is accompanied by an alkaline pH shift in which the bromcresol purple indicator remains purple. If the decarboxylase enzyme is absent from the organism then glucose in the medium is fermented causing an acid pH and yellow color.

1. Inoculate broth.
2. Aseptically overlay the inoculated broth tubes with sterile mineral oil.
3. Incubate at 20 °C for 24 hours to 4 days.

RESULTS: Positive: Purple to yellow-purple color (unchanged)
Negative: Yellow color

QUALITY CONTROL: Positive: *Serratia liquefaciens*
Negative: *Listonella anguillarum*

L. Methyl red (MR)

The methyl red test indicates a change in hydrogen ion concentration (pH) from the acidic end products of glucose fermentation produced by an organism. A MR-positive test indicates a high amount of stable acid product that maintains the acidic pH of the medium. A MR-negative test indicates metabolization of the initial acidic products into more neutral by-products, thereby raising the pH of the medium.

1. Lightly inoculate the MR/VP broth.
2. Incubate at 20 °C for at least 48 hr.
3. Transfer 2.5 ml of an incubated aliquot to a test tube and add five drops of the methyl red indicator.
4. Read color resulting immediately.

RESULTS: Positive: Bright red color
Negative: Yellow color

QUALITY CONTROL: Positive: *Yersinia ruckeri*
Negative: *Enterobacter aerogenes*

M. Motility

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

1. Place a drop of water or PBS onto a clean microscope slide. Inoculate the drop from a single isolated colony that is 24-48 hours old using a sterile loop. Cover with a coverslip. Observe for motility using phase contrast at 1000 x magnification on a compound microscope. Care should be taken not to interpret "drift" or "Brownian motion" as motility.

   If this method fails to show motility then:

2. Inoculate a nutrient broth with the isolate and incubate at 20°C until growth is obtained, usually 24 hours. After incubation use a sterile loop or sterile dropper to place a drop of culture broth onto a clean microscope slide. Cover with a cover slip and observe for motility again at 1000x magnification.
3. Semi-solid motility test medium can also be used. Stab the medium with a small amount of inoculum. Incubate overnight at 20°C. If the bacteria are motile, the medium will become turbid and growth will radiate from the line of inoculum. If the bacteria are non-motile, only the stab line will have visible bacterial growth.

RESULTS: Positive: Straight-line directional movement in a wet mount or cloudy motility test medium beyond the stab line.
Negative: Brownian motion or no movement in a wet mount or growth only along the stab line in the test medium.

QUALITY CONTROL: Positive: *Pseudomonas* sp.
Negative: *Aeromonas salmonicida*

N. 0/129 sensitivity

This test determines the sensitivity of a bacterial organism to the vibriostatic agent 2,4-diamino-6,7 di-isopropylpteridine (0/129).

1. Suspend bacteria in sterile saline.
2. Streak suspension on plate in three planes with a sterile cotton swab.
3. Aseptically place sensitivity disc in the center of inoculum.
4. Incubate at 20°C for 24 hours.

This test can be done on the same plate as the antibiotic sensitivity test.

RESULTS: Sensitive: Zone of inhibition around disc
Resistant: Growth adjacent to disc

QUALITY CONTROL: Positive: *Listonella anguillarum*
Negative: *Aeromonas hydrophila*

O. Oxidase (Cytochrome oxidase)

The oxidase test determines the presence of cytochrome oxidase enzymes.

1. Place a piece of filter paper in a Petri dish.
2. Add 2 to 3 drops of Kovac's reagent (commercially available) to the center of the paper.
3. With a platinum wire or disposable plastic inoculating loop or needle, blot a desired colony onto the reagent-impregnated paper.
4. Alternative procedures include: place an oxidase test disc (TAXO) that is commercially impregnated with test reagent on a clean glass slide and wet with sterile distilled water. Transfer colony material to the moistened disc and read results; or apply reagent with a Spot-Test applicator using filter paper and colony transfer as above or apply directly to agar on the test bacterial colony.

RESULTS: Positive: Purple color within 5-10 seconds
Negative: No purple color

QUALITY CONTROL: Positive: *Pseudomonas* sp.
Negative: *Yersinia* sp.

P. Oxidation/fermentation

Bacteria metabolize carbohydrates by either oxidative and/or fermentative pathways. Oxidation occurs in the presence of atmospheric oxygen (aerobic), whereas fermentation takes place in an anaerobic environment. Metabolization of the carbohydrate dextrose by either an aerobic or anaerobic pathway results in acid production. The resulting acidic environment causes the brom thymol blue pH indicator in the medium to turn from green to yellow. The presence of bubbles in the tube indicate gas production (aerogenic). If no reaction occurs, the medium can remain unchanged or become alkaline (blue).

1. Two tubes are used for this test.
2. With a sterile needle take a small inoculum from an isolated colony and stab to the bottom of one tube, then inoculate the top of the medium with shallow stabs. Repeat with second tube.
3. Overlay the anaerobic tube with sterile mineral oil.
4. Incubate at 20°C for 24-48 hours. Check tubes at 24 hr for acid and/or gas production.

RESULTS: Key: A = acid; AG = acid + gas; N = no change or alkaline

	Open tube	Closed tube
Oxidative	A (surface)	N
Fermentative (aerogenic)	AG	AG
	Open Tube	Closed Tube
Fermentative (anaerogenic)	A	A
Non-reactive	N	N

QUALITY CONTROL: Fermentative: *Aeromonas* sp.
Oxidative: *Pseudomonas* sp.

Q. *Renibacterium salmoninarum* (Rs) isolation and maintenance

1. ISOLATION and MAINTENANCE: Inoculate KDM-2 plates by streaking for isolation from an internal organ, usually kidney. Use a heavy inoculum, preferably from a pustule, and spread out the streak to minimize potential inhibition of Rs growth by adhering fish tissues (Daly and Stevenson 1988). To retain adequate surface moisture, plates should be wrapped and incubated at 15°C for a period of 14 days to 12 weeks. Because certain peptone lots may not provide the necessary growth requirements for Rs cells (Evelyn and Prosperi-Porta 1989), it is necessary to supplement new KDM_2 medium with 1.5% (v/v) of filter sterilized spent KDM_2 broth that has supported growth of Rs cells. This supplementation will also increase sensitivity and reduce the amount of time for initial Rs isolation and allow successful subculturing of Rs stock cultures every 3-5 weeks (Evelyn et al. 1990).

2. FREEZE STORAGE:
   a. Inoculate a pure culture of *R. salmoninarum* in 1 L KDM-2 broth with fetal bovine and 1/2 the normal cysteine HCl. Incubate at 15°C for 7-10 days.
   b. Centrifuge broth and pour off supernatant. Resuspend pellet in 10-20 ml of a peptone-saline solution and triturate to break apart clumps of cells. Place in ice bath for 10 minutes.

c. Transfer 5-10 ml of the suspension into 100 ml of KDM-2 broth with fetal bovine serum and 2% gelatin. To make the broth you can autoclave it with the gelatin and then add calf serum.
d. Store 2-4 ml aliquots in Nalgene® freezer vials at -70°C.

This protocol was contributed by Ron Pascho (retired) of the USGS Western Fisheries Research Center, 6505 N. E. 65th Street, Seattle, WA 98115.

R. Triple Sugar Iron (TSI)

TSI medium can determine the ability of an organism to utilize a specific carbohydrate incorporated in a basal growth medium, with or without the production of gas, along with the determination of hydrogen sulfide (H_2S) production. TSI agar contains the three sugars in varying concentrations: glucose (1X), lactose (10X), and sucrose (10X). It also contains the indicator, phenol red, hence the agar is red. If sugar fermentation occurs, glucose will be used initially and the butt of the tube will be acidic (yellow). After glucose utilization the organism may continue to ferment the remaining sugars. If this occurs the entire tube would become acidic. Certain bacteria are unable to utilize any sugars and will breakdown the peptone present. Peptone utilization causes an alkaline (red) shift in the medium which intensifies the already red color. Blackened medium is caused by hydrogen sulfide production which changes ferrous sulfate to ferrous sulfide. In addition, gas production can be determined by splitting of the medium or presence of bubbles in the butt.

1. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streak the surface of the slant on the way out of the tube. Screw the cap on loosely.
2. Incubate at 20°C. Read after 18-24 hours.

RESULTS:

Key: A = Acid; K = Alkaline; H_2S = Hydrogen sulfide produced; N = No change

Slant/Butt	Color	Interpretation
K/N or K/A	red/red (oxidative) or red/yellow (fermentative)	only peptone utilized only glucose-fermented
A/A	yellow/yellow	glucose, plus lactose and/or sucrose-fermented

Slant/Butt	Color	Interpretation
gas	splitting or bubbles	gas production
H_2S	black butt	Hydrogen sulfide produced

QUALITY CONTROL:

A/A:	± gas:*Aeromonas* sp.	K/A w/gas H_2S:	*Citrobacter freundii*
K/A:	*Listonella anguillarum*	K/N:	*Pseudomonas fluorescens*

S. Voges-Proskauer (VP)

The VP test detects the production of acetoin, a neutral end product derived from glucose metabolism.

PROCEDURE:

1. Lightly inoculate a tube of MRVP broth.
2. Incubate at 20°C for 2-10 days.
3. Transfer 2.5 ml of an incubated aliquot to a test tube.
4. Add the following VP reagents in order:
   a. 0.6 ml (6 drops) of 5% alpha naphthol in absolute ethanol.
   b. 0.2 ml (2 drops) of 40% potassium hydroxide (KOH).
5. Shake tubes.
6. Reaction usually occurs immediately, but allow tube to stand for 10-15 minutes.

RESULTS:	Positive:	Pinkish-red color at the surface of the medium
	Negative:	Yellow or copper color at the surface of the medium
QUALITY CONTROL:	Positive:	*Enterobacter aerogenes* or *A. hydrophila*
	Negative:	*Escherichia coli*

V. Summary and Storage of Quality Control Organisms for Bacteriology Test Media

A. Summary

TEST	POSITIVE CONTROL	NEGATIVE CONTROL
Arginine dihydrolase	*Listonella anguillarum*	*Serratia liquefaciens*
Bile esculin	*Serratia liquefaciens*	*Yersinia ruckeri Types 1 and 2*
Browning on TSA	*A. salmonicida*	*A. hydrophila*
2,3-Butanediol	*Aeromonas hydrophila*	*A. salmonicida*
Citrate	*P. fluorescens*	*A. salmonicida*
Congo red inhibition	*F. psychrophilum*	*Flavobacterium sp.*
Gram Stain	*R. salmoninarum*	*Aeromonas* sp.
Indole	*A. hydrophila*	*Yersinia ruckeri*
Lysine decarboxylase	*Serratia liquefaciens*	*Listonella anguillarum*
Methyl red (MR)	*Yersinia ruckeri*	*Enterobacter aerogenes*
Motility	*Pseudomonas* sp.	*A. salmonicida*
O/F-glucose	*Aeromonas* sp. (fermentative)	*Pseudomonas* sp. (oxidative or non-reactive)
Oxidase test	*Pseudomonas* sp.	*Yersinia* sp.
Sensitivity to 0/129 and novobiocin	*Vibrio* sp.	*Aeromonas* sp.
TSI	*Aeromonas* sp. A/A	*Listonella anguillarum* K/A
VP	*Enterobacter aerogenes*	*E. coli*

B. Storage

Storage of quality control bacteria (and fungi) is accomplished by freezing at -70°C to avoid continual subculturing and potential genetic changes that could alter specific biochemical reactions.

PROCEDURE:

1. Prepare pure 18-24 hr subculture on TSA medium.
2. Scrape the colony growth from the plate with a sterile cotton swab and inoculate heavily into 1 ml of Tryptic Soy/glycerol broth (bacterial freeze media) contained in a 2 ml screw-capped freezer tube (Sarstedt) appropriately labeled.
3. Place tubes in ultracold freezer (-70°C) in a labeled freezer box and enter freezer log information.

VI. Commercial Identification Systems - best if used in conjunction with the Rapid Bacteriological Identification Chart in Appendix C (page 4-26).

A. API; - Two different API kit systems are used to identify Gram negative pathogens in fish tissues. The kits consist of microtubules that contain dehydrated substances. Most tests are inoculated with the test organism suspension which reconstitutes the test substance. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The API 20 NE (product # 20050) is used to identify non-enteric, Gram negative rods and all oxidase positive fish pathogens. The API 20 E (product # 20100) is used for enteric, Gram negative rods and all oxidase negative fish pathogens. Kits can be purchased from:

BioMerieux
P. O. Box 15969
Durham, North Carolina 27704
919-620-2000

B. Bionor: - There are four Bionor Aqua rapid agglutination tests that are useful in the fish pathology labs; Mono-As for *Aeromonas salmonicida* (product # DD 020), Mono-Va for *Listonella anguillarum* (product # DE 020), Mono-Yr for *Yersinia ruckeri* (product # DC 020) and Mono-Vs for *Vibrio salmonicida* (DF 020) These can be purchased from:

Bionor
Strømdaljordet 4,
P.O. Box 1868 Gulset
N-3701 Skien
Norway

These kits contain a test reagent and a control reagent. The test reagents consist of monodispersed particles coated with antibodies which form a granular particle agglutination pattern when mixed with the homologous bacteria. When the bacterial isolate is mixed with the control reagent, no agglutination will appear. See instructions enclosed with each test for complete directions. These tests appear to be very specific.

C. Microtek:- There are seven Microtek latex bead agglutination tests that are useful in the fish pathology labs for identification of *Aeromonas salmonicida* (ADA501), *Flavobacterium psychrophilum* (ADFP01), *Yersinia ruckeri* Types 01 (ADYR01) and 02 (ADYR02), *Listonella anguillarum* 01 (ADVA01) and 02 (ADVA02) and *Vibrio salmonicida* (ADVS01). These kits can be purchased from:

Microtek
6761 Kirkpatrick Crescent
Saanichton, British Columbia
Canada V8M 1Z8
www.microtek-intl.com

VII. Biochemical Characteristics of Common Bacterial Fish Pathogens (see Appendices B and C on pages 4-25 and 4-26)

A. *Aeromonas salmonicida*: Gram-negative rod, non-motile, browning on TSA, cytochrome oxidase positive, ferments O/F glucose, 2,3-butanediol negative, 0/129 resistant. See appendix A for exceptions.

B. *Aeromonas hydrophila*: Gram-negative rod, motile, cytochrome oxidase positive, ferments O/F glucose w/gas, 0/129 and novobiocin resistant, Voges-Proskauer positive. Only organism in the aquatic environment that is 2,3-butanediol positive.

C. *Aeromonas* sp. other than *A. hydrophila*: Gram-negative rod, motility variable, 2,3-butanediol negative, cytochrome oxidase positive, ferments O/F glucose, 0/129 and novobiocin resistant.

D. *Pseudomonas fluorescens*: Gram-negative rod, motile, cytochrome oxidase positive, O/F oxidative, inert or alkaline. Produces fluorescein pigment. Does not grow at 42°C.

E. *Vibrio ordalii*: Gram-negative rod, motile, cytochrome oxidase positive, ferments O/F glucose, 0/129 and novobiocin sensitive, lysine decarboxylase negative, arginine dihydrolase negative. Will grow on TSA supplemented with NaCl.

F. *Listonella anguillarum*: same as *Vibrio ordalii*, except arginine dihydrolase positive.

G. *Yersinia ruckeri*: Gram-negative rod, motile, cytochrome oxidase negative, indole negative, alkaline/acid in TSI, ferments O/F glucose. Bile esculin and salicin negative. Two types, I and II, may be separated by FAT or sorbitol utilization test (negative for Type I).

H. *Serratia liquefaciens*: Gram-negative rod, motile, cytochrome oxidase negative, O/F fermentative, acid/acid or alkaline/acid in TSI, lysine decarboxylase positive, arginine dihydrolase negative, bile esculin and salicin positive.

I. *Renibacterium salmoninarum*: Small, Gram-positive diplobacilli, only grows on KDM-2 medium. Confirm with FAT or PCR.

J. *Flavobacterium columnare* (flavobacteria): Gram-negative, very long thin rod, gliding motility, colony is dry, rhizoid, yellowish on cytophaga agar, often seen as tangled masses on fish gills.

K. *Flavobacterium psychrophilum* (flavobacteria): Gram-negative, medium long thin rod, gliding motility. Cultures produce non-diffusible-yellow pigmented colonies with thin spreading margins. Cells become pleomorphic in older cultures. Confirm with congo red inhibition and PCR.

L. *Flavobacterium* sp.: Gram-negative non-motile rod, yellow, orange, or pink colonies on TSA, cytochrome oxidase variable, O/F negative. Examine gills for clubbing and filamentous rods.

VIII. Bacterial Fish Diseases: Causative Agents and Signs

DISEASE	AGENT/SIGNS
Bacterial Gill Disease:	Causative agents: *Flexibacter* sp., *Flavobacterium* sp. and unclassified filamentous bacteria, other unidentified non-motile rods. **External signs:** gill hyperplasia; swelling, sometimes clubbing, fusing of filaments and lamellae; presence of large numbers of bacteria; listlessness; loss of appetite.
Bacterial Kidney Disease:	Causative agent: *Renibacterium salmoninarum.* **External signs:** exophthalmia; abdominal swelling; sometimes blisters in skin filled with clear amber to cream colored purulent fluid. **Internal signs:** kidneys pale and swollen; abscesses in kidney, liver or spleen; may have ascitic fluid in abdomen; intestine distended, fluid filled.
Bacterial Septicemia:	Causative agents: *Aeromonas* or *Pseudomonas* sp. particularly *Aeromonas hydrophila* and *P. fluorescens*. **External signs:** hemorrhaging at base of fins, eyes and vent; pale gills; exophthalmia. **Internal signs:** bloody or ascitic fluid in body cavity; hemorrhaging of internal organs; kidney soft; spleen and other organs pale.
Cold Water Disease (and other proteolytic skin infections):	Causative agents: *Flavobacterium psychrophilum* and other filamentous bacteria. **External signs:** tail darkening, white or bluish areas behind dorsal or adipose fins; loss of epidermis on dorsal or posterior surface; erosion of the dermis on the peduncle exposing skeletal muscle; loss of caudal peduncle; erosion of jaw or snout; gill hemorrhages and anemia. **Internal signs:** generally not remarkable but sometimes: enlarged spleen with myriad number of filamentous rods; petechial hemorrhages of adipose tissues.

Enteric Redmouth: Causative agent: *Yersinia ruckeri* Types 1 and 2.
External signs: hemorrhaging or erosion around mouth; exophthalmia; swollen abdomen; reddened opercula and fin bases; inflamed hemorrhagic vent.
Internal signs: inflammation and hemorrhaging in most visceral organs; edema in spleen, liver and kidney; liver may be pale and hemorrhagic; fluids may accumulate in abdominal cavity, stomach and intestine; inflamed, hemorrhagic lower intestine with bloody diarrhea.

Furunculosis: Causative agent: *Aeromonas salmonicida.*
External signs: skin blisters or furuncles which may ulcerate; petechiae; erythema of eyes, base of fins and anal vent. In acute cases, bleeding from the gills may be seen.
Internal signs: kidney necrosis; petechiae in mesenteries around pancreatic tissue; localized hemorrhages in intestine and liver; dark, hypertrophied spleens.

Vibriosis: Causative agents: *Listonella anguillarum, Vibrio ordalii* and *Vibrio* sp.
External signs: hemorrhages in the eye; erythema at the base of fins; petechiae in skin and musculature; darkening of dorsal surface; bloating and open penetration of abdominal cavity; bleeding at the vent; pale gills.
Internal signs: petechiae and hemorrhagic areas in internal organs and mesenteries; enlarged spleen and kidney; fluid in the gut.

Air bladder or gut fungus: Causative agents: *Phoma* sp., *Saprolegnia* sp.
External signs: pinched abdomen sometimes bloated anteriorly; erythema of vent and rectal prolapse; occasional exophthalmia; bloated abdomen with open penetration of abdominal cavity.
Internal signs: fluid-filled air bladder with hemorrhages and mycelial mass; hemorrhagic gut with mycelial mass; visceral adhesions with diffuse fungal mycelium; secondary motile bacterial septicemia.

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X. Appendices A, B, C

APPENDIX A: Variations in *Aeromonas* sp.

A. Pigmentation production is variable
   1. 5-10% of the strains of *A. salmonicida salmonicida* do not produce pigment when grown on TSA.
   2. 5-10% of the strains of *A. salmonicida achromogenes* do produce pigment when grown on TSA.
   3. Some strains of *A. salmonicida* will produce pigment on Furunculosis agar (not commercially available) but not on TSA. This is due to mutations which result in the ability of the bacteria to use tyrosine but not phenylalanine.
   4. Most strains of *A. hydrophila* do not produce pigment. However, pigment-producing strains can be found.

B. Motility
   1. Virtually all strains of *A. salmonicida* are non-motile.
   2. Most strains of *A. hydrophila* are motile. However, it is not uncommon to find non-motile strains of this bacteria.

C. How to distinguish between *A. salmonicida* and a non-motile, pigment-producing strain of *A. hydrophila*
   1. When grown at 20°C, *A. salmonicida* has certain components in its cell wall that cause the cells to stick together. This is visible upon wet mount examination as aggregations of bacteria. *Aeromonas hydrophila* does not form these aggregations. Thus, examine 24- or 48-hour cultures grown at 20°C for the ability to aggregate. It is important to grow the cultures at 20°C as *A. salmonicida* loses the ability to aggregate when grown at 25°C.
   2. *A. hydrophila* produces 2,3-butanediol and Indole as metabolic end products, while *A. salmonicida* produces neither.
   3. When growing cultures at 20°C it is important to remember that this is not the optimal growth temperature for the bacteria. Therefore, it is necessary to wait 48 hours before conducting any biochemical analysis in order to avoid any false-negative results.

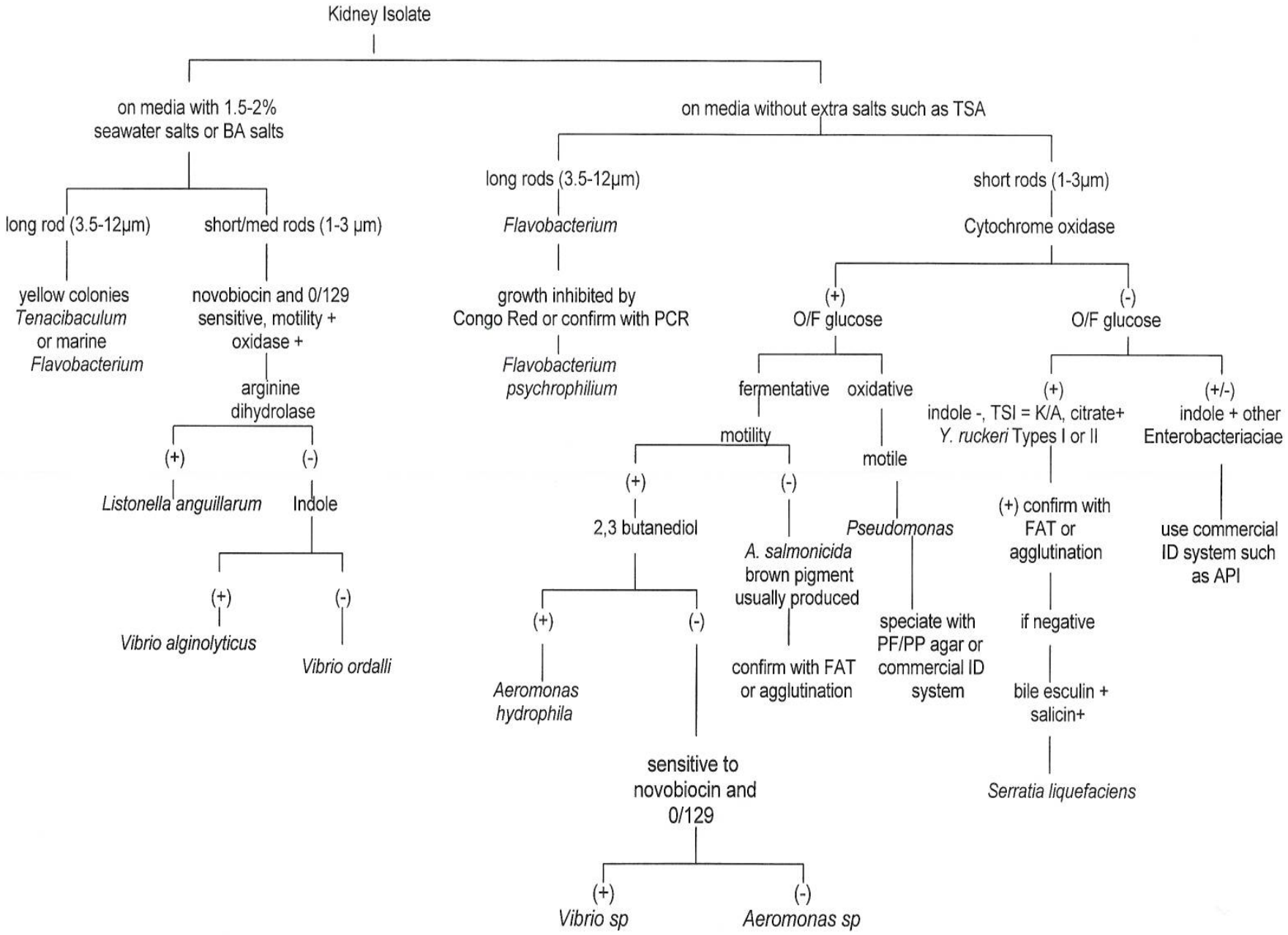
D. Serology
   1. Many antisera to *A. salmonicida* will cross react with *A. hydrophila* due to similarities of the lipopolysaccharides in the cell walls of both bacteria. Thus, any serologic tests could identify the genus *Aeromonas* but not necessarily the species.

E. Test results to confirm the identification of *A. salmonicida*
   1. Non-motile.
   2. Usually produces brown pigment on TSA.
   3. Oxidase positive.
      NOTE: An atypical oxidase negative isolate has been described.
   4. Oxidative and fermentative utilization of glucose (TSI and O/F tubes).
   5. Nitrates reduced to Nitrites.
   6. Usually gelatinase positive
   7. Can utilize esculin and mannitol as carbon sources for growth.
   8. Arginine dihydrolase positive (arginine is oxidized to ornithine, NH_3 and CO_2).
   9. Indole negative.

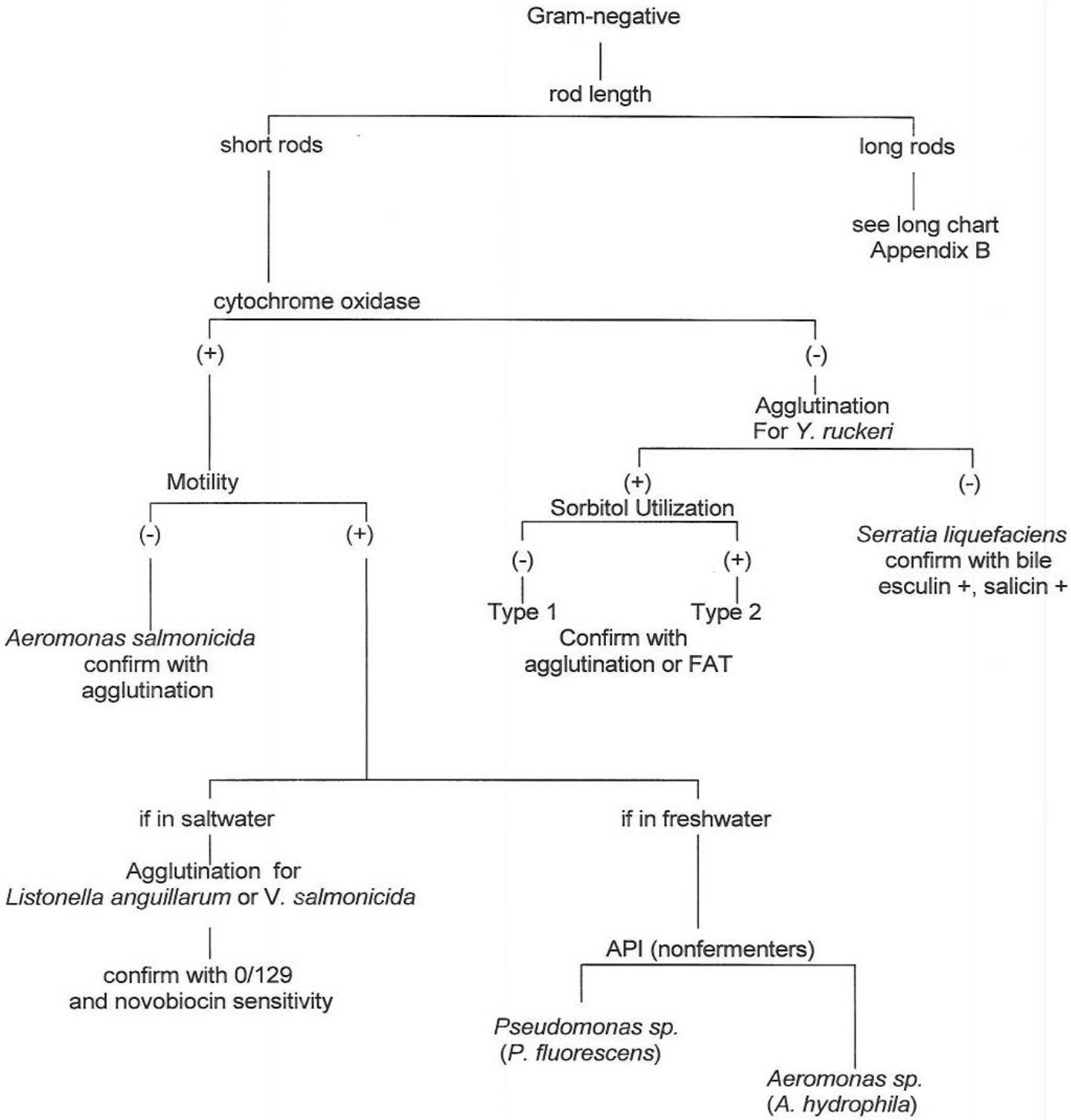
F. Differentiation between *A. salmonicida* subspecies *salmonicida*, *achromogenes* and *masoucida*

salmonicida	*achromogenes*	*masoucida*
brown pigment on TSA or Furunculosis agar	non-pigmented	non-pigmented
gelatinase +	gelatinase -	gelatinase -
Indole -	variable	Indole +
growth on mannitol	no growth on mannitol	growth on mannitol
growth on esculin	no growth on esculin	growth on esculin
many species of salmonids can be the host	originally Atlantic salmon were the only known salmonid host but now other salmonids are included	found in masou salmon in Japan
systemic pathogen	a surface pathogen	a surface pathogen

Appendix B: Identification of Certain Gram-Negative Bacteria Associated with Fish Diseases in Alaska



APPENDIX C: Rapid Bacteriological Identification Chart



CHAPTER 5

Virology and Cell Culture

Tamara Burton, Jill Follett, Joan Thomas, Marie Fried, Sally Short, and Theodore R. Meyers

I. Suggested Tissue Types and Sample Sizes

A. Juvenile fish ≤ 7.0 cm in length
1. Fish 2.5 cm in length - use whole, cut off and discard any visible yolk sac (yolk sac has not produced cell toxicity problems in the FPS labs).
2. Fish 2.5 to 4.0 cm - cut off and discard heads and tails
3. Fish 4.0 to 7.0 cm - use viscera

B. Juvenile fish > 7.0 cm in length
1. IHNV, VHSV samples - kidney and spleen
2. IPNV samples - kidney and spleen
3. Herpesvirus samples - kidney and spleen

C. Adult fish - use spawning or postspawning fish
1. IHNV and VHSV samples
   a. Females - ovarian fluid from spawning or postspawning fish. Generally, if sample is for disease history establishment use postspawning fish if possible, spawning if not possible. Use ovarian fluids from fish spawned for the eggtake when the eggs are to be incubated at a hatchery.
   b. Males - spleen and kidney; seminal fluid can be useful if organs are unavailable.
2. IPNV samples
   a. Females - kidney, spleen and ovarian fluid
   b. Males - kidney, spleen and seminal fluid
3. Herpesvirus samples
   a. Females - ovarian fluid
   b. Males - kidney and spleen

D. Adult molluscs
1. Digestive gland and mantle

E. Sample size
1. Broodstock screening - 60 fish or molluscs per stock. Sockeye samples are processed individually while non-sockeye species or samples for non-IHNV screening are processed in five fish pools. Molluscs are usually processed in 5 shellfish pools.
2. Prerelease inspection - generally 60 fish per lot but may differ depending on circumstances. Samples are processed in pools of five.
3. Diagnostic - minimum of 10 moribund or dead fish per lot. Samples are processed in pools of five.

II. Collecting Ovarian Fluid, Whole Fish, and Tissue Samples in the Field

A. Ovarian fluid samples - field equipment needed
1. Ice chest and blue ice
2. Seventy 10-ml snap cap polypropylene centrifuge tubes
3. Seventy-five clean paper cups
4. Two large plastic bags and tie labels
5. Spawning gloves
6. Paper towels
7. Iodophor, diluted to 100 ppm
8. Centrifuge tube rack
9. Optional - automatic 2-ml pipettor and 70 sterile pipet tips

B. Procedure for collecting ovarian fluid samples (or seminal fluid)
1. Expressing ovarian fluid
a. Disinfect the abdomen of the fish with iodophor and wipe with a clean paper towel to remove any disinfectant or mucous which could drip into the sample.
b. Partially strip ovarian fluid from one female fish into a clean paper cup. If possible, avoid extrusion of blood, fecal material, and nematodes.
c. Crimp edge of paper cup to "strain out" any eggs present and pour 2-5 ml ovarian fluid from each fish into one centrifuge tube per fish. Do not fill tube completely full. Do not palm or warm fluid, which could inactivate low levels of IHNV if present.
d. Tightly cap, number each tube and replace in tube rack. Keep all filled tubes in chest on blue ice while collecting remainder of samples.
2. Drawing ovarian fluid using an automatic pipettor - an alternate method if *in situ* contamination is a problem.
a. Install sterile tip on pipettor.
b. Insert tip into the urogenital opening of the fish while applying light pressure to the body.
c. Draw up a sample of ovarian fluid and place in one sterile centrifuge tube per fish. Discard tips between samples.

C. Transport of ovarian fluid samples
1. Check each centrifuge tube for tightness of cap prior to transport.
2. Place rack with centrifuge tubes in plastic bag. Label bag with number of samples, location of sample collection, date, fish life stage, species and enclose a completed sample submission form (page 1-7).
3. Transport samples in a vertical position on blue ice in the chest. Keep samples cool and avoid sunlight. Do not freeze unless samples cannot be transported to the Fish Pathology Lab(s) (FPL) within 4 days or are otherwise grossly contaminated.

D. Storing ovarian fluid samples in the laboratory
1. Store samples at 4°C if they will be processed within 4 days of collection and are not grossly contaminated.
2. Store samples at -70°C if they will not be processed within 4 days of collection.

E. Whole fish and tissue samples - field equipment
1. Whole fish - alevin or juvenile fish $\leq$7 cm in length
a. Ice chest and blue ice
b. Seventy Whirl-Pak® bags

c. Two large plastic bags and tie labels

2. Tissue samples - fish >7 cm in length
   a. Ice chest and blue ice
   b. Seventy Whirl-Pak® bags
   c. Two large plastic bags and tie labels
   d. Scissors
   e. Seventy sterile tongue depressors, optional
   f. Scalpel and blades
   g. Forceps
   h. Iodophor at 100 ppm or gauze squares soaked in 70% ETOH
   i. Paper towels
   j. Spawning gloves

F. Procedure for collecting tissue samples
   1. Whole alevin or juvenile fish ≤7 cm in length. Place 5 fish into 1 Whirl-Pak® bag per pooled sample. Keep samples cold.
   2. Tissue samples from fish >7 cm in length
      a. Aseptically remove from each fish a piece of the kidney and spleen with forceps, scissors, scalpel and/or tongue depressor. For pooled samples, combine tissue samples from each fish into a single Whirl-Pak® bag. The sample should be at least 1 g of tissue.
      b. Seal Whirl-Pak® bag. Keep these cool while collecting remainder of samples.
      c. Between each fish, clean instruments of any tissue with gauze sponges dipped in 70% ETOH or wipe with iodophor followed by a clean paper towel.
   3. Adult molluscs. Shuck animal and aseptically remove a small piece of mantle and digestive gland. Pool samples in a Whirl-Pak® bag. The sample should be at least 1 g of tissue.

G. Transport of tissue samples
   1. Check seals on bags.
   2. Place Whirl-Pak® bags into large plastic bag. Label bag with number of samples, location sample taken, sample type, date, life stage, species of fish and enclose a completed sample submission form.
   3. Transport all samples on blue ice in the chest. Keep sample cool and avoid sunlight. Do not freeze unless samples can't be transported to the FPL within 4 days.

H. Storing tissue samples in the laboratory
   1. Store samples at 4°C if they will be processed within 4 days of collection.
   2. Store samples at -70°C if they will not be processed within 4 days of collection.

III. Maintenance of Stock Cell Lines-Passage of Confluent Cell Monolayers

A. Allow medium and cell cultures to equilibrate to room temperature.

B. Aseptically decant medium from the flask of cells into a beaker containing diluted bleach.

C. Rinse cell monolayer with 2-8 ml versene-PBS or trypsin-versene (TV), depending on flask size, then decant. Warming the TV to 30-32°C will allow it to work more rapidly on cells such as EPC or BF-2. However, CHSE-214 and PHE cells do not tolerate warm TV.

D. Add 2-8 ml TV or versene-PBS to flask using pipettor and place on rocker for 2-5 min. Older cells require TV, freshly monolayered cells can be removed with versene-PBS. Cells will appear rounded when examined with an inverted light microscope and the monolayer will become opaque and grossly visible.

E. Place sterile centrifuge tubes containing approximately 0.5-1 ml of serum or 2-3 ml MEM-10 in an ice bath, or keep otherwise cold. Serum and cold temperature neutralize the action of trypsin.

F. When cells are sufficiently loosened by the TV, strike the flask lightly to dislodge them. Triturate with a sterile pipet until cells are single or in aggregates of two's and three's.

G. Immediately place cells in the tubes of medium or serum. If any clumps remain, let the clumps settle to the bottom and transfer remaining suspended single cells in the supernatant to a fresh tube. To remove trypsin, spin at 2000 x g (1100 rpm) for 10 minutes in the refrigerated centrifuge and discard supernatant.

NOTE: If cells are removed using only versene-PBS or the TV is decanted from the flask prior to dislodging the cells, centrifugation is unnecessary.

H. Re-suspend cells in growth medium.

I. Dilution of cell suspension:

Volume of medium needed per flask to grow tissue culture cells

Medium volume	Flask size
5 ml	25 cm^2
25 ml	75 cm^2
50 ml	150 cm^2

1. If cells were a confluent monolayer recently passaged but more than 2 days old, EPC cells can be split 1:4 and other cell lines 1:3 or 1:4 depending on viability and flask size. CHSE-214 cells should be split 1:4 every 7-10 days. They should not be allowed to get thick or too old before splitting or they will clump. Thick monolayers of CHSE-214 also may become too acidic in pH. On older flasks, changing the medium the day before splitting will activate the cells and somewhat reduce clumping.
2. Following centrifugation, add enough MEM-10 to re-suspend cells to the appropriate dilution. If cell counts are done they should be approximately 5 x 10^5 to 1 x 10^6 cells/ml. Decant or pipet the correct aliquots of cell suspension into each flask; i.e., when diluting a 75-cm^2 flask of EPC cells 1:4 (1 flask into 4 flasks) add 100 ml MEM-10 to the cells. The cell suspension is then decanted equally into four 75-cm^2 flasks. Good sterile technique will allow re-use of the original flask for several more passages of the same cell line and reduce the consumption of plastic ware.
3. Alternately, following trituration, pipet appropriate aliquots of cell suspension into the flask(s); i.e., if you have 10 ml of cell suspension from a 75-cm^2 flask pipet 2.5 ml into each of four 75-cm^2 flasks. Add MEM-10 in sufficient volume to cover the bottom of the flask.

J. Label the flask(s) with cell line initials, passage number, date, splitting ratio, operator initials and lot #'s of medium and serum used as follows:

EPC p.86 10-17-09 1:4 MPF FBS lot 1163

A notebook log of prepared media that are dated and numbered should be maintained. i.e.,

- date made
- medium recipe
- serum type and lot # used
- operator initials

This information will be very useful for investigating sources of contamination or toxicity and can be cross-referenced directly to the flask of cells. This may prevent the need for wholesale discarding of cells, reagents or media.

K. Incubate cells at the optimum growth temperature and allow to form a cell monolayer without changing the medium.

L. Recommended media for flasks and plates

Cell Line	Flask Media	Plate Media
EPC	MEM 10	MEM 10 Tris
CHSE-214	MEM 10 CHSE	MEM 10 CHSE Tris
RTG-2	MEM 10	MEM 10
BF-2	MEM 10	MEM 10
ASK	MEM 10	MEM 10 Tris
TO	MEM 10	MEM 10 Tris
SSN-1	MEM 10	MEM 10 Tris
PHE	Herring Media	Herring Media
INEM-1	MEM 10	MEM 10 Tris
FHM	MEM 10	MEM 10 Tris

IV. Cell Counting Using the Hemocytometer

Although not done routinely, counting cells by the use of a hemocytometer is a useful method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching coverglass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 ml, the cell concentration/ml is the average count per square x 10^4. Routinely, cells in 10 of the 1 mm squares are recommended for counting (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

When splitting or seeding cells the recommended cell counts for tissue culture lines used at the FPL are 5×10^5 to 1×10^6 cells/ml.

A. Materials
   1. Hemocytometer and cover glass
   2. Pipets
   3. Trypan Blue (0.1% in PBS)
   4. Microscope
   5. Dilution tubes
   6. Cell suspension to be tested

B. Procedure
   1. Remove cells from flask surface following normal procedures.
   2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20-50 cells/mm^2. Dilutions vary depending on age of the cells, cell density and cell aggregation.
   3. Aseptically transfer 0.5 ml of the cell suspension into a dilution tube.
   4. Add 0.5 ml Trypan Blue stain (0.1%).
   5. Gently mix to suspend the cells evenly. Fill the two chambers of the hemocytometer with a Pasteur pipet or capillary tube (without overflow) by capillary action.
   6. Using a microscope with a 10X ocular and a 10X objective count 10 squares (5 from each chamber) as outlined above.
   7. Calculate the # of cells/ml and the total # of cells as follows:

      Cells/ml = $\bar{x}$ (mean) count per square x 10^4 x Trypan Blue dilution factor

      Total cells in flask = cells/ml x total volume of cell suspension

      e.g., total # cells counted in 10 squares = 300 cells

      $\bar{x}$ count/square = 300 cells/10 squares = 30 cells

      cells/ml = 30×10^4 x 2 (dilution factor)
      cells/ml = 60×10^4 cells/ml
      cells/ml = 6.0×10^5 cells/ml

      Total cells = 6.0×10^5 cells/ml x 8 ml (original volume cell suspension)
      Total cells = 48.0×10^5 cells
      Total cells = 4.80×10^6 cells

   If the cells/ml calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

      ml medium needed = (actual cells/ml)(vol. of cell suspension) ***/*** desired cells/ml

      e.g., actual count = 6×10^6 cells/ml

desired count = 1 x 10^6 cells/ml
volume of cell suspension = 8 ml
ml medium needed = x

= ml medium needed = 6 x 10^6 cells/ml x 8 ml / 1 x 10^6 cells/ml

ml medium needed = 48 x 10^6 ml / 1 x 10^6

= 48 ml

Since you have 8 ml already in the flask, you would need to add 40 ml of medium to the flask before splitting to get the recommended seeding cell density for each new culture.

V. Incubating Cell Lines

	Optimum Growth Temperature	Growth Temperature Range
EPC (epithelioma papulosum cyprini)	23-27°C[a]	15-33°C[a]
CHSE-214 (Chinook salmon embryo)	22°C	12-23°C
FHM (fathead minnow)	25-30°C	14-34°C
RTG-2 (rainbow trout gonad)	20°C (RT overnight)	4-26°C
INEM-1 (sheefish embryo)	15-20°C	12-24°C
PHE-184 (Pacific herring embryo)	15-23°C	Not determined
BF-2 (Bluegill fry)	23°C	15-33°C
ASK (Atlantic salmon kidney)	20°C (RT overnight)	Not determined
TO (Atlantic salmon head kidney leucocyte)	20°C (RT overnight)	Not determined
SSN-1 (snakehead fish)	25°C (RT overnight)	Not determined

After a cell layer is confluent, the flask can be transferred to the 15° incubator for holding until use. If they are to be held for extended periods of time the medium should be replaced with MEM-5 and the cells incubated at 4°C.

[a] EPC cells must be thickly monolayered or they will retract, forming holes in the cell sheet when placed at 14-16°C for incubation of virus samples. This makes interpretation of a plaque assay difficult.

VI. Storing Tissue Culture Cells

A. Preparation of cells
1. Change medium on one 150 cm^2 flask and incubate for 24 hours.
2. Split the flask at a 1:2 ratio and incubate for 24 hours.
3. Remove cell monolayers from the flasks using method described in “Maintenance of stock cell lines: passage of confluent cell monolayers”. The cells should be actively growing and do not need to be confluent.
4. After pelleting cells by centrifugation pour off supernatant and re-suspend pellet with 4 ml of freeze medium.

B. Freeze medium is prepared by adding to MEM-10: 10% more FBS and 10% DMSO (v/v); i.e., to 6.4 ml MEM-10 add 0.8 ml FBS and 0.8 ml DMSO. Filter sterilize through a 0.45-µ syringe filter before adding 4 ml to the cell pellet.

C. Pipet four 1 ml quantities of cells suspended in freeze medium into labeled (cell line, passage, date) and cooled freezer vials.

NOTE: Cells should not remain in freeze medium longer than 10 min before or after freezing.

D. Freeze vials in insulating foam blocks at -70°C for 12 hours. Transfer to LN_2 for several days and then remove one and check for viability as described in E below. If not viable, remove other vials and repeat process.

E. Thawing tissue culture cells
   1. Rapidly thaw the cell suspension in 1 vial. When coming from LN_2 place the ampule in lukewarm (30°C) water in a plastic container with 10% Clorox® solution. Wear a plastic face shield for protection against the remote chance that the vial will explode upon thawing from LN_2. This is generally a problem with glass ampules that have not sealed properly. Air leaks into the vial and rapid expansion on thawing causes the explosion. Remove the vial as soon as the ice disappears from the contents.
   2. Resuspend the cells in at least 5 ml of MEM 10. It is very important to adequately dilute the freeze medium for the best cell recovery rate. Centrifuge and re-suspend the cell pellet in 5 ml MEM-10 and pour into one 25-cm^2 flask.
   3. Incubate the flask for 24 hours at the proper temperature for the cell line and observe for normal cell attachment and growth. Change the growth medium.

F. Care of liquid nitrogen dewer
   1. Liquid nitrogen for freezing of cell lines is available at Air Liquide in Anchorage. Normally the transport canister is filled and then emptied into the storage dewer upon return to the office.
   2. The level should be checked bimonthly and when new vials are added. When the level drops to 8 or 9 cm or if the automatic level alarm is activated then the dewer should be refilled. In the Juneau lab, LN_2 is ordered by the Anchorage FPL at the request of the Juneau FPL.
   3. A single filling usually lasts 3-5 months in either lab. Never let the storage dewer go dry, otherwise all stored cells will be lost. If all LN_2 is found to have evaporated, the storage dewer should be taken instead of the transport canister and cooled down prior to filling. Otherwise the LN_2 is rapidly depleted. This is not an option in the Juneau lab.
   4. Appropriate protective clothing including faceshield, gloves and shoes should be worn when handling LN_2. Never use a hollow tube to check the level of the LN_2 as the intense vapor pressure will cause it to squirt out the upper end, possibly in the user's face! A solid wooden yardstick works fine as a volume measuring tool.

VII. Detecting and Avoiding Tissue Culture Contaminants

A. Use autoclave tape on all reagent containers to ascertain effective sterilization. The autoclave should be tested with Killit® ampules to determine proper autoclave settings to achieve complete sterilization. Test the sterility of all tissue culture reagents periodically (everytime new medium is made). Add 0.5 ml of the reagent or medium to be tested to 5 ml thioglycollate broth or TSB. Incubate at 23-25°C (room temperature) for 14 days. Check for growth on day 1, 2, 7, and 14. If contamination is observed in either broth, autoclave and discard. Maintain a log notebook for the periodic quality control testing performed.

B. Discard any reagent or medium in which contamination is observed. Autoclaving is not necessary unless a fungal contaminant is present.

C. Discard any flask or plate in which contamination is observed prior to inoculation of samples. Do not transfer a cell monolayer that is contaminated. After discovering a contaminated flask, thoroughly disinfect tissue culture incubator and tissue culture hood before splitting any more flasks.

D. If contamination obscures results of one or more samples, repeated inoculations will be necessary. The original samples can be filter-sterilized using 0.45-µm disposable syringe filters that have been wetted with serum. Alternatively, if samples were not already in antibiotic cocktail, they can be re-centrifuged and the supernatant added to antibiotic cocktail and incubated for 2 hours at 15°C.

E. Test cell lines for mycoplasma contamination twice a year using the PCR procedure outlined in the molecular chapter and test all newly acquired cells from outside sources for mycoplasma contamination before adding the cells to the regular inventory.

F. Never mouth pipet samples, cell lines or media.

G. Do not reenter a medium bottle with a pipet that has been used; even if it has been used aseptically. For repeated pipetting, pour a small amount of medium into a sterile bottle and pipet from that. Discard any remaining medium when the procedure is completed.

H. Work with only one cell line at a time to prevent cross contamination by cells and/or potential contaminants. Disinfect hood between cell lines.

I. Never handle all cultures of a cell line in one day. This prevents the risk of contaminating an entire active stock of a cell line if contamination is accidentally introduced. Therefore, maintain multiple daughter cultures for a single cell line.

J. All work with un-inoculated or stock cell lines should be done prior to performing virus assays if done at the same location on the same day.

K. Use only sterile tissue culture grade distilled water for all media and reagent preparations.

L. Maintain a detailed log of media and solution preparations.

VIII. Mycoplasma Screening of Continuous Cell Lines

A. Introduction

Mycoplasmas are a common contaminant of cell cultures. They are very small and are characterized by their lack of a rigid cell wall. This lack of a cell wall allows them to pass through 0.2-µ filters and also allows them to be resistant to certain antibiotics. As a general rule, cell cultures in flasks are maintained in media without antibiotics to avoid allowing sloppy cell culture which could allow mycoplasmas to be introcduced. By not using antibiotics, any potential deviation of aseptic technique will be obvious immediately in the flask cultures. Because of their extremely small size and slow growth rate, mycoplasma contamination is not readily detected by routine laboratory methods. Contamination of cell lines can have serious detrimental effects on normal cell growth

characteristics, disrupt nucleic acid synthesis, change antigenicity of cell membranes, interfere with virus replication, and interfere with virus yields.

The cell culture lines in use at the Fish Pathology Laboratories are screened for the presence of mycoplasma contaminants twice yearly using a PCR test. See the molecular chapter for procedure.

IX. Processing Ovarian Fluid, Whole Fish, and Tissue Samples

A. Processing ovarian fluid samples

1. Each ovarian fluid sample should be mixed well on a vortex mixer before centrifugation to thoroughly separate cell debris from potential virus particles and/or possibly reduce viral clumping.
2. For pooled samples, aseptically pipet 1 ml from each of 5 ovarian fluid samples into a sterile 10 ml centrifuge tube. Pool samples with less than 1 ml together at equal quantities, for example, 0.5 ml each. For individual samples, leave in original centrifuge tube.
3. Centrifuge ovarian fluid samples at 6000 x g for 20 min if using polypropylene tubes. If using polystyrene the speed must be reduced to prevent the tubes from cracking (approximately 1200 x g). It is optimal to process in a refrigerated centrifuge at 4°C to prevent warming of the sample. Tubes must be balanced.
   Refrigerated centrifuges:
   - IEC (Anchorage) 36 tube head 3500 rpm 2100-2375 x g
   - IEC (Juneau) 36 tube head 4000 rpm 3200 x g
   - Omnifuge RT (Juneau/Anchorage) 48 tube head 6000 rpm 6000 x g
   - Sorval Biofuge (Juneau) 80 tube head 13000 rpm 16400 X g

   If unable to use a refrigerated centrifuge, use the following centrifuges at top speed to attain maximum g:

   - Clinical centrifuge 12 tube head 3400 rpm 1640 x g
   - HN-S centrifuge 24 tube head 3500 rpm 1525-1800 x g
   - HN-S centrifuge 12 tube head 3650 rpm 1900 x g

4. Continue to process as appropriate for the plaque or quantal assays. Samples are ready for inoculating onto cells unless contamination is expected to be a problem. If any of the ovarian fluids appear unusually cloudy consider treatment to reduce bacterial and fungal concentrations. In this case samples should be filtered through serum-soaked 0.45-µm syringe filters or antibiotics added as indicated below. Ovarian fluids from chum salmon frequently need such treatment.

   a. Antibiotic treatment
      (1) Without disturbing the pellet, aseptically pipet 1.5 ml from each ovarian fluid sample into its respective tube containing 0.25 ml of thawed antibiotic cocktail (see Media section) and vortex. Label tubes.
      (2) Incubate at 15°C for 2-6 h or at 4°C overnight. Ovarian fluid samples (undiluted = 10°) are ready for inoculation onto cells for viral assay.
   b. Treatment with polyethylene glycol (PEG)

For non-sockeye species, prepare a 7% w/v PEG solution in MEM-10 with 3x antibiotics (see Media section). Briefly adsorb 100 µl to each drained monolayer well at room temperature prior to inoculation of samples.

5. If feasible, process within 24-48 hours of sample collection, but samples can be held at 4°C for up to 4 days. Maintain samples at 4°C during processing if possible.
6. Suggested cell lines for virus isolation

Virus	cell lines (from most sensitive to least)
• IHNV, VHSV[a]	EPC, BF-2, FHM, CHSE-214, RTG-2[b]
• IPNV	CHSE-214, BF-2, RTG-2
• Herpesvirus	RTG-2, CHSE-214
• Aquareo & aquabirnavirus	BF-2
• Paramyxovirus	CHSE-214

7. Optimum growth temperature and pH for virus-inoculated cell cultures

Virus	Temperature	Range	pH
IHNV	15-18°C	4-20°C	7.0-7.8
IPNV	15-18°C	4-26°C	7.0-7.8
Herpesvirus	10-12°C	5-15°C	7.0-7.8
VHSV	12-15°C	4-15°C	7.6-7.8[c]

B. Processing tissue samples

1. Weigh approximately 0.5-1 g samples from each fish or mollusc into one 2 oz Whirl-Pak® bag per animal (note sample types to be used in Section I). When using more than one tissue from a fish take approximately the same amount of each tissue. When practical, process fish individually and pool after centrifugation. This will allow re-testing of individual samples to determine prevalence if an individual pool is positive for virus. For pooled samples, 5 alevin fish or a total of 1 g of tissues from 5 fish or molluscs may be pooled into one Whirl-Pak® bag prior to homogenization. The bag can be tared and the tissues weighed directly into it. Keep samples cool at all times using an ice bath. Smaller sockeye fry may require 7-10 fish for a 1-g sample.
2. Add MEM-0-TRIS to equal a 1:10 dilution (w/v). If unable to complete processing in one day the tissues can be refrigerated overnight at this point (DO NOT STORE OVERNIGHT AS HOMOGENATES). Alternatively, the tissues may be homogenized, centrifuged and the supernatants refrigerated overnight.
3. Homogenize samples using a Stomacher® (Virtis® or Contorque® grinders require considerable disinfection of containers between samples). Contorques may need to be used for molluscs or whole small fish to get a thorough homogenization.
4. Centrifuge tissue samples at 6000 x g for 20 minutes. If there is remaining yolk from sac fry, collect the supernatant from below the floating yolk material.

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[a] In examining tissues for VHSV or for IHNV from non-sockeye IHNV-susceptible species, both EPC and CHSE-214 lines should be used concurrently as per recommendations within the AFS "Bluebook" and the Pacific Northwest Fish Health Protection Committee (PNFHPC) Comprehensive Fish Health Protection Program.

[b] RTG-2 not recommended for IHNV assay.

[c] Cell culture pH for North American VHSV IVa isolates can be as low as 7.0 without inhibition of CPE and virus isolation. VHSV IVb requires a pH of 7.4 or above during cell culture assay.

5. Continue to process as appropriate for the plaque or quantal assays. See section IX. A. for suggested cell lines. Samples are ready for inoculating onto cells unless contamination is expected to be a problem. In this case samples should be filtered through serum-soaked 0.45-µm syringe filters or antibiotics added as indicated for ovarian fluids in section IX. A.
   For VHSV samples, pre-treat cell monolayers with PEG as described in section IX. A. 4.

X. Cytopathic Effects (CPE) of Virus Infection in Tissue Culture Cells

A. IHNV-induced CPE
   1. Rounded and granular cells in grape-like clusters.
   2. Margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened).
   3. Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque.

B. IPNV and other aquabirnavirus-induced CPE
   1. Spindle-shaped or "balloon-on-a-stick"-shaped cells.
   2. Pyknosis of nuclei (nuclei shrink in size and chromatin condenses).
   3. Plaques are stellate in a confluent cell monolayer and contain not only live cells but also normal looking cells that have been killed and retain normal shape (these stain lightly).
   4. Little cellular debris.

C. Herpesvirus-induced CPE
   1. Pyknosis of nuclei and cellular fusion (syncytia).
   2. Syncytia produce multinucleated giant cells.
   3. Plaques tend to elongate and follow whorl lines of growth if on RTG-2 cells. They have relatively clear interiors, but living cells extend into the open area.
   4. Little cellular debris.

D. VHSV-induced CPE
   1. The North American VHSV type IVa isolates plaque very similarly to IHNV in EPC cells forming rounded and granular cells in grape-like clusters.
   2. The European VHSV isolates differ from IHNV on RTG-2 cells by having more regular plaque margins with uniformly distributed granular debris within the plaques. Also, affected cells do not show margination of chromatin. Virus titers for Pacific cod North American VHSV IVa in 3 cell lines are indicated in the Appendix Section XXIII.

E. Aquareovirus-induced CPE
   1. Balooning cellular swelling
   2. Cellular vacuolation
   3. Syncytial cell formation

F. Paramyxovirus-induced CPE
   1. Diffuse retracted and rounded cells
   2. Extensive incubation period

G. Number of days following infection with virus that CPE is usually observed in freshly monolayered fish cell cultures:

Virus	Days	
IHNV, VHSV	2-10	If blindpassages are needed, do at 14 days and examine for an additional 14 days.
IPNV and other aquabirnaviruses	2-10	If blindpassages are needed, do at 14 days and examine for an additional 14 days.
Herpesvirus	14-30	Frequently does not appear on the initial culture and must be blindpassaged at 28 days. Examine for an additional 28 days.
Aquareoviruses	3-10	
Paramyxoviruses	14-30	Frequently requires blindpassage.

Toxicity can sometimes mimic viral CPE. Observing the gradual development of CPE pattern over several days is the best way to distinguish viral CPE from toxicity. In questionable cases blindpassaging usually allows the distinction between toxicity and CPE. Blindpassaging dilutes out toxicity which will not reappear but usually builds virus titers with CPE becoming evident more quickly.

H. Intensity of CPE - confluent cell monolayers are examined with an inverted light microscope at low power (40x) to determine intensity of CPE. This scoring is used in the quantal assay.
   1. ± Questionable CPE
   2. +1 At least one focus of CPE in monolayer
   3. +2 CPE in 25% to 50% of monolayer
   4. +3 CPE in 100% of monolayer
   5. +4 CPE with few live cells remaining and/or cell monolayer no longer attached to flask/plate. (Separation or peeling of the cell monolayer from edge of flask/plate can be due to toxicity rather than viral CPE.)

XI. Plaque Assay

The plaque assay (Burke and Mulcahy, 1980) is a virus titration method used for sockeye salmon broodstock screening, all fish virus research experiments, and generally when an accurate viral titer determination is desired without the use of several replicates as required for the $TCID_{50}$ method. A semisolid methylcellulose overlay is added so that discrete plaques are formed and then formalin with crystal violet is added to kill, fix and stain the cells so that the plaques may be counted. Flat-bottomed 24 well plates are used for the plaque assay and samples are not pooled but tested individually. The FPL has been using this assay primarily for IHNV detection in sockeye salmon in which case only the EPC cell line is used.

A. Seeding 24 well (16 mm well) plates
   1. Determine number of plates needed for the assay. Four wells are used per sample along with control wells. Sixty samples can be assayed using 11 plates.
   2. Remove confluent cell monolayer using method described in "Maintenance of stock cell lines: passage of confluent cell monolayers". One confluent EPC cell monolayer in a 75-cm^2 flask can make 4 plates if cells are re-suspended in 100 ml of MEM-10-TRIS. Confluent monolayers in three 75-cm^2 flasks or two 150-cm^2 flasks are needed in a final volume of 300 ml MEM-10-TRIS to make 11 plates. Cell

counts should equal approximately 5 x 10^5 to 1 x 10^6 cells per ml for overnight confluency.

3. Pipet a 1.0-ml cell suspension into the center of each well. It may be necessary to add additional medium or cell suspension (several drops) to the corner wells to offset evaporation. Mix the stock cell suspension frequently to maintain a homogeneous suspension for more confluent monolayers.
4. Cover each plate with the accompanying lid. Mark the side of each plate on both the lid and bottom so they can be matched up if they become separated. Label each plate with seeding date, cell line initials, passage number, and operator initials. Place plate(s) in a Ziploc ® plastic bag or place in an airtight plastic container.
5. Incubate at 20-24°C and allow to reach 100% confluency without changing the medium (2 days maximum). If the cells have been seeded too heavily, the plaques will not open. Again note that the confluency of EPC cells must be thicker than for other cells to avoid retraction when placed at 14-15°C.

B. Inoculating cells with ovarian fluid and tissue samples

1. Materials needed for assay:
   - Appropriate number of 24 well plates. Do <u>not</u> use plates in which cells have been monolayered more than 2 days or cell monolayer may be too thick for well formed plaques to occur.
   - Ovarian fluid and/or tissue samples
   - MEM-0-TRIS dilution blanks (0.9 ml)
   - MEM-10-TRIS (same batch that was used for seeding the plates)
   - Methylcellulose overlay (calf serum may be used instead of fetal bovine serum)
   - 100 lambda MLA pipettor (blue) or Pipetman 100
   - Sterile pipet tips
   - Plastic bag or airtight plastic container
2. Inoculating cells with samples
   a. Label plate(s) with accession number and date of inoculation. Label wells with sample numbers and tube dilutions. This assay is generally done with plates aligned 4 columns across (4 dilutions) and 6 rows down (6 samples). Suggested tube dilutions for:

      sockeye salmon:
      Ovarian fluid:
      10^{-0}, 10^{-1}, 10^{-3}, 10^{-4} or 10^{-5}
      Tissue:
      10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}

      chum salmon:
      Ovarian fluid:
      10^{-0}, 10^{-1}, 10^{-2}, 10^{-4}
      Tissue:
      10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}

   b. Empty medium from wells by inverting plate over bleach bucket or aspirate medium off by tilting the plate and using a pipet, (at this time MEM-10-TRIS should remain on 2 of the control wells) leaving cells covered with a small amount of medium. Be sure there is enough medium to prevent centers of the monolayers from drying out and do not damage the monolayers with the pipet.

c. For testing of non-sockeye species add 100 µl (0.1 ml) of 7% PEG at room temperature to each cell monolayer. At least one control well should go without PEG.

d. Dilutions are made using 0.9-ml MEM-0-TRIS dilution blanks and a 100 lambda MLA pipettor (or Pipetman 100).

(1) Mix contents of dilution blank with a Vortex® mixer. Do not use a pipettor to mix dilution blanks. Monolayered cells are inoculated subsequent to PEG adsorption and concurrently or subsequent to dilution preparation.

(2) Pipet 0.1 ml of each undiluted ovarian fluid or tissue sample (1:10 w/v) into the first well allocated per sample.

(3) Pipet 0.1 ml of sample into one 0.9-ml MEM-0-TRIS dilution blank and mix. Discard pipet tip into bleach bucket. The tube dilution of an ovarian fluid sample is now 10^{-1} and a tissue sample 10^{-2}.

(4) Continue making serial dilutions and inoculating plates with desired dilutions. Unused dilutions may be discarded.

Example:	Tube dilution:	undil	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
	inoculum:	0.1ml	0.1ml	-	0.1ml	-	0.1ml
	Plate dilution:	10^{-1}	10^{-2}	discard	10^{-4}	discard	10^{-6}

(5) Alternately, to inoculate the plate after all of the dilutions have been made use the 100 lambda pipettor to pipet 0.1 ml of each dilution per sample into each well, starting with the most dilute (10^{-4} or 10^{-5}) and finishing with the least dilute. The same pipet tip can be used for inoculating all dilutions when done this way.

(6) When assaying 60 fish, every other plate inoculated has 4 control wells. Aseptically pipet 0.1 ml MEM-0-TRIS into each of 2 sham control wells per control plate. If a different MEM-0-TRIS was used for diluting the samples than was used in the dilution blanks, controls must be made of both batches. Pipet 1.0 ml MEM-10-TRIS into each of 2 monolayer control wells per control plate.

3. Replicates of each dilution per sample are used in some diagnostic assays and throughout research experiments. Number of replicates will depend on statistical analyses to be performed on the results. When using replicates, make 1 set of dilutions per sample as instructed in the procedure. Plate replicates from each dilution blank. Do <u>not</u> make replicate dilution blanks.
4. Cover plates with lids and place in a sealed plastic bag or airtight plastic container. Adsorb samples onto cells for at least 30 minutes (60 minutes standard) at 15°C.
5. Following adsorption, approximately 1.0 ml of methylcellulose overlay is carefully pipetted into each well except the two control wells per control plate containing 1 ml of MEM-10-TRIS. The overlay is added by dripping it into the center of each well or down the side of each well. Discard the pipet between samples if the pipet tip touches the side of any well. Overlay the controls first and then the tube dilutions in the following order: 10^{-5}, 10^{-3}, 10^{-1}, and 10^{-0} (highest to lowest dilution) to minimize potential carry-over of sample. <u>Do</u> <u>not</u> reenter the methylcellulose bottle with a used pipet and do not disturb any bottom sediment in the overlay material. Sediment pipetted onto cells will cause distortion of the monolayer making interpretation difficult.
6. Cover plates with lids. Place plate(s) in a Ziploc ® plastic bag or airtight plastic container.

7. Incubate at 15°C for 7 days for IHNV. Label the bag or plastic container with the date plate is to be fixed and stained, which will be day 7.
8. Freeze ovarian fluid and tissue samples at -70°C until completion of the assay. Autoclave and discard used dilution blanks.

C. Examination of cells
1. Examine plates after 24 hours to determine whether toxicity is present and note which samples are affected. Also, examine plates for plaques and possible contamination once again (day 5) before the 7th day. Severe contamination or toxicity in several samples may require repetition of the assay for those samples.
2. On day 7, examine cell monolayers with an inverted light microscope to determine whether cells within plaques have lifted off the plate(s). A superficial count of plaques for each sample while the cells are still alive is useful for differentiating small plaques from holes. Also, should monolayers peel off in the staining process, these plaque counts make repeating the assay unnecessary. If cells within plaques have lifted off, fix and stain the plate(s) for at least 1 hour by carefully pipetting approximately 0.5 ml of 0.5% crystal violet in 40% formalin to each well (3 drops from a 25-ml pipet works well). If plaques have not opened up, incubate an additional 1-3 days.
3. After the hour of staining, pour off the stain, gently rinse cell monolayers with tap water, invert on paper towels and allow to air dry.

D. Determining viral titer
1. Number of plaques per well are counted to determine viral titer. Wells with more than 200 plaques (depending on the size of the plaques) are not counted and are labeled too numerous to count (TNTC). Record the number of plaques in each well for each sample.
2. Examine any questionable plaques, using an inverted light microscope, and note typical IHNV-induced CPE as described in Section X.
3. Calculate viral titer. Viral titer for each sample is expressed as mean plaque-forming units (pfu) per ml of ovarian fluid or per gram of tissue. The best wells to use for determining titer are those of the highest dilutions with between 20 and 200 plaques.

NOTE: Occasionally one dilution will be TNTC but the next will have no plaques. This can commonly occur when a dilution is skipped. Because TNTC is generally well over 100 viral plaques, the titer estimate could be recorded as likely to be $\geq$ 100 X the uncountable dilution.

a. The following equation is used to express pfu/ml (or gram of tissue) in one well:
plaques x 1/tube dilution x 1/# ml added to well.
e.g. The 10^{-4} well of ovarian fluid sample A has 20 plaques in the cell monolayer.
1/tube dilution = $1/10^{-4} = 10^{4}$
1/# ml = 1/0.1 ml = $1/10^{-1} = 10^{1}$ (omit step if using plate dilution)
20 plaques x 10^{4} x 10^{1} = 2.0 x 10^{6} pfu/ml ovarian fluid

b. Determine mean pfu/ml for a sample as follows:

# Plaques	tube Dilution #	pfu/ml
200	10^{-1}	2.0 x 10^{4}
30	10^{-2}	3.0 x 10^{4}

Mean pfu/ml = $(2.0 \times 10^4) + (3.0 \times 10^4) / 2 = 2.5 \times 10^4$

If replicates of each dilution were plated, determine mean pfu/ml using all countable wells. Record mean pfu/ml or pfu/g for tissues, the latter requiring the initial sample dilution (1:10) in the computation, i.e., x 1/dilution of tissues.

NOTE: The first wells will sometimes contain defective interfering (D.I.) particles or toxicity and their number of plaques will be much lower than expected. If the differential between the first and second dilution is very large, use the number of plaques in the second dilution for determining the titer.

c. Example of data reporting:

Titer pfu/ml	Neg	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8
No. of fish	0	1	5	0	7	7	12	8	2
% total	0	2.3	11.9	0	16.7	16.7	28.6	19.0	4.8

% + $\geq 10^4$ = 36/42 = 85.7%*
% IHNV + = 42/42 = 100%

* High titers are those $\geq 10^4$/g or 10^4/ml based on the bimodality of IHNV titers observed in many Alaskan sockeye salmon populations (Meyers, et al, 1990).

E. Minimum levels of detection
   1. For tissue samples, not pooled or in replicate, it is 100 pfu/g of tissue.
   2. For ovarian fluid samples, not pooled or in replicate, it is 10 pfu/ml of fluid.

F. Two virus-positive ovarian fluid samples should be randomly selected from ultracold storage and inoculated onto cells with a fluid overlay to harvest virus for identification by PCR.

XII. Quantal Assay

The quantal assay (also referred to as endpoint dilution) is used to examine fish when only the presence or absence of a virus needs to be verified, such as in suspected viral epizootics, disease history or broodstock screening of non-sockeye species, screening of fish in new water supplies and pre-release inspections. It is also used to examine adult molluscs for the presence of viruses. No semisolid overlay is added so that the virus can replicate and spread throughout the well for easy recovery. A fluid medium also facilitates blindpassage of negative cultures. Flat-bottomed 24 well plates are used for this assay and samples are nearly always pooled from 2-5 fish. When examining samples from non-sockeye species, both the EPC and CHSE-214 cell lines are used. When examining samples from molluscs, BF-2 cells are used. For determination of the $TCID_{50}$ of a virus sample or isolate, replicate samples are necessary and 96 well plates become more useful. The $TCID_{50}$ assay is not routinely used in the FPL because the number of replicate dilutions required are often not practical. Thus, no such methods will be included in this manual. The procedures for the $TCID_{50}$ assay are described by Reed and Muench (1938) and Rovozzo and Burke (1973).

A. Seeding flat-bottom 24 well plates
   1. Determine number of plates needed for the assay. Remember that non-sockeye samples will require 2 cell lines (EPC and CHSE-214). Samples should be done in duplicate, except routine sockeye salmon fry diagnostics. In samples where a high viral titer may be anticipated, four dilutions are done. When testing samples where negative or low virus levels are anticipated, only two dilutions are necessary.

Additionally, both monolayer and sham controls are needed, at least one set per accession number or more if several plates are used.

2. Remove confluent cell monolayer using the methods described in Section III. One confluent cell monolayer in a 25-cm^2 flask can make one 24 well plate if 26 ml MEM-10-TRIS are added to the harvested cells.
3. Pipet a 1.0-ml cell suspension into each well of the plate(s). Mix the cell suspension frequently to keep the cells homogeneously suspended for confluent monolayers.
4. Add a few extra drops of MEM-10-TRIS to all corner wells to compensate for evaporation.
5. Any liquid spilled between wells may be aspirated off or dried by use of sterile gauze. Cover each plate with the accompanying lid.
6. Label each plate with the date, cell line initials, passage number, and operator initials. Place plate(s) in a plastic Ziploc ® bag or into an airtight plastic container.
7. Incubate at 20-24°C until 100% confluent without changing the medium. The plates should monolayer in 24-48 hrs. If necessary, plates can be made the same day as inoculation but they will have to be seeded with more cells. However, same day inoculation does not necessarily result in any earlier detection of virus.

B. Inoculating cells with ovarian fluid and tissue samples

1. Materials needed for assay
   a. Appropriate number of 24 well plates with 100% confluent cell monolayers (do not use plates in which cells have been monolayered more than 3 days). Again, EPC cell monolayers will require thicker confluency to avoid retraction when placed at 15°C.
   b. Ovarian fluid and/or tissue samples, either in antibiotic cocktail or filtered
   c. MEM-0-TRIS 0.9-ml dilution blanks
   d. One hundred lambda MLA pipettor (blue) or Pipetman P100
   e. Sterile pipet tips
   f. Plastic Ziploc ® bag or airtight plastic container.
2. Inoculating cells with samples
   a. Each sample is generally plated in duplicate. Label each plate with the inoculation date and accession number. The assay is usually done with the plate aligned with 6 wells across the top and 4 wells down. Label wells with sample numbers (facing front side), the dilutions used (across the top end), and identify controls. Use the plate dilution in labeling. Because 0.1 ml of sample is used as inoculum the actual plate dilution is 1 log higher than the tube dilution. For virus other than IHNV or anticipated low IHNV levels, only 10^{-0} and 10^{-1} tube dilutions need to be done.
   b. Empty medium from wells by inverting the plates over a bleach bucket or use a pipet to aspirate off, leaving a small amount of medium on the cells to prevent drying in the center.
   c. Pretreating cells with PEG is necessary for samples from non-sockeye species. Add 0.1 ml of 7% PEG to the drained cells prior to inoculation.
   d. Pipet 0.1 ml of each ovarian fluid or tissue sample into the replicate first wells allocated per sample.
   e. Pipet 0.1 ml of sample into one 0.9-ml MEM-0-TRIS dilution blank and mix well. Discard the pipet tip into the bleach bucket. The tube dilution of an ovarian fluid sample is now 10^{-1} and a tissue sample 10^{-2}.
   f. Always mix contents of the dilution blanks with a Vortex® mixer rather than the pipettor. Monolayered cells can be inoculated concurrently with the dilution preparations or afterwards.
   g. Continue making additional serial dilutions if necessary and inoculate plates.

h. Alternately, to inoculate the plate after all of the dilutions have been made use the 100-lambda pipettor to pipet 0.1 ml of each dilution per sample into each well, starting with the most dilute (10^{-5} or 10^{-6}) and finishing with the least dilute. The same pipet tip can be used for inoculating all dilutions for a single sample when done this way.

i. Inoculating control wells
   (1) Monolayer control wells: Pipet 1.0 ml appropriate plate media into each of two wells if the plate was inverted. If pipetted, leave two wells with the medium on.
   (2) Sham control wells: Pipet 0.1 ml of the MEM-0-TRIS (with antibiotic cocktail) previously used in the preliminary processing of tissues and in dilution blanks into each of two wells.
   (3) Add PEG to one of each of the above wells.

j. Adsorb samples for at least 30 minutes (60 minutes standard) at 15°C.

k. Add approximately 1.0-ml of appropriate MEM-10 medium to each well. Seal in a plastic bag or in an airtight plastic container.

l. Depending upon the virus to be detected, incubate the plate(s) at the appropriate temperature noted in Section IX.

m. Freeze all ovarian fluids and all tissue samples at -70°C until completion of the assay.

n. Minimum levels of detection (assuming replicate wells)
   (1) For tissues, it is 50 infectious particles (I.P.)/g pooled sample.
   (2) For ovarian fluid, not pooled, it is 5 I.P./ml
   (3) For ovarian fluids, pooled, it is 5 I.P./ml pooled sample or 25 I.P./ml/fish for a 5 fish pool.

C. Blindpassage of negative or questionable sample wells

1. 14 days after the first passage any apparently virus-negative wells in the assay are ready to be blindpassaged. Blindpassages take half the number of plates than the first passage. Plates should be made 1 day in advance and incubated at the appropriate temperature for the cell line used.

2. First passage plates should be read and an "X" put on the plate lid over the wells to be passaged. Two first passage plates are passaged to 1 blindpassage plate, because only one dilution is passaged to only 2 wells. The lowest dilution should be selected for blindpassage unless severely contaminated or destroyed by toxic effect or another dilution exhibits some indication of possible viral CPE.

3. Label the new plate, e.g.:

09-543	(case #)
PWS herring	(subject)
Blind pass	(passage)
12/4/09	(date passage started)
1	(Plate #)

   Also, label the bottom edge of the new plate in front of each row of wells with the individual fish numbers, eg: 231, 232, 233, 234, 235, 236 and then on the other side of the plate, eg: 237, 238, 239, 240, 241, 242.

4. Also, label the new plate with the dilution that was passaged. Do this on each individual row of wells unless all dilutions passaged are the same.

5. In the virology hood, place a first passage plate in front of you with the new plate behind it. Use a separate 10 ml "stubby" pipette for each series of sample wells to triturate the cells in the overlay medium in the first passage plate and pipette 100 µl of old medium and cells into the same numbered well on the new plate. Repeat for

the replicate. Select a new sterile pipette and repeat the process with the next sample well.

6. When the entire blindpassage is completed the first passage plates may be retained for a few days more at 15ºC for continued observation if necessary and as a backup source for another blindpassage should the new plate(s) prove unsatisfactory due to contamination, poor cell growth, etc. When appropriate, discard the first passage plates by placing into a bucket of bleach solution for 30 minutes and drain afterwards for disposal.
7. Overlay the new blindpassage plate(s) with MEM-10 medium, place in a lidded plastic container, label the outside and place at 15ºC for 2 weeks.
8. Examine the new plates at 7 and 14 days and record results on the lab worksheet in the virology folder. If all wells are negative discard the plates as already indicated.
9. If there are any wells exhibiting CPE, save 2 representative samples of the virus isolates as described in XIII and freeze at -70ºC in the virus freezer box labeled for that year. The virus isolates will be identified or confirmed by PCR.
10. Depending on the type of sample, original samples in a virus-positive pool may be thawed and individually re-tested to determine virus prevalence and/or titer.

XIII. Storing: Freezing and Thawing Virus Isolates

A. Preparation of virus isolates for freezing

1. Virus samples - suspected virus isolates from all fish species are frozen after completion of viral assays. At least 2 viral isolates (if 2 or more samples produce CPE) are frozen per accession number, preferably from wells having 4+ CPE.
2. Aseptically pipet 1.5-2 ml of tissue culture fluid and cell debris from the wells representing each isolate into four labeled freezer vials. Seal tightly.

B. Freezing virus isolates

Freeze vials at -70°C. Virus should not be frozen in any LN_2 dewer that contains the stock cell lines. An exception would be if there is only one dewer and a herpesvirus is strongly suspected (i.e., the virus in whole cells could be more easily lost at -70°C). Log each isolate in the freezer notebook. Label each freezer vial per isolate with the accession number, isolate number, number of passages through which cell line, fish stock and species, original sample type (ovarian fluid or tissue sample) and date frozen as follows:

09-085 #5
P1 through EPC (Passage 1 through EPC cells)
Hidden Lake
O. nerka O/F
10-14-09

C. Thawing virus samples

1. One vial of the virus should be thawed and tested for viability before freezing all samples if the identity and stability of the isolate is unknown.*****
2. Always thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.
3. Decant MEM-10 from the required number of 1 to 2-day-old monolayers in 25-cm^2 flasks. Pipet 0.1-ml virus sample onto each cell monolayer.
4. Allow virus to adsorb for 30-60 minutes at 15°C.
5. Add 5 ml MEM-10 to each flask and incubate at the appropriate temperature until all cells lift off each flask (4+ CPE).

* This may not be feasible for unknown virus isolates requiring long incubation times to produce CPE.

XIV. IHNV Concentration in Water Samples

In order to learn more about the epizootiology of IHNV, it may be desirable to attempt detection of the virus in water samples. The virus requires concentration by tangential flow filtration (TFF) using the Pellicon Cassette System apparatus, followed by polyethylene glycol (PEG) precipitation. The technique may be used to evaluate a hatchery water supply or the effluent. Reportedly, the technique can detect virus as low as 3 infectious particles per 10-L sample (Batts and Winton, 1989).

A. Filtration unit
Detailed specifications can be found in the accompanying user manual.
- Pellicon cassette system, xx42-02K-60
- 2 gpm tubing pump system
- Two filters (5 ft^2)
- Intake tubing-silicone 3/8"; retentate and filtrate 1/4" about 5 feet.
- Operate pump at speed setting 3.

B. Collection of the water sample
1. Up to 40 L of water should be collected in clean containers that have not been exposed to virus. Carboys may be obtained from the lab or large plastic bags can be utilized.
2. Virus should be stabilized by immediately adding 0.1% FBS or BCS.
3. Keep the sample cool (4°C) and transport to FPL the same day.

C. Preparations to complete
1. Prepare 5 L PBS: 1 for pretreating filter, 4 for cleaning the filter.
2. Prepare 15 L cold, virology-grade (VG) water.
3. Prepare 1 L 0.1-M NaOH (24 g/6 L).
4. MEM-0 for diluent.
5. Weigh sterile containers to use for centrifuging retentate.
6. Prepare fresh EPC monolayers in 24 well plates for viral assay.
7. Weigh and chill a sterile 250-ml serum bottle for collecting retentate.
8. Set up two 100-ml containers with sterile PBS for flushing out the sample.

D. Preparation of the filtering apparatus
1. Flush out formalin or NaOH with 5 L VG water with pump running clockwise (cw).
2. Place retentate and filtrate lines into 5 L container of VG water and reverse flow for a few minutes.
3. Pump through another 3 L VG water with flow cw.
4. Pump through 1 L PBS cw.

E. Running the filtration
Allow particulates to settle out in the water sample. Then set up a separate vessel to collect retentate and do not pull the sample off bottom of the original container.
1. Run the sample through cw with pump set on 3 and recycling retentate until the volume is reduced to approximately 200 ml. Keep the sample on ice during filtration.

2. Shorten the tubes and move into a 250-ml serum bottle. Pump retentate through and reduce the volume to approximately 50 ml.
3. Close filtrate lines and pump out any remaining sample.
4. Flush 100 ml PBS through cw and add to the retentate.
5. Flush 100 ml PBS through the retentate line (counter cw).
6. Weigh to measure the total volume.
7. Initiate cleaning procedures for the filters (see below).

F. Processing of the retentate
1. Many samples will require processing to remove excess silt or debris prior to PEG precipitation. If the retentate is cloudy or dark, one of these alternatives should be considered.
   a. Centrifuge at 6,500 x g for 1 hour in a refrigerated centrifuge. Retain the supernatant.
   b. Filter the retentate using a disposable 150-ml 0.45-µm filter and a vacuum pump. Pass about 10-20 ml of BCS through filter prior to the sample. After processing, transfer the sample to a 250-ml Erlenmeyer flask.
2. PEG precipitation used to further concentrate the virus.
   a. Add 7% PEG-20,000 (M.W. 15,000-20,000) to the sample using the following formula: (sample volume x 0.070) = grams of PEG to add.
   b. Stir overnight on a mixer in a refrigerator. Use an ice-water bath or insulating material under the flask to prevent heating of the sample.
   c. Afterwards, antibiotics may be added (not necessary if sample has been filtered as above) to reduce microbial contamination during the viral assay at levels not to exceed the following:

      gentamycin -2,000 µg/ml
      Fungizone -400 IU/ml
      Pen/strep -800 IU/ml and 800 µg/ml
3. Centrifuge the samples in two weighed 250-ml sterile bottles at 6,500 x g for 60-90 minutes in a refrigerated centrifuge.
4. Pour off the supernatant, retaining some to test for virus if desired. Weigh bottles to determine the weight of the pellet. Re-suspend the pellet in a small (3-5 ml) measured quantity of MEM-10.
5. Test for virus using a quantal assay with multiple dilutions to determine titer by $TCID_{50}$ (Reed and Muench 1938; Rovozzo and Burke 1973). Observe the assay for at least 14 days and blindpassage the lowest dilution for 14 days if no virus is detected.
6. When reporting results, indicate that a negative finding does not necessarily mean that virus was not present in the water sample. Minimum detectable levels are reported to be 3 infectious particles per 10-L sample (Batts and Winton 1989).

G. Cleaning of the filters
1. Flush with 4 L PBS to remove protein.
2. Draw in 1 L of 0.1-M sodium hydroxide and retain it for storage of filters.
3. Alternately, flush out the NaOH and draw in 1% formalin in PBS. Plug the intake and retentate lines.

XV. Alkaline Phosphatase Immunohistochemical (APIH) Procedure for the Identification of IHNV in Tissue Culture Cells, Paraffin and Frozen Sections, and Kidney Tissue Smears

The APIH provides rapid identification of IHNV by staining viral nucleoprotein a bright red color that is visible by eye and by light microscopy in tissue culture cells and in tissues. The following procedure was adapted from Drolet et al. (1993).

A. Preliminary processing
   1. Tissue culture plates are emptied of medium by inversion over or aspiration into a solution of bleach. The monolayers are fixed by adding 1 ml of 10% formalin to each well. After 1 hour, remove fixative, rinse gently with tap water and invert over paper towel to air dry. For previously stained tissue culture plates, de-stain the wells with 70% ethanol and allow to dry. Destaining may require a long soak-time and several changes of ethanol.
   2. For paraffin-embedded tissue deparaffinize as follows:

histoclear	5 minutes
histoclear	5 minutes
100% ETOH	30 seconds-1 minute
95% ETOH	5 minutes
95% ETOH	5 minutes
70% ETOH	5 minutes

   3. For cryostat sections, air dry the sections and fix in acetone or methanol for 15 minutes immediately before staining.
   4. For kidney smears, air dry and fix in acetone or methanol for 15 minutes.

B. Staining procedure
   1. Hydrate in water for 10 minutes and then in PBS for 20 minutes.
   2. Block with 5% non-fat powdered milk in PBS for 20 minutes.
   3. Wash in PBS two times for 5 minutes each.
   4. Apply primary antibody (anti-IHNV) for approximately 1 hour.
   5. Wash in PBS two times for 5 minutes each.
   6. Apply secondary biotinylated antibody for 30 minutes.
   7. Wash in PBS two times for 5 minutes each.
   8. Apply alkaline phosphatase avidin-biotin complex (ABC-AP) for 1 hour.
   9. Wash in PBS once for 5 minutes.
   10. Apply substrate buffer (Tris-HCl) for 2 minutes.
   11. Apply Vector Red phosphate substrate with levamisole for 20-30 minutes. Keep in the dark.
   12. Wash in running water for 5 minutes.
   13. Counterstain in hematoxylin for 3 minutes.
   14. Wash in running water.
   15. Put in Ammonia 70% ETOH for 3 minutes.
   16. Mount slides.

C. Reagents
   1. PBS

NaCl	7.2 g
Na2HPO4	1.48 g
KH2PO4	0.43 g
dH_2O	1000 ml
pH	7.4

   2. Blocking agent - 5% non-fat powdered milk in PBS
   3. Primary Antibody - Anti-IHNV mouse monoclonal antibody (several MAbs available against G and N proteins of Western Regional Aquaculture Consortium strain of IHNV)

No dilution necessary

4. Secondary antibody - Biotinylated Goat anti-mouse in PBS
   Vectastain Mouse IgG alkaline phosphatase kit AK-5002
   Vector Laboratories (www.vectorlabs.com)
   Add 1 drop (50 microliters) to 10 ml PBS for working dilution
5. Avidin Biotin Complex - alkaline phosphatase in PBS
   Vectastain Mouse IgG alkaline phosphatase kit
   Vector Laboratories - same kit as above
   Add 2 drops of Reagent to 10 ml PBS and then add 2 drops Reagent B and mix immediately. Let stand 30 minutes before using.
6. Substrate buffer - 100 mM Tris-HCl pH 8.2
7. Substrate - Vector Red plus Levamisole
   Alkaline Phosphatase Substrate Kit I SK-5100
   Levamisole Solution SP-5000
   Vector Laboratories
   Add 1 drop levamisole to 5 ml substrate buffer (Tris-HCl). Immediately before use add 2 drops Reagent 1 and mix, 2 drops of Reagent 2 and mix, and 2 drops of Reagent 3 and mix.
8. Ammonia 70% ETOH - Ammonium Hydroxide 0.02% in 70% ETOH

XVI. Biotinylated DNA Probes for the Detection of IHNV and Distinction Between the European and North American Strains of VHSV

Biotinylated DNA probes for IHNV and both European and North American strains of VHSV were developed by Batts et al. (1993). The probes hybridize with different sequences within the messenger RNAs of the nucleoprotein (N) gene elicited by each of the viruses that is extracted from tissue culture cells that have been infected for 24-48 hours. The probe for North American VHSV IVa hybridizes specifically with a nearly unique 28-nucleotide sequence following the open reading frame of the N gene mRNA. The probe for European VHSV recognizes a 29-nucleotide sequence near the center of the N gene common to both American and European strains of VHSV. A third probe recognizes a 30 base sequence unique only to IHNV. The following procedure was adapted from Batts et al. (1993) who graciously supplied the probes and PCR controls for our use.

A. DNA probe test set-up
   1. Two days before running DNA Probe Test:
      a. Prepare a 24-well microtiter plate with EPC or CHSE-214 cells so that it will be confluent by the next day.
      b. If necessary, make up DEPC-treated water at a concentration of 1 ml DEPC to 1 L of distilled water. Mix on stir plate until thoroughly mixed. Make at least 5-6 L for treating glassware.
      c. Rinse needed glassware with DEPC treated water and let dry. Store on shelf in an area dedicated for this use.
      d. Sterilize distilled water and make up solutions that need to be autoclaved (solutions f, g, l, m, n).
   2. One day before running DNA probe test:
      a. Inoculate viral isolates onto cell monolayers in 24-well plate. Use several wells per isolate. Inoculate 2 wells with MEM-10-TRIS to use as a negative control. There should be no CPE when mRNA is extracted. Use dilutions if CPE occurs in 24-48 hrs. Incubation of virus on cells may require up to 48 hrs for adequate mRNA from certain isolates.

b. Prepare all other solutions needed to run the test. Adjust pH of final products carefully.
c. Fill both water baths and turn on. Adjust to 55°C and 65°C.
d. Get out rotator, Hybridot, and Seal-a-Meal apparatus and make sure they are operational.

B. DNA probe test procedure
1. Extraction of mRNA from infected cells:
a. Preparation
(1) Place crushed ice in a tray with microcentrifuge racks.
(2) Make sure water baths are at 55°C and 65°C.
(3) Always wear latex gloves.
(4) Label tubes to be used.
b. Pipet off the infectious medium above cells and add 0.5 ml RNAzol B to each well. Replace lid and put on rocker for 5-10 minutes at room temperature to digest cells.
c. During step b put 50 µl cold chloroform/iso-amyl alcohol into labeled siliconized 1.7-ml tubes and keep on ice.
d. Triturate the cell debris in each well with a 1-ml pipet five times and transfer solution into the labeled chloroform/iso-amyl tubes. Vortex the tubes 3 seconds each and store on crushed ice for 5 minutes to allow phase separation.
e. Centrifuge the suspension at 10,000 rpm for 15 minutes. The RNA will remain in the clear aqueous phase and the DNA and protein will be left in the lower blue phenol phase.
f. During step e, put 0.25 ml of cold absolute isopropyl alcohol into new labeled tubes and store on ice. Keep the alcohol at -20°C until ready for use.
g. Transfer the aqueous phase containing the RNA (0.25 ml, no blue fluid) into the tube with 0.25 ml absolute isopropyl alcohol. Vortex for 1 second and chill tubes on ice for 15 minutes to precipitate RNA.
h. Centrifuge for 15 minutes at 10,000 rpm and remove as much fluid as possible from pellet. When you centrifuge, put the hinge of the microtube on the top. The pellet will be on that side and may be very difficult to see.
i. During step h, prepare nitrocellulose membrane:
(1) Wet membrane in distilled water for 1 minute. Wet by capillary action at an angle.
(2) Pour water off.
(3) Soak for at least 5 minutes in 10X standard saline citrate (SSC).
j. For each probe used, heat approximately 140 µl of North American VHSV, Common VHSV and IHNV PCR products for 1 minute in boiling water to denature the DNA. Transfer to ice. If only two probes are used, heat about 250 µl of product.
k. Warm prehybridization buffer to 55°C in water bath.
l. Add 170 µl autoclaved distilled water to RNA pellets. Mix by flicking bottom of tube and warm tubes in 65°C water bath for 15-20 minutes. RNA pellets should dissolve. Mix again. Pellets appear as small white or brown flakes.
m. Add 170 µl of 20X SSC into tubes containing dissolved RNA pellets and store on ice.
n. During step k put wetted membrane in Hybridot. Attach vacuum pump hoses to blotting device.
o. Add 200 µl of 10X SSC to each well of blotting device. Membrane should not be dry when RNA is added. Try to avoid trapping air in the wells of the Hybridot.

p. Mix gently and add 100 µl of each RNA solution to wells of Hybridot which contain 200 µl of 10X SSC. Blot PCR products last.
q. Apply vacuum at 5 psi. After all solutions are added leave vacuum on 10-15 psi for about 1 minute. Turn off vacuum. Poke holes with pipet tip into empty wells for easy cutting of membrane.
r. Dismantle apparatus and remove membrane with forceps. Transfer membrane to thick filter paper wetted with 10X SSC.
s. Cut membrane into sections and label.
t. Transfer membranes to dry sheet of blotting paper and cover with a second sheet. Microwave for 60 seconds on high to attach nucleic acids to membrane. Weights can be placed on sides of the blotting paper to keep it from curling up.

2. Hybridization of probes with RNA on nitrocellulose membrane:
   a. For prehybridization, place membranes spot-side-up into separate Seal-A-Meal® pouches. Add 10 ml prehybridization buffer to each pouch, remove air bubbles, and seal. Prehybridize for 30 minutes to 24 hours at 55°C in water bath.
   b. Thaw the probe solutions and heat to 50-55°C. Cut off edge of pouches and pour off the prehybridization buffer. Add 10 ml of each probe solution (prediluted in buffer) to the respective pouch and re-seal. React membranes in probe solutions for 1 hour to 24 hours at 55°C in water bath. If you are using probes that are not prediluted, do not pour off the prehybridization buffer and add 100 µl of probe.
   c. Remove probe solutions from pouches and store in tubes at -20°C for reuse up to 5 times.
   d. Transfer membranes into 40 ml post-hybridization solution in a buffer dish. Wipe forceps between each membrane. Discard solution and add 40 ml fresh post-hybridization solution and wash for 15 minutes on rocker at RT. Wash two more times with 40 ml buffer for 15 minutes each on the rocker at RT.
   e. Put dish with membranes and pre-warmed post-hybridization buffer into 55°C waterbath for 15 minutes. Cover dish with parafilm.
   f. Warm color development buffer to RT.
   g. Rinse membranes briefly with 40 ml of Buffer A.
3. Color development of biotinylated probe:
   a. Incubate membranes in a solution containing 40 µl streptavidin/alkaline phosphatase conjugate in 40 ml Buffer A for 30 minutes on rocker at RT. The conjugate can be used up to five times.
   b. Rinse membranes briefly in 40 ml Buffer A and then wash twice in 40 ml Buffer A on the rocker for 7 minutes at RT.
   c. Wash twice in 40 ml Buffer B on the rocker for 7 minutes at RT.
   d. Immediately before use, add 0.4 ml alkaline phosphatase (AP) color reagent A and 0.4 ml AP color reagent B to 39.2 ml color development buffer warmed to RT.
   e. Add 40 ml color development solution to the dish containing the membranes. Store in the dark for 15 minutes on the rocker at RT. The rocker can be placed under a box for this step.
   f. Wash membranes in distilled water for 10 minutes with at least one change of water. Store membranes in distilled water until ready to photograph.

4. Solutions needed for DNA probe dot blot procedures:

   All glassware should be Cleaned with DEPC-treated water and autoclaved before use. This is to prevent RNA-ase contamination. This water is available

from Invitrogen (catalog #10813-012). See DNA probe set-up section for preparing your own DEPC water for rinsing glassware.

(a) PREHYBRIDIZATION BUFFER

Distilled-deionized water	69.5 ml
10x Denhardt's solution	10 ml of 100x stock (#4)
2x SSC	10 ml of 20x stock (#6)
1% SDS	10 ml of 10% stock (#8)
0.1 mg/ml SSS DNA (Invitrogen)	0.5 ml of 20 mg/ml stock (#5)

(b) HYBRIDIZATION SOLUTION

Prehybridization buffer	10 ml (#1)
Biotinylated DNA probe	100 ng/ml

(Store at -20°C; may reuse up to 5 times)

(c) POST-HYBRIDIZATION SOLUTION

2x SSC	50 ml of 20x stock (#6)
0.1% SDS	5 ml of 10% stock (#8)
distilled-deionized water	up to 500 ml

(d) DENHARDT'S SOLUTION

(Commercial product (Invitrogen) purchased at 50x stock concentration)
(Catalog #750018 for 100-ml size)

A 10x solution contains:
1% bovine serum albumin
1% polyvinylpyrrolidone 360
1% ficoll 400

(e) SHEARED SALMON SPERM DNA (SSS DNA)

(Commercial product (Invitrogen) purchased at 20 mg/ml)
(Catalog #15632-011 for 5-ml size)

Procedure
Transfer 0.5 ml of SSS DNA into 10 vials (with gaskets). Place vials into boiling water for 10 minutes. Cool vials in crushed ice, then transfer to -20°C freezer until needed. When needed, add 0.5 ml to prehybridization buffer (see #1)(final concentration of 0.1 mg/ml).

(f) 20x STANDARD SALINE CITRATE (20X SSC)

NaCl (Sigma #S-3014, 3 M final concentration)	87.65 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	44.11 g

distilled-deionized water	up to 500 ml

(Adjust to pH 7.0 with HCl, AUTOCLAVE)

(g) **10x STANDARD SALINE CITRATE (10X SSC)**

NaCl (Sigma #S-3014, 3 M final concentration)	43.82 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	22.05 g
distilled-deionized water	up to 500 ml

(Adjust to pH 7.0 with HCl, AUTOCLAVE)
OR
Dilute 1:2 from 20x SSC. Combine equal volumes of 20x SSC with distilled-deionized water, AUTOCLAVE.

(h) **10% SODIUM DODECYL SULFATE (10% SDS)**

Lauryl sulfate sodium salt (Sigma #4390)	10.0 g
sterile distilled-deionized water	up to 100 ml

(Adjust to pH 7.2. **Do not autoclave this solution!**)

(i) **STREPTAVIDIN/ALKALINE PHOSPHATASE CONJUGATE (SA/AP)**

0.1 µg/ml streptavidin/alkaline phosphatase conjugate (Bio-Rad #170-3554), store vial at 4°C.

Prepare by diluting SA/AP 1:1000 in Buffer A:
(Example: 30µL SA/AP stock added to 30 ml of Buffer A)

(May reuse this solution up to 5 times, store at 4°C)

(j) **BUFFER A**

0.1 M Tris (pH 7.5)	50 ml of 1 M stock (#14)
0.1 M NaCl	10 ml of 5 M stock (#12)
2 mM $MgCl_2$ (Sigma #M-1028, 100 ml size)	1 ml of 1 M stock
0.05% Triton X-100 (BIORAD, Catalog #161-0407)	0.25 ml
distilled-deionized water	up to 500 ml

(k) **BUFFER B**

0.1 M Tris (pH 9.5)	50 ml of 1 M stock (#13)
0.1 M NaCl	10 ml of 5 M stock (#12)
50 mM $MgCl_2$ (Sigma #M-1028, 100 ml size)	25 ml of 1 M stock
distilled-deionized water	up to 500 ml

(l) **5 M NaCl**

NaCl (Sigma #S-3014)	146.1 g
distilled-deionized water	up to 500 ml

(AUTOCLAVE THIS SOLUTION)

(m) **1 M TRIS BUFFER (pH 9.5)**

Tris base (Sigma #T-8524)	54.7 g
Tris HCl (Sigma #T-7149)	7.6 g
distilled-deionized water	up to 500 ml

(Adjust to pH 9.5, then AUTOCLAVE!)

(n) **1 M TRIS BUFFER (pH 7.5)**

Tris base (Sigma #T-8524)	11.8 g
Tris HCl (Sigma #T-7149)	63.5 g
distilled-deionized water	up to 500 ml

(Adjust to pH 7.5, then AUTOCLAVE!)

(o) **CHLOROFORM/ISOAMYL ALCOHOL MIXTURE**

chloroform (J.T. Baker #9180-03)	24 ml
iso-amyl alcohol (J.T. Baker #9038-1)	1 ml

(Mix together and store at -20°C until needed)

(p) **ISOPROPYL ALCOHOL**

2-Propanol (isopropyl alcohol), (J.T. Baker #9084-03),

(Use undiluted for precipitation of RNA)

(q) **PUREZOL RNA ISOLATION REAGENT**

RNA isolation solvent, store at 2-8°C in the dark. Bio-Rad Catalog #732-6890 is 100-ml size. Contains phenol and thyocynate compounds.

(r) **ALKALINE PHOSPHATASE CONJUGATE SUBSTRATE KIT**

(**NOTE:** This product contains dimethylformamide. Use in area with good ventilation.)
(BIORAD Catalog #170-6432)

- Dissolve AP color development buffer in 1 L volume of distilled-deionized water.
- Filter-sterilize then store at 4°C until needed.
- Immediately before use, add 0.3 ml of AP color reagent A and 0.3 ml AP color reagent B to 29.4 ml color development buffer at RT.

XVII. Plaque Reduction Serum Neutralization Assay

Serum neutralization is one method of confirming the serological identity of a virus isolate. When a known concentration of a virus from tissue culture is incubated with a known dilution of specific neutralizing antiserum against that virus, the ability of the virus to then produce CPE when inoculated onto cells is significantly reduced (neutralized). This neutralization is often temporary such that with time the antigen-antibody complex (virus and antibody combined) breaks apart, freeing the virus, allowing it to again infect a cell. This is called "breakthrough" which can confound results if a neutralization test is not read soon enough. Generally, the results of the unknown virus dilutions can be read when the positive control of known virus is significantly neutralized (at least one $\log_{10}$ in titer or 80% plaque reduction).

There are at least two general variations of the virus neutralization test; constant virus concentration exposed to varying antiserum dilutions or varying virus concentrations exposed to a constant antiserum dilution. The latter type of test is used here because it requires the least amount of antiserum and has less inherent error in preparation. The following method will apply for those viruses that will produce plaques under a semi-solid overlay (rhabdoviruses, birnaviruses, aquareoviruses, and some herpesviruses).

The plaque reduction serum neutralization assay – This test was used to confirm the identity of suspected IHNV and VHSV isolates and is presented here for a historical perspective. However, the PCR assay is now the standard test used to identify all presumptive IHNV and VHSV isolates.

A. Preparation of plates
   1. Determine number of plates needed for the assay. You will need three 24-well plates to run one unknown virus against one antiserum.
   2. Prepare the plates and allow monolayers to form the day before you want to run the assay.

B. Preparing dilutions of known virus, unknown virus, antiserum and normal serum
   1. Dilute antiserum to appropriate dilution with MEM. Various dilutions of antiserum will have to be tested against the control virus beforehand to determine the optimum neutralizing dilution. You will need 1.5 ml of diluted antiserum to run one unknown against one antiserum.
   2. Dilute normal serum with MEM to the same dilution as the antiserum. You will need 1.5 ml of diluted normal serum to run one unknown against one antiserum.
   3. Dilute known virus and unknown virus to approximately 1×10^5 pfu/ml with MEM. You will need 1.5 ml of diluted known and unknown virus.

C. Setting up neutralization test and controls
   1. Label a sterile empty 24-well plate appropriately, as in example.
   2. Aseptically pipet 200 µl of diluted antiserum into appropriate wells.
   3. Aseptically pipet 200 µl of diluted normal serum into appropriate wells.
   4. Aseptically pipet 200 µl of MEM into appropriate wells. Add 400 µl of MEM into tissue control well (MEM only).

5. Aseptically pipet 200 µl of each known and unknown virus into appropriate wells.
6. Incubate for one hour at room temperature on the rotary shaker.

EXAMPLE

	A Known Virus	B Known Virus	A Unknown Virus	B Unknown Virus	MEM
Antiserum	KV + AS	KV + AS	UV + AS	UV + AS	MEM + AS
Normal Serum	KV + NS	KV + NS	UV + NS	UV + NS	MEM + NS
MEM	KV + MEM	KV + MEM	UV + MEM	UV + MEM	MEM

KV Known virus
UV Unknown virus
AS Antiserum
NS Normal serum
MEM Minimum essential media

D. Performing the assay
1. Label the three 24-well plates to be used in the plaque assay. Three replicate tests are run on one plate, i.e., plate 1 may contain:

e.g., KV + AS; UV + AS; KV + NS

KV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
UV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
KV + NS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}

The antiserum, normal serum and MEM controls can be run on the third plate using only 10^{0} and 10^{-1} dilutions.

2. Dilute 0.1 ml of the solution from each test well in the incubated 24-well plate 10^{-0} to 10^{-3} in 0.9 ml MEM dilution blanks. Dilute 0.1 ml of the mixture from each control well 10^{-0} to 10^{-1} in 0.9 ml MEM dilution blanks.
3. Overlay EPC cells with 100 µl of 7% PEG for a few minutes prior to inoculations. PEG solution should be made up in MEM-10.
4. Pipet 100 µl of each dilution into appropriate well of PEG-treated EPC cells.
5. Incubate for 30 minutes at room temperature to allow virus adsorption.
6. Overlay wells with 1 ml of methylcellulose overlay medium.
7. Incubate at 15°C for 7 days in a sealed plastic bag or plastic container.
8. Fix and stain plates by pipetting approximately 1 ml of 0.5% crystal violet in 40% formalin into each well and let stand for 1 hour.
9. Pour off stain, rinse monolayers with water and allow plates to air dry.
10. Count and record numbers of plaques.

E. Interpretation
1. The tissue control wells (MEM only), the AS + MEM wells and the NS + MEM wells should not have any plaques present. Plaques would indicate that the medium,

antiserum or normal serum were contaminated with virus and the test must be repeated.

2. An 80% or greater reduction of plaques is considered a positive serum neutralization test and confirms the identity of the virus. The known virus control should always show an 80% or greater reduction for the test to be valid.
3. When determining if there is an 80% reduction of plaques, first look at the virus control wells. Determine the dilution where countable numbers of plaques are present. Calculate the mean pfu of the duplicate wells and compare this value to that of the virus + AS wells at that same dilution. Subtraction of the latter value from the control value will provide the pfu/ml of virus remaining after neutralization.
4. The normal serum + virus wells should not show any plaque reduction as compared to the virus positive MEM wells. If there is significant plaque reduction in the normal serum wells, this indicates that there is some nonspecific neutralization occurring with the virus and the serum.

Another method of expressing neutralization is the neutralization index (NI). This value is calculated by subtracting the $\log_{10}$ pfu/ml value of the neutralized virus remaining from the value of the same unneutralized virus in MEM. Example:

Antisera	$\log_{10}$ pfu/ml Remaining Virus			
	IHNV	**NI**	VHSV	**NI**
IHNV	2.0	**2.8**	4.4	**0.4**
VHSV	4.8	**0**	<1.0	**>3.8**
MEM	4.8	**0**	4.8	**0**

Using $\log_{10}$ pfu, a smaller value denotes greater neutralization.

After conversion to NI the opposite is true, i.e., the larger the NI value the greater the neutralization.

XVIII. Fluorescent Antibody Staining

Immunofluorescence assay for IHNV – can be used as another rapid serological test for confirming the identity of a viral isolate such as IHNV. The assay uses a primary mouse anti-IHNV monoclonal antibody and a goat anti-mouse IgG FITC conjugate. The assay is performed in microwells of standard FAT slides as described in the FAT chapter of this manual (see Chapter 8).

XIX. PCR for IHNV and VHSV

All VHSV and IHNV isolates are routinely confirmed with reverse transcriptase nested PCR tests. Routine confirmations are done several times a year in batches. These tests are also periodically run on unusual isolates or isolates from uncommon fish species. Rhabdovirus isolates are always run on both the IHNV and VHSV tests in parallel. See the molecular chapter for procedures.

XX. Virus Characterization Tests

Non-routine virus isolates are identified using electron microscopy (see electron microscopy chapter). In addition the following other tests are also used to identify virus characteristics.

A. $TCID_{50}$ assay - to determine tissue culture infective dose prepare serial dilutions of a virus suspension to be titered to 10^{-8} in MEM. Inoculate virus dilutions into 96 well plates with confluent cells using 6 replicates of each dilution. Use a cell line where the virus appears to be growing best (subsequently called primary cell line). Read inoculated wells for CPE and determine the 50% infectivity end point, i.e. the highest dilution at which 50% of inoculated wells are positive for virus indicating viral titer. See Reed and Muench (1938) for procedural details.

B. Replication in various cell lines- to test the ability of various cell lines to support virus growth. Grow cells to be tested in 96 well plates and incubate at appropriate temperature until confluent. Titrate virus to 10^{-8} and inoculate cell cultures (6 replicates) with virus, incubate and observe daily for CPE for 14 days. Record CPE at 1+ to 4+ every 2 days. After incubation determine viral titers using $TCID_{50}$ calculations for the replicates of each cell line. Include primary cell line as a control for comparison purposes.

C. Optimal temperature- to determine the optimal growth temperature for viral replication. Grow up primary cell line in 96 well plates at appropriate temperature until confluent. Inoculate cell cultures with 10 fold dilutions of virus to 10^{-8}. Incubate cultures at 5, 10, 15, 20, and 25°C for 14 days. Determine viral titers by $TCID_{50}$ calculations for the replicates at each temperature.

D. Thermostability- to determine the stability of the virus at various temperatures. Incubate viral suspensions in MEM at 4, 16, 37, and 56°C. Remove samples of the suspensions at 1, 6, 24 hours and 7 days incubation. Determine viral titers using 10 fold dilutions of virus for $TCID_{50}$ assay in primary cell line.

E. pH stability- to determine stability of the virus at various pH values. Make MEM adjusted to pH 2, 3, 5, 7, 9, and 11 by adding HCl or NaOH and filter sterilize. Add 0.1 ml of virus suspension from infected primary cell line to 0.9 ml aliquots of MEM at each pH. Mix and incubate at 4°C for 3 hours. Determine the virus titer at each pH level using 10 fold dilutions of virus for $TCID_{50}$ assay in primary cell line.

F. Chloroform sensitivity- to determine sensitivity of virus to chloroform indicating presence or absence of a lipid envelope. Take 2 ml of virus suspension from infected primary cell line and divide in half. One ml will serve as a non-treated virus control. Add 0.05 ml chloroform to 1 ml of virus suspension in a glass tube. Shake control and chloroform mixture vigorously for 10 minutes at RT and centrifuge at 1000 x G for 15 minutes at 4°C. Remove supernatant from chloroform-treated virus and place in sterile tube. Let sit at 4°C for 1 hour with a loose cap to evaporate residual chloroform. Determine viral titers of the control and chloroform treated material using 10 fold dilutions of virus for $TCID_{50}$ assay in primary cell line.

G. Size via membrane filtration- to determine if unknown agent passes through a filter. Pre-treat 0.45 and 0.25 micron filters with fetal bovine serum until saturated. Filter viral suspension through each of the two filter sizes. Determine viral titers before and after filtration using 10 fold dilutions of virus for $TCID_{50}$ assay in primary cell line.

H. IUDR- to determine nucleic acid type (DNA/RNA) using the DNA inhibitor 5-iodo-2-deoxyuridine. Prepare 96 well plates of primary cell line to confluency. Aspirate media and add MEM-10 to one set of 6 replicates (untreated) and MEM-10 with 100 ug IUDR/ml to the other set for over night. Aspirate and inoculate plates with virus titrated to 10^{-8} in MEM-10

and MEM-IUDR. Additional MEM-10 and IUDR media (0.2 ml) can be added to each well for incubation. Incubate at appropriate temperature for 14 days and determine viral titers using $TCID_{50}$ calculations.

XXI. Washing Glassware

It is important to clean and rinse tissue culture glassware thoroughly. Improperly cleaned glassware may be toxic to tissue culture cells.

A. Bottles and lids
1. Empty reagents and media from bottles. Immediately fill bottles with hot tap water. Replace lids and place on the bench top or a cart near the sink in a plastic dish pan appropriately marked tissue culture only.
2. When enough bottles have accumulated, wash by hand or in a dishwasher. If a dishwasher is used, empty all bottles and place in the dishwasher inverted. Lids are placed in the basket on the top rack of the dishwasher.
3. For hand washing use Liqui-nox® detergent (phosphate free), a brush used only for tissue culture utensils and hot tap water. After washing, rinse at least twice with hot tap water and twice with tissue culture-grade water and drain dry. Mark with autoclave tape and sterilize the glassware in the autoclave.
4. When using the dishwasher use Alcojet® or Labtone® and run on a normal cycle.
5. Run the distilled water cycle 5 times to rinse bottles and lids. Remove bottles and lids from the dishwasher.
6. Rinse each bottle and lid at least 2 times in tissue culture grade water.
7. Drain bottles, replace lids loosely, mark with autoclave tape and autoclave.

B. Contorques® and Virtis® grinders
1. For non-IHNV samples or when extra cleaning is needed.
   a. Immediately after use immerse grinding instruments in a bleach solution of at least 200 ppm and leave overnight.
   b. Drain instruments of bleach solution. Rinse with copious amounts of tap water.
   c. Clean each piece individually with Liqui-nox® and a brush.
   d. Rinse again with copious amounts of tap water.
   e. Rinse 3 times in laboratory-grade water and at least 2 times in tissue culture-grade water.
   f. Place into trays, let dry, cover with aluminum foil, label and autoclave.
2. For IHNV samples when Contorques® are in heavy use. (If only washing occasionally, disinfection with bleach is suggested.)
   a. After use immerse grinding instruments in a bucket of laboratory-grade water (no disinfectant).
   b. Pour off water and rinse instruments thoroughly with laboratory-grade water. If unable to remove all tissue or oils from the instruments, add Liqui-nox®, brush and rinse thoroughly two times in tap water and two times with tissue culture-grade water.
   c. Place in trays, let dry, cover with aluminum foil, label and autoclave.

XXII. Media

NOTE: All chemicals should be reagent or tissue culture grade. Use only glassware which is either new, used only for tissue culture, or has been acid washed (see Chapter 9).

A. 1x MEM-TP (incomplete cell culture medium)
1. 1 (10L) pkg powdered Minimal Essential Medium (MEM)

	29.6 g	tryptose phosphate
	10 L	tissue culture-grade water
2.	1 (1L)pkg	MEM
	2.95 g	tryptose phosphate
	950 ml	tissue culture-grade water (see instructions on package)

Reconstitute powdered MEM and tryptose phosphate in tissue culture-grade water. Dispense medium in 450-ml quantities into clean bottles and autoclave. Label bottles MEM-TP and store at 4°C. If you anticipate having to make herring medium, add water before adding tryptose phosphate and remove the needed quantity of medium without tryptose phosphate. Reduce the amount of tryptose phosphate in recipe based on how much medium remains after removal of herring medium.

B. 2x MEM-TP (incomplete cell culture medium)

1.	1 (10L) pkg	powdered MEM
	29.6 g	tryptose phosphate
	5 L	tissue-culture grade water
2.	1 (1L) pkg	MEM
	2.95 g	tryptose phosphate
	475 ml	tissue culture-grade water (see instructions on package)

Reconstitute powdered MEM and tryptose phosphate in tissue culture-grade water. Dispense in 250-ml quantities into clean bottles and autoclave. Label bottles "2x MEM-TP and store at 4°C. Both 1x and 2x MEM-TP can be made at the same time by first making 2x MEM-TP and dispensing a portion of it and then adding an amount of tissue culture-grade water equal to the remainder of MEM to make it 1x MEM-TP; i.e., dispense 1.5 L of 2x MEM-TP (6 bottles) leaving 3.5 L. Add 3.5 L tissue culture-grade water, mix and dispense as above. Store at 4°C.

C. MEM-10 (complete cell culture medium for closed systems)

450 ml	MEM-TP
50 ml	fetal bovine serum (FBS) or bovine calf serum (BCS)
5 ml	L-glutamine (200 mM)
5-8 ml	7.5% $NaHCO_3$

Do not add $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. For all MEM-based media, proper pH is 7.2-7.6, which results in an orange-red color. Store at 4°C. Cells cultured in medium containing the less expensive BCS may lift off flasks, in time, starting at 2 weeks to a month. (See BCS comments in section XX. V).

D. MEM-10-TRIS (complete cell culture medium for open plates or closed systems)

450 ml	MEM-TP
50 ml	FBS
5 ml	L-glutamine (200 mM)
0.5 ml	gentamycin (50 mg/ml)
5 ml	Fungizone (250 µg/ml)
7.5 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

Do not add Tris and $NaHCO_3$ until all other components have been mixed together, so the proper pH can be determined. Store at 4°C. If for European VHSV or North

American VHSV type IVb assay, media pH should be 7.6-7.8 and 7.4-7.8, respectively, as indicated by some purple color in the medium. However, North American strains of VHSV type IVa will produce CPE at a pH as low as 7.0.

E. MEM-5-TRIS (for quantal assay overlay)

450 ml	MEM-TP
25 ml	FBS or BCS
5 ml	L-glutamine (200 mM)
0.5 ml	gentamycin (50 mg/ml)
5 ml	Fungizone (250 µg/ml)
7.5 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

Do not add Tris or $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. Store at 4°C. If for European VHSV or North American type IVb assay, media pH should be 7.6-7.8 and 7.4-7.8, respectively, as indicated by some purple color in the media. See previous comments about North American strains of VHSV.

F. MEM-0-TRIS (for dilution tubes)

450 ml	MEM-TP
0.5 ml	gentamycin (50 mg/ml)
5 ml	Fungizone (250 µg/ml)
7.5 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

Do not add Tris or $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. Store at 4°C.

G. MEM-10 CHSE (complete cell culture medium for closed systems)

450 ml	MEM-TP
50 ml	fetal bovine serum (FBS) or bovine calf serum (BCS)
5 ml	L-glutamine (200 mM)
5 ml	100mM sodium pyruvate
0.5 ml	100x NEAA (non-essential amino acids)
5-8 ml	7.5% $NaHCO_3$

Do not add $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. For all MEM-based media, proper pH is 7.2-7.6, which results in an orange-red color. Store at 4°C.

H. MEM-10 CHSE Tris (complete cell culture medium for open or closed systems)

450 ml	MEM-TP
50 ml	fetal bovine serum (FBS) or bovine calf serum (BCS)
5 ml	L-glutamine (200 mM)
5 ml	100mM sodium pyruvate
0.5 ml	100x NEAA (non-essential amino acids)
0.5 ml	gentamycin (50 mg/ml)
5 ml	Fungizone (250 µg/ml)
7.5 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

Do not add Tris or $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. For all MEM-based media, proper pH is 7.2-7.6, which results in an orange-red color. Store at 4°C.

I. Herring Medium (MEM-10 without tryptose phosphate)

450 ml	MEM without tryptose phosphate added
50 ml	fetal bovine serum (FBS) or bovine calf serum (BCS)
5 ml	L-glutamine (200 mM)
5-8 ml	7.5% NaH CO_3

Store at 4°C. Use for PHE cells.

J. 2x Methylcellulose

15 g	methylcellulose
985 ml	tissue culture-grade water

Place ingredients in flask. Add stir bar. Tape lid on loosely and autoclave. Allow to cool. Place on magnetic stirrer to dissolve (4-24 hours). Aseptically dispense 250 ml into each of four clean, sterile 500-ml bottles. Label bottles 2x Methylcellulose and store at 4°C.

K. Methylcellulose Overlay (used for plaque assay)

250 ml	2x MEM-TP
250 ml	2x Methylcellulose
25 ml	FBS or BCS
1.5 ml	gentamycin (50 mg/ml)
7.5 ml	Fungizone (250 µg/ml)
7.5 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

Do not add Tris or $NaHCO_3$ until all other components have been mixed together so the proper pH can be determined. Label bottles Methylcellulose Overlay and store at 4°C.

L. Standard final antibiotic concentrations for MEM-10

0.5 ml	gentamycin (50 mg/ml) in 500 ml of MEM-10	= 50 µg/ml
5.0 ml	Fungizone (250 µg/ml) in 500 ml of MEM-10	= 2.5 µg/ml
2.5 ml	mycostatin (10000 IU/ml) in 500 ml of MEM-10	= 50 IU/ml
5.0 ml	penicillin (10000 IU/ml) in 500 ml of MEM-10	= 100 IU/ml
5.0 ml	streptomycin (10000 µg/ml) in 500 ml of MEM-10	= 100 µg/ml

M. Antibiotic cocktail preparation

Basic recipe including all antibiotics in cocktail

20 ml	Fungizone (250 µg/ml)	= 20 µg/ml with sample
5 ml	gentamycin (50 mg/ml)	= 1020 µg/ml with sample
10 ml	pen/strep (10,000 IU/ml & 10,000 µg/ml)	= 408 IU or µg/ml with sample

Dispense 0.25 ml into snap cap tube. Add 1.5 ml sample.
Store by dispensing 15 ml into each of 8 sterile tubes (each tube for a 60 fish sample at 0.25 ml antibiotic cocktail/sample).

Make the cocktails in large batches mixing the antibiotics and antifungals before pipetting out the aliquots, rather than individually pipetting each antibiotic into each tube (sterile).

Because the Fungizone is a saturated solution you must continually mix the cocktail mixture while dispensing. Freeze in upright position and store at -20°C.

N. Versene-PBS or EDTA (ethylene-diamine-tetra-acetic acid)-PBS

0.2 g	Na versenate (EDTA)
8.0 g	NaCl
0.2 g	KCl
1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4
1 L	tissue culture-grade water

Combine ingredients in flask and stir to dissolve. Dispense 100-ml quantities into clean bottles and autoclave. Label bottles Versene-PBS and store opened bottles at 4°C and unopened bottles at room temperature.

O. Trypsin-EDTA or TV

1. Trypsin-EDTA
   (10X), lyophilized
   0.5 g/L of Trypsin (1:250) and 8.5 g/L of NaCl

   Rehydration: Rehydrate with 20 ml of sterile, distilled water.

2. Earle's Balanced Salt Solution (EBSS) (1X), liquid
3. Prepare a (1X) solution using EBSS

Trypsin (1 part)		EBSS (9 parts)
20 ml	+	180 ml
or 40 ml	+	360 ml
or 60 ml	+	540 ml
or 120 ml	+	1,080 ml

Take six 20-ml bottles (Trypsin-EDTA) and rehydrate with 20 ml/bottle of sterile tissue culture-grade water. Aseptically mix the 120 ml trypsin-EDTA with 1,080 ml of EBSS. Aliquot into 250-ml bottles. Label T/V Working Solution and freeze at -20°C.

An alternative procedure is: make versene-PBS using method described in N above. Rehydrate 1 vial of 2.5% Trypsin with 20 ml of tissue culture-grade water. After cooling versene-PBS, aseptically add 4 ml Trypsin into each of 5 bottles of 100 ml sterile versene-PBS. Final Trypsin concentration is 0.1 %

P. 0.5% Crystal violet in 40% formalin

2.5 g	crystal violet
200 ml	formalin
300 ml	deionized water

Combine ingredients in 500 ml stock bottle (labeled 0.5% Crystal Violet in 40% Formalin) containing stir bar. This will require several hours of mixing to dissolve.

Q. 7.5% $NaHCO_3$

75 g	$NaHCO_3$
1 L	tissue culture grade water

Combine ingredients in a flask and stir to dissolve. Dispense 50-ml quantities into clean bottles and autoclave. Label bottles 7.5% $NaHCO_3$ and store at room temperature.

R. 1 M Tris buffer

106.4 g	Tris-HCl (MW 158)
39.4 g	Tris (MW 121.1)
1 L	tissue culture-grade water

correct pH should be 7.80-7.93

Combine ingredients in a flask and stir to dissolve. Dispense 50-ml quantities into clean bottles and autoclave. Label bottles 1 M Tris and store at room temperature; opened bottles may be stored at 4°C.

S. L-glutamine

L-glutamine is received lyophilized in 50-ml quantities. Rehydrate with sterile tissue culture-grade water. Aseptically pipet 5 ml into each of 10 sterile 5-ml test tubes with snap caps. Label tubes and freeze at -20°C in vertical position until use.

T. Polyethylene Glycol (PEG 20,000 MW.)

Polyethylene Glycol (PEG)

7% W/V of MW 20,000
7 g/100 ml of MEM-10 with 3x antibiotic
12 plate assay needs 2.19 g/31.25 ml
6 plate assay needs 1.095 g/15.6 ml
4 plate assay needs 0.732 g/10.4 ml
2 plate assay needs 0.365 g/5.2 ml
1 plate assay needs 0.183 g/2.6 ml

Prepare a 7% solution by dissolving 7 g PEG in 100 ml MEM-10 (3x antibiotic). Because the PEG isn't autoclaved, mixing with the MEM should be done just prior to use.

U. Fetal bovine serum (FBS)

Fetal bovine serum is generally received frozen in 500-ml quantities. Keep frozen until needed and at that time, thaw and aseptically dispense 50 ml FBS into each of 10 clean, sterile 100-ml bottles. Label bottles with FBS, date dispensed, lot number and re-freeze at -20°C.

V. Bovine calf serum (BCS)

To reduce costs, bovine calf serum can be used for propagating EPC and CHSE-214 cells and in overlay media. It should not be used when preparing monolayers in plates. Prepare for storage as for FBS. It is generally more deficient in nutrients for cell growth than FBS and may result in cells lifting off flasks over a period of time. If cells are lifting off or unhealthy switch to FBS. EPC cells on BCS are equally susceptible to IHNV as when on FBS.

NOTE: When purchasing a lot # of serum it used to be necessary to request a small 100 ml sample of the same lot # from the vendor for pre-testing of potential toxicity to <u>all</u> cell lines. Today this practice is not necessary because the characterized and defined FBS (Hyclone brand) have shown no cell toxicity regardless of lot #.

XXIII. Appendix

$TCID_{50}$ levels of Pacific cod Type IVa VHSV in 3 fish cell lines 8 days post-inoculation at 14°C.

Fish cell line	Virus titer ($TCID_{50}$/ml)
EPC	6.39×10^7
CHSE-214	1.61×10^6
BF-2	3.76×10^7

EPC and BF-2 cells were most susceptible of the 3 lines tested. CHSE-214 cells do not detect all isolates of North American Type IVa VHSV.

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XXV. Glossary

BCS - Bovine calf serum taken from newborn calves.

Blindpassage - Transfer of supernatant and inoculated tissue culture cells which are not demonstrating CPE to another plate containing fresh cells in order to dilute out possible inhibitors of viral expression and/or allow possible early viral replication, due to low concentrations of virus particles, to progress to detectable CPE.

Closed System - A system of incubating cells which is sealed against the transfer of air, i.e., a flask.

Confluent Monolayer (100%) - A single layer of tissue culture cells in which the cells have filled in all the spaces between them.

Controls

A. Monolayer control: tissue culture cells are grown in presence of growth medium MEM-10-TRIS. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

B. Sham control: diluent (MEM-0-TRIS) used for suspension of samples or dilution blanks is added to cells. After adsorption, an overlay medium or MEM-10-TRIS is added. If CPE appears in sham control wells, test is invalidated and must be repeated. The MEM or methylcellulose overlay should first be checked for contamination.

Cytopathic Effects (CPE) - Changes in the morphology and metabolism of tissue culture cells due to suspected viral infection.

Defective Interfering Particles (D.I. Particles) - Defective or incomplete virus particles which cannot replicate but may prevent expression of the infectious virus by attaching to the tissue culture cell receptor sites thereby blocking infectious particles. This can be a problem at low dilutions of tissue or ovarian fluid, particularly with the North American strain of VHSV Type IVa.

FBS - Fetal bovine serum taken in utero from unborn calves.

Monoclonal Antibody (MAb) - Antibody produced by tissue culture cell lines derived from the spleen lymphocytes of immunized mice that have been fused (hybridoma) with mouse myeloma tumor cells. Hybridoma cells are cloned to select specific populations of cells, each producing a single antibody against one epitope or antigenic determinant site on one antigen molecule among those used to immunize the mice.

Open System - A system of incubating tissue culture cells which is open to the transfer of air, i.e., a plate. Requires a closed plastic bag or container or a medium which is buffered against rising pH from air exchange.

Overlay - A medium used in the plaque assay that is placed over a virus-inoculated cell monolayer to prevent physical spreading of viral particles except by cell-to-cell release of

infectious particles. The overlay contains a semisolid ingredient such as methylcellulose or gum tragacanth.

PCR - Polymerase Chain Reaction

Plaque - A hole or focus of degenerate or dead tissue culture cells in the cell monolayer caused by viral replication. One discrete plaque is assumed to be caused by infection with one infectious particle or aggregate (called one plaque-forming unit = pfu).

Polyclonal Antibody - The entire population of antibodies produced in the sera of immunized animals that are directed against many epitopes on many of the antigenic molecules used for immunization. Most immunogens injected are whole cells or viruses that are composed of many different antigen molecules. Each antigen molecule may have more than one epitope. See "Monoclonal Antibody".

Serum neutralization - Antibody molecules in the antiserum neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the homologous virion (antigen). This prevents virion attachment to and subsequent penetration of host tissue culture cells or virus replication once inside the cell. Neutralization of viruses by antibodies is specific and used to confirm viral identity. Neutralization may be reversible.

Subculture - Transfer of inoculated tissue culture cells and supernatant from one plate to another which contains fresh cells. Used for suspected positive cultures to confirm presence of viral CPE as opposed to toxicity or contamination. Also used to replicate more virus for storage, etc.

$TCID_{50}$. Denotes fifty percent tissue culture infective dose. This is the reciprocal of the highest dilution of virus which causes CPE in 50% of the wells inoculated with that dilution of infectious materials. This is determined by the Reed and Muench (1938) method.

Tissue Culture-Grade Water - High-quality water (low in ions, minerals and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells. In the Anchorage lab water is obtained using a Millipore Milli-Q system. City water is pre-filtered, goes through a reverse osmosis filter, a carbon filter and 2 deionizing filters. In the Juneau lab city water is pre-filtered and double glass distilled in a Barnstead F1 system.

Titer - The number of infectious units or plaque-forming units per unit of sample, i.e., per g or ml.

Toxicity - Changes in cell morphology or metabolism caused by toxic substances in the medium or inoculum. This can either cause cell death or interfere with cell metabolism, thereby reducing or preventing replication of the virus. These effects may have arisen through sample toxicity, bacterial or fungal contamination, improper glassware cleaning or improper media preparation. Usually toxicity can be distinguished from viral CPE by how rapidly it occurs (1 day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

NOTE: Inoculation of very high-titered suspensions of certain viruses can cause an apparent toxic effect within 24 hours. If there is any doubt to whether disruption of the cell layer was caused by toxicity or CPE, a subculture should be made. This is especially true for some inocula which can produce toxic effects that may take 5-7 days for development.

Trituration - The act of dispersing tissue culture cells for transfer by repeatedly drawing the cell suspension into a pipet and expelling it back into the flask. This should be done until the cells are in groups of no more than three when examined with an inverted light microscope.

Trypsin - A proteolytic enzyme used to disaggregate cells and cause their release from the culture surface. It is neutralized by serum proteins and its action slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - Ethylene di-amine tetra acetic acid is a chelating agent which binds divalent cations active in forming cell cement (hyaluronic acid) causing cells to round and release from the culture surface.

CHAPTER 6

Histology for Finfish and Shellfish

Sally Short and Theodore R. Meyers

I. Preparation of Tissue

Preparation is different depending on species and will be addressed in the following sub-headings:

- Bivalve molluscs (oysters, clams, scallops, mussels) - adult, spat, and larvae
- Crustacea
- Finfish - adult and juvenile

A. Bivalve molluscs

NOTE: Only live or moribund bivalves will be suitable for processing. Tissues in dead bivalves autolyze very quickly and will mask antemortem changes. Do not collect and process dead bivalve molluscs. Keep molluscs alive in containers of seawater or live wells if they must be transported to the processing site. Do not over-ice animals such that tissues freeze while in transit. Frozen tissues are unacceptable for histological examination. Also, do not allow melt water to contact marine shellfish because freshwater will cause mortality and tissue artifact. Animals are anesthetized first by placing at 4°C for 35-40 min.

1. Soft tissues should be preserved in Bouin's fixative. The volume of fixative should be ten times the volume of the tissue. This is important since less fixative may result in tissue autolysis and unacceptable samples. Replace Bouin's fixative with alcohol to prevent tissues from becoming too hard and brittle when in this fixative for long periods.
2. The sample size for a disease history per site and species is 30 bivalves, live or moribund.
3. Bivalves less than 6 cm in length (shucked) can be fixed whole by dropping into preservative. Animals must be shucked cleanly from the shell by severing adductor muscles (Figs. 1 and 2, pgs. 6-20 and 6-21) prior to fixation. It is imperative that no shell fragments remain attached to soft tissues. Discard the shell unless there is a shell deformity or otherwise abnormal valve. In such a case, the shell should be included with the donor animal by wrapping both in gauze.
4. For good fixation, larger bivalves require 3 incisions (anterior, mid, posterior) made across the surface of the animal about mid-way through the tissues. Do not cut completely through the animal so that individual specimens remain intact and tissues do not become mixed.
5. Tissue and shell abnormalities must be noted on a sample submission form (Pg. 1-7) and the particular animal identified (bag in gauze and label, if necessary). The submission form will also contain the label information discussed below and must accompany the samples in a separate Ziploc® bag.
6. If samples are collected in the field for shipment to the FPL a label with bivalve species, size range and life stage, date of sample, location of sample, and contact person's name, address, and telephone number must be placed within each of the

sample jars. Use a pencil with soft lead for labeling so that the writing remains legible.

7. Do not mix samples of different species within the same jar of fixative. Each species must be placed in a separate sample jar(s). If samples are being shipped use plastic jars or containers with adequate packing material in approved Haz-mat packaging. Be sure all lids on jars or containers are tight and do not leak. Alternatively, after 48 hr of fixation animals or tissues may be placed into Ziploc® bags with paper towels soaked with tap water to avoid shipping hazardous fixative. Once at the FPL, samples should be placed into 70% alcohol.
8. After fixation bivalve tissues are firm enough to section for embedment. Each animal body is cut through the anterior, middle and posterior areas (Figs. 1 and 2, pgs. 6-20 and 6-21) resulting in 4 separate pieces of tissue. One section, about 2 mm in thickness, is shaved with a razor blade from each of the faces of the tissues representing the 3 major body areas. The sections are placed within 1-3 tissue cassettes depending upon their size for dehydration and embedment. See Chapter 10 on labeling histology samples. Small bivalves can either be embedded whole or cut longitudinally on the median axis and both tissue halves placed face down within a cassette.

   Spat - Process according to size. Those large enough to shuck are fixed as above and embedded whole usually 2-4 animals per cassette. The smaller spat are fixed in Bouin's fixative for 2-3 days. They do not need to go into a decal solution since the acidic Bouin's fixative achieves the same decalcifying effect. Otherwise, soaking in decal solution will be necessary.

   Larvae - Fix in a test tube of Bouin's fixative, then centrifuge @ 1,500 rpm for 10 minutes. Discard supernatant and embed the larvae in an agar plug. Remove the plug from the test tube for dehydration and embedment in wax in the usual manner (trim if necessary).

B. Crustacea

1. King Crabs, Dungeness crabs, Tanner crabs (see Johnson 1980 for figure illustrations).

   NOTE: Only live or moribund crabs will be suitable for processing. Tissues in dead crabs autolyze very quickly and will mask antemortem changes. Do not collect and process dead crabs. Keep crabs alive in containers of seawater or live wells if they must be transported to the processing site. Do not over-ice animals such that tissues freeze while in transit. Frozen tissues are unacceptable for histological examination. Also, do not allow meltwater to contact marine shellfish because freshwater will cause mortality and tissue artifact. Animals are anesthetized first by placing at 4°C for 35-40 min.

   a. Soft tissues should be preserved in Bouin’s fixative. The volume of fixative should be ten times the volume of the tissue. This is important since less fixative may result in tissue autolysis and unacceptable samples. Replace Bouin’s fixative with 70% alcohol to prevent tissue from becoming too hard and brittle when in this fixative for long periods.
   b. The sample size for a disease history per site or species is 30 crabs, live or moribund.

c. Prior to tissue collection, a hemolymph smear should be prepared from each live crab. Insert a 1-cc syringe with a 20-gauge needle into the articular membrane of any walking leg. The third joint of either cheliped works best. Express a large drop of hemolymph from the syringe onto one end of a clean, frosted-end glass slide and use another slide to make the smear as described in chapter 3 (fig. 1) on page 3-6. Allow to air dry, label the frosted end with an assigned crab number, date and store in a small slide box with the samples below. An alternative to the syringe would be to snip off the dactyl (tip) of a walking leg and allow not more than 1-2 drops of hemolymph to fall onto the slide.

   Do not let saltwater mix with the blood on the slide, as it will cause blood cell lysis. Be sure to allow slides to completely dry to prevent decomposition and growth of mold.

   NOTE: King crab blood clots very fast, so make the smear quickly.

d. The chitinous exoskeleton of large crustacea prevents adequate penetration of any fixative by simple immersion. Consequently, major organs and tissues of crabs must be dissected and dropped into fixative. This procedure is described by the following:
   (1) The carapace over the visceral cavity of the crab must be removed using tin snips, bone snips, or otherwise heavy duty serrated scissors.
   (2) Once the carapace is removed, the pigmented epidermis may come off with it or remain overlying the viscera. Snip a small 5-mm portion of the epidermis overlying the heart and save for fixation, then proceed to uncover the visceral cavity. Also, fix a 1-cm-square piece of the carapace.
   (3) Once the cavity is exposed, the heart, cardiac stomach, hepatopancreas, gonads (posterior to heart in Alaskan crab species), and gills become obvious.
   (4) Remove the right rear gill arch and take a 0.5-cm portion thereof.
   (5) Remove a 0.5-cm portion of the heart, which will be beating if the animal has been freshly killed.
   (6) Remove a 0.5-cm square of hepatopancreas to the left of the heart.
   (7) Remove both antennal glands (green glands). Each lies on either side against the frontal carapace of the crab and is surrounded by urinary bladder and hepatopancreas. This can be a difficult organ to find and should be retrieved early on before other tissues are disturbed and landmarks are lost.
   (8) Remove the entire GI tract starting with the esophagus, which is ventral and anterior to the cardiac stomach continuing with the entire stomach and intestine ending with the rectum that terminates at the vent on the ventral surface of the abdominal apron or flap underneath the crab. The intestine is long, curling down posterior to the heart and extending anteriorly into the abdominal flap. It is fragile and requires some digging with forceps and cutting away from hepatopancreas with scissors to free the specimen. Remove 0.5-cm portions of the esophagus, cardiac stomach, pyloric stomach, midgut, hindgut, and rectum.
   (9) Remove a 0.5-cm section of the gonads, also located posterior to the heart on either side. Gonads are part of the tissues that obstruct the extraction of the intestine.

Ovaries are large diameter, tubular organs that can be white, yellow, blue, or dark brown in color, depending upon the crab species.

Testes and vasa deferentia are thin, very white, twisted threads containing viscous gametogenic material. Remove a 0.5-cm portion of testes dorsal to hepatopancreas and lengths of anterior, mid, and posterior vasa deferentia.

(10) Expose the thoracic ganglion, which lies beneath the heart on the floor of the body cavity, by removing the residual hepatopancreas. Remove a 0.5-cm portion of the thoracic ganglion. The correct organ has been obtained if severance of the radiating peripheral nerves causes violent twitching of the respective walking leg of the crab in a freshly killed animal.

(11) In female Dungeness crabs, the paired seminal receptacles will be located below and on either side of the thoracic ganglion. Remove the right organ for fixation.

(12) Remove both eyestalks and the cerebral ganglion (brain) appearing as a white, pea-sized organ located at the juncture of the eyestalks. This all can be removed as one piece by snipping out with a pair of scissors.

Y-organ and mandibular gland. These are undefined in king crabs, Dungeness, and Tanner crabs - their locations are unknown!

(13) All tissues removed from a single crab should be placed into tissue-processing cassettes, 4-5 tissue samples to one cassette. (See next page for cassette designations for the different crab species.) Each cassette must be labeled with the accession and animal number from which the tissues were collected. Cassettes are then placed within large sample jars containing Bouin's fixative.

(14) Behavioral, external, and internal abnormalities must be noted on a sample submission form (Pg. 1-7) and the respective animal cassettes noted for identification. Sample submission forms will also contain the label information below and must accompany the samples in a separate Ziploc® bag. Be sure to include tissue from a lesion if one is observed--this includes shell lesions as well.

(15) If samples are collected in the field for shipment to the FPL a label with crab species, size range and life stage, date of sample, sample location, and contact person's name, address, and telephone number must be placed within each of the sample jars. Use a pencil with soft lead for labeling so that the writing remains legible. For shipment use plastic jars or containers with leak-proof lids in approved Haz-mat packaging. Alternatively, after 48 hr of fixation animals or tissues may be placed into Ziploc® bags with paper towels soaked with tap water to avoid shipping hazardous fixative. Once at the FPL, samples should be placed into 70% alcohol.

(16) Do not mix samples of different crab species within the same jar of fixative. Each species requires a separate jar(s).

Cassette and block label designation for king crabs

A = heart, gill
B = hepatopancreas, brain, epidermis
C = antennal gland, thoracic ganglion
D = pyloric stomach (decalcify)
E = cardiac stomach (decalcify)
F = gut (esophagus, midgut, hindgut, rectum)
G = eyestalks (decalcify)
H = ovaries or testes, vas deferens, sponge(eggs) (chelate with EDTA)
I = urinary bladder, muscle (usually leg)

Removal of the carapace from a king crab (*Paralithodes sp. Lithodes sp.*) is done with a scalpel blade eased under the back edge and pointed up underneath the carapace. Then using a pair of bone scissors, a large flap can be cut away, ending forward at the eyestalks, which are removed and fixed.

Removal of the carapace for adult Tanner crabs (*Chionoecetes bairdi* and *C. opilio*) is the same as for king crabs but the adult body of a Tanner is smaller and therefore more tissues fit into fewer cassettes.

Cassette and block label designation for Tanner crabs:

A = gill, heart, hepatopancreas, epidermis, urinary bladder
B = testes or ovaries, vas deferens, thoracic ganglion, antennal gland, sponge (chelate with EDTA)
C = pyloric stomach (decalcify)
D = cardiac stomach (decalcify), gut, leg muscle
E = eyestalks, brain (decalcify)

The carapace of an adult Dungeness crab *(Cancer magister)* requires use of a pair of bone scissors. An additional organ is the paired seminal receptacle in female crabs as indicated earlier.

Cassette and block label designation for Dungeness crabs:

A = gill, heart, seminal receptacles
B = hepatopancreas, brain, epidermis
C = antennal gland, thoracic ganglion
D = pyloric stomach (decalcify)
E = cardiac stomach (decalcify)
F = gut
G = eyestalks (decalcify)
H = gonads
ovaries and sponge - separate into EDTA for one week
testes and vas deferens
I = leg muscle, urinary bladder

Cassette and block label designation for juvenile Dungeness crabs:

A = heart, gill, hepatopancreas, epidermis, urinary bladder
B = antennal gland, thoracic ganglia, testes/vas deferens
C = pyloric stomach, gut (midgut, hindgut, rectum, and esophagus) - (decalcify)
D = cardiac stomach (decalcify)
E = eyestalks and brain (decalcify)

F = ovaries, sponge (chelate with EDTA), seminal receptacles

2. Shrimp (See Bell and Lightner 1988 for figure illustrations)

NOTE: Only live or moribund shrimp will be suitable for processing. Tissues in dead shrimp autolyze very quickly and will mask antemortem changes. Do not collect and process dead shrimp. Keep shrimp alive in containers of seawater or live wells if they must be transported to the processing site. Do not over-ice animals such that tissues freeze while in transit. Frozen tissues are unacceptable for histological examination. Also, do not allow meltwater to contact marine shellfish because freshwater will cause mortality and tissue artifact. Animals are anesthetized first by placing at 4°C for 35-40 min.

a. Soft tissues should be preserved in Bouin's fixative. The volume of fixative should be ten times the volume of the tissue. This is important since less fixative may result in tissue autolysis and unacceptable samples. Replace Bouin's fixative with alcohol to prevent tissue from becoming too hard and brittle when stored in this fixative for long periods.
b. The sample size for a disease history per site or species is 30 shrimp, live or moribund.
c. The chitinous exoskeleton of shrimp prevents adequate penetration of any fixative by simple immersion. Consequently, the fixative must be injected into strategic internal areas of each animal prior to dropping the whole shrimp into the fixative. Inject fixative into the shrimp using a 10-ml syringe and appropriately sized needle, depending upon the size of the animal (small shrimp; i.e., small-gauge needle). This procedure is described by the following:

(1) First inject laterally into the hepatopancreas; i.e., cephalothorax region.
(2) Then inject dorsally into the region anterior to the hepatopancreas; i.e., between the thorax and the eyestalks.
(3) Inject the posterior abdominal region.
(4) Inject the anterior abdominal region. Inject more of the fixative into the hepatopancreas than the other sites but overall use about 5%-10% of the shrimp's body weight.
(5) Immediately after injection, slit the cuticle of the animal from the last (6th) abdominal segment to the base of the rostrum. The incision in the cephalothoracic region should be just lateral to the dorsal midline and that in the abdominal region should be mid-lateral. Do not cut too deeply into the underlying tissue. The objective is to break the cuticle to allow fixative penetration.
(6) SHRIMP LARGER THAN 12 GRAMS
Same as above, but a transverse cut should be made at the abdomen/cephalothorax junction and again midway across the abdominal area.

d. After injection and body incisions, the animal may be dropped into the fixative.
e. External abnormalities and unusual behavior must be noted on the sample submission form (pg. 1-7), and the specimen jar numbered for identification.
f. If samples are collected in the field for shipment to the FPL a label with shrimp species, size range and life stage, date of sample, location of sample, and contact person's name, address, and telephone number must be placed within

each of the sample jars. Use a pencil with soft lead for labeling so that the writing remains legible.

g. Do not mix samples of different shrimp species within the same jar of fixative. Each species requires a separate jar(s). Transfer shrimp to 70% ethyl alcohol after 48 hrs.

h. Place sample jars and a separate Ziploc® bag containing the sample submission form data into a suitable Haz-mat shipping package with adequate packing material to prevent breakage. Plastic jars or containers for fixative and samples should be used . Be sure lids are tight and do not leak.
Alternatively, after 48 hr of fixation animals or tissues may be placed into Ziploc® bags with paper towels soaked with water to avoid shipping hazardous fixative. Once at the FPL, samples should be placed into 70% alcohol.

i. Preparation of Tissues for Cassettes (see Bell and Lightner 1988 for figure illustrations)
   (1) Remove preserved shrimp from 70% ethyl alcohol and place on a cutting surface.
   (2) Using a single-edge razor blade or scalpel, bisect shrimp transversely (for shrimp greater in length than 3.0 cm) at the junction of the cephalothorax and the abdomen (Fig. 5a; Bell and Lightner 1988).
   (3) Longitudinally bisect the cephalothorax just lateral of the midline (Fig. 5b; Bell and Lightner 1988), or if possible, the whole specimen for shrimp less that 3.0 cm in length (Fig. 5c; Bell and Lightner 1988).
   (4) From the half of the cephalothorax without the mid-line, remove, with a diagonal cut starting at the distal surface, the branchiostegal region containing the gills (Fig. 5d; Bell and Lightner 1988).
   (5) Remove the distal 80% of head appendages if these are not to be studied or if the appendages would get in the way during embedding (Fig. 5e; Bell and Lightner 1988).
   (6) Utilizing a razor blade, separate abdominal segments #1, 3 and 6 from the remainder of the abdomen and remove distal ends of the uropods (Fig. 5f; Bell and Lightner 1988).
   (7) Longitudinally bisect the 6th abdominal segment, as in the manner of the cephalothorax (Fig. 5g; Bell and Lightner 1988).
   (8) Depending on the size of the shrimp, the available tissue blocks and the size of the anticipated embedding mold, place either all or any number of the following tissue blocks into histological embedding cassettes (Fig. 5h; Bell and Lightner 1988).

      - half of complete shrimp with mid-line, cut-side-down (less than 3 cm).
      - half of cephalothorax with mid-line, cut-side-down.
      - branchiostegal region, cut-side-up.
      - other half of complete shrimp, or cephalothoracic region from this half, (without the mid-line), cut-side-up (less than 3 cm).
      - other half of the cephalothorax (without the mid-line) placed with the cut-side-up.
      - transverse block(s) of abdominal segments #1 and/or #3.
      - longitudinal block of 6th abdominal segment (with mid-line) placed cut-side down.

   (9) Tissue blocks should not exceed ¼" in thickness.

C. Finfish - salmonids and marine species

NOTE: Only live or moribund fish will be suitable for processing. Tissues in dead fish autolyze very quickly and will mask antemortem changes. Do not collect and process dead fish. Keep fish alive as long as possible during transport to the site of necropsy. Do not over-ice fish such that tissues freeze while in transit. Frozen tissues are unacceptable for histological examination. Animals are euthanized in a solution of MS-222.

a. Tissues should be preserved in Bouin's fixative or 10% buffered formalin. The volume of fixative should be ten times the volume of tissue. This is important since less fixative may result in tissue autolysis and unacceptable samples. After 48 hours, the Bouin's or 10% buffered formalin fixative may be poured off and replaced with 70% ethyl alcohol for transport and storage to prevent tissues from becoming too hard and brittle when in acid fixatives for long periods. Also, the fixative poured off may be saved and strained of tissue fragments and used several more times for other samples if necessary.
b. Fish less than 3 cm may be fixed whole by dropping into preservative.

   NOTE: Important! Remove egg yolk from sac fry before fixation. (Do it **now** - it doesn't work later!)

c. Fish 4 cm-10 cm should have the abdomen opened with a scalpel or scissors, the intestine detached at the vent, and the internal organs pulled out slightly for proper fixative penetration.
d. Larger fish (11 cm-20 cm) will require on-site excision of 0.5-cm sections of major tissues and internal organs (Fig. 3, pg. 6-22) as listed. Do not send whole fish.
e. Excise the head (from just behind the opercular opening) and 0.5-cm samples of liver, air bladder, head and mesonephric kidney, spleen, GI tract (esophagus, stomach, pyloric cecae, anterior and posterior intestine with attached adipose tissue and pancreas), heart and gonads. Also, take a 0.5-cm square of musculature and attached skin intersected by the lateral line midway between the head and tail on the right side of the fish. Take a second 0.5-cm section of muscle and skin from the body wall covering the viscera from the right side of the fish.
f. Organs and tissue samples from a single fish should be placed in tissue-processing cassettes, 4 to 5 tissue samples to one cassette. Label each cassette with the accession and animal number from which it was taken. Place cassettes and the fish head in a jar of fixative. A head may be numbered with a tag through the jaw.
g. Fish larger than 20 cm also require that 0.5-cm portions of each major organ be utilized (if larger than 0.5 cm) and the whole head will be eliminated from the sample unless a lesion is present or brain is to be examined. In this case the head may be cut in half longitudinally to include any lesion and tagged for identification. If the head is not kept, the first right gill arch is excised and fixed before discarding the head. Organs and tissues from a single fish are placed in tissue-processing cassettes as above or otherwise numbered.
h. External and internal abnormalities must be noted on the sample submission form in Chapter 1 (pg. 1-7), and the particular fish sample identified. Be sure and include tissues from a lesion area if there is one observed. The sample submission form will also contain the label information below and must accompany the samples in a separate Ziploc® bag.

i. If samples are collected in the field for shipment to the FPL a label with fish species, size range and life stage, date of sample, location of sample, and contact person's name, address, and telephone number must be placed within each of the sample jars. Use a pencil with soft lead for labeling so that the writing remains legible.

j. Do not mix samples of different fish species within the same jar of fixative. Each species requires a separate sample jar(s).

k. If shipping collected material, place sample jars containing fixative and tissues and the Ziploc® bag containing sample submission data into a suitable Haz-mat shipping package with adequate packing material to prevent breakage. Plastic jars or containers for fixative and samples should be used. Be sure lids are on tight and do not leak. Alternatively, after 48 hr of fixation animals or tissues may be placed into Ziploc® bags with paper towels soaked with tap water to avoid shipping hazardous fixative. Once at the FPL, samples should be placed into 70% alcohol.

l. Special Procedures - for fingerlings, fry, and sac fry:

Decalcify fingerlings and fry - Chelate sac fry with EDTA to soften any residual yolk sac.

Cassette and block label designation for finfish

Adults:

A = gill arch (decalcify), thyroid - thyroid follicle present beneath gill arches
B = liver, gallbladder, spleen
C = heart, thymus (excised from beneath opercula where they join the head)
D = kidney, head kidney, air bladder
E = gut (esophagus, anterior intestine, posterior intestine, rectum), pyloric caeca, pancreas
F = stomach (cardiac and pyloric)
G = brain, eye
H = gonads

Juvenile:

A = halved head (thymus, thyroid, heart, eye, brain, gill, head kidney)
B = liver, gallbladder, spleen, kidney, gonads, air bladder
C = stomach, pyloric caeca, intestine, rectum, pancreas

NOTE: Proper sectioning of the brain and eye in smaller fish will require that the head be halved longitudinally after fixation using a very sharp razor blade. Both halves are laid face down in the cassette for embedment after decalcification.

II. Fixation and Decalcification

A. Shellfish

All shellfish are fixed in Bouin's fixative for 48 hours. Spat are fixed in Bouin's for 2-3 days.

B. Finfish

Finfish adults, juveniles, fingerlings, fry and sac fry are all fixed in Bouin's (buffered formalin may be used as a substitute fixative) that is usually prepared in 10-L quantities. Under usual circumstances soft tissues should be removed from Bouin's after 24-48 hr to reduce brittleness from acidity of the fixative. For larger whole fry it is advantageous to leave in the fixative for up to 2-3 days since the acidity will decalcify bones, allowing for whole sectioning.

NOTE: Small whole swimup fry do not need to be decalcified and become too brittle unless removed after 4-8 hours of fixation in Bouin's. The removal of picric acid from the tissues cannot be overemphasized. Rinse tissues in running tap water for 1 hr prior to transfer to alcohol. One or two changes of 50% alcohol may be necessary prior to storage of samples in 70% alcohol.

Bouin's Fixative (10 L)

dH_2O	7.07 L
Formalin (37-40%) (CH_2O)	2.37 L
Glacial acetic acid (CH_3COOH)	476 ml
Picric acid (2,4,6-$(NO_2)_3C_6H_2OH$)	71.0 g

Dissolve picric acid (see comments on picric acid in Safety, Chapter 16, pg. 16-15) in water, then add other ingredients and leave on magnetic stirrer overnight.
DO NOT HEAT! Complete Bouin's fixative solution is also available commercially.

10% Buffered Formalin

	1 L	20 L
37-40% formalin (CH_2O)	100 ml	2 L
Sodium phosphate (monobasic) $NaH_2PO_4H_2O$	4 g	80 g
Sodium phosphate (dibasic)(Na_2HPO_4)	6 g	120 g
dH_2O	900 ml	18 L

Decalcification Solution (Additional decalcification procedures for Whirling Disease are in Chapter 11, pg. 11-4.)

Solution A =	Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	50.0 g
	dH_2O	250 ml
Solution B =	Formic acid 88% (HCOOH)	125 ml
	dH_2O	125 ml

① Mix A & B in equal portions for use - (decalcify tissues for 8-12 hours) (oyster spat for two days).
② Wash in running tap water for 2-4 hours.
③ Place tissues in tissue processor for usual cycle.
Alternately, place in a commercial decalcifying reagent (VWR) for 24 hours. Rinse in running water 3-4 hours and process as usual.

C. Fixative for fish and crab eggs (A. K. Hauck, unpublished)

① Use 5% DMSO (Dimethyl sulfoxide $\{CH_2\}_2SO$) in 10% neutral formalin.
② When eggs (fish) are hard - open the membrane with a needle and tease the yolk out.
③ Process routinely, or try chelation if DMSO fixation procedures are not sufficient.

Chelation (used routinely for crab ovaries)

① Fix as usual (without DMSO).
② Place ovaries/alevins in 5.5 g EDTA and 100 ml 70% alcohol for 1 week.

NOTE: the EDTA disodium salt does not dissolve = saturated solution.

III. Tissue Dehydration and Infiltration (all tissues)

A. After the tissues are in cassettes, preserved with fixative and moved into 70% alcohol, they are dehydrated in the tissue processor. The 12 baths in the processor should already contain the following solutions:

#1 70% alcohol
#2 85% alcohol
#3 95% alcohol
#4 95% alcohol
#5 95% alcohol
#6 100% alcohol
#7 100% alcohol
#8 100% alcohol
#9 xylene substitute or xylene
#10 xylene substitute or xylene
#11 melted paraffin
#12 melted paraffin

These solutions will evaporate over time and should be topped up as needed. The temperature for the paraffin baths should be set at 58-60°C. Depending on the paraffin product, exceeding a temperature of 62°C may result in polymerization. This will produce hard blocks resulting in difficult or impossible sectioning. For quality control, maintain a log attached to the tissue processor that documents when reagents were changed and temperature validation of the paraffin by thermometer.

B. Place the cassettes into the processor basket and attach the basket to the processor. Line the basket up so that it will begin the cycle in the 70% alcohol bath #1.

C. Program the dehydration cycle by setting the clock timer for 24 hrs at 12 two hr intervals. The processor will now advance the basket through the 12 baths at the two hour intervals when the cycle is activated. NOTE: Before starting the processor, think about the timing. The cycle will end in 24 hours. Make sure that you will have sufficient time to embed the tissues immediately following the completion of the cycle. An example would be to start the cycle at 1 PM on Monday for a completion time of 1 PM on Tuesday. This would leave enough time Tuesday morning to allow melting of the paraffin in the embedder and to embed two baskets of cassettes in the afternoon. Do not allow the tissues to remain in melted paraffin any longer than necessary. Excessive time in melted paraffin can cause tissues to become brittle and difficult to cut.

IV. Embedding Tissues into Paraffin Blocks

A. Turn on the paraffin bath in the embedder several hours prior to the time that the tissue processor cycle will end. The internal timer may also be set to automatically turn the embedder on to allow the paraffin time to completely melt.

B. Shortly before the scheduled embedding, turn on the cold plate so that it becomes well chilled.

C. When the tissue processor cycle ends, remove the basket from the final paraffin bath and pour the cassettes into the melted paraffin bath of the embedder.

D. Now you are ready to begin embedding.

1. Dispense enough paraffin into an embedding block mold to just cover the bottom. Place the mold on the hot plate of the embedder.
2. Remove a cassette from the paraffin bath of the embedder and place it on the hot plate.
3. Open the cassette and discard the lid. Using forceps, transfer the tissue sample(s) from the cassette to the mold.
4. Place the mold on the cold plate. Using rounded forceps, gently press each tissue piece to the bottom of the mold. This must be done quickly. Allow the paraffin to set up just enough to hold the tissues in place. DO NOT ALLOW THE PARAFFIN TO COMPLETELY HARDEN.
5. Quickly move the mold back to the hot plate and place the cassette bottom onto the mold like a cap.
6. Fill the mold with melted paraffin from the dispenser of the embedder.
7. Return the mold to the cold plate to cool.

E. Once the block has completely solidified, it may be popped out of the mold and stored on the cold plate or in the freezer until you are ready to cut.

V. Cutting Paraffin Blocks and Mounting Sections on Glass Slides

A. Preparation of materials

1. About an hour prior to cutting blocks, turn on the cold plate of the embedder and the water bath (start with fresh water each day). The water bath should be set at 45°C-48°C.
2. Sprinkle gelatin crystals (using a salt shaker) over the surface of the water bath. The gelatin is used to adhere the tissue section to the slide and helps provide a wrinkle-free bond between the tissue and the slide. If a heated stain is to be used, do not use gelatin. Instead use slides previously dipped in 5% Elmer's® glue solution.

   Recipe for Elmer's® glue dipped slides (for use with heated stains):
   a. Make 5% glue solution with distilled water (from commercial bottle of Elmer's® glue).
   b. Heat in microwave.
   c. Allow air bubbles to disperse.
   d. Dip slides and allow to air dry.

3. Store glass slides from the box in 100% alcohol and wipe clean as needed. Using <u>clean</u> glass slides can not be overemphasized.

4. Place blocks to be cut on the cold plate.

5. Check the blade in the microtome and replace if nicked or scratched. Use disposable blades, they are <u>always</u> sharp and clean. Because disposable blades

are at a fixed angle, there is little tissue loss when refacing a block if recutting is required.

B. Cutting sections

1. Clamp a block securely into the microtome chuck and begin cutting. Ideal sections will be between 2-6 microns thick.
2. Once you are able to cut a flat ribbon of whole sections, transfer the ribbon to the water bath, taking care to gently stretch out the wrinkles as the ribbon makes contact with the warm water surface.
3. Tissue's fixed in Bouin's tend to fragment during cutting (due to the picric acid). If you are having difficulty obtaining good sections, try placing a gauze pad that has been soaked in 2% ammonia on the face of the block for a few minutes. Then recut. If fragmentation or compression still occurs, place the block and the ammonia soaked gauze in a sealed bag and leave overnight. Recut.
4. Sections that “pile up” on the edge of the knife or otherwise do not flatten out may indicate that the block has warmed and is too soft to cut. Chill the block for a few hours or overnight in the freezer and recut.
5. Separate the desired sections from the ribbon by gently pulling the ribbon apart using two small, fine bristled paint brushes. Stubborn sections may be separated with a dissecting needle dipped in xylene substitute or a tool heated in an open flame which will “cut “ the sections apart.

C. Mounting sections

1. Submerge a clean glass slide into the water bath under the desired sections. Gently pull the slide out of the water at an angle holding the sections in place on the slide with the paint brush.
2. Lean the slide upright to drip dry for a few moments. Heat fix the slide by placing it on the slide warming area of the water bath (or any slide warming tray) for a second or two. Caution! You do not wish to melt the paraffin in the section, just warm the slide.
3. Label the slide with the accession number, block number, slide number, date and your initials using special labels printed from the computer.
4. Place the slides in a staining rack. Typically, sections for three slides are cut from each block. Two of these slides are stored in a rack for standard H&E staining at a later date while the third slide is stored in a second rack and will not be stained with the other two. Instead, this third slide is used as a back-up should there be a staining problem or need for a special stain.
5. All the slides now in racks should be dried overnight at 40-50°C prior to staining. This step helps to prevent wash-offs during the staining process. If the paraffin in the sections melts, the temperature is too high and tissue artifact will occur.

VI. Routine Staining of Paraffin Sections - Hematoxylin and Eosin

A. Hematoxylin Solution (Harris Formula)
Purchase already prepared; it is inexpensive and gives reproducible results (500 ml is less than $10). It contains no mercury and is available in both acidified and unacidified formulations.

Eosin Y

1% stock solution

Eosin Y, water soluble	1 g
dH2O	20 ml

Dissolve and add:

Alcohol 95%	80 ml

Working Solution

Eosin stock solution	1 part
Alcohol 80%	4 parts

Just prior to use add 0.5 ml of glacial acetic acid to each 100 ml of Hematoxylin and the working Eosin solution.

B. General H & E staining
The basic procedure includes getting rid of the paraffin in the sections (deparaffinization) and rehydration of the tissue so that the H & E stains may be used. This is followed by dehydration again so that the stained section may be mounted in a permanent medium under a glass coverslip.

Standard H&E schedule

#1 Xylene - 10 minutes or xylene substitute- 15 minutes (at least). Change the xylene substitute after several racks (check slides).
#2 Xylene - 10 minutes or xylene substitute- 15 minutes (at least)
#3 100% alcohol - 1 minute
#4 100% alcohol - 1 minute
#5 95% alcohol - 1 minute
#6 95% alcohol - 1 minute
#7 tap H_2O - 10 minutes
#8 Hematoxylin - 10 minutes (varies with section thickness and animal species)
#9 tap H_2O - 4 dips
#10 Acid alcohol - 3 - 10 dips
#11 Tap H_2O - 4 dips
#12 Ammonia water - 3 - 5 dips
#13 Tap H_2O - 20 minutes
#14 Eosin - 2 seconds to 2 minutes (varies with section thickness and animal species)
#15 95% alcohol - 2 minutes
#16 95% alcohol - 2 minutes
#17 100% alcohol - 3 minutes
#18 100% alcohol - 3 minutes
#19 Xylene or xylene substitute - 2 minutes
#20 Xylene or xylene substitute - 2 minutes

Staining times will vary with thickness of sections, age of stain, and animal species. Thinner sections will require increased staining times. This staining schedule is based on sections of fish tissues 2-3 μ thick.

VII. Special Staining of Paraffin Sections

A. Giemsa Stain

1. Solutions/stains

Stock Giemsa

Giemsa Powder	1.0 gm
Glycerin	66 ml
Absolute methyl alcohol	66 ml

Mix glycerin and Giemsa powder. Place in 60°C oven for 2.5-3 hours, stirring often. Cool mixture and add methyl alcohol. Store stock Giemsa in refrigerator.

Working Giemsa

Stock Giemsa	14 ml
D H_2O	180 ml

Make fresh each use. Mix together into staining container.

Stock Jenner's

Jenner's Dye	1.0 ml
Absolute methyl alcohol	400 ml

Mix all of Jenner's dye and all of methanol in beaker and place on an automatic stir plate. Stir until dye is dissolved in alcohol. Store stock Jenner's in refrigerator.

Working Jenner's

Stock Jenner's	100 ml
D H_2O	100 ml

Make working solution fresh weekly. Mix together into staining container.

1% Acetic Acid

Acetic Acid	2 ml
D H_2O	198 ml

2. Giemsa Staining Procedure

#1 Xylene - 10 minutes or xylene substitute - 15 minutes (at least). Change the xylene substitute after several racks (check slides).
#2 Xylene - 10 minutes or xylene substitute - 15 minutes (at least)
#3 100% alcohol - 1 minute
#4 100% alcohol - 1 minute
#5 95% alcohol - 1 minute
#6 95% alcohol - 1 minute
#7 tap H_2O - 10 minutes
#8 Methyl alcohol - 3 minutes

#9 Methyl alcohol - 3 minutes
#10 Working Jenner's - 6 minutes
#11 Working Giemsa - overnight
#12 D H_2O - 10 dips
#13 1% acetic acid to differentiate - 10 dips
#14 D H_2O - 10 dips
#15 95% alcohol – quick dip to rinse
#16 95% alcohol – quick dip to rinse
#17 100% alcohol - 1 minute
#18 100% alcohol - 1 minute
#19 Xylene or xylene substitute - 3 minutes (at least)
#20 Xylene or xylene substitute - 3 minutes (at least)
#21 Xylene or xylene substitute - 3 minutes (at least)
#22 Mount

B. Grocott's Methenamine Silver (GMS) Stain for fungus

1. Solutions/stains

<u>4% Chromic acid</u>

Chromic acid	4.0 gm
D H_2O	100.0 ml

<u>1% Sodium bisulfite solution</u>

Sodium bisulfite	1.0 gm
D H_2O	100.0 ml

<u>Methenamine silver nitrate stock solution</u>

5% silver nitrate	5.0 ml
3% methenamine	100.0 ml

<u>5% Silver nitrate</u>

Silver nitrate	0.25 gm
D H_2O	5.0 ml

<u>3% Methenamine</u>

Methenamine	3.0 gm
D H_2O	100.0 ml

A white precipitate can form but immediately dissolves on shaking. Clear solution remains usable for months. Store in refrigerator.

<u>Methenamine silver nitrate working solution</u>

Methenamine silver nitrate stock	25.0 ml
D H_2O	25.0 ml
5% borax	2.0 ml

Make this solution fresh.

<u>5% Borax</u>

Borax	5.0 gm
D H_2O	100.0 ml

0.1% Gold chloride solution

Gold chloride 1% solution	10.0 ml
D H_2O	90.0 ml

This solution may be used repeatedly.

2% Sodium thiosulfate solution

Sodium thiosulfate	2.0 gm
D H_2O	100.0 ml

0.2% Light green solution stock

Light green, SF yellowish	0.2 gm
D H_2O	100.0 ml
Glacial acitic acid	0.2 ml

Light green solution working

Light green stock	10.0 ml
D H_2O	50.0 ml

2. Grocott's Staining Procedure

#1 Deparaffinize and hydrate to distilled water
#2 Oxidize in 4% chromic acid solution for 1 hour
#3 Wash in tap water for a few seconds
#4 Sodium bisulfite solution for 1 minute to remove any residual chromic acid
#5 Wash in running water for 5 -10 minutes
#6 Rinse with 3 or 4 changes of distilled water
#7 Place in freshly mixed working methenamine silver nitrate solution in oven at 58 to 60°C for 60 minutes or until section turns yellowish brown
#8 Rinse in 6 changes of distilled water
#9 Tone in gold chloride solution for 2 – 5 minutes
#10 Rinse in distilled water
#11 Remove unreduced silver with sodium thiosulfate solution for 2 – 5 minutes
#12 Wash thoroughly in tap water
#13 Counterstain with working light green solution for 30 – 45 seconds
#14 Dehydrate in 95% aocohol, 100% alcohol and clear in xylene, 2 changes each
#15 Mount

C. Brown and Brenn Gram Stain

1. Solutions/stains

1% Crystal violet solution

Crystal violet	1.0 gm
D H_2O	100.0 ml

5% Sodium bicarbonate solution

Sodium bicarbonate	5.0 gm
D H_2O	100.0 ml

Gram's Iodine solution

Iodine	1.0 gm
Potassium iodide	2.0 gm
D H_2O	300.0 ml

Ethyl ether – acetone solution

Ethyl ether	50.0 ml
Acetone	50.0 ml

0.25% Basic fuchsin solution stock

Basic fuchsin	0.25 gm
D H_2O	100.0 ml

Basic fuchsin solution working

Basic fuchsin solution stock	10.0 ml
D H_2O	100.0 ml

Picric acid – acetone solution

Picric acid	0.1 gm
Acetone	100.0 ml

Acetone – xylene solution

Acetone	50.0 ml
Xylene	50.0 ml

2. Brown and Brenn Staining Procedure

#1 Deparaffinize and hydrate to distilled water
#2 Place slides on a staining rack. Pour on approximately 1 ml (20 drops) of crystal violet solution and add 5 drops of sodium bicarbonate solution and stain for 1 minute.
#3 Rinse with tap water
#4 Flood slides with Gram's iodine solution for 1 minute
#5 Rinse in tap water and blot with filter paper to complete dryness
#6 Decolorize with ethyl ether-acetone solution dropped on slides until no more color runs off
#7 Working basic fuchsin solution for 1 minute. Wash in water. Blot gently but do not allow sections to dry completely
#8 Dip in acetone to start differentiation reaction
#9 Differentiate immediately with picric acid-acetone solution until sections are yellowish pink
#10 Rinse quickly in acetone and then in acetone-xylene solution
#11 Clear in xylene, several changes
#12 Mount

VIII. References

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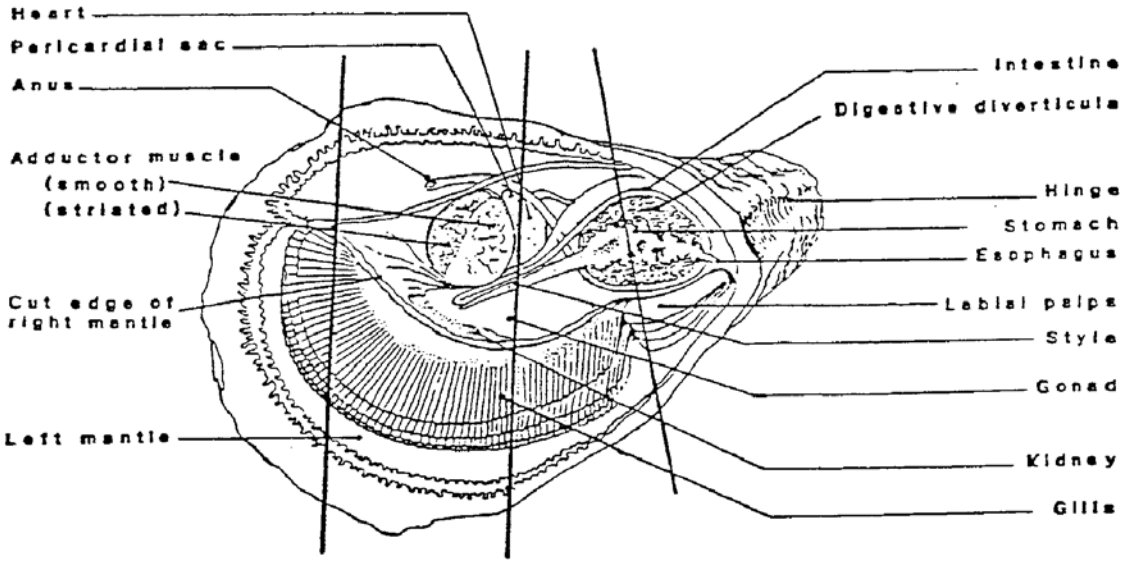
Figure 1. Oyster anatomy and cross section (adapted from Howard, Lewis, Keller and Smith, 2004). Tissue sections are taken in areas of the bold lines.



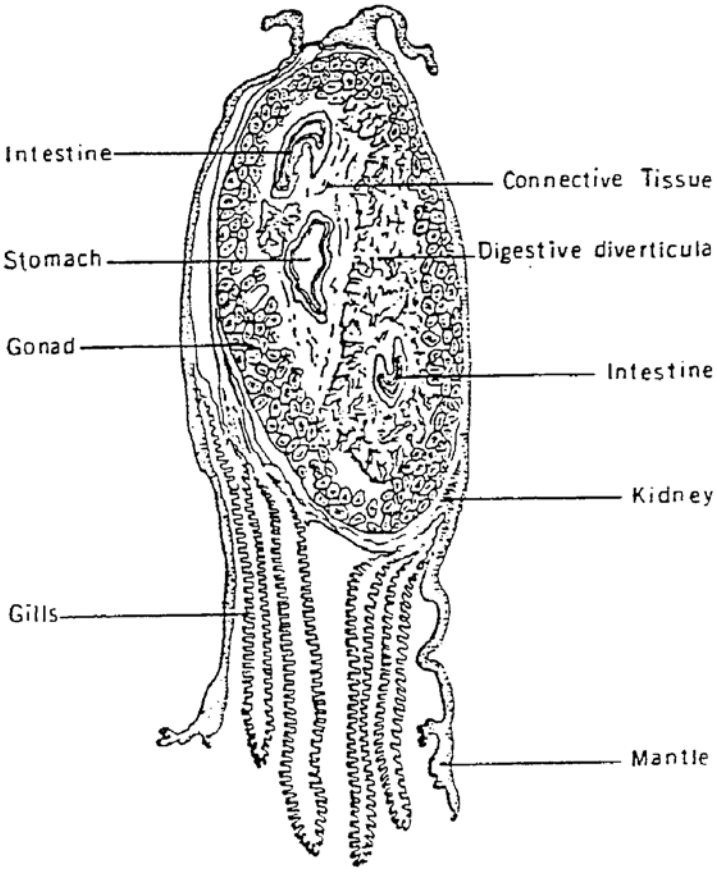
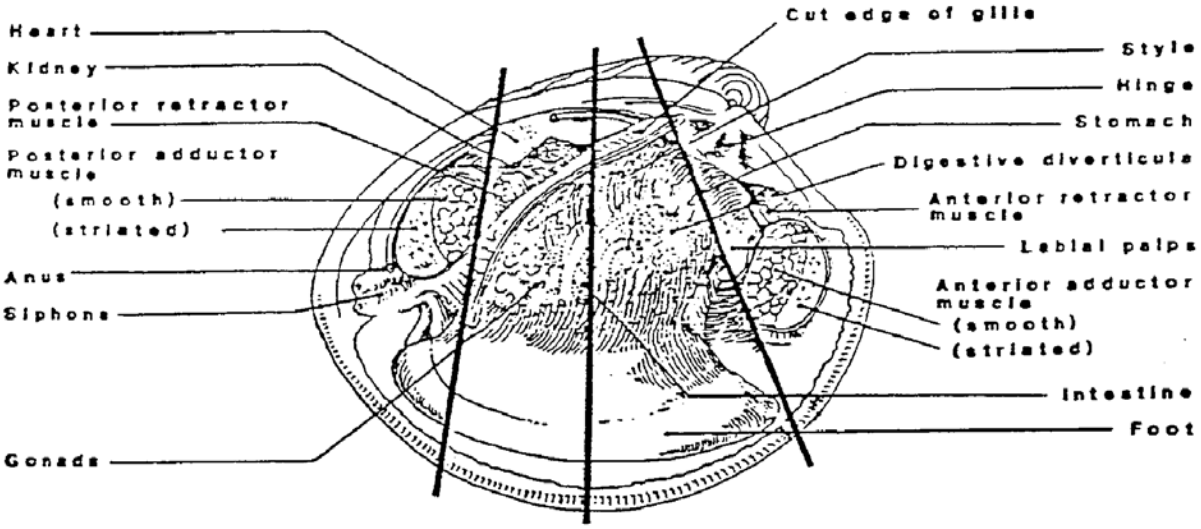


Figure 2. Clam anatomy and cross section (adapted from Howard, Lewis, Keller and Smith, 2004). Tissue sections are taken in areas of the bold lines.



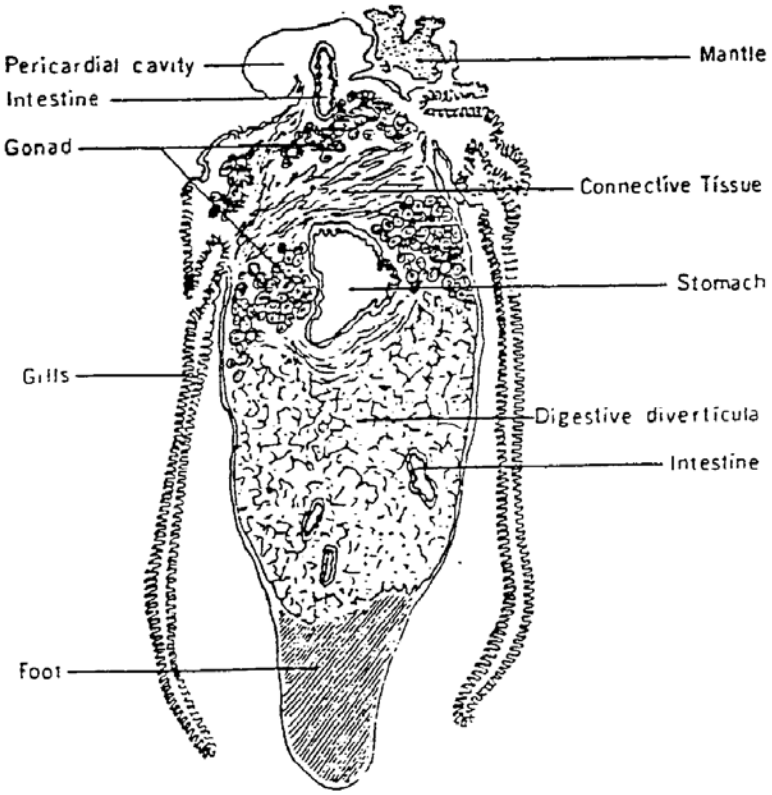
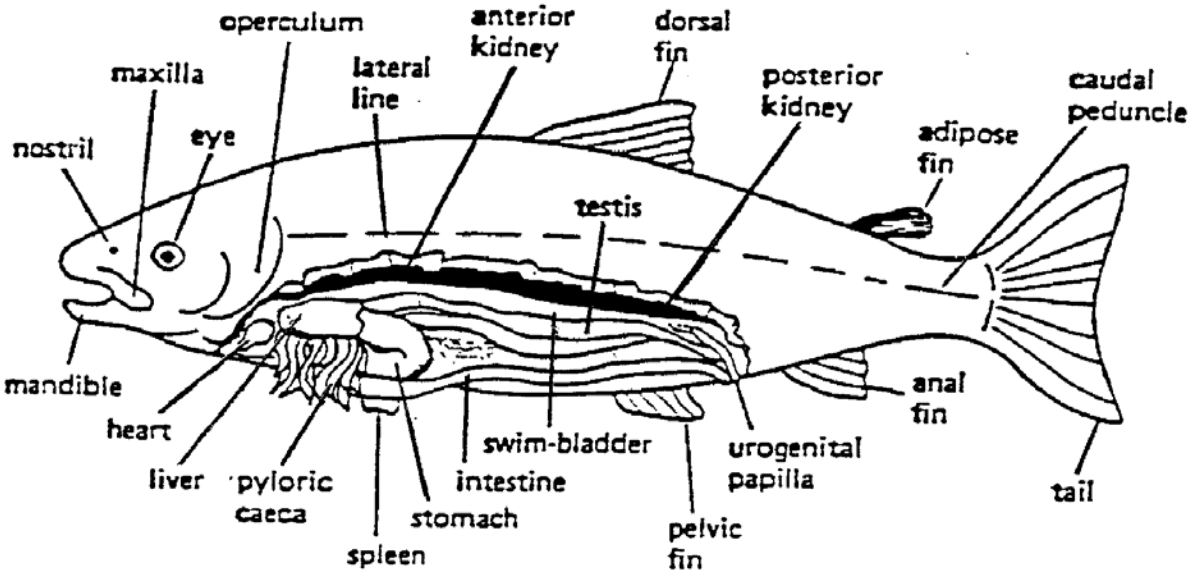


Figure 3. Salmonid anatomy (adapted from Roberts and Shepherd, 1974)



CHAPTER 7

Transmission Electron Microscopy: Fixation and Sample Staining Procedures

Theodore R. Meyers

I. Fixation and Embedment of Tissues from Vertebrates

A. Cut a piece of tissue no larger than 1-mm-square and fix within 4% glutaraldehyde in 0.1-M cacodylate buffer overnight at 4° C.

B. Rinse once in 0.1-M cacodylate buffer and postfix in 1% osmium tetroxide in 0.1-M cacodylate buffer for 1-2 hrs at 4° C.

C. Rinse 2 times again in cacodylate buffer before transferring to 70% ethanol for 5-10 min at room temperature (RT). All the osmium must be rinsed off or it will form a precipitate with any organic solvent such as alcohol. This is a good point at which to stop if tissue needs to be stored rather than be processed further. Store in 70% ethanol indefinitely at 4° C.

D. To continue, rinse in 95% ethanol for 5-10 min at room temperature (RT).

E. Rinse in 100% ethanol for 10 min at RT.

F. Rinse a second time in 100% ethanol for 10 min.

G. Infiltrate with 1:1 100% ethanol and complete Spurr's resin (Spurr 1969) for 1-2 hrs at RT on a rotary mixer. This may also be done overnight if more convenient.

H. Infiltrate with complete Spurr's resin on a rotary mixer for 2-6 hrs depending upon density of tissue, i.e., pelleted cells may be infiltrated in 2 hrs or less.

I. Place tissue in a Beem capsule and orient into the tip of the mold with a probe. Add fresh complete resin and a strip of white paper with the penciled case number.

J. Place the Beem capsule containing the tissue in a rack at 70° C for 8-16 hrs cure time depending upon mixture recipe of the resin (Table 1).

K. Once cured, cut the outer Beem capsule away from the block with a razor blade. The specimen is now ready for rough trimming of the block or subsequent file storage.

NOTE: Tissue culture cells, protozoa or other particulates will have to be centrifuged prior to each reagent change in order that fluid may be poured off without losing the material of interest. An alternative would be embedment of fixed cells (postfix in osmium and rinse with buffer beforehand) in an agar plug which could then be transferred to reagents as if a standard piece of tissue.

II. Fixation and Embedment of Tissues from Marine Invertebrates

NOTE: Same as with vertebrates except that the glutaraldehyde is diluted with seawater or Millonig's buffer to maintain proper osmolality.

III. Retrieval and Embedment of Cut Sections from Histological Slides (modified from Van den Bergh Weerman and Dingemans 1984; Yau et al. 1985)

Circumstances may require going back to tissues on histologically prepared glass slides if no fresh or preserved samples are available or if the subject of interest is so infrequent as to make random thin sectioning of embedded tissues impractical.

A. Select a stained histological slide containing the area of interest (i.e., lesion, cellular or tissue inclusion bodies, organism, etc.). Etch a circle or square around the target area on the back of the glass slide, i.e., permanent marking ink will dissolve when soaked in xylene below.

B. Place slide in a Coplin jar with xylene to remove the coverslip, usually takes 2 hrs to 2-3 days.

C. This step is optional since any added electron density from osmication of previously paraffin-embedded tissues is often marginal. Under a hood, place the slide into xylene containing 1% osmium tetroxide and postfix for 1-2 hrs. This eliminates the additional rehydration steps in other protocols for postfixation in aqueous buffered osmium and prevents further tissue artifact. The xylene will turn black due to the osmium. Do not reuse.

D. Trim excess section material away from the target zone using a scalpel. Rinse the slide in 3 changes of 100% ethanol, 5 minutes each.

E. Place the slide in a petri dish and flood surface with a 1:1 mixture of Spurr's embedding resin and 100% ethanol for 1 hr at RT.

F. Pour off resin mixture and replace with complete resin for 1 hr at RT.

G. Pour off resin and replace with new resin and cure at 70^{o} C for 8-16 hrs depending upon resin recipe (Table 1). Do not add too much resin such that it flows from the slide into the petri dish. Resin overflow will cement the slide to the petri dish making further processing very difficult.

H. Examine the cured slide on a compound microscope to locate the etched area of interest and mark on the surface of the resin with a marking pencil or razor blade. The originally stained histologic detail will be unchanged.

I. Locate the marked area of interest on the slide using a dissecting microscope and begin cutting into the resin around the area with a razor blade or scalpel. The cut area of resin containing the area of interest can be gradually lifted from the surface of the slide by flooding distilled water between the slide and the resin-embedded section as the resin is teased from the surface with a small, narrow scalpel blade. The tissue will come off cleanly with the resin. If the area of interest is in the center of the section, large areas can be removed by the earlier trimming in part D to gain access to the desired target zone.

The chip of resin containing the tissue of interest should not be larger than 1 or 2 mm^2 and should be flat. A diagram of the tissue field in the chip is helpful in locating landmarks while ultrasectioning.

J. Place the tissue chip into a Beem capsule containing complete resin and orient the chip so that it lays flat, tissue-side-down, on the bottom at the capsule tip. Flat chip orientation is critical to allow easy location of the target zone when sectioned.

K. Cure at 70^o C for 8-16 hrs depending upon resin recipe (Table 1).

L. Care must be taken in rough trimming the block for thick and thin sectioning so as not to cut away the chip or into the tissue section which will be from 3-6 µm thick. The final trimming is better done by thick sectioning on the ultramicrotome. The original histologic staining of the tissue should allow adequate visibility for orientation.

M. Make serial thick sections, retrieving and staining every 5th to 10th section for microscopic examination. When the target zone is located, take several thin sections to ensure against section loss during staining or possible blocking of target zones by TEM grid wires.

IV. Negative Staining of Virus Particles

A. Tissue culture fluid from a large flask (75 cm^2) of cells exhibiting complete destruction of the monolayer is clarified of cell debris at low speed centrifugation, i.e., 6-7000 X g for 20 min. If much cell-associated virus is expected, the suspension should be sonicated for at least 20 sec prior to clarification.

B. The clarified supernatant is collected and spun in an ultracentrifuge to pellet the virus. A suggested reference point is 81,000 X g for 3 hrs in a Spinco SW 27 (Beckman) swinging bucket rotor.

NOTE: Fragile viruses may require a sucrose (50%) pillow to cushion the pellet and reduce damage to virus capsids.

C. Re-suspend the virus pellet with 0.5-1 ml of distilled water. Buffer will cause salt crystals to form on the dried final preparation, possibly obscuring or causing artifacts in virus structure. However, some viruses, such as herpesvirus, may also be sensitive to un-buffered preparations.

D. Prepare a 2% (w/v) solution of phosphotungstic acid (PTA) by adding 2 g of PTA to 100 ml distilled water. Raise the pH to 7.0 with 1N KOH.

E. Mix 0.02 ml of virus suspension with an equal volume of PTA on a glass slide.

F. Touch a formvar-coated copper grid (purchased or coated in the lab) to this virus/stain mixture and allow the resultant film to air dry on the grid surface. Doubling dilutions of the virus stain mixture may be necessary if the stain or virus concentration is too thick. An alternate method is to allow the film to dry 1-2 minutes and then draw off the remaining moisture with a dry filter paper.

G. After air drying, the grid is ready to be examined on the transmission electron microscope (TEM).

V. Staining Thick Sections for Light Microscopy

A. Two to three thick sections cut from a block are collected from the knife boat in a loop and placed onto a cleaned (with ethanol) glass slide.

B. Gently heat the slide over a flame or on a slide warmer before the water drop containing the sections can dry. The heat will cause the sections to flatten out, eliminating wrinkles and resultant artifact.

C. Air dry and heat fix the sections over a flame or slide warmer to provide better adherence to the slide.

D. Flood the sections with buffered toluidine/methylene blue stain and heat gently over an open flame or slide warmer for 2-3 minutes. Do not allow the stain to dry. Deplasticizing is not necessary with Spurr's resin but will reduce the stain time. Sections can be deplasticized for 10-30 seconds by flooding with a supersaturated solution of KOH in 95% ethanol and rinsing with 95% ethanol. Rinse with water and stain as above.

NOTE: Over-exposure to the KOH will destroy the tissue section.

E. Rinse the slide with tap water from a squeeze bottle and air dry for examination on the light microscope.

VI. Staining Thin Sections for TEM

A. Collect gold, silver or gray sections from the knife boat using a loop and transfer to a drop of water on a glass slide for gentle heating over an open flame to flatten the sections, as above.

B. Retrieve the sections from the water drop with the loop and transfer back to the knife boat and collect each section from the water surface on a cleaned, 300-mesh, slotted copper grid. Grids are pre-cleaned by swirling in a beaker of chloroform. The chloroform is poured off and the grids are allowed to dry. Cleaning in acetone causes discoloration of the grids when stained.

C. Allow the grid and section to air dry on filter paper in a covered plastic petri dish.

D. Grids are stained in 4% uranyl acetate for 90 min by transporting each grid with a pair of forceps section side down onto a drop of stain. Paraffin melted into the bottom of a covered petri dish works well as a surface for holding drops of stain.

E. Retrieve grids from the stain. Dip 5 times in a beaker of distilled water, then dip 5 more times in a second rinse of distilled water and draw off excess moisture with a tissue. Adequate rinsing is critical to remove excess stain that would otherwise precipitate on drying and obscure ultra-structural detail.

F. Allow grids to air dry in a covered petri dish lined with filter paper.

G. Grids are counterstained in drops of lead citrate for 3-5 min in a covered paraffin-lined petri dish as above, containing 2-3 pellets of NaOH. The NaOH absorbs atmospheric CO_2, preventing the precipitation of lead carbonate on the section.

H. After staining, grids are again rinsed in two changes of distilled water as above and air dried.

I. Store stained dry grids in a slotted grid box for later examination.

VII. Reagents

A. 0.1 M Cacodylate Buffer

Cacodylate Acid Sodium Salt MW = 214.02

0.1 M = 21.4 g/1 L dH_2O
= 2.14 g/100 ml dH_2O

Adjust to pH 7.4 with 0.1 N HCl

B. Millonig's Phosphate Buffer

Stock Solutions

Millonig's buffer (0.4 M)

Sodium phosphate (monobasic)	11.08 g
Sodium hydroxide	2.85 g
dH_2O	200 ml

The pH should be about 7.6 with osmolality of 420 mM

Salt solution (0.34 M)

Sodium chloride (MW = 58.45)	19.87 g/1 L dH_2O
or	3.97 g/200 ml dH_2O

C. Millonig's Buffered 2.5 % Glutaraldehyde (for marine invertebrates)

Solution A	25 ml
25% glutaraldehyde	5 ml
0.34 M Sodium chloride	20 ml

Solution B

0.4 M Phosphate buffer (Millonig's)	25 ml

Complete fixative

Mix 25 ml solution A with 25 ml Solution B

2.5% glutaraldehyde	275 mM
0.2 M Millonig's buffer pH 7.6	420 mM
0.14 M Sodium chloride	275 mM

D. Seawater Buffered 4% Glutaraldehyde (for marine invertebrates)

25% glutaraldehyde	10 ml
Filtered seawater (0.45 µ)	50 ml

E. Cacodylate Buffered 4% Glutaraldehyde (for vertebrates)

25% glutaraldehyde	10 ml
0.1 M cacodylate buffer	50 ml

F. Buffered Toluidine/Methylene Blue Stain (for thick sections)

Solution 1

Sodium borate	1 g
Methylene blue	1 g
dH_2O	100 ml

Solution 2

Toluidine blue	1 g
dH_2O	100 ml

Complete stain

Just before use mix equal parts of Solutions 1 and 2. Mix only what will be needed for a single staining session.

G. Buffered 1% Osmium Tetroxide (for postfixation)

Osmium tetroxide (crystalline)	0.5 g
0.1 M cacodylate buffer	50 ml

Osmium comes in a glass ampule with a scored top. Once the top is removed the ampule should be dropped into the buffer contained in a glass stoppered bottle. The osmium will dissolve from the ampule slowly. The bottle must be acid cleaned and organic contamination with solvents etc. should be avoided or the osmium will turn black and form a precipitate. Osmic acid is an extremely strong oxidant and is dangerous regarding skin exposure or inhalation. All handling should be done under a fume hood.

H. Lead Citrate Stain (for thin sections)

Lead nitrate	0.22 g
Sodium citrate	0.29 g
dH_2O	5 ml

Shake vigorously for 1 minute and intermittently for 1/2 hour to change the lead nitrate to lead citrate. Add 1.33 ml of carbonate-free 1N NaOH (fresh). Dilute with distilled water to 8.33 ml and mix by inversion, pH = 12.0. Solution should be clear. If turbidity exists, centrifuge and throw out precipitate. (Make sure that all glassware used is extremely clean, acid wash if possible. Use volumetric flask.)

Stain grids for 3-5 minutes. This stain is used after the uranyl acetate stain (below). Rinse the grids thoroughly in distilled water, drain water off and allow to air dry.

I. Uranyl Acetate Stain (for thin sections on grids)

Make up a 4% aqueous uranyl acetate solution. Dissolve 0.4 g/10 ml distilled water for 10-15 minutes until clear (sonicate for faster dissolution). For larger volumes of stain, a drop of glacial acetic acid added per 60 ml will preserve stain activity.

VIII. References

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research 26:31-43.

Van den Bergh Weerman, M. A., and Dingemans, K. P. 1984. Rapid deparaffinization for electron microscopy. Ultrastructural Pathology 7:55-57.

Yau, W. L., S. B. Or, and H. K. Ngai. 1985. A "free floating" technique for re-processing paraffin sections for electron microscopy. Medical Laboratory Science 42:26-29.

TABLE 1

SUGGESTED MODIFICATIONS OF SPURR'S RESIN (from manufacturer)

Ingredient (weight in grams)	A Firm	B Hard	C Soft	D Rapid Cure	E Longer Pot Life[a] Lower Viscosity[a]
VCD	10.0	10.0	10.0	10.0	10.0
D.E.R. 736	6.0	4.0	7.0	6.0	6.0
NSA	26.0	26.0	26.0	26.0	26.0
Dimethyl-aminoethanol (DMAE)	0.4	0.4	0.4	1.0	0.2
Cure schedule at 70°C (hr)[b]	8	8	8	3	16
Pot life[c] (days)	3-4	3-4	3-4	2	7

[a] As compared with standard medium A.

[b] Cure for minimum hours indicated or longer.

[c] Time between initial mixing and end point for convenient use at room temperature. Complete resin should be stored at -10°C which will extend the pot life for weeks.

NOTE: Do not add DMAE until ready to use. Mix the Spurr's resin in ½ weight quantities in two 50-ml screw-cap conical tubes. Freeze both tubes that are labeled "without DMAE". When needed, take one tube from the freezer, warm to room temperature and add 1/2 the amount of DMAE as indicated in Table 1. Label "DMAE added" and the date when re-freezing the leftover resin. Loss in viscosity of frozen resin compared to freshly made indicates polymerization in which case the resin should be discarded.

CHAPTER 8

Fluorescent Antibody Staining for Bacteria and Viruses

Sally Short, Craig Farrington and Theodore R. Meyers

(Filtration FAT Procedures from Dr. Diane Elliot,
Western Fisheries Research Center, Seattle)

I. Fluorescent Antibody Methods for Bacteria

The Fluorescent Antibody Test (FAT) is one serological method for testing fish tissues for Gram-negative bacteria including *Aeromonas salmonicida* (furunculosis), *Yersinia ruckeri* (enteric redmouth) and the Gram-positive bacterium *Renibacterium salmoninarum (Rs)*, the causative agent of bacterial kidney disease (BKD). FAT of tissue smears is a good qualitative test allowing for specific or presumptive (in the case of *A. salmonicida*) identification of bacterial agents but is poor in assessing the degree of infection or numbers of bacteria present in a kidney sample from any individual fish. The test may miss low-level positive fish in that at least 10^6 organisms must be present per gram of tissue to detect just one fluorescing organism (Bullock et al. 1980). Hence, conventional FAT was never intended as a survey tool for screening asymptomatic carriers of bacterial organisms. The following are the FAT procedures for tissue smears developed by the Commercial Fisheries pathology labs. In order of presentation there are four basic steps: collecting and preparing the kidney (or other tissue) sample; making kidney smears on multiple-well glass slides; staining of the slides with reagents; reading and interpreting the slides.

A. Sample Collection and Processing

Kidney samples for FAT most often are collected in the field as samples for Rs ELISA and then sent to the laboratories for processing (see ELISA chapter for more detail).

In the field, the anterior and posterior kidney are sampled and each should be the size of a thumbnail (about 1.5 cm each or 1-3 g total tissue). Both samples are placed into a single 2-oz. white label Whirl-Pak® bag (VWR 11216-772), appropriately labeled with hatchery and stock identification with consecutive enumeration of each bag. The recommended aseptic procedure is to wash instruments and surgically gloved hands in a 3% solution of iodophor between samples from each fish. The physical process of washing, as well as the disinfectant removes kidney tissue and/or bacteria. Careful entry into the fish is necessary to prevent or minimize the chances of cross contamination of samples. Sterile wooden tongue depressors or other disposable utensils used between samples are an alternative to instrument disinfection.

At the lab (or receiving facility), the bagged samples are checked for completeness of labeling (a case number is assigned) and then processed or frozen for later processing and/or shipment. Processing requires homogenizing the bagged kidney tissues into a

thick slurry using a Stomacher®. Once homogenized, the sample is ready for making a kidney smear.

B. Kidney Smears

The kidney smears are made on labeled multiple-well glass slides (Figure 1 Erie Scientific Cat. #10- 2255 -white). A drop of 0.45 µm filtered phosphate buffered saline from a sterile Pasteur pipet is added to each of 10 wells per slide for a total of six slides (60 wells). To each well a minuscule amount of the homogenized kidney is applied and stirred via a sterile applicator stick. A distinctly brown solution is too much and some of the solution should be drawn off with a sterile Pasteur pipet. The slides are air dried and heat-fixed @ 60° C so that the slide is mildly hot to the touch (not too hot). Next, the slides are fixed in anhydrous methanol in a Coplin jar for five minutes. The slides dry quickly when removed and are ready for staining. Methanol can be reused if it is first filtered through a 0.45-µm filter. Acetone is a second choice for fixative but more often causes smears to float off the wells. Smears that are too thick are also prone to float off during washing.

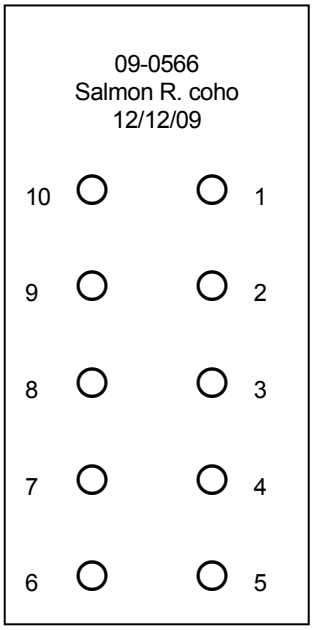


Fig. 1

C. Staining - There are two types of staining procedures; indirect (IFAT) and direct (DFAT).

1. IFAT requires two reagents: Antiserum #1 specific for the bacterial agent and Antiserum #2 (conjugate) specific for the antiserum #1. Antiserum #1 binds to the bacteria and the conjugate, containing fluorescein dye, binds to the molecules of antiserum #1 causing the bacteria to fluoresce. Chicken or rabbit-derived IgG antisera against a specific pathogen and a rabbit or goat anti-IgG conjugate at concentrations of 1:50 are commonly used. Rhodamine is added to the conjugate at a final dilution of 1/200 as an orange to brown counterstain to reduce background non-specific fluorescence. Alternatively, 0.01% Evan's blue in phosphate buffered saline (PBS) can be used to wash off the conjugate and then

allowed to incubate on the smear for 10 minutes to act as a counterstain. A third reagent, serum taken from a normal rabbit or chicken (not injected with the bacterial agent), is used for a negative control by using it in place of the primary antiserum on a known positive kidney smear. Phosphate buffered saline (PBS) can also be used in place of the primary antiserum as another negative control. Positive controls consist of a known positive kidney smear stained using both the primary antiserum and conjugate. The negative and positive controls are placed on a separate slide.

The stains are filtered, using a 0.45-µm syringe filter (VWR #28145-481) and dropped onto slide wells from a sterile Pasteur pipet as follows:

NOTE: Repeated filtering of the conjugate will eventually remove the rhodamine, thus requiring replenishment.

a. Drop normal serum and PBS on the negative control wells.
b. Drop antiserum #1 dilution on the unknowns and positive controls.
c. Wait five minutes.
d. Rinse slides in 0.45 µm filtered distilled water in Coplin jars for five minutes and shake dry.
e. Drop dilution of conjugate onto all wells.
f. Wait five minutes.
g. Rinse slides in 0.45 µm filtered distilled water in a Coplin jar for five minutes, rinse again in a second Coplin jar and shake dry.
h. Remove and coverslip with a minimum amount of FA mounting fluid (pH 8.6). Too much mounting fluid causes excessive elevation of the coverslip and difficulty in focusing the oil immersion lens.

NOTE: If using Evan's blue instead of rhodamine as a counterstain, rinse conjugate off with 0.01% Evan's blue in PBS and incubate smear in counterstain for 10 minutes. Rinse with distilled water and coverslip with mounting fluid as above.

Read the slides on a compound fluorescent microscope @ 1000x.

2. DFAT requires only one reagent because the antiserum #1 specific for the bacterial agent is also conjugated with fluorescein dye. Hence, the first incubation and rinse steps are eliminated (steps b-d) and the antiserum-conjugate in step (e) is not added to the negative control wells. Otherwise all other steps are the same as above. An alternative negative control would be the conjugate-antiserum added to a smear of a heterologous organism, i.e., Rs conjugate added to a smear of the furunculosis agent. However, the latter example must be tested beforehand such that some Rs conjugate will cross-react with *A. salmonicida*.

D. Steps Required for Good Results

1. Filtration of all reagents to reduce background debris that may fluoresce nonspecifically, causing difficulty in reading and interpretation.
2. Do not make smears too thick. Thick smears will not stain properly and will wash off most of the time.
3. Allow for even distribution of the PBS and kidney material in each well.

4. Be sure to heat-fix the slides prior to fixing in methanol. If there is not an adequate way available to heat-fix the slides they can be air dried and sent to the lab without fixation.
5. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination.
6. Use anhydrous methanol. A lesser grade methanol produces lower fluorescence intensity. Acetone often causes washoffs depending upon the sample.

E. Reading and Rating

Slides are read at 1000x on a compound fluorescent microscope. The positive and negative controls on the control slide are read first. This has two purposes: (1) to ascertain that the staining process has indeed worked (positive control should have myriad numbers of fluorescing bacteria, the negative control none); and (2) to inspect bacterial size, shape, and magnitude of the fluorescence of bacteria in the positive control. The reader then uses the positive control as a reference to read the unknowns and rates each sample using the following rating system developed by the Commercial Fisheries pathology staff.

F. Fluorescent Antibody Technique Rating Guide

Standard rating criteria for interpretation of the FAT on tissue smears based upon a minimum of 30 fields examined at 1000x.

Negative (-)

No organisms observed in thirty fields examined.

Plus/minus (±)

Organisms observed with questionable fluorescence or morphology not typical of the target organisms.

Total of one typical organism observed but suspected of not originating from the sample examined, i.e., wash over from a high-level positive sample.

One plus (1+)

One to 5 organisms observed. If only one organism is found, examination of up to 100 fields continues in an attempt to find a second organism that would confirm the 1+ status. If no other organism is detected the final +/- or 1+ interpretation is at the discretion of the individual reader.

Two plus (2+)

Six to 50 organisms observed in which some fields will be negative and some will typically contain several organisms.

Three plus (3+)

Fifty-one to 150 organisms observed with a typical field containing a dozen or more organisms.

Four plus (4+)

Greater than 150 organisms with no more than 200 organisms in an average field.

Clinical (C5+)

Greater than 200 organisms in an average field. Gross lesions are likely to be observed in the sampled kidneys from this category.

G. FAT Material Suppliers

VWR Scientific (Whirl-Pak® bags)
355 Treck Dr.
Seattle, WA 98188-7603
800-932-5000

Erie Scientific Company
20 Post Road,
Portsmouth, NH 03801-5649
(603) 431-8410

Kirkegaard and Perry Laboratories, Inc. (conjugates and Rs reagents)
910 Clopper Road
Gaithersburg, MD 20878
(800) 638-3167
www.KPL.com

Microtek Intl., Inc. (antisera)
6761 Kirkpatrick Crescent
Saanichton, British Columbia
Canada V8M 1Z8
www.microtek-intl.com

H. Reagents

Phosphate buffered saline, pH 7.2 (PBS)

NaCl (sodium chloride, MW 58.44)	7.20 g
Na_2HPO_4 (sodium phosphate, anhydrous dibasic, MW 141.96)	1.48 g
KH_2PO_4 (potassium phosphate, anhydrous, monobasic, MW 136.1)	0.43 g

Bring components to 1 L with distilled water. Adjust pH to 7.2 with 1 M NaOH or HCl.

Rhodamine

Rehydrate vial with 5 ml sterile distilled water
Dilute 1 part with 9 parts of PBS for 10% working stock solution
Add to conjugate for a final dilution of 1:200 (1 part into 20 parts conjugate)

Repeated filtration through a syringe filter (0.45 µ) will remove the rhodamine from the conjugate

Evan's blue (0.01%)

Dissolve 1 part of Evan's blue with 99 parts of PBS for 1% working stock solution
Add 1 part of stock to 100 parts PBS for 0.01% working solution

I. Determination of Antiserum and Conjugate Working Dilutions for FAT

In most cases commercially prepared antisera and conjugates are lyophilized in a concentrated state. Each should be reconstituted according to the manufacturer's instructions which usually include injection with needle and syringe of 1-5 ml of sterile distilled water through the rubber stopper of the vial. The concentrated stock solution can then be withdrawn into the syringe and expressed into a convenient sterile test tube and diluted 1:10 with PBS. Aliquots of 0.5 ml can be frozen for later dilution into a working solution of the reagent. Reagents are more likely to retain their potency longer if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum staining in terms of brightness of fluorescence with a dark background. Generally the working dilution is recommended by the manufacturer and usually is about 1:40 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases the proper working dilution must be established by each laboratory and the recommended dilution serves as a starting point.

Antisera and conjugates should be diluted using two tenfold dilutions on either side of the recommended working dilution. The following situation is an illustrative example. An indirect FAT is to be done using a new antiserum and conjugate. The recommended working dilution from the manufacturer is 1:40 for the antiserum and 1:50 for the conjugate.

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.
2. The conjugate should be diluted 1:30, i.e., the lowest of the two tenfold dilutions below that recommended.
3. The IFAT is performed on replicates of a known positive control, each replicate using a different dilution of the antiserum and the same conjugate dilution. In this way the endpoint or working dilution of the antiserum can be determined, i.e., the highest dilution that still provides a bright specific fluorescence with little or no background staining. Non-specific background fluorescence may be high at this lower conjugate dilution which can be corrected once the working dilution is established for the conjugate.
4. The working dilution of the antiserum is then used against varying dilutions of the conjugate starting at 1:30 to 1:70.
5. Once the working dilutions have been established for both antiserum and conjugate, the test should be run again using these working dilutions with both

positive and negative controls. When results are satisfactory add the rhodamine counterstain to the working dilution of the conjugate which should result in a bright apple green fluorescence of the organism with a light red to brown background due to the rhodamine. A bright yellow background is evidence of excessive nonspecific staining indicating that additional dilution of either the conjugate or primary antiserum is necessary.

6. Direct FAT obviously requires only the titration of the conjugate to determine the working dilution.

NOTE:

a. Monoclonal antibodies for FAT often must be used undiluted or at very low working dilutions as they are not concentrated when taken directly from cell culture.
b. Non-commercial antisera and conjugates will require a wider range of test dilutions to determine the best working dilutions. If tenfold dilutions do not produce an endpoint then doubling dilutions may be necessary (i.e., 1:40, 1:80, 1:160) depending upon the potency of the reagent.

II. Microwell Fluorescent Antibody Test for IHNV

A. Materials

1. cleaned FAT slides and coverslips (Erie Scientific)
2. disposable petri dishes
3. gauze swabs
4. airtight plastic container
5. filtered (0.45 μm) distilled water
6. MEM-10-3X (3x antibiotic)
7. EPC cells
8. MAb mouse monoclonal anti-IHNV antiserum (or other suitable antiserum)
9. Goat anti-mouse IgG FITC conjugate
10. known and unknown virus isolates

B. Setup

1. Work is done in a tissue-culture hood.
2. Clean FAT slides in 70% alcohol and wipe with gauze swabs.
3. Place the clean slides in the sterile petri dishes (chambers), 1 slide per dish. Cut 2 strips from a gauze swab and lay in the dish alongside the slide. (Soak the gauze with distilled water for humidity.)
4. Seed each of the necessary wells with EPC cells in MEM-10-3X of sufficient density to monolayer overnight (1 drop per well from 5-ml pipet). Put all chambers into an airtight container for incubation @ 21^{o}C overnight.

C. Sample Inoculation and Incubation

1. When cells are ready, the medium is dumped from each slide into a waste beaker containing bleach.
2. Add a tissue-culture isolate of suspected IHNV to cells, 1 drop per well. Replicate slides are prepared, one for each incubation period; - 8, 12, 24 and 48 hours. The suspect IHNV isolate should be taken from a culture having 3-4+ CPE.
3. Replicate controls are prepared at the same time, on different slides, for identical treatment and incubation periods. Place experimental and control slides each in separate chambers @ 15^{o}C for the prescribed times. Controls should include: a known IHNV isolate stained with and without the primary antiserum (these could

be on the same slide) followed by the conjugate; uninfected cells stained with all reagents.

For a single tested isolate there would be: 4 slides of cells infected with unknown virus, each in a single chamber for the 4 incubation periods; 4 slides of cells infected with known IHNV in another chamber; and 4 slides of uninfected cells in a third chamber. Hence, there would be 3 slides (unknown, known, uninfected) removed for staining at each incubation interval. The optimum sample will be the incubation interval just prior to early CPE.

D. Fixation
   1. At scheduled incubation periods, an experimental and the 2 control slides are washed for 5 minutes in a Coplin jar containing cold PBS.
   2. Slides are fixed in methanol for 10 minutes.
   3. Slides can be stored @ 4^{o}C until ready to stain.

E. Staining
   1. Undiluted MAb is added to appropriate wells (except negative IHNV control) and allowed to incubate for 5 minutes.
   2. Rinse slides in 0.45 µm filtered distilled water for 5 minutes and gently shake free of water.
   3. Goat anti-mouse conjugate diluted 1:160 (or as determined) is added to each well for 5 minutes.
   4. Rinse in filtered distilled water for 5 minutes as above and shake off excess water.
   5. Remove and coverslip with a minimum amount of FA mounting fluid and observe at 1000X for cytoplasmic fluorescence in the known positive control. No fluorescence should be observed in the negative controls. Read results for the unknown samples.

III. Ovarian Fluid Filtration FAT for Detection of *Renibacterium salmoninarum* - (Elliot and Barila 1987; with some modifications made by Commercial Fisheries pathology staff).

A. Mix 0.5 ml of ovarian fluid with 0.5 ml of PBS-Triton and 0.5 ml of trypsin solution in a small centrifuge tube. Mix vigorously on a Vortex mixer for 20-30 seconds. A P1000 Pipetman is useful for pipetting samples. Run 5-8 ovarian fluid samples in a group - each group takes about 3 hours to complete.

B. Heat ovarian fluid mixture in a 50°C water bath for 10 minutes.

C. Withdraw each sample from the centrifuge tube using a 3-ml syringe with a 22-gauge needle. Triturate the sample a few times with the syringe to break up any remaining clumps of material.

D. Attach each syringe containing its sample to a syringe filter holder (Millipore #SX0001300) containing a 13 mm, 0.2-µm Nucleopore (#110406) polycarbonate filter and a 13 mm, 5.0-µm Nucleopore (#140413) Membra-Fil cellulosic filter (see Note below). Force the sample through the filter. If samples won't filter, centrifuge at 4500-8800 x *g* for 10 minutes. Discard the supernatant and resuspend the pellet to the original volume in PBS-Triton, and filter. This step is required for most ovarian fluids from Alaskan salmon. If samples still filter very slowly, set them aside for a few

minutes, then try again. If this is still unsuccessful, discard the sample and start again, making dilutions of the ovarian fluid as follows:

Ovarian fluid		PBS		
1 ml	+	1 ml	=	1:2
1 ml of 1:2 dilution	+	1 ml	=	1:4
1 ml of 1:4 dilution	+	1 ml	=	1:8

Keep diluting until sample filters properly. (May go as far as 1:8.)

NOTE: The polycarbonate filter should be placed shiny-side-up (toward the syringe) in the filter holder. This is the smoother surface and is, therefore, the best surface for counting bacteria. To identify the upper surface in case the filter is accidentally turned over after it is removed from the holder, write a word such as "up" on this side of the filter near the edge before placing the filter in the holder (write carefully with a ball-point pen so the filter is not damaged). When loading, the filter is face down in ½ inch Swinney adaptor. Polycarbonate filters are thin, and frequently develop "ridges" from the ridges on the support screens of the filter holders. Placement of a thicker cellulosic filter between the polycarbonate filter and the support screen prevents the formation of extreme "ridges" on the polycarbonate filter and, therefore, makes the filter surface flatter for easier observation and counting of bacteria. The large (5 µm) pore size of the cellulosic filter does not affect filtration, and the presence of this filter helps to ensure an even flow of materials through the polycarbonate filter.

To use a two-filter system: Place the polycarbonate filter in the upper half of the filter holder as described above (make certain the silicon gasket is secured in the upper half of the Millipore holder before placing any filters). Place the cellulosic filter on top of the polycarbonate filter (this filter will be on the "downstream" side from the polycarbonate filter). Make certain both filters are flat. Screw on the bottom half of the filter holder. Make certain that the filter holder is screwed tightly together, so it won't leak. Follow the remaining steps of the protocol as for a one-filter system, but separate and discard the cellulosic filter before placing the polycarbonate filter on a glass slide (see step I).

E. Rinse each filter with 3 ml of PBS-Triton (force through the filter with a syringe). But first remove the filter holder to allow withdrawal of the plunger, then use a P5000 Pipetman to fill the syringe barrel with the PBS-Triton and replace the plunger.

F. Open the filter holder but leave the filter in the holder, and drop on 100 µl of FITC-conjugated anti-*Renibacterium salmoninarum* antiserum at the optimum working dilution using a P100 Pipetman. Tilt the filter to ensure the entire surface is covered. Cover the top of the filter holder with Parafilm® and incubate upright in the dark at room temperature for 1 hour. During this incubation the filter holder may be rested on an opened test tube (labeled) within a test tube rack.

G. Following incubation, close the filter holder and rinse each filter with 3 ml of PBS-Triton by forcing through the filter with a syringe as described above.

H. Counterstain by forcing 1 ml of Eriochrome black T suspension (1:20,000) through the filter with a syringe. Again, fill the syringe barrel using a P1000 Pipetman after

removing the filter holder and plunger. See Section IV. D. on pg. 8-11 before using stored stain.

NOTE: For many samples, Eriochrome can be added directly to PBS and included in the Step G rinse, but for samples which prove difficult to filter, fewer problems with filter breakage and leakage are encountered when two separate rinsing steps are employed.

I. Remove the 0.2-µm polycarbonate filters from their holders, and place on microscope slides to air dry. First separate the polycarbonate filters from the cellulosic filters, and discard the cellulosic filters. Place a drop of pH 8.6 glycerol mounting medium in the center of each filter, and mount with coverslip. Examine using a microscope equipped for FITC epifluorescence.

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

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

The conversion factor is the filtering surface area divided by the area of a single field at the magnification used. One can calculate the theoretical sensitivity of the technique for any desired number of fields to be examined by entering "1" in the equation for the total number of bacteria counted. The detection limit is reported to be less than 10^2 bacteria/ml of coelomic fluid (Elliott and Barila 1987).

IV. Reagents

A. PBS, pH 7.1

1. 1X concentration (1 L)

NaCl	8.50 g
Na_2HPO_4 (dibasic anhydrous)	1.07 g
$NaH_2PO_4 \times H_2O$ (monohydrate)	0.34 g
dH_2O to	1 L

Preserve the solution by adding 10 ml of a 1% thimerosal solution to 1 L (1:10,000 dilution thimerosal).

2. 5X concentration (2 L of 5X makes 10 L of 1X PBS)

NaCl	85.0 g
Na_2HPO_4 (dibasic anhydrous)	10.70 g
$NaH_2PO_4 \times H_2O$ (monohydrate)	3.45 g
dH_2O to	2 L

Preserve 5X solution for storage by adding 1.0 g thimerosal powder to 2 L of PBS. To make 1X PBS solution, mix 200 ml 5X solution with 800 ml dH_2O.

B. PBS-Triton (Work in hood with Triton X-100)

Triton X-100	5.0 ml
PBS, pH 7.1 to	1 L

(Dilute 100 ml 5X PBS with 400 ml H_2O and 2.5 ml Triton X-100.)

C. Trypsin solution

Trypsin powder, 1:250 (Difco)	1.0 g
dH_2O to	100 ml

Mix trypsin with water at 4°C. Clarify solution by filtering through Whatman No. 1 filter paper, then a 0.2-µm filter. Dispense in small aliquots and freeze at -20°C or colder. (May be stored at -20°C for several weeks.)

D. Eriochrome black T, 1:20,000 (counterstain)

Eriochrome black T suspension: Eriochrome black T (Sigma) diluted 1:20,000 in PBS, pH 7.1. (Make a 1:1000 or 1:2000 stock suspension of Eriochrome black T in PBS, pH 7.1. Filter through Whatman No. 1, then Whatman No. 2 filter papers to remove large particulates of stain.)

Individual lots of Eriochrome black T powder may be somewhat variable (the stain from Sigma is approximately 65% pure). For some lots, a 1:20,000 dilution of the powder does not produce a dark enough stain on filters. In these cases, a 1:2000 dilution may give a better stain. Filters should show a definite purple color after counterstaining.

Store in a dark (or foil-covered) bottle.

Use 1:20,000
1. 0.5 g Eriochrome black T in 500 ml PBS pH 7.1 (1:1000)
2. 1 ml - 1:1000 dilution in 1 ml PBS pH 7.1 (1:2000)
3. 1 ml - 1:2000 dilution in 9 ml PBS pH 7.1 (1:20,000)

NOTE: Stain precipitates over time. Do not use once it starts making a darker than normal filter.

E. pH 8.6-9.0 mounting medium with DABCO

1. PBS, pH 7.4 (0.15 M NaCl, 0.01 M phosphate)

Solution A: KH_2PO_4, 0.5 M (anhydrous, 68.04 g/L dH_2O)	3.36 ml	or 1.68 ml
Solution B: K_2HPO_4, 0.5 M (anhydrous, 87.09 g/L dH_2O)	16.0 ml	or 8.0 ml
NaCl	8.5 g	or 4.25 g
dH_2O	1 L	or 500 ml

2. Glycerol mounting medium

Glycerol	90 ml
1, 4-diazabicyclo-(2,2,2)-octane (DABCO)	2.5 g
PBS, pH 7.4	10 ml

Add DABCO to glycerol (solubilize DABCO by heating DABCO-glycerol mixture gently in a water bath). Then, add PBS. Adjust pH to 8.6-9.0 by addition of 0.1 N HCl or 0.1 N NaOH.

Mounting medium may be stored at room temperature in a dark bottle.
See Elliott and Barila (1987) for further information on this technique.

V. References

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

Elliot, 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.

CHAPTER 9

ELISA for the Detection of Antigen of *Renibacterium salmoninarum* (Rs) in Fish Tissues

Theodore R. Meyers, Sally Short, Karen Lipson and Craig Farrington

The following section contains detailed protocols used for the Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for detection of the Rs antigen in fish tissues (Fig. 1). Details include: reagents, reagent preparation, materials and equipment lists; use of equipment; raw sample preparation (tissue, ovarian fluids and blood); performance of the ELISA; and interpretation of results. Much of the protocols and figures are adapted from the methods of Ron Pascho (retired) and Dr. Diane Elliott (personal communication) of the USGS Western Fisheries Research Center Laboratory in Seattle, Washington, and have been published in Meyers et al. (1993 a, b).

I. Reagents (KPL, i.e., Kirkegaard and Perry Laboratories, except where indicated)

Catalog #:

- 01-96-91 Affinity purified antibody to *R. salmoninarum*
- 04-96-91 Peroxidase labeled affinity purified antibody to *R. salmoninarum*
- 50-84-01 Coating solution concentrate
- 50-63-00 Wash solution and tissue diluent - PBS with Tween 20 is a substitute that can be made in the laboratory
- 50-82-01 Milk diluent/blocking solution concentrate
- 50-62-00 ABTS peroxidase substrate system - A and B
- 50-85-01 ABTS peroxidase stop solution
- 50-96-91 *R. salmoninarum* positive control antigen

Kirkegaard and Perry Laboratories, Inc.
910 Clopper Road
Gaithersburg, MD 20878
(800) 638-3167 www.KPL.com

II. Reagent Preparation and Formulae (see reagent worksheets for 2-, 4-, 6-, 8- or 10-plate assays at the end of this chapter)

A. Coating antibody (KPL)

Coating antibody is prepared in goats immunized with whole cells of Rs. Thus, it is a polyclonal antiserum. It comes as a 1-mg lyophilized product that is reconstituted in

Figure 1. Schematic illustration of the steps within the double sandwich ELISA.

The Double Antibody Sandwich ELISA

The specific antibody for *R. salmoninarum* antigen adsorbed to plate

Wash

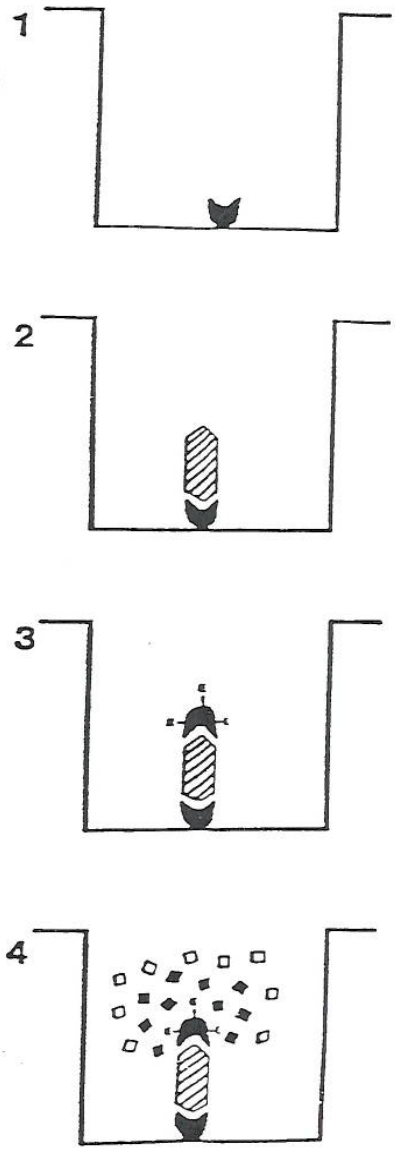
Fish tissues suspected of containing antigen are added to well

Wash

Add enzyme labeled specific antibody

Wash

Add enzyme substrate



Amount of hydrolysis (color intensity) = amount of antigen present

1 ml of 50% glycerol. Before reconstitution, thimerosal can be added to the 50% glycerol as a preservative:

10 µl of 1% thimerosal solution per ml of 50% glycerol

Although the stock coating antibody can be stored at minus 10°C for at least 1 year, activity is more stable if aliquots of 0.5 ml are frozen at -70°C and thawed when needed for assays. Use thawed antibody within 3-4 months and store at minus 10 °C. The working concentration of coating antibody may vary depending on the quality but generally begins at 1 µg/ml (1:1000 up to 1:3000) obtained by dilution of the stock in the coating buffer (see II.J.3). To determine the amount of coating antibody needed at a working concentration the following example is given (refer to worksheet for 8-plate assay).

In an 8-plate ELISA run there are 720 wells requiring coating antibody.

The calculation for the working solution of coating antibody needed is:

720 wells X 200 µl/well = 144 ml

144 ml + 8 ml (surplus) = 152 ml working coating antibody

The 8-ml surplus is excess needed for loss in filling and wetting the dispenser head. If this is not included you will not have enough antibody to coat 8 plates.

Rounding off to make a 1:1500 working coating antibody solution take:

<u>103 µl stock coating antibody + 155 ml coating buffer solution</u>

In order to make up the working coating antibody above you first must determine the amount of coating buffer needed to dilute the stock antibody.

B. Coating buffer (KPL)

Coating buffer (0.1 M phosphate buffered saline) is used for dilution of the coating antibody and comes as a 10X concentrate which must first be diluted 1:10 with distilled water. The number of plates, and hence the number of wells, each requiring 200 µl of coating antibody, must be calculated as above in order to determine the amount of coating buffer needed; 16 other wells on plates 1 and 7 containing the substrate chromogen and conjugate controls do not require coating antibody but they do require coating buffer as the overnight incubation solution. Consequently, in the calculations for the needed buffer solution the 16 wells (i.e., 3.2 ml, or 4 ml rounded off) must be included.

The calculation for the needed coating buffer is:

720 wells X 200 µl = 144 ml

144 ml + 4 ml (controls) + 8 ml (surplus) = 156 ml

Rounding off to make a simple 1:10 dilution of buffer concentrate take:

16 ml buffer concentrate + 144 ml distilled water = 160 ml coating buffer

The 5 ml of extra coating buffer is used for the conjugate and substrate chromogen controls on plates 1 and 7, leaving 155 ml for the dilution of the stock-coating antibody above.

C. Conjugate (KPL)

Peroxidase labeled affinity purified polyclonal antibody is the same product as the coating antibody except for the peroxidase conjugation. Production of both the coating antibody and the conjugate from the same animal source reduces nonspecific reactions in the assay. Conjugate comes lyophilized in a 0.1-mg quantity that is reconstituted in 1 ml of 50% glycerol. Thimerosal may be added as a preservative to the 50% glycerol before reconstitution of the conjugate but is not necessary if stored at minus 10°C:

10 µl of 1% thimerosal solution per ml 50% glycerol

To maintain activity this stock conjugate must be stored at minus 10°C for up to one year, but activity is more stable if aliquots of 0.5 ml are frozen at -70°C and thawed when needed for assays. Use thawed conjugate within 3-4 months. Currently, conjugate is used at a 1: 3000 up to 1:4000 working dilution with milk diluent (see II.J.3). The amount of conjugate needed using the 8-plate assay as an example is as follows.

728 wells X 200 µl/well	=	146 ml
146 ml + 8 ml (surplus)	=	154 ml working conjugate
	=	156 ml (2 ml added for margin of error)

52 µl stock conjugate + 156 ml working milk diluent = 156 ml working conjugate

D. Milk diluent (KPL)

Milk diluent (2% nonfat dry milk in borate buffer) is used to dilute the conjugate to its working concentration and acts as a blocking solution. A blocking solution binds to any remaining protein-binding sites after the primary Rs antigen is bound (or not bound) to the solid phase surface via capture antibody. Milk diluent comes as a 20X concentrate and is stable in the refrigerator for at least one year if not contaminated.

Since 156 ml of conjugate is needed, a convenient rounded total volume for a 1:20 dilution of milk diluent is 160 ml.

8 ml milk diluent concentrate + 152 ml distilled water = 160 ml

Use 4 ml for the control wells, leaving 156 ml of working milk diluent used above for making the working conjugate dilution.

E. Substrate chromogen (KPL)

Peroxidase substrate chromogen is added to the double sandwich to react with peroxidase enzyme in the conjugate that might have attached to any antigen captured on the plate. Reaction of the substrate with peroxidase in the attached conjugate produces a product that reacts with the chromogen producing a color of blue-green. The intensity of color is dependent upon the amount of bound conjugate and in turn the amount of captured antigen in the sample. The chromogen comes in two solutions, A & B, which must be warmed to RT and mixed together just before use. Discard any leftover solutions of A & B.

The calculation for the needed substrate chromogen for 8 plates is:

736 wells X 200 µl/well = 148 ml

148 ml + 8 ml (surplus) = 156 ml working substrate chromogen

78 ml Solution A + 78 ml Solution B = 156 ml working substrate chromogen

F. Stop solution (KPL)

The stop solution is used to stop the enzyme-substrate reaction after a precise incubation period. Consequently, stop insures that the incubation period and, thus, the amount of color development is standardized over time to allow comparison of different unknown samples. The stop chemical is sodium dodecyl sulfate (SDS) and is in a 5X concentrate.

The calculation for the needed stop for 8 plates is:

736 wells X 100 µl = 74 ml

74 ml + 8 ml (surplus) = 82 ml

Round off to 85 ml needed working stop

The stop concentrate is made to dilute to 1X delivered in a 200-µl quantity which would overflow the wells (capacity = 300 µl) at this point of the assay, i.e., 200 µl of substrate chromogen + 200 µl stop = 400 µl.

To make the recommended working dilution of 1X, dilute the stop to a 2X solution delivered in 100 µl which provides the same concentration of stop as 200 µl of 1X.

2X = 1 ml concentrate + 1.5 ml distilled water, thus:

34 ml stop concentrate (5x) + 51 ml distilled water = 85 ml working (2X) stop

G. Wash buffer and tissue diluent (PBS T-20 made in the lab)

Wash buffer is used to wash plates after each reagent incubation to get rid of any unbound materials. Tissues for testing can also be diluted in this buffer; consequently, large quantities of this buffer must be on hand. A convenient batch amount is 50 L.

PBS T-20 (pH 7.4 + 0.05% Tween 20)

NaCl	400 g
KH_2PO_4	10 g
Na_2HPO_4-$12H_2O$	145 g
KCl	10 g
Tween 20	25 ml
Thimerosal	5 g[a]
dH_2O to	50 L

or the KPL wash buffer concentrate may be used as indicated in the 8-plate worksheet. Reagent preparation for 2-, 4-, 6-, or 10-plate assays are calculated similarly as above and are provided in the worksheets at the end of this chapter.

Note: With the current KPL coating antibody and conjugate, the detection signal (OD value) of Rs antigen appears to be stronger when the wash buffer is aged for several weeks, possibly due to the amount of Tween 20 used in the FPL buffer. Therefore, buffer should be made well in advance of need. Also, large quantities of buffer remaining after one season can be saved for use during the next ELISA season.

H. Positive control antigen (KPL)

The positive antigen control, as with the negative control, is necessary to determine if the assay is working properly. The antigen is a lyophilized product of heat-killed cells of the Rs agent in 1 ml of dextrose solution with an approximate packed wet-cell volume of 0.5%. This is equivalent to about 420 µg of protein per vial as determined by the Lowry Assay (KPL, personal communication). The vial is rehydrated with 1 ml of distilled water and is added to ELISA-negative raw kidney homogenate according to the Table 1 and instructions below:

Table 1.

Dilution	Raw kidney	Diluent	Total	KPL Rs control	Pipettor
1:25,000	22.50 g	67.50 ml	90 ml	3.6 µl	P10
1:5,000	22.50 g	67.50 ml	90 ml	18 µl	P20
1:1,000	21.25 g	63.75 ml	85 ml	85 µl	P100
1:100	22.50 g	67.50 ml	90 ml	900 µl	P1000
Total	88.75 g	266.25 ml	355 ml	1006.6 µl	
Amount for all four dilutions	90 g	270 ml	360 ml		

1. Use ELISA-negative kidneys ranging in approximate OD values from 0.040 to 0.048.
2. Weigh out 90 g (or appropriate amount for dilutions being made) of raw, frozen kidney tissues.
3. Cut kidney tissues into small pieces and place into two large Whirl-Pak® bags.

[a] A stock 1% (1% = 1 g per 100 ml distilled water) solution of thimerosal (merthiolate) may be made and 10 ml per liter added to the PBS T-20 buffer (final 0.01%).

4. Use Stomacher to further break up the kidney tissues.
5. Add 25 ml of diluent to each bag and homogenize in the Stomacher.
6. Pour the slurry from the bags into a wide-mouth sterile bottle and add remaining diluent (220 ml for a full batch of 4 dilutions) to make the standard 1:4 w/v (1 part to 3 parts) dilution of kidney tissues. Mix well.
7. Use a graduated cylinder to divide the slurry into 4 bottles according to the aliquots needed.
8. Add the necessary amount of KPL Rs-positive control to each bottle and mix well.
9. Divide each bottle of the spiked slurry into two 50 ml screw capped centrifuge tubes.
10. Boil the tubes containing the slurry for 15 min.
11. Centrifuge the tubes at 6000 x g for 25 min.
12. Transfer the supernatants into color coded 2 ml Sarstedt tubes and fill each tube completely to the top. This is important because if not completely filled one tube will not provide enough positive control for the 2 control plates in a 10 plate assay.
13. Place the tubes into two freezer boxes labeled with the positive control dilutions and freeze at minus 70^{o}C.
14. The amounts listed in the Table above will provide well over twenty 10 plate ELISAs but if individual dilutions need to be made on a smaller scale, the volumes can be adjusted using the Table 2 below.

Table 2.

Dilution	Negative kidney slurry	KPL Rs-positive control
1:25,000	30 ml	1.2 µl
1:5,000	6 ml	1.2 µl
1:1,000	1 ml	1.0 µl
1:100	1 ml	10.0 µl

15 Any remaining antigen concentrate can be frozen in one of the freezer boxes for later dilution. However, avoid repeated freeze/thaws of this material by dispensing aliquots of the concentrate into multiple tubes. Repeated freeze/thaws increase the optical density values as the antigen is degraded.

16. ELISA-negative kidney tissues "spiked" with a known amount of positive control antigen have shown this assay to detect between 21 and 42 ng/ml of Rs antigen (Table 3) based upon minimum positive optical density (OD) values of 0.095 and 0.1, respectively. This sensitivity is slightly less than the probable sensitivity using the pure antigen due to interference by the kidney tissues. This data was based on use of the Bio-Tek EL 310 autoreader and the protein analysis data provided by KPL. Replacement of this reader with a Bio-Tek ELx 800 autoreader with different optics changed the negative-positive threshold OD values to $\geq$ 0.068. New protein determinations done on the KPL Rs-positive control whole cell antigen indicated this OD threshold provides a detectable limit of 10-16 ng/ml of Rs antigen. This corresponds to about a 0.095 OD value on the previous EL 310.

Standard curve of ELISA optical density (OD) values for various protein concentrations of *Renibacterium Salmoninarum* antigen added to negative control kidney tissues. Protein concentration of stock antigen is 420 µg/ml. Positive OD values are ≥ 0.095. Negative kidney OD values were 0.075 prior to addition of antigen.		
Antigen dilution	**Protein concentration (µg/ml)**	**Average OD value**
1:100	4.20	1.056
1:1,000	0.42	0.441
1:2,000	0.21	0.196
1:5,000	0.084	0.139
1:10,000	0.042	0.110
1:20,000	0.021	0.094
1:25,000	0.017	0.087
1:30,000	0.014	0.083

Table 3.

I. Negative control tissues (provided by lab)

Uncooked, homogenized diluted kidney tissues left over from samples shown to be negative on ELISA with mean OD values between 0.070 and 0.075 (using EL 310) or 0.040 and 0.048 (using ELx 800) may be pooled and processed to completion (boiled, centrifuged, tubed and frozen @ -70^{o}C) for a stock supply of negative control material. Tubes with negative kidney supernatant should be filled to the very top to provide enough material for at least two control plates. PCR may also be used to confirm the absence of Rs antigen in negative control tissues (Chase and Pascho 1998).

Negative control ovarian fluids may be pooled and processed as described but should be checked by both ELISA and filtration fluorescent antibody testing (FAT) to rule out false ELISA-negative results unique to ovarian fluids as described later on.

J. Cautions in reagent preparation and storage

1. All glassware (storage bottles and graduated cylinders) should be acid washed, rinsed in distilled water and autoclaved. This reduces risk of cross-reacting antigen contamination.
2. Similarly, each of the 4 major reagents (coating antibody, conjugate, substrate chromogen, stop) including distilled water, should have their own glassware that is labeled accordingly. Do not mix the glassware. Separate dispenser heads should also be used for each of the 4 reagents.
3. Standardization of coating antibody and conjugate reagents may be necessary to minimize variations in OD values resulting from minor differences in reagent activity from vial to vial or between lot numbers. This can be done by first pooling several

vials of each reconstituted stock reagent followed by freezing into aliquots of 0.5 ml at -70°C. Pool only reagents from the **same** lot #, otherwise test first before pooling to avoid mixing reagents of variable quality. Aliquots of each reagent are thawed and different concentrations of each are tested against known negative and positive control samples. Dilutions tested for the coating antibody include: 1:1000, 1:2000 and1:3000. Dilutions tested for the conjugate include: 1:1000, 1:2000, 1:3000 and 1:4000. The reagent dilutions are selected that provide an acceptably low OD value for the negative control while still providing adequately high values for the positive controls. This selection is based on previous ELISA performance for each laboratory. Approximate target ELISA OD values used by the Fish Pathology Section are provided in VI. A. on pg. 9-23.

4. The stop concentrate and sometimes the coating buffer concentrate will crystallize in the refrigerator and must be warmed to room temperature to re-dissolve before diluting to working solutions.
5. The stability of the substrate chromogen once aliquots of A & B are mixed is short-term. The complete substrate must be used soon after mixing (preferably within 20 min) solutions A & B and care must be taken to not contaminate stock contents of bottle A with any of bottle B or vice versa. Allow both bottles A & B to warm to RT beginning in the early morning prior to mixing and use. If quantities needed for the assay are significantly less than what is present in the stock bottles, aliquot the necessary volumes separately for warming to RT. Stability of solutions A & B in their respective bottles is about one year. Do not stockpile large quantities of solution A & B substrate chromogen that cannot be used within 8 months to a year.
6. Beware of contaminating reagents with antigen from either sample unknowns or positive antigen controls which is especially critical of the distilled water and its container. Most all reagents, glassware, etc., come in contact with the distilled water.
7. To minimize risk of accidental contamination prepare positive control antigen dilutions away from any area where other reagents and glassware are used. Clean up any spills with 3% iodophor or bleach and do not prepare antisera or conjugate dilutions at the same time as the positive control antigen dilutions.
8. Do not use sodium azide as a preservative in buffers as it will inactivate the peroxidase in the conjugate.
9. Distilled water and wash buffer in their respective reservoirs should contain thimerosal as a preservative. Both distilled water and wash buffer reservoirs and all hoses should be acid cleaned after the ELISA season is over.
10. Milk diluent concentrate is stable for at least one year and should be examined for potential contamination before use.

III. ELISA Materials and Equipment

A. Materials

1.	2 oz. Whirl-Pak®, white label bags #11216-772	Van Waters and Rogers (VWR) (800) 932-5000
2.	Polyethylene Transfer Pipets #14670-103	" "
3.	Parafilm #52858-000	" "

4.	ELISA plates, Falcon #62406-321 #62407-867	" "
5.	Screw-cap Microtubes w/caps for boiling and storage of samples #72.694.007	Sarstedt (800) 257-5101
6.	Styrofoam tube-storage boxes #95.064.249	" "
7.	Screw-cap Micro tube racks #93. 848.100	" "
8.	Adhesive plate sealers, pre-cut, pressure sensitive acetate # 62402-921	VWR (800) 932-5000
9.	Pipet-aid # 53498-001	VWR " "
10.	Pipetman P-20 (for antigen preparation) #F-123600	Rainin (800) 445-7661
11.	Pipetman P-200 (one per person for sample loading)# F-123601	" "
12.	Pipet-tips Pakrak refill 200 µl # 30128--376	VWR
13.	Gauze pads #82004-740	VWR
14.	2 Timers, 8-Channel alarm #23609-192	VWR
15.	Plastic container with lid to hold, carry, and incubate plates	Rubbermaid (local purchase)
16.	Hot plate (capable of boiling enough water to accommodate at least 2 Sarstedt tube racks)	Local purchase

B. Equipment

1.	Automatic dispenser (or repeating pipettor) for applying coating antibody and conjugate. It is highly advisable to use an automatic dispenser for beginning the substrate-chromogen reaction, and adding the stop solution.	Titertek, MTX Lab System
2.	Microplate ELISA reader and (optional) computer with interface cable. **NOTE:** Some of the microplate readers may only print out values up to an absorbance of 2.0, yet still output higher values to a computer.	Bio-Tek, Flow labs, etc.
3.	Microplate washer for disposing of reagents and washing plates after various steps of the assay.	Bio-Tek, Flow labs, etc.

4.	37°C incubator for substrate-chromogen reaction.	VWR (800) 932-5000
5.	Omnifuge RT centrifuge or other equivalent capable of ≥ 6,000 x g. #C-1730-1	VWR
6.	Stomacher® homogenizer	Tekmar Co.

C. Equipment use (be familiar with all user manuals for operation/maintenance details)

1. The ELISA Reader/Spectrophotometer
   a. Be sure to place plates in the reader carrier correctly oriented with well #1A at the top left hand corner or where noted on the carrier.
   b. The reader should be set on a wavelength of 405 nm for this particular substrate chromogen.
   c. Both reader and computer should be turned on at least 1 hr prior to reading the first plate. The software program should be ready on standby to read the first plate.
   d. Most readers are capable of various functions. Be familiar with the user manual and particularly with any self-check troubleshooting capability. Periodically run any self checks on the reader, especially when first purchased or serviced.

2. The ELISA Plate Washer and Accessories
   a. Keep a plate with distilled water in the washer at all times and recharge the water once a week (Friday) to keep the plate manifolds wet. For long term storage, purging and cleaning of the system may be necessary to prevent obstruction from crystallization of dried buffer in the washer system.
   b. Take exceptional care to not damage the plate manifolds (192 stainless tubes that dispense and aspirate buffer in the wells) when handling plates in or out of the washer.
   c. It is important to adjust plate height on the washer so that the aspirator tubes (longest) do not physically contact, and therefore scratch, the optical surface of the plate wells. This may allow more residual buffer to remain in some wells after washing that must be shaken out. Different plate brands may require readjustment of the plate height.
   d. Assay settings on the plate washer should be for 5 cycles, a 30-second soak time and 0.25-µl fill volume. Some washers have a shaking cycle that can be programmed during the soak time – the shaking cycle is not necessary.
   e. Label buffer and distilled water reservoir hoses conspicuously at their points of valve connection to the washer to prevent accidental use of the wrong reagent.
   f. Provide an adequate moisture trap to the excurrent hose leading to the vacuum pump from the second vacuum jar. Moisture in an oil vacuum pump will cause it to seize up requiring disassembly and cleaning.

3. Plate Dispenser
   a. Separate dispenser heads are used for coating antibody, conjugate, substrate chromogen and stop. Each are labeled as such with a lab marker and stored dry in similarly labeled plastic containers with lids to protect from dust and stray contamination.

b. After each use, the dispenser heads are rinsed at least three times with distilled water and allowed to dry on absorbent paper towels overnight before storage in their plastic containers.
c. Be sure dispenser volumes are set correctly for the proper reagent. The coating antibody, conjugate and substrate chromogen are set at 200 µl/well. The stop must be set for 100 µl.
d. After first wetting and filling the dispenser head, always dispense two columns worth of reagent back into the reservoir trough. This allows for reagent filling and purging of air bubbles from the dropper tubes. This volume can be pipetted back into the reservoir for the dispenser head.

IV. Raw Sample Preparation

A. Kidney or other organ tissues

1. Extract 1- x 1-cm pieces of kidney (less than thumbnail size) from each fish: 1 anterior, 1 posterior. Put both into the same bag, and label well with a black lab marker (use 2 oz. white stripe Whirl-Pak® bags). Total weight of the two pieces of tissue = 1-3 g.

   NOTE: It is very important to emphasize to the client the correct weight desired for kidney samples. Tissue samples weighing 1-3 g are easiest to work with. The absolute minimum amount of sample is 0.3 g which will allow only one ELISA assay replicate. If too much tissue is submitted (i.e., 8-10 g) the samples will overflow their bags when diluted and homogenized in the Stomacher®. Such large samples will require that each sample be reduced to the correct weight and placed into a new bag - this is unnecessary effort, especially when hundreds of samples are involved. Individual sample bags should be clean of blood and disinfectant, organized numerically in order into groups of 10 with rubber bands and placed into larger plastic bags rather than all tossed into a single container. This organization allows more efficient processing in the lab and any missing sample numbers can be more easily identified.

2. Instruments - Use a small metal teaspoon, with the edges sharpened for tissue extraction. (It's easier than a scalpel to clean.)
   a. wipe (to remove organic material)
   b. scrub in 3% iodophor solution with a brush
   c. wipe clean of disinfectant
3. The spoon must be wiped and disinfected between fish.
4. Tissues may be kept cold or frozen for later processing.
5. Kidney samples are assigned an ELISA number which is written on the sample bag before anything else is done. Numbering of tubes and record keeping is facilitated by assigning a unique ELISA number to each sample (recorded in an ELISA log book) which can be cross-referenced to the original user sample number in the accession log book and on the ELISA plate map. ELISA numbers are preceded by one capital letter to designate the fish species. Samples within each species begin with #1. A separate ELISA numbering system is critical since clients submitting samples have their own numbering systems which often produce duplicate numbers within a single assay when samples are tested from several different sources. ELISA sample numbers within each species designation should be in sequence during the calendar year. When a new year begins, numbering is repeated starting

with #1. Each ELISA assay should also have its own number. These numbers are sequentially continuous across calendar years which allows for easy location of particular sets of samples for any year using the ELISA log book. For example, in 2008, the year finished with ELISA assay # 367 that included ELISA sample #'s (coho) C 1118-1549 on a 10-plate assay. In 2009 , the first ELISA assay will be # 368 , also 10 plates, with ELISA sample #'s (pink) P 1-200, (sockeye) S 1-50, (chum) D 1-30, (chinook) K 1-20, (trout) T 1-132.

6. Kidney tissues are weighed and the 3X w/v dilution calculated. Write both values on each bag.

   NOTE: Kidney weight; ELISA number; and the 3X weight (ml of diluent to be added) should now be on the bottom of the sample bag from left to right. All writing on the bag must be done on the white label using a black Sharpie® waterproof marking pen. Ink from other colors and brands of pens will not adhere to the bag when placed into the Stomacher®.

7. Each sample is then diluted 1:4 w/v (1 part to 3 parts) with PBS T-20 and homogenized within their individual 2 oz white-label Whirl-Pak® bags using a Stomacher® (Tekmar Company).
8. The tissue slurry for each sample is pipetted into a pre-numbered screw-capped Sarstedt tube using a disposable fixed bulb large bore polyethylene transfer pipet (VWR). Remaining homogenized tissue can be frozen within individual Whirl-Pak® bags at -5 to -10^{o}C for later retrieval if necessary.
9. Tightly capped tubes with samples are placed into plastic Sarstedt tube racks and boiled for 15 minutes in a large pan of water heated by a hot plate. The hot plate should be large enough to hold a pan that will accommodate at least two tube racks. Boiling for longer periods of time does change the optical density values but in some cases not significantly (Table 4). However, for strict standardization of all assays performed, the 15-minute boiling time should be closely adhered to. Samples are boiled to denature all nonspecific heat labile protein, leaving the heat stable soluble antigens of *Renibacterium salmoninarum* in infected samples which are serologically related to the antigens found in various tissues of salmonids infected with the same agent (Pascho and Mulcahy 1987).
10. The boiled samples are then frozen in styrofoam Sarstedt tube storage boxes at -5 to -10°C for performance of the assay at a later time.
11. In summary, the sequence of events is generally to assign ELISA numbers, weigh all kidneys and calculate diluent volumes first, then go back to label tubes and finally dilute, homogenize, boil and freeze the samples. For planning of time, a 10-plate assay (432 samples) generally takes about 17 man hours of sample preparation or about 4 man hours/100 samples.

B. Ovarian fluids and serum

1. Raw ovarian fluids (without eggs or other debris) are diluted 1:2 v/v (1 part to 1 part) with PBS T-20 and blood serum is diluted 1:4 v/v (1 part to 3 parts).
2. The samples are placed in labeled tubes and boiled for 15 minutes as described above and can be frozen at this point.
3. When thawed, they are centrifuged and treated as described for kidney tissues during the remainder of the assay.

ELISA #	STOCK	O.D. Tube boiled 15 min.	O.D. Tube boiled 60 min.
P127	Ward Cove Pinks	0.080/0.081=0.080	0.083/0.083=0.083
P145	" " "	0.086/0.082=0.084	0.082/0.090=0.086
P146	Margaret Lake Pinks	0.113/0.112=0.112	0.096/0.099=0.097
P162	" " "	0.093/0.088=0.090	0.107/0.106=0.106
P174	" " "	0.104/0.100=0.102	0.110/0.101=0.105
P186	" " "	0.464/0.435=0.449	0.454/0.527=0.490
P206	" " "	0.076/0.076=0.076	0.069/0.072=0.070

Table 4: Comparison of average optical density values of kidney samples boiled for 15 minutes and 60 minutes.

4. Occasional ovarian fluid samples will give false ELISA-negative results despite having many Rs organisms per ml as determined by filtration FAT (FFAT).

   The mechanism causing this discrepancy is not understood but is suspected to be a natural protein in ovarian fluid that must block or destroy receptor sites of the coating antibody on the plate. This will prevent any potential Rs antigen from being captured and produce a negative result. This protein would vary in concentration from fish to fish, i.e., those infected fish with low or no levels of interfering protein are ELISA-positive. Reducing the boiling time of ovarian fluids was examined to determine if the Rs antigen was being denatured prematurely in ovarian fluids. Results in Table 5 (fish #s 231 and 587) indicated this was not the case.
   A possible alternative sample preparation which may reduce this interference phenomenon is as follows:

   a. Centrifuge the raw ovarian fluid samples for 20 minutes at 6,000 x g to pellet any Rs organisms.
   b. The supernatant in each sample is gently withdrawn and the pellet re-suspended in the exact original volume using PBS T-20.
   c. The samples are then processed as above.

   Although this method needs adequate testing, the concept is that the blocking protein will be discarded (as will any soluble Rs antigen) in the supernatant and any Rs organisms will be retained for boiling and detection. Further concentration of antigen may be accomplished by re-suspending the pellet in a volume of buffer less than the original volume of ovarian fluid.

NOTE: Kidney and ovarian fluids from the same female fish do not always agree as both Rs-negative or -positive (Table 5). Because of ELISA-false negatives, ovarian fluids must be confirmed as negative by FFAT.

			FAT		ELISA OD VALUES			
Accession #	**Species**	**#**	**Kidney**	**Ovarians**	**Kidney**	**Ovarians***		
						A	**B**	**C**
89-0516	Chinook	116	2+	ND	.761	.076	.077	.076
					.625	.076	.074	.075
	"	143	2+	ND	1.309	.077	.073	.070
					1.179	.073	.069	.070
	"	424	2+	ND	.621	.095	.098	.101
					.590	.095	.098	.095
	"	106	Neg	TNTC	.077	ND	ND	.563
89-0518	Chinook	224	1+	ND	.236	.074	.073	.074
					.277	.073	.072	.072
	"	231	2+	TNTC	1.423	2.363	2.25	2.330
					1.553	2.327	2.30	2.404
	"	234	1+	ND	.106	.071	.075	.078
					.115	.069	.078	.077
89-0555	Coho	82	2+	ND	1.203	.092	.104	.106
					1.203	.097	.107	.106
	"	178	2+	ND	.311	.069	.070	.070
					.332	.069	.069	.069
	"	201	1+	ND	.854	.082	.081	.079
					.811	.081	.081	.081
	"	587	1+	TNTC	.220	.575	.596	.583
					.197	.608	.573	.649
	"	706	2+	ND	1.024	.072	.072	.068
					1.047	.073	.073	.068
	"	831	2+	ND	1.043	.068	.071	.070
					1.095	.071	.070	.069
	"	876	2+	ND	.118	.075	.076	.075
					.121	.075	.075	.075

Table 5 : Results of fluorescent antibody tests (FAT) on selected raw ovarian fluids and kidney tissues from female chinook and coho salmon infected with *Renibacterium salmoninarum* compared with ELISA optical density (OD) values from the same kidney material (boiled 15 min) and ovarian fluids boiled for *(A) -5, (B) -10, and (C) -15 minutes. TNTC = too numerous to count. OD values ≥ 0.095 were considered positive for the Rs antigen.

V. ELISA Preparation and Performance

A. ELISA plates to be used should be pre-examined in the spectrophotometer for scratches, burnishes or other imperfections on the optically clear plate bottom. This can be done weeks ahead of time but no later than the morning of the day before an assay so the plates may be coated with primary antiserum in the afternoon. Acceptable plates are each covered on the bottom optical surface with a precut piece of parafilm to protect it from possible scratches and fingerprints that will occur due to repeated handling during the assay. This minor step saves considerable time in eliminating the need to wipe plates of fingerprints every time they are handled which also tends to burnish the plates. Latex gloves worn on the hands as an alternative eliminates fingerprints but does not protect the plate bottom from other stray contamination.

B. One day prior to the assay the tubed samples to be tested are thawed and centrifuged at 6,000 x g at 4^{o}C for 20 minutes to pellet the tissue debris. Coho salmon kidney tissue tends to not pellet as tightly requiring longer spinning times and finesse to not disturb the pellet during sample withdrawal, i.e., the clear colorless to amber-colored supernatant is the ELISA sample. Kidneys that require more centrifugation for pelleting will swell again while in the refrigerator overnight. The supernatant from these samples can be collected immediately after centrifugation and placed into new tubes if the problem is severe enough to warrant this step.

C. A plate map must be drafted (EXCEL software) before the day of the assay. Because the numbering sequence for each rack of samples is identical to the plate map (Fig. 2) for a given ELISA plate, it is critical to sequence the samples correctly within the Sarstedt rack. Error here will cause considerable confusion as to what sample went where on the plates. The plate map is of some help in sorting out such problems and is always used as a cross check for determining exactly where each sample is on a given plate and to identify the original sample number, fish stock, etc. at a quick glance. A plate map is indispensable.

D. Centrifuged samples are placed back into Sarstedt tube racks according to the plate map. Sarstedt racks each hold 48 tubes and are specified here because: one rack holds enough samples (run in replicate) for one ELISA plate; they are specially made to grip the Sarstedt tubes via grooves around the tube collar. This grip allows the cap to be removed with one hand without removing the tube from the rack, thus greatly reducing the time spent in pipetting samples and loading into the ELISA plates. Each Sarstedt rack of samples is numbered as plate 1-10 with a stick-on label and placed in the refrigerator until the assay the next day.

E. Also, one day before the assay, positive antigen and negative kidney control samples should be removed from -70^{o}C and placed into a refrigerator to thaw overnight. If two control plates are to be used, be sure to thaw enough control materials for two replicates.

F. Late in the afternoon on the day before (see Note) a scheduled assay, plates must be coated with coating (capture) antibody. This is done by dispensing (automatic dispenser or repeating pipettor) 200 µl of the working dilution of affinity-purified antibody to *R. salmoninarum* into all wells for sample unknowns and certain controls as specified below (Table 6). Plates with antibody are incubated in a closed plastic container placed in the refrigerator overnight (16 hr).

NOTE: For a summary of pre-assay preparations see Table 7. Also, Monday is not an acceptable day for ELISA due to the advanced preparations necessary the day before.

Figure 2. Example of an ELISA plate map for the control plate #1. See Table 6 for definitions of control wells. In rows 7-12 sample unknown wells each have a unique ELISA # at the top with the client's submitted sample # on the bottom. Fish stock, case # and sex (changes at number change) are also listed above the sample wells.

ELISA 377 Plate 1

2010-031
Ship Creek coho ♂ ♀

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	N		1:100	1:5000		C283 101	287 105	291 109	295 113	299 117	303 65
B	CC	N		1:100	1:5000		284	288	292	296	300	304
C	CC	N		1:100	1:5000		285	289	293	297	301 119	305
D	CC	N		1:100	1:5000							
E	SC	B		1:1000	1:25000		286	290	294	298	302 64	306 68
F	SC	B		1:1000	1:25000							
G	SC	B		1:1000	1:25000							
H	SC	B		1:1000	1:25000							

Well Designation	Step 1 Coating Antibody (overnight)	Step 2 Test Samples (3 hours)	Step 3 HRP-IgG Conjugate (2 hours)	Step 4 Substrate-Chromagen (20 minutes)	Step 5 Stop and Read
Blank (B)	Coating Ab	PBS-T20	HRP-IgG	Substrate-Chromagen	2x SDS
Conjugate Control (CC)	Coating Buffer	PBS-T20	HRP-IgG	Substrate-Chromagen	2x SDS
Substrate-Chrom. Control (SC)	Coating Buffer	PBS-T20	Milk diluent	Substrate-Chromagen	2x SDS
Positive Controls 1:100-25,000	Coating Ab	Purified Antigen	HRP-IgG	Substrate-Chromagen	2x SDS
Negative Control (N)	Coating Ab	Rs-Negative Tissue(s)	HRP-IgG	Substrate-Chromagen	2x SDS
Unknowns	Coating Ab	Unknowns	HRP-IgG	Substrate-Chromagen	2x SDS

Table 6. Summary of steps in the double sandwich ELISA for the antigen of *Renibacterium salmoninarum*.

- Samples thawed 1 day in advance and centrifuged
- Positive and negative controls thawed 1 day in advance
- Clean centrifuge buckets and inserts in preparation for next run
- Microplates inspected and covered
- Sufficient wash buffer reserved (4L)
- Plate maps drafted (prior to centrifuging the samples)
- Glassware washed, acid cleaned, and autoclaved (bottles, cylinders)
- Sufficient quantity of sterile pipet tips reserved
- Night before, coating antibody on plates

Table 7. Pre-assay summary preparations for ELISA

G. It is imperative in any ELISA to include the necessary reagent controls to determine if the assay results are valid. Assays of 8-10 plates require 2 control plates, arbitrarily chosen as #s 1 and 7. When fewer than 8 plates are examined, plate #1 is sufficient as the control plate. Half of a control plate(s) is used for positive and negative control samples loaded in the manner illustrated by Figure 2 and Table 6. During overnight incubation of capture antibody in the refrigerator, wells in column 2 A-D, which will contain the known negative control kidney samples, receive coating antibody. Wells in column 2 E-H, which will contain the blank samples, receive coating antibody. Wells in column 1 A-D, which

will contain the conjugate controls, receive coating buffer. Wells in column 1 E-H, which will contain the substrate chromogen controls, receive coating buffer (see Note). Wells in column 3 are left empty as a buffer zone against cross-contamination. Wells in column 4 A-D and E-H, which will contain the 1:100 and 1:1000 dilutions, respectively, of positive antigen control receive capture antibody. Wells in column 5 A-D and E-H, which will contain the 1:5000 and 1:25,000 dilutions, respectively, of positive antigen control, receive capture antibody. Wells in column 6 are left empty as a buffer zone against cross contamination. Sample unknowns begin in columns 7 through 12; i.e., 24 replicate samples per control plate receiving coating antibody. The control wells are repeated on plate 7 in the same manner.

NOTE: The coating buffer applied to wells of column 1 will have to be hand-pipetted (Pipetman 200) because the dispenser head will already be filled with coating antibody.

H. Plates are numbered and covered with adhesive mylar plate sealers which are also numbered the same as their respective plates (to avoid mix ups with other plates). Plates are placed into a plastic container with lid and incubated in a refrigerator overnight. Unless contaminated during the assay, the sealers are reused on their respective plates through each step of the assay until read.

I. On the day of the assay the plates incubating overnight with the coating antibody are removed from the refrigerator in the early a.m. and washed with PBS T-20 on the plate washer. The washer is set for 5 cycles of washing, each with a 30-second soak time, with a fill volume of wash buffer at 0.25 µl. When each plate is finished, any excess buffer is shaken out (3 times) and the mylar adhesive cover is re-seated. Empty covered plates are placed into a plastic container with lid at room temperature until loaded with samples.

J. Sample unknowns previously prepared in the Sarstedt racks are removed from the refrigerator and 200 µl of each are loaded onto their respective plates using a Pipetman 200 according to the plate map. In a 10-plate assay it is helpful to have at least two to three people loading plates in order that samples can be loaded on all plates in about an hour. This prevents prolonging the assay past normal working hours due to a long first washing cycle caused by strung-out incubation times of all the plates. Hence, at least 2-3 Pipetman 200s are needed. Replicates of each unknown sample are paired in the following manner within each of the 12 vertical 8-well columns to normalize values against temperature differences occurring at the outer plate margins during incubation and handling: A & D; B & F; C & G; E & H. After each plate is completely loaded, the same mylar seal is placed tightly back onto the plate and the time at which the plate is to be washed is written onto the white border of the seal. This time will be 3 hours from the time the loading of the plate was finished. Plates are left in the covered plastic container at room temperature for the 3-hour sample incubation period.

K. On the control plates the blank, conjugate and substrate-chromogen control wells receive 200 µl of PBS T-20. The negative kidney control wells receive the negative kidney supernatant and the positive antigen controls receive their respective antigen dilutions.

CAUTIONS IN SAMPLE APPLICATION:

1. Care should be taken to minimize the introduction of air bubbles or tissue pellet particles into the sample wells. Also, avoid splash to other wells from bursting air bubbles when the last of the sample is expressed into a well from the pipet tip.
2. The Pipetman bore should be wiped on an alcohol swab after the ejection of each tip to prevent potential contamination of the next sample should the previous sample fluid have been inadvertently splashed or sucked onto the bore. This can easily occur should the plunger of the Pipetman be pushed too far to the sample expression stop prior to the next sample withdrawal from the tube. Change alcohol swabs frequently. Alternatively, more expensive cotton-plugged pipet tips eliminate the need to wipe the bore.
3. Once the samples are loaded onto plates, care is needed to prevent any splash onto the seal from the sample wells caused by any sudden movement of the plates. This can cause possible cross-contamination when the seal is pulled off. If this should occur, the seal should be carefully removed and replaced with a new one.
4. Be sure that each plate is oriented so that the column 1A well is in the top left hand corner receiving the first sample. Otherwise, the samples will be applied backwards unless the error is discovered.
5. When loading control plates, the PBS T-20 for the blank, conjugate and substrate-chromogen controls is usually applied first followed by the negative control kidney, then the positive control antigen dilutions in order, with the sample unknowns last.
6. Record any pipetting errors or other aberrations in sample application on the plate map for future reference.

L. Place the conjugate dispensing head onto the plate dispenser and load with conjugate.

M. Each plate is washed in PBS T-20 at the end of its respective 3-hour sample incubation according to the time written on the mylar cover. The plate washer settings are as indicated above. As each plate completes washing it is shaken free (3 times) of any excess buffer and placed into the dispenser for application of the conjugate. The removed mylar cover for each plate is set aside until this step is completed.

N. In the dispenser, 200 µl of peroxidase labeled affinity purified Rs antiserum conjugate is added into all unknown sample wells and those of the blanks, conjugate, positive antigen and negative kidney controls. Be sure the plate orientation is such that column 1 is to the left. A working concentration of milk diluent is added to the substrate-chromogen control wells (Table 6) by hand pipettor. The mylar cover for each plate is again pressed firmly onto the wells taking care to realign with original well impressions. The conjugate is incubated for 2 hours at room temperature with the next wash time again written on the margin of the mylar cover.

O. Remove the conjugate head from the dispenser after rinsing with distilled water and air dry.

P. At the appropriate time, each plate is washed again with PBS T-20 in the plate washer, excess buffer shaken out and the mylar cover again re-seated. At this stage plates are collected as they are washed and resealed with dry wells until all plates are finished washing. This allows for the closer coordination of plate incubation times in numbered plate sequence for the application of substrate-chromogen.

Q. After the last plate is washed and sealed, the head labeled “substrate chromogen” is placed on the dispenser. Substrate-chromogen (warmed to RT and freshly made about 5-20 minutes before use) is loaded into the dispenser head and dispensed at 200 µl per well into plate #1 which is placed into a 37^{o}C incubator for 20 minutes exactly. After about a 90-second pause, substrate-chromogen is added to plate #2 and so on until all plates are in the incubator. It is mandatory to have at least two automated multiple-alarm timers to facilitate tracking the incubation time of each plate in order that the 20-minute development time with the substrate-chromogen can be strictly adhered to for all plates. The 90-second pause between each plate prevents plates from stacking up after the incubation period awaiting to be read on the spectrophotometer.

R. With these 90 second pauses, there are approximately 4 minutes left before the first plate is read. This time is used to replace the substrate-chromogen head of the dispenser with the head labeled "stop" and to load with reagent.
NOTE: For whatever reason, should there be a delay in applying stop to plates beyond the 20 minute incubation the increased development time will NOT increase background or Rs negative fish OD values. However, the OD values of Rs-positive fish will increase. This may be useful information in saving an assay should there be a power or mechanical failure at this critical time in performing the ELISA.

S. At the end of incubation for each plate, 100 µl of stop solution is dispensed into each well.
NOTE: the wells only hold 300 µl so do not forget to change the dispenser to a 100-µl volume.

T. After stopping a plate, remove the parafilm "bootie" from the bottom optical surface and insert into the plate reader so that well 1A is in the correct orientation as specified by the carrier of the plate reader. Continue in this manner reading all plates until finished. Positive wells will have varying shades of blue-green color. The plate reader is set at a wavelength of 405 nm.

U. ELISA results are saved into the computer and printed out after the assay is completed (Figure 3). The data should be exported into a software database (Windows KC3 and EXCEL) that can provide, at a minimum, the means and standard deviations of paired samples.

V. The substrate and stop dispenser heads are rinsed with distilled water and air dried.

VI. Interpretation of ELISA Results

A. ELISA controls

A typical ELISA printout for the EL 310 is illustrated in Figure 3 for control plate #1 where the threshold value for an Rs-positive sample was 0.095. The negative kidney controls, blanks, substrate chromogen and conjugate controls should range near 0.070 to 0.085 while the same controls using the ELx 800 range lower near 0.036 to 0.047. Dilutions of whole cell antigen and negative kidney tissues spiked with Rs antigen are relatively close in OD values at the high end of antigen concentration regardless of the reader used but fall lower with the ELx800 at lower antigen concentrations that are closer to the negative-positive threshold value.

BIO-TEK MICROPLATE READER 12/21/ 2008 AT 02:46 PM 00000778

ASSAY # 367

PLATE # 1 OPERATOR

NOTES

PROGRAM MODE #4 SINGLE WAVELENGTH: 405

TABLE OF ABSORBANCE VALUES

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.072	0.078		2.321	0.688		0.083	0.074	0.075	0.072	0.078	0.076
B	0.071	0.082		2.190	0.713		0.078	0.073	0.079	0.074	0.079	0.087
C	0.073	0.080		2.286	0.670		0.077	0.075	0.074	1.993	0.078	0.074
D	0.072	0.082		2.270	0.639		0.079	0.074	0.075	0.072	0.077	0.077
E	0.072	0.076		1.130	0.319		0.078	0.071	0.073	0.074	0.082	0.073
F	0.072	0.076		1.194	0.351		0.076	0.075	0.078	0.073	0.078	0.087
G	0.073	0.075		1.238	0.353		0.078	0.072	0.077	2.059	0.076	0.073
H	0.071	0.076		1.204	0.373		0.078	0.071	0.073	0.073	0.080	0.072

Figure 3: ELISA plate reader printout of control plate #1 from ELISA # 367 . Note paired positive unknown sample in column 10 C&G.

2A-D = negative kidney control; 2E-H = blank control; 1A-D = conjugate control; 1E-H = substrate-chromogen control; 3 and 6 = empty columns; 4 and 5 positive antigen controls; 7 - 12 sample unknowns. In this assay, positive samples were $\geq$ 0.095 in optical density value.

Table 8. Optical density (OD) values from two different models of ELISA readers for whole cell Rs antigen and negative kidney tissue controls "spiked" with Rs antigen.

OD values for whole cell Rs-antigen (KPL)

Rs dilution	EL 310	ELx 800
1:100	2.0 to 2.2	2.0 to 2.1
1:1000	0.68 to 0.77	0.66 to 0.76
1:2000	0.55 to 0.53	0.48 to 0.52
1:5000	0.23 to 0.24	0.19 to 0.20

OD values for kidney "spiked" with Rs-antigen (KPL)

Rs dilution	EL 310	ELx 800	Rs-antigen concentration (µg/ml)
1:10	1.971	1.986	40.000
1:100	1.371	1.329	4.000
1:1000	0.598	0.584	0.400
1:2000	0.427	0.397	0.200
1:5000	0.248	0.209	0.080
1:10,000	0.151	0.117	0.040
1:20,000	0.127	0.099	0.020
1:25,000	0.111	0.080	0.016
1:30,000	0.104	0.073	0.013
1:40,000	0.098	0.067	0.010
Unspiked	0.085	0.053	

Protein concentration of whole cell Rs antigen = 400 µg/ml

All control values should be within expected ranges or the validity of the assay is questionable. However, the values above are only guidelines and will vary somewhat with the individual conditions of the assay at different laboratories using different equipment. Aberrant replicates are also possible due to scratches on the plate surface (despite pre-inspection), air bubbles or chance cross-contamination from handling errors. Except for the odd aberrant wells, replicate OD values should be within 0.011 for negative samples. If not, check for handling, reagent or equipment errors. Replicate values for positive samples will be much farther apart, their ranges tending to increase with increasing antigen levels and OD values. This is likely due to layering of antigen within the samples that cannot be thoroughly mixed which would disturb the pellet.

The OD values may rise and fall from day-to-day variation in the assay within the same lab. If this variation is significant, the average sample unknown OD values can be corrected by the computer using a formula from Pascho et al. (1987) that compares positive antigen control values from a given assay with a normalized absorbance curve derived from many positive antigen controls within the accumulated database.

The statistic is: 1-A where:

$$A = 1/4 \left(\frac{\bar{x}100 - \bar{X}100}{\bar{x}100} + \frac{\bar{x}\,10 - \bar{X}10}{\bar{x}10} + \frac{\bar{x}\,1 - \bar{X}1}{\bar{x}1} + \frac{\bar{x}\,0.1 - \bar{X}0.1}{\bar{x}\,0.1} \right)$$

$\bar{x}_i$ = daily mean absorbance of four test wells at each antigen concentration i,

$\bar{X}_i$ = the grand mean absorbance at antigen concentration i over all days.

If the sum of the squared errors for the corrected absorbance curve is less than that for the uncorrected curve, then the correction factor is applied to the optical densities for all the samples for an assay on a particular day.

If the sum is greater than that for the uncorrected curve then the correction factor is not applied.

B. Positive-negative thresholds

Other ELISA protocols have used two standard deviations from the average OD value of the negative kidney controls as the threshold positive value. Any sample unknown with a value equal to or greater than this threshold is considered Rs antigen-positive.

However, this may be too conservative by representing a very small selected number of individuals with lower OD values that do not reflect the greater normal variation in a population of negative fish. Considerable data points have been statistically analyzed by Commercial Fisheries pathology staff to determine if a more valid method of setting a threshold level is available. These results indicated that the threshold optical density value for an antigen-positive sample should be $\geq$ 0.095 on the EL 310 reader (Meyers et al. 1993b) which corresponds to an approximate OD value of 0.065 to 0.068 on the ELx 800 instrument. These OD values may vary within the databases of other laboratories. Hence, a more standard method of comparison would be an OD threshold value equivalent to a minimum detection level of 10 to 16 ng of Rs antigen per ml of tissue homogenate as indicated by the OD comparisons in Table 8 of the previous page.

VII. Miscellaneous – Protocol for acid washing glassware

A. Ensure you are wearing the proper personal protective equipment including goggles, suitable rubber gloves and a lab coat.

B. Wash glassware that is to be acid washed with soap and water, rinse with tap water to remove soap.

C. Take 1 N hydrochloric acid solution and measure just enough into a beaker to coat all surfaces of the glassware to be acid washed.

D. Pour acid into the cleaned glassware and swish around to ensure all surfaces have been coated with the acid. Continue for approximately 2 minutes or more, depending on the size of the glassware being washed.

E. Pour used acid into a beaker and add enough liquid neutralizer until the solution is completely neutralized per product instructions. Turn on the tap water in the sink and pour the neutralized solution down the drain allowing the water to flow for at least two minutes to ensure adequate flushing of the neutralized acid.

F. Using distilled water, rinse the glassware three times thoroughly to ensure removal of all contaminants and remaining acid.

G. The glassware is ready to be dried and sterilized.

VIII. References

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

IX. ELISA worksheets

NOTE: Exact numerical quantities of reagents needed were calculated first with surplus for error added on. However, the final dilution ratios used may be further adjusted upward to provide whole, even numbers. This provided adequate reagent with insignificant waste and ease of computation. Coating antibody and conjugate working dilutions are provided as examples but may differ in other laboratories depending on the potency of the reagents and/or equipment optical density readings.

Reagent Dilutions for ELISA - 2 Plates

Coating Antibody (168 wells; 200 µl) (72 fish samples)

Plate 1 - 72 wells (24 controls, 48 unknowns)
2 - 96 wells
168

168 wells for antibody (minus the 8 wells of SC, CC)

Coating Buffer (10 x) 168 wells x 200 µl = 34 ml
34 + 2 ml (controls) + 8 ml (surplus) = 44 ml

5 ml buffer concentrate + 40 ml dH_2O = 45 ml buffer solution (save 2 ml)

Coating Ab - 168 wells x 200 µl = 34 ml + 8 ml (surplus) = 42 ml

42 µl coating Ab + 42 ml coating solution

Conjugate - 180 wells x 200 µl = 36 ml + 8 ml (surplus) = 44 ml

Milk diluent (20 x) = 2.2 ml concentrate
41.8 ml dH_2O
44.0 ml
Conjugate 0.1 mg/ml = 100 µg/ml
1 µl : 1999 µl milk diluent = 2 ml 44/2 = 22

22 µl conjugate + 44 ml milk diluent

Substrate Chromogen - 184 wells x 200 µl = 36 ml + 8 ml (surplus) = 44 ml

22 ml Solution A 22 ml Solution B 44 ml

Stop -(5x) Need 2 x solution at 100 µl for 184 wells
2 x = 1 ml concentrate + 1.5 ml dH_2O

Need 184 x 100 µl = 18 ml + 8 ml (surplus)
= 26 ml

11 ml (5x) + 16.5 ml dH_2O

Wash Buffer - (20x)

4 L = 200 ml concentrate + 3,760 ml dH_20 + 40 ml 1% Thimerosal

Reagent Dilutions for ELISA - 4 Plates

Coating Antibody (360 wells, 200 µl) (168 fish samples)

Plate 1 - 72 wells (24 controls, 48 unknowns)
2 - 96 wells
3 - 96 wells
4 - 96 wells
360 wells

360 wells for antibody (minus 8 wells of SC, CC)

Coating Buffer - (10 x) 360 wells x 200 µl = 72 ml
72 + 2 (controls) + 6 ml[1] (surplus) = 80 ml

8 ml buffer concentrate + 72 ml dH_2O = 80 ml buffer solution (save 2 ml)

Coating Ab - 360 wells x 200 µl = 72 ml + 8 ml (surplus) = 80 ml

80 µl coating Ab + 80 ml coating solution

Conjugate - 364 wells x 200 µl = 72 ml + 8 ml (surplus) = 80 ml
Milk diluent (20 x) = 4 ml concentrate
= 76 ml dH_2O
80 ml

Conjugate 0.1 mg/ml = 100 µg/ml
1 µl : 1999 µl milk diluent = 2 ml 80/2 = 40

40 µl conjugate + 80 ml milk diluent

Substrate Chromogen - 368 wells x 200 µl = 73 ml + 8 ml (surplus) = 81 ml

41 ml Solution A 41 ml Solution B 82 ml

Stop -(5x) Need 2 x solution at 100 µl for 368 wells
2 x = 1 ml concentrate + 1.5 ml dH_2O

Need 368 x 100 µl = 37 ml + 8 ml (surplus)
= 45 ml

Stop - 18 ml (5x) + 27 ml dH_2O

Wash Buffer - (20x)

4 L = 200 ml concentrate + 3,760 ml dH_2O + 40 ml 1% Thimerosal

[1] Surplus changed to 6 ml due to wear of this particular dispenser head.

Reagent Dilutions for ELISA - 6 Plates

Coating Antibody (552 wells, 200 µl) (264 fish samples)
Plate 1 - 72 wells (24 controls, 48 unknowns)
2 - 96 wells
3 - 96 wells
4 - 96 wells
5 - 96 wells
6 - 96 wells
= 552 wells for antibody (minus 8 wells of SC, CC)

Coating Buffer - (10 x) 552 wells x 200 µl = 110 ml
110 + 2 ml (controls) + 8 ml (surplus) = 120 ml

13 ml buffer concentrate + 112 ml dH_2O = 125 ml buffer solution (save 3 ml)

Coating Ab- 552 wells x 200 µl = 110 ml + 8 ml (surplus) = 118 ml

122 µl coating Ab + 122 ml coating solution

Conjugate - 556 wells x 200 µl = 111 ml + 8 ml (surplus) = 119 ml

Milk diluent (20 x) = 6 ml concentrate
114 ml dH_2O
120 ml

Conjugate 0.1 mg/ml = 100 µg/ml
1 µl : 1999 µl milk diluent = 2 ml 120/2 = 60

60 µl conjugate + 120 ml milk diluent

Substrate Chromogen - 560 wells x 200 µl = 112 ml + 8 ml (surplus) = 120 ml

60 ml Solution A 60 ml Solution B 120 ml

Stop - (5x) Need 2 x solution at 100 µl for 560 wells
2 x = 1 ml concentrate + 1.5 ml dH_2O

Need 560 x 100 µl = 56 ml + 8 ml (surplus) = 64 ml

Stop - 27 ml (5x) + 40 ml dH_2O

Wash Buffer - (20x)

4 L = 200 ml concentrate + 3,760 ml dH_2O + 40 ml 1% thimerosal

Reagent Dilutions for ELISA - 8 Plates

Coating Antibody (720 wells, 200 µl) (336 fish samples)

Plate
1 - 72 wells (24 controls, 48 unknowns)
2 - 96 wells
3 - 96 wells
4 - 96 wells
5 - 96 wells
6 - 96 wells
7 - 72 wells (24 controls, 48 unknowns)
8 - 96 wells
720 wells for antibody (minus 16 wells of SC, CC)

Coating Buffer - (10 x) 720 wells x 200 µl = 144 ml
144 + 4 ml (controls) + 8 ml (surplus) = 156 ml

16 ml buffer concentrate + 144 ml dH_2O = 160 ml buffer solution (save 5 ml)

Coating Ab - 720 wells x 200 µl = 144 ml + 8 ml (surplus) = 152 ml

155 µl coating Ab + 155 ml coating solution

Conjugate - 728 wells x 200 µl = 146 ml + 8 ml (surplus) = 154 ml

Milk diluent (20 x) = 8 ml concentrate
152 ml dH_2O
160 ml

Conjugate 0.1 mg/ml = 100 µg/ml
1 µl : 1999 µl milk diluent = 2 ml 156/2 = 78

78 µl conjugate + 156 ml milk diluent

Substrate Chromogen - 736 wells x 200 µl = 148 ml + 8 ml (surplus) = 156 ml

78 ml Solution A
78 ml Solution B
156 ml

Stop - (5x) Need 2 x solution at 100 µl for 736 wells
2 x = 1 ml concentrate + 1.5 ml dH_2O

Need 736 x 100 µl = 74 ml + 8 ml (surplus)
= 82 ml

Stop - 34 ml (5x) + 51 ml dH_2O

Wash Buffer - (20x)

4 L = 200 ml concentrate + 3,760 ml dH_2O + 40 ml 1% Thimerosal

Reagent Dilutions for ELISA - 10 Plates

Coating Antibody (912 wells, 200 µl) (432 fish samples)
Plate
1 - 72 wells (24 controls, 48 unknowns)
2 - 96 wells
3 - 96 wells
4 - 96 wells
5 - 96 wells
6 - 96 wells
7 - 72 wells (24 controls, 48 unknowns)
8 - 96 wells
9 - 96 wells
10 - 96 wells
912 wells for antibody (minus 16 wells of SC, CC)

Coating Buffer - (10 x) 912 wells x 200 µl = 183 ml
+ 4 ml (controls) + 8 ml (surplus) = 195 ml

20 ml buffer concentrate + 180 ml dH_2O = 200 ml buffer solution (save 5 ml)

Coating Ab - 912 wells x 200 µl = 183 ml + 8 ml (surplus) = 191 ml

195 µl coating Ab + 195 ml coating solution

Conjugate - 920 wells x 200 µl = 184 ml + 8 ml (surplus) = 192 ml

Milk diluent (20 x) = 10 ml concentrate
190 ml dH_2O
200 ml

Conjugate 0.1 mg/ml = 100 µg/ml
1 µl : 1999 µl milk diluent = 2 ml 196/2 = 98

98 µl conjugate + 196 ml milk diluent

Substrate Chromogen - 924 wells x 200 µl = 185 ml + 8 ml (surplus) = 193 ml

98 ml Solution A 98 ml Solution B 196 ml

Stop - (5x) Need 2 x solution at 100 µl for 928 wells
2 x = 1 ml concentrate + 1.5 ml dH_2O

Need 928 x 100 µl = 93 ml + 8 ml (surplus)
= 101 ml

Stop - 44 ml (5x) + 66 ml dH_2O

Wash Buffer - (20x)

4 L = 200 ml concentrate + 3,760 ml dH_2O + 40 ml 1% Thimerosal

CHAPTER 10

Labeling Procedures for Laboratory Specimens

Joseph R. Sullivan

Case material in use for more than a few moments needs to be sufficiently labeled so that any other diagnostician/technician can understand its significance. Inadequately labeled case material may be irretrievably confused with other samples if its location within a group of similar tests is shifted. Some general concepts are provided below, followed by specific degrees of labeling to be applied to histology blocks and slides, FAT and general bacteriology material. Additional comments regarding other tests and samples can be found in their respective chapters.

General

All material that is useful must have the case accession number and specimen number. An inoculated tube of bacteriologic medium, for example, would have the accession number, the sample number, a single colony isolate (SCI) code (which could include an organ code and a number specifying a particular isolate), the date inoculated (very important for reading test results) and the initials of the person performing the test. A motility slide, conversely, may have the sample number, the SCI code and nothing else. Such slides are only momentarily useful, then discarded, and more than one case would not typically be processed at a time.

All controls must be labeled with the cases to which they apply. Control samples only apply to unknown samples processed the same day with the same reagent material.

I. FAT

A. Bags of kidneys or fish
   1. Accession number
   2. Brood year, stock, and species
   3. Sample date
   4. Initials of the labeler
   5. Name of person assigned to perform the tests (labeler should contact this person)

B. Individual sample bags of kidneys or fish
   1. Unique sample number
   2. Sex (if appropriate)

C. FAT sample slide
   1. Accession number
   2. Test specified
   3. Date processed
   4. First, fifth, sixth, and tenth or last sample on each slide (1, 5, 6, 10, 11, 15, 16, 20, 21, 25, 26, 30, etc.)
   5. Sex (if appropriate)
   6. Organ/lesion designation (if not kidney)
   7. Single colony isolate designation (if from bacterial culture)

D. FAT Control
    1. Applicable accession numbers
    2. Test specified
    3. Marked locations of control material

E. Flats or boxes for storing slides
    1. Accession number
    2. Personnel initials
    3. Date prepared

F. Antisera/conjugates (store in Whirl-Pak® bags with extra labels for attachment to work sheets)
    1. Substance identification (including species source of antiserum, organisms for which it is specific and whether with or without FITC, etc.)
    2. Manufacturer
    3. Lot number
    4. Date reconstituted
    5. Dilution of stock solution
    6. Rhodamine dilution
    7. Status of filtering
    8. Initials of responsible personnel

G. Worksheets
    1. Accession number
    2. Brood year, stock, species
    3. Date sample read
    4. Direct or indirect test
    5. Label of antiserum and conjugate from Whirl-Pak® bags
    6. Control results
    7. Test results

II. Bacteriology

A. Agar plate/slant tube for primary isolation
    1. Medium acronym
    2. Accession number
    3. Specimen number
    4. Organ sampled
    5. Date struck
    6. Personnel initials

B. Agar plates struck for single-colony isolation
    1. Medium acronym
    2. Accession number
    3. Specimen number
    4. Single-colony isolate (SCI) designation; may be sequential with no letters repeated within a specimen number regardless of the organ sampled or derived as an acronym from the organ using a numerical sequence (e.g., 880099-4C is the third colony isolated from fish 4 in 880099; 880086-3K2 is the second colony isolated from the kidney of fish 3 in 880086); label colonies selected on original streak plate with this designation

5. Date struck
6. Personnel initials

C. Tube tests/ multitest strips
   1. Accession number
   2. Specimen number
   3. SCI designation
   4. Date inoculated
   5. Personnel initials

D. Slide tests
   1. Specimen number
   2. SCI designation
   3. Accession number

E. Flats or boxes for storing slides
   1. Accession number
   2. Personnel initials
   3. Date stored

F. Controls
   1. Accession number
   2. Identity of the organism (including ATCC or FPS code as well as genus and species)
   3. Date inoculated

G. Identification sheets
   1. Accession number
   2. Specimen number
   3. Organ struck
   4. SCI designation
   5. Initial isolation medium
   6. Incubation temperature
   7. Incubation time prior to selecting SCI
   8. Test results

NOTE: Particularly with respect to bacteriology, a log of events and their dates should be kept in a lab notebook. If, for example, 10 kidneys are sampled but nothing grows on 9 of them, there will be no bacterial organism sheets for these 9 fish. However, it is important to note that they were sampled. Also, SCI codes must be explained in the lab notebook. Otherwise, no one will be able to interpret what has been done.

III. Histology

Histology blocks and slides need a standard labeling scheme. Two different formats are used for labeling slides; one for temporary diagnostic use and the other for reference collections. The following guidelines should be used when labeling histology blocks and slides.

A. Labeling blocks

The paraffin block number will consist of the accession number followed by the block number. In some cases the block number will be followed by a capital letter. The following guideline should be used in assigning block "numbers".

1. Finfish
    a. When small fish or animals are embedded whole, assign each block a number (but no letter) in addition to the accession number. There may be one or more animal(s) per block. This will not affect the block number.
    b. When portions of a large animal are embedded into several blocks, each block receives a number, which corresponds to the animal, and a letter, which represents the contents of the block. An explanation of the block codes should be recorded on the laboratory worksheet for that case. An example might help illustrate this: (see Chapter 6)

        Parts of the kidney, gills and liver of an adult fish are to be embedded into three different blocks. The blocks would be labeled with the accession number followed by a number and a letter. (e.g. "96-0000-1A", "96-0000-1B" and "96-0000-1C"). The 1A, 1B, and 1C indicate that each block contains a portion of animal "1" and the letter (A, B, or C) represents the kidney, gills and liver. The key to the letters is recorded on the Laboratory Worksheet for that case. Additional information regarding the sample, such as which raceway it came from or state of the animal (e.g. moribund, healthy, normal), etc. should be recorded on the laboratory worksheet, not on the blocks or slides.

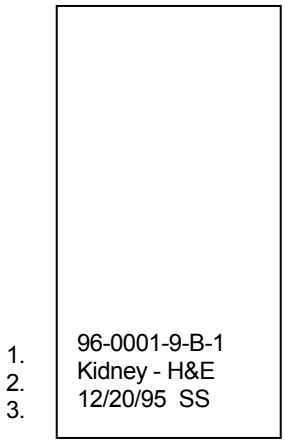
        The main point is that blocks with the same number followed by different letters are parts of the same animal.
2. Bivalve Molluscs

    Adult bivalve molluscs are cut into four pieces, of which thick sections from three are embedded (from the same animal). See Figures 1 and 2 in histology Chapter 6 for further clarification. It is often possible to put 2 sections into one block. It is much more efficient to fill a block with 2 or more sections rather than using smaller blocks to keep each section separate. Each animal receives a number and if multiple blocks are used for that animal, each block will have a unique letter that follows the animal number. This letter only means that the block is one of 2 or more blocks for that animal. Unlike the fish illustration in Section A.1.b above, the letters will not designate a specific part of the animal.
3. Crustacea (see Chapter 6)

B. Slide Labels for Diagnostic Use

Most slides that are labeled will be for diagnostic purposes. Generally, two or three lines of labeling on each slide will be sufficient. The information should be presented as follows (See example 1): Label with a soft lead pencil which will not wash off in solvents. After section has been stained use a slide label printed from computer.

Line 1: Accession number, dash (or space), block number/letter, dash, slide number (relative to other slides made from the same block) (e.g., 960001 - 10B-1)
Line 2: (Optional) This line may contain additional information helpful to the reader. Examples of such information are: types of tissue, description of section or stain used
Line 3: Date slide was made and initials of technician

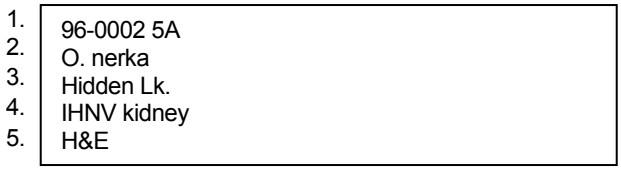


Example 1. Diagnostic Slide Label

C. Reference Slide Collection

1. Label slide with a computer slide label. The label should be placed so that it is readable on the left side of the slide. There are software programs available that will produce small print on small labels for slides.
2. Information provided on the slide should minimally include the following (See example 2):

Line 1: Accession Number (top line) - include block number and letter
Line 2: Species
Line 3: Stock (river, stream, lake, etc.)
Line 4: Tissue and/or feature of interest (e.g., IHNV in kidney or kidney/normal)
Line 5: Stain (e.g. H&E, Gram's)



Example 2. Reference Collection Slide

CHAPTER 11

Digest Methods for Detection of the Whirling Disease Agent (*Myxobolus cerebralis*)

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I. *Myxobolus cerebralis* Survey Procedure (Modification)

A. Remove head including all gill arches for fish less than 6 inches total length. For larger fish a wedge of tissue is cut in the following manner:
A triangular-shaped wedge is cut posterior to the orbit at the dorsal surface almost to the ventral edge of the opercula. The top (dorsal) part of the wedge should measure 1.5 cm. Then, 1/2 of wedge may be placed in fixative should later confirmation be necessary.

B. Optional, only when clinically infected fish are suspected. Check muscle and brain tissues for spores. Cut 1 cm x 1 mm thickness from skin above lateral line and from brain and squash on slides for temporary wet mount. Observe for large cysts.

C. Thaw heads the night before processing, pool them as needed, and heat as follows:
juvenile to adult trout in 60°C waterbath for 10 minutes in a Whirl-Pak® bag.
salmon adults (size may preclude pooling) - Autoclave in autoclave bag for 15 minutes (one layer deep). Autoclaving is not necessary if wedges are sampled.

D. Samples are ground in a strainer using a spatula and water spray to remove soft tissues (skin, eyes, lower jaw, muscle). Discard soft tissues.

E. Weigh cleaned samples and blend with pepsin solution (20 ml/g) in a sterile electric blender. Decant into a beaker and stir for at least 30 minutes at 37°C. This is a good stopping point: after placing pepsin on heads, place in refrigerator overnight or until able to continue with procedure the same day. Warm up in water bath when continuing. Digestion may require 2-4 hrs.

F. Centrifuge an aliquot of pepsin digest at 1200 x g for 10 minutes. Pour supernatant into concentrated bleach. Check digest material for spores using a wet mount. If negative, proceed to next step.

G. Add approximately 20 ml trypsin solution/g of undigested specimen to centrifuge tube. Shake tube, then add it to original beaker with remaining specimen. Adjust pH to 8.5 with 1 N NaOH. Digest at room temperature for minimum of 30 minutes. (Be sure to sanitize pH probe after use).

H. Pass digested specimen through cheese cloth and save fluid. Autoclave cheesecloth. Centrifuge fluid at 1200 x g for 10 minutes. Re-suspend pellet in volume of 10% buffered

formalin small enough to suspend and observe for spores (if sample is from spore-positive area).

I. If no spores are observed in previous steps, layer approximately 1 ml of formalinized sample over 8 ml of 55% dextrose solution (sucrose or percol are good substitutes) 1 cm depth of sample to 5 cm depth of dextrose. Centrifuge in a swinging bucket rotor at 1200 x g for 30 minutes.

J. Examine pellet for spores. A malachite green stain of air-dried material may help in spore detection at any of the above steps.

*Acknowledge Dr. Rich Holt, Oregon Department of Fish and Wildlife, for providing initial procedure, 1987.

SOLUTIONS

Pepsin:	To 1 L water add 5.0 g pepsin and 5 ml concentrated hydrochloric acid.
Trypsin:	Make a 1-L solution of distilled water containing 0.2 g EDTA, 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g $NaHPO_4$ and 5.0 g Trypsin.
Malachite Green:	1% solution in water. Stain air-dried smear with Malachite for 1 min, wash with water then destain in 70% ethanol for 30 sec and 100% ethanol for 30 sec. Let slide dry and coverslip with immersion oil.

II. Identification of *Myxobolus cerebralis*

Myxobolus cerebralis can easily be confused with its congeners in the genus *Myxobolus*; therefore, the following should facilitate identification. In wet mount and, in some cases, stained preparations from digested specimens, it is difficult to distinguish these species. A combination of several species may be detected in one sample.

Myxobolus cerebralis: ovoidal front, lenticular profile; 2 pyriform polar capsules at anterior end. Sporoplasm without iodinophilous vacuole; therefore, no stain is taken up by the sporoplasm and the entire spore stains the same color using Lugols iodine. Vacuole staining is not a very accurate method due to variation within the species. Some spores have unusual processes but generally are 2/3 the size of *Myxobolus kisutchi*. *M. cerebralis* is about 6-10 µm and is histozoic in cartilage/bone tissue, primarily in the head, but also in the spinal column. Capsules are about 2/5 of spore length. Some common hosts of *M. cerebralis* include *Oncorhynchus nerka*, *O. clarki*, *O. mykiss*, *O. aquabonita, Salmo salar, Salvelinus fontinalis*, and *O. tshawytscha*. Some refractory hosts include *O. kisutch* and *S. trutta, Salvelinus namaycush* and *splake*.

Myxobolus kisutchi: Ovoidal with 2 polar capsules at anterior end. Sporoplasm with iodinophilous vacuole. Sometimes with posterior prolongation of shell. It is about 7-8.5 µm and is histozoic in or adjacent to nervous tissues. Hosts include *O. kisutch*, *O. tshawytscha*, and *O. mykiss*. *Myxobolus insidiosus* spores are pyriform or tear-drop shaped and the long

axis is longer than *M. kisutchi*. The iodinophilous vacuole stains dark orange if the spore is young and the storage area has not been used up. Giemsa stains vacuoles very well. Skeletal muscle is infected and white patches on the skin have been associated with heavy infections.

Other species of Myxosporidia that may confuse diagnosis of *M. cerebralis* in salmonids include:

Myxobolus dermatobius : in *O. kisutch*: spores 8-10 µm in size. In skin under epithelium or scales. Narrow ends of polar capsules widely apart. Produces ulcers.

Myxobolus squamalis: in *O. kisutch, O. mykiss*. Found in scale pockets. Scales are raised, giving the appearance of warts. Fixed spores are 8-9 µm in diameter, uniform and have equal polar capsules with a narrow ridge paralleling either side of suture ridge.

Myxobolus neurobius: in *O. kisutch*, *O. nerka, Thymallus arcticus, S. trutta*, *S. alpinus, Salmo salar*. In spinal cord, brain, and nerves. Fixed spores pyriform 8 x 10-12 µm. Polar capsules occupy less than half of the spore length. Fresh spores are larger (8-14 µm).

Myxobolus arcticus: in central nervous system of *O. kisutch, O. nerka, S. malma, S. neiva* (Russian char), *T. arcticus, S. alpinis,* and *Coregonus clupeaformis.* Fresh spores are large, 7.5 x 14-16 µm, with elongated polar capsules.

M. insidiosus: in muscle of *O. clarki, O. tshawytscha,* and *O. kisutch* of Western U. S. Fresh spores are similar in size and shape to *M. arcticus* (9-11 x 12-17 µm).

III. Confirmatory Diagnosis of *Myxobolus cerebralis*

A. Confirmatory diagnosis may be accomplished by histology: Fresh or frozen heads are fixed in Bouin's or Davidson's. These fixatives are preferred over 10% neutral buffered formalin because the acetic acid assists in decalcification. Decalcify as specified in Section V, then paraffin embed, section and stain (May-Gruenwald Giemsa or Toluidine blue). Scan cartilage tissues at 200-400x magnification. The presence of spores in cartilage confirms diagnosis.

B. Although the direct FAT method has been a suggested confirmatory means (Markiw 1992), experience suggests this technique should be treated with caution because of cross reactivity with other *Myxobolus* spp. from Pacific Northwest salmonids. (Ancillary note: FAT conjugates against BKD may also cause fluorescence with *Myxobolus* spores)

C. An alternative to homogenization of head and cartilage tissues is the performance of PCR (Andree et al. 1998) on 1/2 of each sample and preserve the remaining 1/2 for histology. Preserved tissues from the PCR-positive samples would then be processed for histology to further confirm the presence of parasite stages (see Molecular Techniques chapter).

IV. References:

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V. Decalcification Procedure for Detection of Whirling Disease

A. Purpose

Tissue sectioning of large fish heads for the presence of *Myxobolus cerebralis* spores can be facilitated by chemical decalcification of fixed bone/cartilage and of frozen samples which are later placed into a fixative. Only heads or wedges from fish over 8" should be decalcified. Wedges from fish 6-8" in length are adequately decalcified in Bouin's fixative.

B. Equipment/supplies

Lerner D-Calcifier solution (Hydrochloric acid, polyvinylpyrrolidone)
Dissecting needles
pH paper
Forceps
Beakers
Pipets
Magnetic stirring rods
Stirring plate
Graduated cylinders
Watch glasses
0.1 N NaOH

Scalpels
5% Ammonium Oxalate solution (NH) C O H O

C. Procedure

1. A 2½ - x 1½- x ¼-cm section of the skull (Bouin's fixed) is removed with scalpel and forceps from an area encompassing the otolith/auditory canal. The section is rinsed, weighed and placed in a cassette. The size of the section may vary according to the size of cassettes, molds, etc., that are available in the lab.
2. The cassette is placed in a beaker with a magnetic stirring rod and sufficient D-calcifier to provide a ratio of 20 ml D-calcifier to 1 g of tissue sample. Cassettes from the same case number or lot of fish may be pooled into one beaker. Care must be taken not to overload the beaker with cassettes, or the stirring rod will not function. Stirring assists in decreasing the decalcification time by increasing the permeation of the specimen with the solution. Cassettes should be labeled with a marker that will withstand acidic solutions, or identity of the specimens will be lost. The beakers should be covered to reduce evaporation and for safety reasons.
3. The covered beakers are placed onto a stirring plate for 4-16 hours at room temperature. The time depends on the thickness of the specimen and the amount of bone/cartilage present. If laboratory personnel cannot be present to monitor this process during these hours, then the cassettes must be removed from the solution, rinsed with distilled water, and submerged in a beaker of distilled water until the next day when the digestion procedure can be resumed with fresh decalcifier.
4. The degree of remaining calcification is tested by a combination of physical and chemical testing during the decalcification process.

D. Physical test

A dissecting needle is pressed against the bone/cartilage area to test its softness. Puncturing is not suggested since this alters the integrity of the specimen. This physical test will give some indication of the progress of the digestion and should be performed approximately 3 hours after the initiation of digestion and periodically thereafter.

E. Chemical test

Decalcification is complete when the chemical test cannot detect any calcium in the decalcification solution.

1. This test requires removal of 5 ml of the decalcifying solution from the beaker of specimens after 3 hours of decalcification, and also every hour thereafter; and placing that amount into a small beaker with a piece of pH paper.
2. The solution is neutralized (pH 7.0) with 0.1 N sodium hydroxide as indicated by the pH paper which is immediately removed with forceps.
3. 1 ml of ammonium oxalate solution is added to the neutralized solution and mixed. This solution is allowed to stand for at least 15 minutes to determine if a precipitate forms. The precipitate is calcium oxalate and indicates that the decalcification is incomplete. Decalcification should be continued until no calcium oxalate precipitate can be detected.
4. After decalcification is complete, the specimens are rinsed in distilled water and loaded into a tissue processor.

F. Discussion

This procedure was developed for the histological examination of large heads that were fixed or frozen/fixed. A 10% nitric acid solution was found to be essentially equal to the D-calcifier in decalcification time and effectiveness, but required a larger ratio of solution per gram of specimen (50:1). The D-calcifier was selected as the agent of choice due to its commercial availability. Other commercial products, especially those that do not require overnight monitoring of the decalcification process, and formic acid may also be suitable but have not been tested.

Giemsa stains of decalcified tissue sections are not as intense as normally fixed samples, but *Myxobolus cerebralis* spores are still evident. Specimens have also been "partially" decalcified before loading them into the tissue processor and these specimens, although a little more difficult to section, appeared to retain better staining properties.

CHAPTER 12

Mycology

Tamara Burton

I. General Information

A. Organisms in the kingdom Fungi are multicellular, eukaryotic organisms, commonly having hyphae (mycelium) and whose nutritional mode of living is by absorptive nutrition.

B. In various stages and under various conditions, fungi exist as amoeboid or flagellated cells, have defined size and body shape, exist as yeasts, or are composed as individual threadlike structures called hyphae.

C. Fungi are found in almost any aerobic habitat. In general fungi are aerobic organisms with a few that can be facultative anaerobes.

D. In general, fungi reproduce both sexually and asexually with the spore as the dissemination product. An exception to this is the imperfect fungi that have no known sexual reproduction. In general, fungi produce incredibly large numbers of spores.

E. The systematics of fungi are based on the type of spore, how it is produced and the type of spore producing structures.

F. Large numbers of fungi are being discovered and described all the time and standard classification is constantly changing in this kingdom.

G. Fungi are difficult to classify because of their ability to change the way they look and act depending on their environment and their life stage. On different medias, fungi may express very different characteristics so when identifying fungi, it is imperative that you use the media that was used when the fungus was described in the reference you are using.

H. Because of their hyphal wall and because they are common symbionts of plants, fungi sometimes are associated with botany, however fungi do not contain chlorophyll and are NOT plants.

I. Many fungi are able to live as parasites of animals where they can cause disease, however the majority of fungi are saprobes, primarily living on dead organic matter.

II. Classification and some genera isolated from fish

The true fungi are divided into 5 subdivisions based on the type of reproduction.

A. Mastigomycotina (aquatic fungi) – This subdivision is basically the water molds*. Hyphae are aseptate and relatively large in size. Both sexual and asexual reproduction occurs. Spores are flagellated and are capable of locomotion. Baited water cultures may be necessary to identify these to the species level (see baiting on page 12-4).

1. *Saprolegnia*
2. *Achlya*
3. *Dictyuchus*
4. *Aphanomyces*

*Note: Based on genetic sequencing, water molds (oomycetes) have been tentatively re-classified as the class Oomycota in the phylum Heterokontophyta more closely related to photosynthetic brown algae and diatoms.

B. Zygomycotina - The hyphae are large and mostly aspetate. These fungi generally grow quickly and develop into fluffy usually light colored mycelium.

1. *Basidiobolus*
2. *Mortierella*
3. *Rhizopus*
4. *Amylomyces*
5. *Actinomucor*
6. *Absidia*
7. *Mucor*

C. Ascomycotina (sac fungi) –This is a large group that includes many yeasts, lichens, and some mushrooms. Reproduction can be sexual with the formation of spores in sacs called asci or asexual by the formation of conidia or by budding.

1. *Mycosphaerella*
2. *Penicillium*
3. *Chaetomium*

D. Basidiomycotina (club fungi) – This group includes many mushrooms. They form sexual spores in club shaped structures called basidia or reproduce asexually by forming conidia.

1. *Sporidiobolus*

E. Deuteromycotina (imperfect fungi) – This is the group that includes most fish fungal pathogens. Hyphae are septate and mostly thin and fine. Some "imperfect" yeasts are in this group. No sexual reproduction is known to occur. Reproduction is accomplished asexually through the formation of conidia in various types of spore producing structures.

1. *Phoma*
2. *Aspergillus*
3. *Cladosporium*
4. *Exophiala*
5. *Phialophora*
6. *Scolecobasidium*
7. *Bullera*
8. *Candida*
9. *Trichosporon*
10. *Sepedonium*
11. *Chrysosporium*

III. Media and Reagents

A. Corn Meal Agar (CMA)
Suspend 17.0 grams of dehydrated medium in 1 liter of distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C. Final pH = 6.0 ± 0.2 at 25°C.

B. Potato Dextrose Agar (PA)
Suspend 39 grams of the dehydrated medium in 1 liter of distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. To reduce bacterial growth on primary isolation add 1.6 ml of sterile 10% tartaric acid per 100 ml medium. After addition of acid, do not heat the medium . Cool medium to 45°C and pour into petri plates. Final pH = 3.5 ± 0.2 at 25°C.

C. Oatmeal Agar (OA)
Suspend 72.5 grams of dehydrated medium in 1 liter of distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C.

D. Sabouraud Dextrose Agar (SAB)
Suspend 65.0 grams of dehydrated medium in 1 liter of distilled water and heat to boiling to dissolve completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C. Final pH = 5.6 ± 0.2 at 25°C.

E. Pond Water/Seawater Agar
Pond water or seawater can be coarsely filtered with filter paper. Then add 2% agar and bring to a boil to dissolve agar. Autoclave for 15 minutes at 15 pounds pressure. Add 2 g/L ampicillin or chlorophenicol before pouring into petri plates.

F. V-8 Juice Agar

V-8 Juice	200.0 ml
$CaCO_3$	3.0 g
Agar	15.0 g
Water	800.0 ml

Mix and heat above until dissolved completely. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C. Final pH = 7.2 ± 0.2 at 25°C.

G. Potato Carrot Agar

Potatoes	20.0 g
Carrots	20.0 g
Agar	15.0 g
Water	1000.0 ml

Dice potatoes and carrots and cook in water for 30 minutes. Remove potatos and carrots and add agar. Autoclave for 15 minutes at 15 pounds pressure. Pour into petri plates. Store at 2-8°C.

H. Hemp and Sesame Seed Baits
Boil hemp seeds for 15 minutes to sterilize and rupture the ovary and seed coat. After cooling the white hypocotyl should be visible but if not, aseptically pinch the seed to rupture the coat. Sesame seeds are prepared by autoclaving. Baits can be stored in a sterile container at 2-8°C until needed.

I. Shear's Mounting Medium

Glycerine	120 ml
Ethyl Alcohol 95%	180 ml
Potassium acetate (2%) in Quink (Parker blue fountain pen ink)	6 ml
Distilled water	300 ml

IV. Isolation and Sub-culture Techniques

A. Direct hyphae transfer method.
A small sample of fungal hyphae is removed from a lesion and teased apart. Aseptically force the hyphae into the agar; sterile, curved forceps work well for this procedure. Several hyphae can be placed around the periphery of the same plate.

B. Tissue explant
Place a small piece of infected tissue in the center of a medium plate and press firmly onto the agar. Fungal hyphae will grow out from the explant on the fungal medium.

C. Sub-culture
To sub-culture or isolate cut out a small area of agar containing the hyphae or growth of interest using a sterile scalpel. Avoid obvious contaminating bacteria or other organisms. Mount the agar upside down (hyphae side down) by putting it onto the center of a new medium plate and pressing gently. When spore structures are visible (e.g. pycnidia) the fungus can be sub-cultured by taking a sterile needle to collect some spores which can be placed onto a new medium plate. If spores are dry, you may need to push the needle into the agar so the spores adhere to the medium.

D. Baiting
This technique can be successfully used to culture water molds from a water sample by providing a specific substrate for fungus colonization. Water molds (e.g. *Saprolegnia*) are strongly attracted to hemp seeds and sesame seeds. Prepared seeds (as described in media section) are placed into the container with the water sample. Baits are examined until hyphae are visible. This technique can also be used to grow fungal hyphae from a lesion or culture. The seeds are put into sterile water and hyphae are aseptically added to the water.

V. Culturing and Identification

A. Inoculation and Incubation

1. Media must be used that will allow for growth and isolation of the fungi and also encourage sporulation since classification is based on the morphology of spore producing structures. For routine cultures, use PA and either OA or CMA. NaCl can be added at a concentration of 1.5% if the donor is a marine fish or the fungus is suspected to be of marine origin.
2. Fungal cultures should be kept for a minimum of a month before being terminated as negative. Many fungi grow very slowly on artificial medias. Some fish fungi will grow fine at room temperatures, however for routine culturing of fish fungi from arctic species, use 16°C for a standard incubation temperature.
3. If a fungus fails to produce spore structures on the initial medium, try other medias, e.g. sabouraud dextrose agar, V-8 juice agar, or potato carrot agar. Remember you must grow the fungus on the medium that was used when it was described, since fungi exhibit very different characteristics on different media.

4. If sporulation does not occur, some alternatives can be tried to induce sporulation. Try different media, different incubation temperatures, and alternating incubation in periods of light and dark.
5. For aquatic fungi such as *Saprolegnia*, CMA, pond water agar, or a water culture baited with seeds may be necessary.

B. Reading of Cultures

1. Cultures used for identification should be free of contaminants such as other fungi and bacteria. Antibiotics can be added to fungal medias to reduce or eliminate bacterial contamination. Sub-culture as necessary to obtain a pure culture.
2. Identify the isolates as soon as possible after initial isolation. Continued sub-culturing can cause the fungi to become sterile or mutated.
3. Examine cultures several times a week for growth.
4. When present, describe the gross appearance and growth characteristics of the fungal colonies making sure to note medium type. Note obverse and reverse colony color, describe hyphae, mold or yeast structure, pigments, visible spore structures, etc. Re-describe over time as characteristics of fungi readily change as they age in culture.

C. Microscopic examination

1. If the fungus is an aquatic fungus, prepare a wet mount in sterile water. Shear's Mounting Medium can also be used if staining is desired or you want to make a semi-permanent mount by sealing the edges of the coverslip with clear fingernail polish or Permount.
2. If the fungus is terrestrial, use water or sometimes a wetting agent makes a better wet prep. 3% Triton X-100 or Kodak Photo-Flow work well. Shear's can also be used for wet mounts and semi-permanent mounts.
3. Describe the hyphal characteristics including if they are septate or aseptate, appearance, color, size, shape, branching, straight, curved, etc.
4. Describe any reproductive structures and spores present. If none are present, re-incubate, or try some of the techniques described to induce sporulation.
5. Based on observations above, try to classify the fungus into one of the sub-divisions. Use available references and keys to identify the fungus.

VI. Commercial Identification Systems – Fungi that exhibit only yeast forms can be identified using the API 20 C AUX Yeast Identification System. Follow procedures as outlined in the kit.

VII. Maintenance/Preparation of Stock Cultures

A. Stock cultures are kept in the -70C freezer with an assigned fungus "F" number. Each new isolate, in addition to the case number, is assigned a fungus number and the data is added to the fungus data spreadsheet.
B. To prepare an isolate for the isolate collection, sub-culture the fungus in pure culture to a fungus medium slant that is prepared in a 6 dram, flat bottomed screw cap tube. Allow the fungus to grow close to the edge of the agar slant. Store in the fungus isolate boxes in the ultracold freezer.

VIII. Safety Measures

A. Never smell a fungus culture as fungal spores are readily transferred through the air. Many fungi are irritating to mucus membranes and some can be opportunistic pathogens.

B. Only open petri plates when necessary to sub-culture or make slides. Do so quickly and immediately close the plate lid.
C. If cultures are held for a long period in petri plates, the edges of the plate can be sealed with laboratory tape.
D. Autoclave all cultures before discarding. Do not allow old fungal cultures to accumulate.
E. For long term holding or storage of fungal cultures, use agar slants in wide mouth screw-cap tubes.
F. Disinfect all bench tops and working areas immediately after use.

IX. References and Keys

A. Identification Manual for Fungi From Utility Poles in the Eastern United States. C. J. K. Wang and R. A. Zabel, eds. 1990. ATCC. Kansas.
B. The Genera of Fungi Sporulating in Pure Culture. J. A. Von Arx. 1981. J. Cramer. Germany.
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CHAPTER 13

Molecular Techniques
Polymerase Chain Reaction (PCR)

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I. Introduction

The polymerase chain reaction (PCR) technique is used in the Anchorage Fish Pathology Laboratory primarily as a confirmatory procedure and in a few cases as a screening tool. We use a variety of different PCR techniques. Traditional PCR uses oligonucleotide primers to amplify DNA segments of genes for target pathogens. Reverse transcriptase PCR (RT-PCR) uses an initial reverse transcriptase step so that complimentary DNA can be amplified from viral RNA. A nested PCR technique is also used in some tests where initial amplified products are re-amplified using a second round of PCR to increase test sensitivity. In general, DNA or RNA is extracted from various fish tissues or laboratory assay products (viral isolates, bacterial isolates, etc.) and amplified using forward and reverse primer sets. PCR amplified products, or amplicons, are either visualized using DNA stains and gel electrophoresis which provide a qualitative result or by using quantitative or real-time PCR which detects the quantity of amplified product as it is produced using a fluorescent signal.

II. Quality Assurance and General Guidelines for PCR

A. General Information

1. Separate areas of lab space are used to reduce risk of contamination. Contamination most often is caused by amplicons from previous assays which can easily be transported by aerosols, clothing, hands or equipment. We use separate dedicated areas for PCR work. Samples are collected in the middle lab most often used for necropsy. PCR reagents are dispensed in the Airclean 600 PCR Hood. Samples are dispensed in the Environmental Air Control Hood which is also used for setting up viral assays. The PCR thermocycling and gel electrophoresis is done on the counter of the virology laboratory. DNA staining and visualization is done in the DNA staining room. In general, separate lab coats should be used for each area and gloves changed prior to entering or exiting.
2. Always wear disposable gloves for all stages of the PCR process. Change gloves often. Always use fresh gloves and lab coat when moving into the “clean” PCR area.
3. Always dispose of gloves, PCR products and consumables into the PCR waste container. The container should be emptied daily during PCR work and should only be used for PCR disposables.
4. Decontaminate all work surfaces with 10% chlorine bleach or a commercial reagent like DNA Away before and after use. Use plastic-backed laboratory bench liners during the testing procedures and dispose of used liners into a PCR waste container post amplification. Remember that alcohol is often used to preserve DNA and WILL NOT effectively decontaminate DNA from utensils and surfaces.
5. Disinfect sample racks, pipettes and other PCR equipment with 10% bleach solution after every use. Products such as DNA or RNAase Away may also be used and are sometimes preferable since they can be less caustic to delicate equipment.

6. Turn on the UV light in the clean PCR hood (AirClean) for at least 30 minutes after use. The UV will essentially "UV crosslink" potential DNA contaminants making them unamplifiable in future PCR runs.
7. The main PCR bench work area is equipped with an overhead UV light which turns on automatically every night for 60 minutes. Occasionally check to make sure the unit is operating properly.
8. Only use aerosol resistant pipette tips and separate designated pipettors for each aspect of PCR preparations. Pipettors are labeled accordingly. At a minimum, separate dedicated pipettors for clean reagents, samples, and amplified products are absolutely mandatory.
9. Use screw cap microcentrifuge tubes where possible as the snap top tubes can cause aerosolization.
10. Always briefly centrifuge tubes and plate strips prior to opening to reduce aerosolization of contents.
11. When preparing master mix, prepare enough extra volume for positive, negative and no template controls. Always add enough for a few extra reactions.
12. Minimize freeze-thaw cycles of working primer solutions.
13. Keep reagents on ice while preparing working stocks or master mix.
14. Use a PCR worksheet (gel map or plate map) for each assay to keep track of sample locations, reagents, concentrations used, etc. After the assay is completed, all worksheets are kept in the individual notebook for the type of test performed. See Appendix A for worksheet examples.
15. All original worksheets along with printed photo documentation of gel results are kept indefinitely for historical reference in the notebooks, even though original gel pictures are also saved on the computer.

B. Sample collection and extraction --general considerations
1. Collect tissue samples for PCR on a clean benchtop which has been disinfected using 10% bleach or DNA Away. If in the field or if disinfection is not possible, use a disposable work surface (foil, plastic-backed paper, etc.) and replace between samples.
2. Use sterile collection tools between samples. Alcohol will not effectively decontaminate DNA from utensils. If disposable utensils are not possible, use alcohol and flame between samples to effectively remove DNA contaminants from previous samples.
3. Collect samples from fresh tissues if possible or tissues that have been frozen at -70ºC.
4. Always keep samples cold and freeze as soon as possible until processing can be done.
5. Since RNA can be very sensitive to breakdown, tissue samples for fish virus testing (IHNV, VHSV) should be immediately placed into RNAlater to store until processing.
6. Follow individual protocols for specifics concerning sample handling and extraction for the various tests performed.
7. Always use micro-centrifuge tubes with screw-cap lids (PCR tubes) and that are RNA/DNA free.
8. Use amount of tissue suggested in the extraction kit protocol for the specific type of tissue you are using.
9. Use a designated set of calibrated pipettors for sample extraction.
10. Extract a known positive tissue sample and a known negative tissue sample from the start of the extraction process through amplification whenever possible. These controls will allow for detection of contamination and successful extraction of the target product.
11. If a protocol suggests or if extraction quality is suspect, determine that an appropriate amount of DNA or RNA was extracted using a spectrophotometer. A good reference for this is the Blue Book procedure for analysis of extracted DNA using a UV spectrophotometer (Blue Book section 6.5).
12. Freeze extracted DNA at -70ºC if PCR testing will not be done within a week.
13. When opening and closing tubes containing DNA samples of any kind, avoid touching the inside of the cap and rim of the tube to prevent contamination of the samples.

C. Equipment

1. Thermal cyclers – The laboratory maintains two Bio-Rad iCyclers. One of the units is dedicated to PCR assays that use gel electrophoresis for amplicon detection and the second unit is equipped with the iQ5 real time detection system and is used for quantitative real time assays. The bases of the two instruments are the same and thus interchangeable.
   a. Calibration Module for Real Time PCR **-** Instrument calibration is required for the real time thermal cycler at a minimum of every six months or if a new reaction volume, dye layer, vessel type or seal is used in the assay. A calibration log is maintained within the program and recorded by the technician in a log book. The software will only alert to an expired calibration prior to initiating a run. Therefore, it is important to review current calibrations before each run.
      1). Calibration files can be viewed by selecting Calibration Data from the View menu.
      2). The background factors file is in the Background Factors folder in the iQ5 Program Folder.
      3). Persistent Well Factors are in the Well Factors folder in the iQ5 Program Folder.
      4). Pure Dye Calibrations will be found in the RMEData folder in the iQ5 Program Folder.
   b. Performing Calibrations for the real time iQ5 instrument - Refer to the iQ5 Manual for stepwise preparation and calibration of the iQ5 instrument. You will need the following to calibrate the iQ5 camera and prepare the external well factor plate, the background calibration plate, and the pure dye calibration plate.
      1). iCycler iQ Calibrator Dye Solution Set
      2). iCycler iQ External Well Factor Solution (found in the iQ Calibrator Dye Solution Set)
      3). 3 x 96 well PCR plate or preferred reaction vessel
      4). Optical quality sealing tape or preferred sealing method

2. Centrifuges - Separate centrifuges are maintained for sample extraction and pre or post PCR "zip spins". A mini centrifuge is available for tube strips and a plate rotor assembly for the 96 well format. Using centrifuges helps to reduce aerosols when tube caps are opened and helps ensure contents are at the bottom and bubbles are minimized. Centrifuges are to be balanced and operated according to manufacturers' specifications and decontaminated with RNAase away (or similar product) after use.

3. Spectrophotometer – The laboratory has a Bio-Rad SmartSpec Plus spectrophotometer which can be used to provide quantification and purity readings for extracted DNA and RNA The DNA/RNA assay will automatically collect absorbance readings at 260 and 280 nm and will provide the concentration of the nucleic acid sample. The purity of the nucleic acid preparations may be measured with this assay by toggling the 260:280 keypad. The SmartSpec has other assays useful in the analysis of PCR sample preparations which is sometimes used in our bacterial PCR assays. See complete protocols in the SmartSpec Technical manual.

4. Electrophoresis chamber and Power source – We use a Bio-Rad Power Source with a submarine gel system.
   a. Submerge gel in 1X TAE buffer before loading product and connecting to the power source. Place the lid on the electrophoresis chamber carefully, matching the red and black banana jacks on the lid with those on the base. Turn on the power source, select the "run" button to begin and adjust voltage to desired level using up or down arrows.
   b. It is important to keep the wire electrode in the caster submerged with TAE buffer when not in use or rinse the chamber and electrode with distilled water before storing.
   c. Inspect chamber, electrode wires, cables and all parts for build up of dried salts, dirt, cracks, and loose connections before use.
   d. Decontaminate gel unit with bleach or other suitable decontamination product after use.
   e. Always use the same batch of TAE buffer for making a gel and running the same gel.

5. UV transilluminator system with attached camera - this unit is located in the DNA staining isolation room where all gel staining and reading of final gels is performed.
    a. Clean transilluminator plate with paper towels frequently. Clean entire unit occasionally with bleach.
    b. After reading and photograph documentation is complete on gels, all materials should be discarded into the ethidium bromide (EtBr) waste container beside the transilluminator.

D. Controls
1. PCR control material is stored in a -70ºC freezer separate from where reagents are stored.
2. A minimum of one known positive, one known negative and a no template control are included with each PCR run. The no template control should be master mix and serves to QC all of the reagents except the template.
3. Preparing a PCR run of controls only prior to testing a large number of unknowns is desirable if the positive control material has not been tested within one year to insure proper functioning of the control and test reagents.
4. It is a good idea to place positive controls at the end of the tube strips or at the farthest corner on the plate and add them last.
5. Quantified dilutions of control material should be used to generate standard curve for real time PCR, such that QPCR results can be quantified.

E. Waste Disposal
1. PCR waste generated from assay preparations through thermocycling must always be quickly disposed of into dedicated labeled PCR waste containers. At the end of each day that PCR tests are performed, the biohazard bags are sealed and placed on the autoclave cart for sterilization and disposal.
2. Ethidium bromide (EtBr) stain waste is collected each day of use into a waste carboy in the DNA staining room. When the carboy is full, remove EtBr stain from buffer following the outlined procedure. All waste from the DNA staining room is disposed of in the PCR incinerator waste box located inside the DNA staining room. When full, this box is sent to hazardous waste for incineration.
3. EtBr and other DNA stain disposal – We use an extractor device that was developed by Schleicher and Schuell as a way to safely remove EtBr or similar nucleic acid stains from waste buffer and to provide for easy disposal. It consists of two layers of specially formulated activated carbon filters sealed into a polypropylene housing. Using a simple vacuum filtration procedure >99% of DNA stains are removed from up to 10 liters of buffer solution through adsorption to the activated carbon matrix. The decontaminated filtrate can then be safely disposed of down the drain.
4. DNA stain removal using extractor device
    a. Gather necessary materials for filtration of 10 liters or more of waste liquid including a 2 liter Erlenmeyer flask, extraction devise, incineration disposal container, vacuum pump capable of 14 psi, approximately ten feet of vacuum tubing, nitrile gloves, dedicated lab coat and protective eyewear.
    b. Place the Erlenmeyer flask on a bucket in front of the staining table with the extractor devise on top. Place the glass fiber filter inside the Extractor device.
    c. Attach the vacuum hose from the vacuum pump to the extractor devise inlet.
    d. Place the carboy with the waste on the table directly above the extractor device. Place the outlet tube that is attached to the carboy valve inside the funnel of the extractor devise. Be careful as not to damage the glass filter inside. Slowly open the valve to allow just enough liquid to pass into the Extractor with out overflowing the device. Adjust the flow rate as needed to prevent spillage.
    e. Turn on the vacuum pump and adjust pressure to 14 psi. If functioning properly, the vacuum will hold the extractor devise securely on top of the Erlenmeyer flask.

f. When the Erlenmeyer flask is almost full, close the carboy valve and let the remaining waste inside the extractor filter into the flask.
g. After the Extractor is empty, turn the vacuum pump off, remove extractor device from the flask and empty the filtered decontaminated waste down the dedicated drain.
h. Keep track of the volume of waste filtered on the log sheet. Replace filter when 10 liters have been filtered. Dispose of used filter in the PCR waste incinerator container located inside the DNA staining room.

III. PCR Protocols – The Anchorage Fish Pathology Laboratory currently performs a variety of confirmatory and screening PCR protocols for viruses, bacteria, protozoans, and *Mycoplasma*. Infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) isolates are confirmed with reverse transcriptase nested PCR protocols. Single round traditional PCR is used for the confirmation of *Flavobacterium psychrophilum* isolates, in the confirmation and screening for *Ichthyophonus hoferi* and for the detection of *Mycoplasma* contamination in the continuous cell cultures. A nested PCR is used in screening for *Myxobolus cerebralis*. In addition we also have real-time quantitative TaqMan assays used in screening for *Myxobolus cerebralis* and *Renibacterium salmoninarum.*

A. RT-PCR for Infectious Hematopoietic Necrosis Virus (IHNV), nested protocol

1. Viral RNA preparation
   a. This test is performed directly on suspect tissue culture isolates without extraction.
   b. To release RNA from a virus solution, dilute each sample 1:20 and 1:100 in sterile deionized water in 200 µl microcentrifuge tubes.
   c. Place the diluted samples into the thermal cycler set to the RNA release program which heats the preparation to 95^{o}C for 2 minutes. Then store on ice or at 4^{o}C for up to 30 minutes.

2. Reverse transcription and round 1 PCR
   a. Using the Qiagen Taq PCR core kit, thaw 10x PCR buffer, dNTP mix, primer solutions, 25 mM $MgCl_2$, DNA ladder and loading dye. Keep on ice.
   b. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a total of 45 µl master mix per sample and control reaction including a little extra. Use two IHNV isolates for positive controls, uninfected EPC cells and a VHSV isolate for negative controls and a no template control in the calculation.
   c. Master mix volume for each 50µl reaction:
      30.75 µl of DEPC water
      5µ l of 10x buffer w/1.5mM $MgCl_2$
      2 µl 25 mM $MgCl_2$
      1 µl 10 mM dNTP
      2.5 µl of upstream primer at 20 pmoles/µl (5'-TCA AGG GGG GAG TCC TCG A-3')
      2.5 µl of downstream primer at 20 pmoles/µl (5'-CAC CGT ACT TTG CTG CTA C-3')
      0.5 µl Taq Polymerase at 5 U/µl
      0.5 µl AMV reverse transcriptase at 9 U/µl
      0.25 µl RNAsin at 39 U/µl
   d. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
   e. Dispense 45 µl of the master mix into each 200 µl microtubes. Use 50 µl for no template control.
   f. In sample loading area, add 5 µl of template RNA to each microtube. Add controls to control wells. Centrifuge the tubes for 10 seconds to 1 minute (zip spin) at 1000 rpm to make sure the contents are at the bottom.
   g. Place the microtubes in the thermal cycler and start the appropriate program. Be sure

volume is set at 50 µl.
1). 1 cycle at 50°C for 15 minutes
2). 1 cycle at 95°C for 2 minutes
3). 25 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds
4). 1 cycle at 72°C for 7 minutes
5). 1 cycle at 4°C indefinitely
Total run time: Approximately 2 hours

h. Visualize and photograph the 786 bp PCR amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.
i. If the round 1 PCR does not provide sufficient amplified product, continue to the nested round 2 PCR for additional DNA amplification.

3. Nested round 2 PCR
   a. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a total of 48 µl master mix per sample including a little extra.
   b. Master mix volume for each 50 µl reaction:
      34.5 µl of DEPC water
      5 µl of 10x buffer w/1.5 mM $MgCl_2$
      2 µl 25 mM $MgCl_2$
      1 µl 10 mM dNTP
      2.5 µl of upstream primer at 20 pmoles/µl (5'-TTC GCA GAT CCC AAC AAC AA-3')
      2.5 µl of downstream primer at 20 pmoles/µl (5'-GCG CAC AGT GCC TTG GCT-3')
      0.5 µl Taq Polymerase at 5U/µl
   c. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
   d. Dispense 48 µl of the master mix into each 200 µl microtube. Use 50 µl for a no template control.
   e. In sample loading area, add 2 µl of round 1 template to each microtube. Centrifuge the tubes for 10 seconds to 1 minute at 1000 rpm to make sure the contents are at the bottom.
   f. Place the microtubes in the thermal cycler and start the appropriate program. Be sure volume is set at 50 µl.
      1). 1 cycle at 95°C for 2 minutes
      2). 25 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds
      3). 1 cycle at 72°C for 7 minutes
      4). 1 cycle at 4°C indefinitely
      Total run time: Approximately 1 hour
   g. Visualize and photograph the 323 bp PCR amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.

4. Reference:

Arakawa, CK, RE Deering, KH Higman, KH Oshima, PJ O'Hara, and JR Winton. 1990. Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms 8:165-170.

Bill Batts and James Winton, personal communication. Western Fisheries Research Center, Biological Resources Division, USGS, 6505 NE 65th St. Seattle WA 98115

Deering RE, CK Arakawa, KH Oshima, PJ O'Hara, ML Landolt, and JR Winton. 1991. Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms 11: 57-65.

B. RT-PCR for Viral Hemorrhagic Septicemia Virus (VHSV), nested protocol

1. Viral RNA preparation
   a. This test is performed directly on suspect tissue culture isolates without extraction.
   b. To release RNA from a virus solution, dilute each sample 1:20 and 1:100 in sterile deionized water in 200 µl microcentrifuge tubes.
   c. Place the diluted samples into the thermal cycler set to the RNA release program which heats the preparation to 95°C for 2 minutes. Then store on ice or at 4°C for up to 30 minutes.

2. Reverse transcription and round 1 PCR
   a. Using the Qiagen Taq PCR core kit, thaw 10x PCR buffer, dNTP mix, primer solutions, 25 mM $MgCl_2$, DNA ladder and loading dye. Keep on ice.
   b. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a total of 45 µl master mix per sample and control reaction including a little extra. Use two VHSV isolates for positive controls, uninfected EPC cells and a IHNV isolate for negative controls and a no template control in the calculation.
   c. Master mix volume for each 50µl reaction:
      30.75 µl of DEPC water
      5 µl of 10x buffer w/1.5mM $MgCl_2$
      2 µl 25 mM $MgCl_2$
      1 µl 10 mM dNTP
      2.5 µl of upstream primer at 20 pmoles/µl (5'-TCT CTC CTA TGT ACT CCA AG-3')
      2.5 µl of downstream primer at 20 pmoles/µl (5'-TTC CGG TGG AGC TCC TGA AG -3')
      0.5 µl Taq Polymerase at 5U/µl
      0.5 µl AMV reverse transcriptase at 9U/µl
      0.25 µl RNAsin at 39U/µl
   d. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
   e. Dispense 45 µl of the master mix into each 200 µl microtubes. Use 50 µl for no template control.
   f. In sample loading area, add 5 µl of template RNA to each microtube. Add controls to control wells. Centrifuge the tubes for 10 seconds to 1 minute at 1000 rpm to make sure the contents are at the bottom.
   g. Place the microtubes in the thermal cycler and start the appropriate program. Be sure volume is set at 50 µl.
      1). 1 cycle at 50°C for 15 minutes
      2). 1 cycle at 95°C for 2 minutes
      3). 25 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds
      4). 1 cycle at 72°C for 7 minutes
      5). 1 cycle at 4°C indefinitely
      Total run time: Approximately 2 hours
   h. Visualize and photograph the 950 bp PCR amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.
   i. If the round 1 PCR does not provide sufficient amplified product, continue to the nested round 2 PCR for additional DNA amplification.

3. Nested round 2 PCR
   a. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a total of 48 µl master mix per sample including a little extra.
   b. Master mix volume for each 50µl reaction:
      34.5 µl of DEPC water
      5 µl of 10x buffer w/1.5mM $MgCl_2$

2 µl 25 mM $MgCl_2$
1 µl 10 mM dNTP
2.5 µl of upstream primer at 20 pmoles/µl (5'-ATG GGC TTC AAG GTG ACA C-3')
2.5 µl of downstream primer at 20 pmoles/µl (5'-GTA TCG CTC TTG GAT GGA C-3')
0.5 µl Taq Polymerase at 5U/µl

c. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
d. Dispense 48 µl of the master mix into each 200 µl microtube. Use 50 µl for no template control.
e. In sample loading area, add 2 µl of round 1 template to each microtube. Centrifuge the tubes for 10 seconds to 1 minute at 1000 rpm to make sure the contents are at the bottom.
f. Place the microtubes in the thermal cycler and start the appropriate program. Be sure volume is set at 50 µl.
   1). 1 cycle at 95°C for 2 minutes
   2). 25 cycles at 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds
   3). 1 cycle at 72°C for 7 minutes
   4). 1 cycle at 4°C indefinitely
   Total run time: Approximately 1 hour
g. Visualize and photograph the 558 bp PCR amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.

4. References:

Batts, Bill and James Winton, personal communication. Western Fisheries Research Center, Biological Resources Division, USGS, 6505 NE 65th St. Seattle WA 98115.

Batts WN, CK Arakawa, J Bernard, and JR Winton. 1993. Isolates of viral hemorrhagic septicemia virus from North America and Europe can be detected and distinguished by DNA probes. Diseases of Aquatic Organisms 17:67-71.

Einer-Jensen K, NJ Olesen, N Lorenzen, PEV Jorgensen. 1995. Use of the polymerase chain reaction (PCR) to differentiate serologically similar viral haemorrhagic septicaemia (VHS) virus isolates from Europe and America. Veterinary Research 26:464-469.

C. PCR for *Flavobacterium psychrophilum*

1. Preliminary set-up
   a. This PCR targets specific sequences within the 16S rRNA gene of *F. psychrophilum.* When using a bacterial culture, it is not necessary to use a DNA extraction kit such as Qiagen.
   b. Turn on thermal cycler and check desired program parameters. Turn on PCR (Airclean) hood and wipe down with DNAerase. Turn on the Bio-Rad SmartSpec and blank with diluent used to prepare cultures.

2. Control and sample preparation
   a. Prepare positive controls using Spectrophotometer or McFarland standards. After blanking spectrophotometer with buffer or water, adjust control suspension to have an O.D. value of 0.3-0.6 at 525 nm or visually approximate a 5 McFarland standard.
   b. Prepare the PCR template using a fresh culture of the unknown bacteria.
      1). Dispense approximately 2-3 ml of TE buffer or molecular grade water to sterile, glass 15 x 120 mm culture tubes labeled with sample numbers.
      2). Take a loop of bacteria and add to the respective labeled tube of TE buffer and thoroughly mix. The bacterial suspension density is not critical, however, a 5 McFarland or an O.D. value as described previously has given the clearest result band in the gel. For easier handling, transfer 0.5 ml of sample to a 1.5 ml corresponding microcentrifuge tube.
3. PCR Protocol
   a. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube. Use a total of 49 µl

master mix per sample. Be sure to include positive, negative and no template controls in the calculation.

b. Master mix volume for each 50 µl reaction:
   31.75 µl of DEPC water
   5 µl of 10x Buffer with 1.5 mM $MgCl_2$
   1 µl 25 mM $MgCl_2$
   1 µl 10 mM dNTP
   5 µl upstream primer at 20 pmoles/µl (5'-CGA TCC TAC TTG CGT AG-3')
   5 µl downstream primer at 20 pmoles/µl (5'-GTT GGC ATC AAC ACA CT-3')
   0.25 µl Taq Polymerase at 5U/µl
c. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
d. Dispense 49 µl of the master mix into each 200 µl microtube. Be sure to label tube strips.
e. Add 1 µl of bacterial suspension to each microtube. Centrifuge the tubes for about 10 seconds to ensure the contents are at the bottom.
f. Place the microtubes in the thermal cycler and start the appropriate program.
   1). 1 cycle of 95° C for 4 minutes
   2). 30 cycles of 95° C for 45 seconds, 55° C for 60 seconds, 72° C for 90 seconds
   3). 1 cycle of 72° C for 4 minutes
   4). Hold at 4° C indefinitely
   Total run time approximately 2 hours and 45 minutes.
g. Visualize and photograph the 1100 bp amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.

4. References:

NWFHS Laboratory Procedures Manuel, Third Ed., June 2005. Chapter 7, pg. 21, Section 2, PCR for *Flavobacterium psychrophilum, Yersinia ruckeri, and Aeromonas salmonicida.*

Taylor, PW and JR Winton. 2002. Optimization of nested polymerase chain reaction assays for the identification of *Aeromonas salmonicida, Yersinia ruckeri and Flavobacterium psychrophilum*, Journal of Aquatic Animal Health (14)216-224.

D. QPCR for *Renibacterium salmoninarum*, TaqMan Assay

1. Sample preparation and extraction
   a. When using a bacterial culture, extract DNA using Qiagen DNeasy Tissue Kit procedure for Gram positive bacteria.
   b. For ovarian fluids and kidney or other tissues, extract DNA using Qiagen DNeasy Tissue Kit procedure for animal tissues including a few modifications developed by WFRL, USGS personnel.
   c. For ovarian fluids and spiked PBS (for controls and standard curve material) aliquot 50 µl into small tubes. Add 180 µl of ATL buffer and 20 µl of Proteinase K solution to the tubes and incubate at 55°C until completely lysed. Vortex occasionally. This usually takes about 1 hour.
   d. For kidney and other tissues aliquot 50 mg into small tubes. Add 180 µl of ATL buffer and 20 µl of Proteinase K solution. Incubate at 55°C for 1 hour or at 37°C for 1-16 hours or until tissue is completely lysed. Vortex occasionally. Overnight incubation of kidney samples at 37°C is usually effective for complete tissue lysis.
   e. Allow samples to cool to 37°C or below and add 50 µl of 4x lysis buffer (see recipe below). Incubate at 37°C for 1 hour. Vortex occasionally.
   f. Add 4µl RNase A, vortex and incubate at room temperature for 2 minutes.
   g. Add 250 µl of buffer AL (without ethanol) and incubate at 70°C for 10 minutes.
   h. Centrifuge kidney samples for 1 minute at full speed to pellet any melanin in the kidney. Transfer the supernatant to a new tube containing 250 µl of 95% ethanol. Add 250 µl 95%

ethanol to ovarian fluid or PBS samples. Triturate samples to thoroughly mix.

i. Place a spin column in a 2 ml collection tube. Carefully add the mixture to the spin column without moistening the rim, close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 minute.
j. Place the spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
k. Carefully open the spin column and add 500 µl of buffer AW1. Centrifuge at 6000 x g for 1 minute. Place the spin column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
l. Carefully open the spin column and add 500 µl of buffer AW2. Centrifuge for 3 minutes and at full speed.
m. Place the spin column in a clean 1.5 ml microfuge tube and discard the collection tube containing the filtrate.
n. Add 400 µl of buffer AE preheated to 70°C to the spin column and incubate for 1 minute at room temperature. Centrifuge at 6000 x g for 1 minute.
o. If extracted DNA will be used within a week, store in the refrigerator at 4°C. For extended holding, extracted DNA can be frozen indefinitely at -20°C.
p. 4x Lysis Buffer preparation (from Gram positive bacteria section of Qiagen DNeasy Tissue Kit procedure):

Tris-HCl, pH 8.0, 1M	16 ml
EDTA, pH 8.0, 0.1M	16 ml
Triton X-100	9.6 ml
Lysozyme, Sigma #L7651	16 g

Slowly bring up to 200 ml in VG water (~158.4 ml; this tends to foam very badly). Aliquot into small tubes, freeze and store at -20ºC. You can re-freeze unused portions. You may need to warm to get back into solution after thawing.

2. PCR Protocol
   a. Prepare master mix for the number of samples to be analyzed. Work under clean PCR hood and wear gloves.
   b. The master mix for one 24 µl reaction is as follows:
      12 µl ABI TaqMan universal PCR master mix (2x)
      0.48 µl (45 µM) forward primer RS1238
      (5'-GTG ACC AAC ACC CAG ATA TCC A-3')
      0.48 µl (45 µM) reverse primer RS1307
      (5'-TCG CCA GAC CAC CAT TTA CC-3')
      1.0 µl (6 µM) TaqMan probe RSProbe1262MGB
      (6FAM-CAC CAG ATG GAG CAA C-MGBNFQ)
   c. Aliquot 14 µl of master mix to each well. Add 10 µl of water to no template controls (NTC) wells.
   d. Move to sample hood and add 10 µl of extracted DNA to each test well. Be sure to include positive and negative controls. Cover plate.
   e. Centrifuge the plate to eliminate air bubbles in the wells.
   f. Initiate proper protocol on thermal cycler for BKD quantitative PCR as follows:
      1). 50°C for 2 minutes
      2). 95°C incubation for 10 minutes
      3). 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds

3. Quantification
   a. For quantification, prepare a standard curve of serially diluted *R. salmoninarum* DNA representing a range of 5 to 5 x 10^6 cells per reaction.
   b. Run standard curve analysis in triplicate.
   c. To confirm the concentration of *R. salmoninarum* cells, perform either membrane-filtration

FAT or plate counts. To prepare bacterial suspensions, grow *R. salmoninarum* in KDM-2 media with 10% FBS at 15°C, spin bacterial suspension at 5000 x g for 20 minutes, and resuspend cells in 0.01 M PBS, pH 7.4 or filtered ovarian fluid.

4. Data Analysis Guidelines for BKD QPCR
   a. In Bio-Rad iQ5 software go to workshop and results and select data file of interest and then data analysis.
   b. View colors associated with all wells. Change white colors by right clicking, define trace style, select new color, click selected wells, select white well to change to selected color.
   c. View data in PCR baseline subtracted.
   d. Go to log view.
   e. Find highest of "background noise" of wells not amplifying in the linear phase on the graph.
   f. Click results, amplification data tab and find well from e. above and determine highest value (likely at late cycle).
   g. Set threshold one RFU above the background value, right click on graph, baseline threshold, user defined and then add one RFU to number determined and enter new value. Click OK to save.
   h. Remove any problem wells from analysis if necessary.
   i. Reanalyze data with new adjusted threshold.
   j. Select file save. The O drive copy only is saved with new colors and threshold.
   k. Go to reports and select PCR Quant Detailed to print.
   l. Check the following on the report:
      1). Check well factors.
      2). Check validity of background, RME, and well factors.
      3). Check Ct for all unknowns and negative controls which should be N/A if negative. If they are not negative, repeat test to confirm.
      4). Check Ct of BKD controls and compare to standard curve values. Values should be close.
      5). Check standard deviation of control repeats. If SD is not < 1.0, re-run due to poor repeatability of controls
      6). Check defined threshold.
   m. Put results onto datasheet in pink or red.
   n. Save report to file and re-name report to Report PCR Quant Detailed plus date of run.

5. References:

Applegate, Chip, Western Fisheries Research Center, USGS. Personal communication.

Applegate, LynnMarie, Diane Elliott, Maureen Purcel, Connie McCibben, James Woodsen and Samantha Badil. 2009. Comparison of three quantitative polymerase chain reaction (qPCR) methods to detect and enumerate *Renibacterium salmoninarum.* Poster presented at 50th Western Fish Disease Workshop, Park City, Utah.

Bio-Rad Technical Support Staff Webinar and Teleconference on Data Analysis, April 2008.

Chase, Dorothy M., Diane G. Elliott and Ronald J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. Journal of Veterinary Diagnostic Investigation, Brief Communications. 18:375-380.

DNeasy Tissue Handbook. Insert received with Qiagen Uneasy Tissue Kit.

Pascho, Ronald J., Dorothy Chase and Connie 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.

E. PCR for *Ichthyophonus hoferi*

1. Preparation and extraction
   a. This PCR is for the detection of *Ichthyophonus* from tissues using primers Ich7f and Ich6r specific for the small subunit rDNA of the parasite.
   b. Fresh or frozen animal tissues work best. Tissue fixed in ethanol should be washed twice with PBS before processing.
   c. Using a disposable single edged razor blade or scalpel and a piece of coated weigh paper, dice 50 mg tissue into small pieces. Put pieces into a 1.5 ml microcentrifuge tube, and add 180 µl buffer ATL. Use new razor blade and paper for each sample. If using a scalpel, clean between samples by washing with soap and water, place into 20% bleach for 30 minutes and rinse in water or immerse in alcohol and flame between samples.
   d. To lyse, add 40 µl Proteinase K and mix by vortexing for 5 seconds. Place samples on their sides on a rocking platform and tape down to hold in place. Agitate briskly on a rocking platform in a 37°C incubator overnight or in a 55ºC incubator for four hours. Check for complete lysis. Fluid should be clear without visible chunks of tissue present when lysis is complete. After lysis, samples can be stored at 4°C indefinitely.
   e. To continue with extraction, vortex for 15 seconds. Add 200 µl buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70°C in water bath for 10 minutes. Vortex sample several times during the 10 minutes.
   f. Add 200 µl ethanol (96 – 100% not denatured) to the sample, and mix thoroughly by vortexing.
   g. Pipet the mixture (approximately 610 µl) into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 minute at 8000 rpm with hinges pointed down. Discard the filtrate and collection tube. This step is to get rid of the alcohol.
   h. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl buffer AW1 and centrifuge for 1 minute at 8000 rpm. Discard the filtrate and collection tube.
   i. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl buffer AW2 and centrifuge for 1 minutes at 20000 x g (14000 rpm), empty collection tube, using the same collection tube spin again for 3 minutes at 14000 rpm. Discard the filtrate and collection tube.
   j. Place the DNeasy Mini spin column in a clean 1.5-2 ml microcentrifuge (Eppendorf) tube and pipet 100 µl buffer AE directly onto the DNeasy membrane. Use separate tips. You can use up to 200 µl buffer AE for this step. Incubate at room temperature for 1 minute and centrifuge for 1 minute at 6000 x g (8000 rpm) or greater to elute. Yields approximately 100 µl DNA template.
   k. If extracted DNA will be used within a week, store in the refrigerator at 4°C. For extended holding, extracted DNA can be frozen indefinitely at -20°C.

2. PCR Protocol
   a. The PCR master mix preparation is made using the Qiagen Taq PCR core kit.
   b. Thaw 10x PCR buffer, dNTP mix, primer solutions, and 25 mM $MgCl_2$. Keep on ice.
   c. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube. A total of 23 µl master mix/sample. Be sure to include positive and negative controls in the calculation.
   d. Master mix volume/reaction:
      18.375 µl of DEPC water
      2.5 µl 10x buffer
      1 µl of 25 mM $MgCl_2$
      0.5 µl 10 mM dNTP mix
      0.25 µl of Ich7f at 50 pmol/µl
         (5'-GCT CTT AAT TGA GTG TCT AC-3')
      0.25 µl of Ich6r at 50 pmol/µl
         (5'-CAT AAG GTG CTA ATG GTG TC-3')

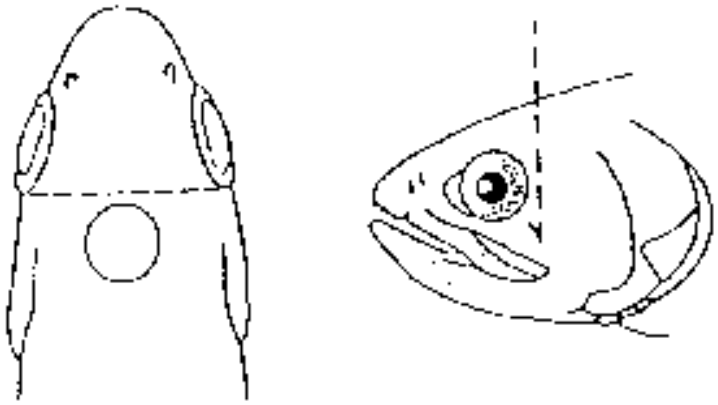
0.125 µl of Taq DNA polymerase at 5 U/µl

e. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
f. Dispense 23 µl of master mix into 200 µl microtubes. Label tubes.
g. Add 2µl of template DNA and controls to microtubes containing the master mix. Centrifuge microtubes for 1 minute at 1000 rpm to make sure all the ingredients are at the bottom.
h. Place the microtubes in the thermal cycler and start the appropriate program.
   1). 1 cycle at 95°C for 3 minutes
   2). 35 cycles at 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 60 seconds
   3). 1 cycle at 72°C for 7 minutes
   4). 1 cycle at 4°C indefinitely
   Total run time: Approximately 2 hours
i. Visualize and photograph the 371 bp PCR amplicon by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.

3. Reference:
Whipps, Chrostopher, Tamara Burton, Virginia Watral, Sophie St-Hilaire and Michael Kent. 2006. Assessing the accuracy of a polymerase chain reaction test for *Ichthyophonus hoferi* in Yukon River Chinook salmon, *Oncorhynchus tshawytscha.* Diseases of Aquatic Oganisms 68(2):141-147.

F. PCR Protocol for the Detection of *Myxobolus cerebralis*, nested protocol

1. Reagent preparation and DNA extraction is performed using purification of total DNA from rodent tail protocol in the DNeasy Tissue Handbook with USFWS modifications.
   a. Check AL and ATL buffers for precipitates. If precipitates are present, warm buffers at 55ºC until fully dissolved.
   b. Add appropriate amounts of 100% pure ethanol to buffers AW1 and AW2 as indicated on the bottles.
   c. Mix buffer AL with ethanol for use below. Mix 200 µl with 200 µl of ethanol per preparation. The AL-ethanol mixture is stable for at least 3 months when stored at room temperature.
   d. The water bath temperature should be 55ºC for tissue lysis.
   e. Sever head from freshly dead fry or fingerlings with sterile scalpel or disposable razor blade. If using scalpel use 70% alcohol and flame between fish heads.
   f. Place fish heads onto separate tared weigh paper. If fish is less than 2 gm use whole head, if greater than 2 gm split head and use half. For large fish, biopsy punch of caudal ventral portion of the skull and defleshing of tissue is necessary (see American Fisheries Society Fish Health Section Blue Book diagram below). Place pooled bone pieces in plastic, round bottom 15 ml culture tubes adding enough water to cover material. Place tubes in 95°C waterbath for up to one hour. Pour off water and dump contents onto weigh paper and deflesh.
   g. Always mince head tissue well after defleshing. If pooling (five fish) the same paper may be used for all fish in the pool. Place 25 mg of minced fish head pieces into a labeled 1.5 -2 ml microcentrifuge tube. Use new razor blade and paper for each sample or pool. If using a scalpel, clean between samples by washing with soap and water, place into 10-20% bleach for 30 minutes and rinse in water or immerse in alcohol and flame utensils between samples. If available disposable utensils like single edged razor blades may be used and discarded between samples or pools.
   h. Add 180 µl buffer ATL to 25 mg sample.



i. To lyse add 40 µl Proteinase K and mix by vortexing for 5 seconds. Ensure tissue is completely submerged in buffer and pro K. Put in a 55ºC shaking waterbath for 6-8 hours (or overnight). Fluid should be clear without visible chunks of tissue present when lysis is complete. If lysate appears gelatinous, reduce the amount of starting material and cut into smaller pieces for future preparations. After lysis, samples can be stored at 4°C indefinitely.
j. After lysis is complete, vortex for 15 seconds. Add 410 µl of the buffer AL-ethanol mixture to the sample and mix thoroughly by vortexing,
k. Pipet all the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 minute at 8000 rpm with hinges pointed down for one minute. Discard the filtrate and collection tube.
l. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl buffer AW1, and centrifuge for 1 minute at 8000 rpm. Discard the filtrate and collection tube.
m. Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl buffer AW2, and centrifuge for 3 minutes at 6000 x g (8000 rpm).
n. Carefully remove the DNeasy mini spin column and place in a clean 1.5-2 ml microcentrifuge (Eppendorf) tube. Pipet 200 µl of prewarmed (70°C) buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 minute and centrifuge for 1 minute at 6000 x g (8000 rpm) or greater to elute. Do not discard the filtrate.
o. As an optional step you can add another 200 µl of pre-heated buffer AE. Incubate for 1 minute and centrifuge for 1 minute at 6000 x g (8000rpm). Note: for higher yield of DNA, you may run the same 400 µl of AE buffer through the spin column one more time.
p. Discard spin column. If extracted DNA will be used within a week, store in the refrigerator at 4°C. For extended holding, extracted DNA can be frozen indefinitely at -20°C. Recommended to store in elution buffer because storing DNA in water may cause acid hydrolysis.

2. Round 1 PCR
   a. Thaw 10x PCR buffer, dNTP mix, and primer solutions. Keep on ice.
   b. Turn on thermal cycler and check program parameters and cycles.
   c. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a 50 µl total reaction volume. Prepare 47µl master mix/sample. Be sure to include positive, negative and no template controls in the calculation.
   d. Master mix volume/reaction:
      35.6 µl of DEPC water
      5 µl 10x buffer with 1.5 mM $MgCl_2$
      2 µl 25 mM $MgCl_2$
      2 µl 10mM dNTP mix
      1 µl of Tr3-16 primer 40 µM (5'-GAA TCG CCG AAA CAA TCA TCG AGC TA -3')
      1 µl of Tr5-16 primer 40 µM (5'-GCA TTG GTT TAC GCT GAT GTA GCG A- 3')
      0.4 µl of Taq DNA polymerase at 5 U/µl

e. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
f. Dispense 47 µl of master mix into 200 µl microtubes for each reaction. Label end of tubes with row number.
g. Move to template loading workstation (virology hood). Add 3 µl of template DNA and controls to microtubes containing the master mix. Centrifuge microtubes for 1 minute at 1000 rpm to make sure all the ingredients are at the bottom of the microtube.
h. Place the microtubes in the thermal cycler and start the appropriate program.
   1). Denature at 95^{o}C for 5 minutes
   2). 35 cycles at 95^{o}C for 60 seconds, 65^{o}C for 2.5 minutes, 72^{o}C for 1.5 minutes
   3). 1 cycle at 72^{o} C for 10 minutes.
   4). 1 cycle at 4^{o}C indefinitely
   Total run time: Approximately 3.5 hours
i. Centrifuge microtubes for 1 minute at 1000 rpm.

3. Nested round 2 PCR
   a. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a total of 49 µl master mix per sample including a little extra.
   b. Master mix volume for each 50 µl reaction:
      37.6 µl of DEPC water
      5 µl 10x buffer with 1.5 mM $MgCl_2$
      2 µl 25 mM $MgCl_2$
      2 µl 10mM dNTP mix
      1 µl of Tr3-17 at 40 µM (5'-GC ACA CTA CTC CAA CAC TGA ATT TG -3')
      1 µl of Tr5-17 at 40 µM (5'-GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3')
      0.4 µl of Taq DNA polymerase at 5 U/µl
   c. Mix the master mix thoroughly by vortexing the microcentrifuge tube.
   d. Dispense 49 µl of the master mix into each 200 µl microtube. Use 50 µl for no template control.
   e. In sample loading area, add 1 µl of round 1 template to each microtube. Centrifuge the tubes for 10 seconds to 1 minute at 1000 rpm to make sure the contents are at the bottom.
   f. Place the microtubes in the thermal cycler and start the appropriate program. Be sure volume is set at 50 µl.
      1). Denature at 95^{o}C for 5 minutes
      2). 35 cycles at 95^{o}C for 60 seconds, 65^{o}C for 2.5 minutes, 72^{o}C for 1.5 minutes
      3). 1 cycle at 72^{o} C for 10 minutes.
      4). 1 cycle at 4^{o}C indefinitely
      Total run time: Approximately 3.5 hours
   g. Visualize and photograph the 1300 bp amplicon (round 1) and the 425 bp PCR amplicon (round 2) by electrophoresis of the product in a 2.0% agarose gel with ethidium bromide staining. Observe using UV transillumination.

4. References:
American Fisheries Society Fish Health Blue Book, 2005; Chapter 5.2
Andre, Karl B. (1998) A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*, Diseases of Aquatic Organisms Vol 34:145-154.
Idaho Fish Health Center, Laura Kessel (USFWS) nested Mc PCR procedure.
Kelly, Garry, et al (2004), Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*, Journal of Veterinary Diagnostic Investigation 16:202-211.

G. QPCR Protocol for the Detection of *Myxobolus cerebralis*, TaqMan Assay

1. Reagent preparation and DNA extraction is performed using the purification of total DNA from rodent tail protocol in the DNeasy Tissue Handbook with USFWS modifications. Follow the DNA extraction protocols previously discussed in the nested PCR for *Myxobolus cerebralis* section.

2. PCR Protocol
   a. Remove reagents (primers Myx 18-909f, Myx 18-996r, BSA, TaqMan probe working solution) from freezer. Keep on ice. Remove ABI Master Mix (2x) and water from the refrigerator.
   b. Turn on thermal cycler and camera. Start Bio-Rad iQ5 program and check program parameters and cycles for Mc real time assay.
   c. Prepare the master mix in a clean 1.5 or 2 ml microcentrifuge tube for a 20 µl total reaction volume. Prepare 16 µl master mix/sample.
   d. Master mix volume/reaction:
      3.58 µl of DEPC water
      10 µl 2x ABI universal PCR master mix
      0.5 µl 100x BSA
      0.8 µl Myx18-909f forward primer 10 pmoles/µl
      (5'-CTT TGA CTG AAT GTT ATT CAG TTA CAG CA-3')
      0.8 µl Myx18-996r reverse primer 10 pmoles/µl
      (5'-GCG GTC TGG GCA AAT GC-3')
      0.32 µl of TaqMan probe Myx18-953 6000 pmols
      (6FAM-ACC GGC CAA GGA CTA ACG AAT GCG-TAMRA)
   e. Mix the master mix gently by vortexing the microcentrifuge tube. Zip spin.
   f. Dispense 16 µl of master mix into 96 well PCR plate and 20 µl master mix into wells designated for NTC.
   g. Move plate to template loading workstation (virology hood). Add 4 µl of template DNA and controls to 96 well plate using a plate map. Run samples in triplicate. Carefully add plastic film to top of plate, holding only the edges. Centrifuge plate for 1 minute at 900 rpm to make sure all the reagents are at the bottom of the plate wells.
   h. Place the 96 well plate in the thermal cycler and start the appropriate program (Myx. cerebralis RT) Reporter=FAM and Quencher= TAMRA
      1). 50°C for 2 minutes
      2). 95°C for 10 minutes
      3). 50 cycles of 95° for 15 sec and 60° C for 60 seconds.
   i. In the Bio-Rad iQ5 software, select plate and protocol for run (run set) and begin run. Save file as Mc date.
   j. Go to Data analysis and select report type and "save file" to O drive QPCR, *M. cerebralis*, results.

3. Data Analysis Guidelines for QPCR *M. cerebralis*
   a. In Bio-Rad iQ5 software go to workshop and results and select data file of interest and then data analysis.
   b. View colors associated with all wells. Change white colors by right clicking, define trace style, select new color, click selected wells, select white well to change to selected color.
   c. View data in PCR baseline subtracted.
   d. Go to log view.
   e. Find highest of "background noise" of wells not amplifying in the linear phase on the graph.

f. Click results, amplification data tab and find well from e. above and determine highest value (likely at late cycle).
g. Set threshold one RFU above the background value, right click on graph, baseline threshold, user defined and then add one RFU to number determined and enter new value. Click OK to save.
h. Remove any problem wells from analysis if necessary.
i. Reanalyze data with new adjusted threshold.
j. Select file save. The O drive copy only is saved with new colors and threshold.
k. Go to reports and select PCR Quant Detailed to print.
l. Check the following on the report:
   1). Check well factors.
   2). Check validity of background, RME, and well factors.
   3). Check Ct for all unknowns and negative controls which should be N/A if negative. If they are not negative, repeat the test.
   4). Check Ct of Mc controls and compare to standard curve values. Values should be close.
   5). Check standard deviation of control repeats. If SD is not < 1.0, re-run due to poor repeatability of controls
   6). Check defined threshold.
m. Put results onto datasheet in pink or red.
n. Save report to file and re-name report to Report PCR Quant Detailed plus date of run.

4. References

Arsen, Leyla, (2006) Thesis: Potential for Dispersal of the Non-native Parasite *Myxobolus cerebralis*: Qualitative Risk Assessments for the State of Alaska and the Willamette River Basin, Oregon, Oregon State University.

Bio-Rad Technical Support Staff Webinar and Teleconference on Data Analysis, April 2008.

Oregon State University, Sascha Hallett. (2007). *Mxyobolus cerebralis* QPCR Long Version and QPCR Short Version, documents prepared by J. Dubanoski.

H. PCR for Mycoplasma

1. General information

   Cell cultures used at the Fish Pathology Laboratories are screened for the presence of Mycoplasma contaminates by PCR twice yearly. Several PCR based Mycoplasma detection kits are available. We are currently using the Sigma-Aldrich VenorGeM (MP0025) PCR based kit. This PCR targets the 16S rRNA coding region of the Mycoplasma genome, which allows for the detection of all *Mycoplasma, Acholeplasma* and *Ureaplasma* species which are usually encountered as contaminants in cell cultures. The detection kit should come with a positive control and an internal control preparation to indicate a successful PCR. General guidelines for sample preparation and PCR protocol are presented below, however it is important to follow the technical instructions that are included in the kit selected.

2. Sample and reagent preparation
   a. Cell lines should be cultured in the absence of antibiotics for several days prior to testing. Cultures should be 90-100% confluent. Inhibitory substances may accumulate in the medium of older cultures and it is strongly suggested to perform an extraction of the cell culture sample. The Blood Gnomonic DNA Kit (Sigma NA2000) and extraction protocol included in the kit is the recommended choice for this procedure.
   b. Before rehydrating the kit tubes, centrifuge to ensure that the lyophilized contents are spun down to the bottom of tube.
   c. Add the appropriate amount of deionized, DNA-free water to the labeled reagent tubes. Use chart provided in the package insert to determine amount of water per reaction.

d. Incubate reagents for five minutes at room temperature.
e. Vortex tubes and centrifuge again to insure contents are well mixed and at bottom of tube.
f. Keep reagents on ice while using and store at -20ºC after rehydration.

3. PCR Protocol
   a. Master mix volume/reaction:
      15.1 µl of DEPC water
      2.5 µl of 10x Buffer (Blue cap) with 3.0 mM $MgCl_2$ final concentration
      2.5 µl Primer/nucleotide mix (Red cap)
      2.5 µl Internal control (Yellow cap)
      0.4 µl Taq Polymerase (Catalog Number D9307 Jumpstart taq) at 2.5 U/µl
   b. Mix the master mix thoroughly by vortexing the microcentrifuge tube. Dispense 23 µl of master mix preparation into 200 µl microtubes.
   c. Add 2 µl of deionized, DNA-free water as a negative control into appropriate reaction tube and seal.
   d. Pipette 2 µl of positive control DNA into positive control tube.
   e. Add 2 µl of sample to each microtube. Centrifuge the tubes for about 10 seconds to insure the contents are at the bottom.
   f. Place the microtubes in the thermal cycler and start the following cycle:
      1). 1 cycle of 94^o C for 2 minutes
      2). 39 cycles of 94^o C for 30 seconds, 55^o C for 60 seconds, 72^o C for 30 seconds
      3). Cool down to 4-8^o C.
      Total run time: 3 hours
   g. Use 1.5-2.0% standard agarose gel. Load each PCR reaction, mixed with bromophenol blue loading buffer. Stop electrophoresis after 2 cm run distance at 100V.

3. Gel evaluation
   a. A distinct 191 bp band should appear (internal control) in every lane indicating a successfully performed PCR.
   b. Interpretation of possible band patterns:

Band pattern	Interpretation
Band at 191 bp	Negative sample
Band at 270 bp and 191 bp	*Mycoplasma* positive
Strong band at 270 bp	*Mycoplasma* positive, heavy contamination
No bands	PCR inhibition, insufficient polymerase activity

5. References:
VenorGeM Mycoplasma Detection Kit, PCR based Sigma-Aldrich MP0025 technical bulletin.

IV. General guidelines for preparing and running electrophoresis gels

A. Preparation of 1.5% - 2.0% agarose electrophoresis gels
   1. To prepare a 2% gel, add 3 gm agarose to 150 ml 1x TAE buffer in an Erlenmeyer flask. To prepare a 1.5% gel, add 2.25 gm agarose to 150 ml 1x TAE buffer. Heat in microwave on high for 3 minutes. Make sure agarose is completely dissolved, if not continue to microwave until dissolved.
   2. Allow agarose to cool to the touch or temper in 55ºC waterbath. Slowly pour into gel holder being careful to not form bubbles. Place appropriate comb(s) into the liquid gel and let harden for approximately 1 hour. Remove combs.

B. Preparing DNA amplicons and running gel

1. Add 2.5 µl loading dye to each 25 µl amplicon. Adjust accordingly for different amounts of product.
2. Add 2.5 µl loading dye to 12 µl of 100 bp DNA ladder (enough for 2 wells).
3. Mix the dye and sample by flicking bottom of microtube.
4. Centrifuge the samples for about 10 seconds at 1000 rpm (zip spin) so contents are at the bottom of the tube.
5. Remove gel from holder and slowly place into electrophoresis chamber. Cover with 1x TAE buffer so the gel is totally submersed.
6. Load 12 µl sample and 6 µl ladder into sample wells of the gel following the map.
7. Connect electrical terminal with the negative (black) on top and the positive (red) on the bottom.
8. Set voltage at 100 volts and set time for 60 minutes.
9. Turn on power unit and watch for bubbles in the TAE buffer indicating that the current is flowing. Do not touch the TAE buffer while the power unit is on.
10. After about 2 minutes, check to see if the samples and dye are running in the right direction, down toward the positive terminal.
11. Run for the set time checking occasionally so the DNA does not run too far.
12. Turn off power supply, and carefully remove gel to transfer dish.

V. General preparations for gel staining and visualization of PCR products

A. Staining gels with ethidium bromide (EtBr)
   1. Staining using DNA stains is done in the separate DNA staining room. Stained gels and staining reagents are not taken out of this room.
   2. Use all established laboratory precautions when using EtBr. Dedicated lab coats, gloves, and eyewear should be worn without exception in this room. Disposable dedicated lab coats are left in the room upon exit. Periodically change out lab coats and dispose of old in the EtBr disposal box.
   3. The gel to be stained is brought into the room in the gel transfer tray dedicated for this purpose.
   4. Pour TAE buffer into a dedicated staining dish. Usually 400 ml of TAE buffer is sufficient to cover the gel.
   5. Add 5 µl of EtBr/100 ml of TAE buffer. Mix by rocking the dish.
   6. Gently add gel to staining liquid, rock staining dish every 5-10 minutes.
   7. Staining time varies from 10 minutes to 1 hour depending on gel thickness, 15 minutes works well for 100-150 ml TAE gels.
   8. If necessary de-stain for 10–30 minutes in de–ionized water. Repeat with fresh water if needed.

B. Visualization and photo documentation of PCR products
   1. Enter “Doc-it” program on PCR computer and select tab for Advanced Capture.
   2. Set optical zoom to 11.2mm, 18.5 x 14.0 cm.
   3. Remove gel from staining solution or water and view with ultraviolet transilluminator at 300 nm.
   4. Click on “Capture” button on computer to transfer the image to the computer.
   5. Save picture to O drive, DCF, PATHLOG folder under gel pictures.
   6. Discard staining liquid and rinse into dedicated waste containers.
   7. Dispose of gloves and gel into biohazard box.
   8. Remove dedicated lab coat and eyewear and leave in DNA staining room.

VI. Reagent List

Reagent	Supplier/Catalog number
TAE Buffer 25x	VWR EM 8750
TE Buffer	VWR EM 8890
SYBR Green Supermix	Bio-Rad 170-8880
(ABI) TaqMan Master Mix	Applied Bios stems 4304437
iCycler iQ Dye Calibration Set	Bio-Rad 1708792
Taq Core Kit	Qiagen/Operon 201225
TaqMan Probes	Applied Biosystems 450025
AMV Reverse Transcriptase	VWR PAM5101
DNeasy Tissue Digestion Kit	Qiagen/Operon 69506
Ethanol / Ethyl Alcohol, Pure 200 proof	VWR EM-4450
Proteinase K	Qiagen/Operon 19133
Ethidium Bromide Solution	VWR PAH5041
Agarose	Promega/VWR PAV 3121
6x Loading Dye	Promega PAG1881
DNA Ladder 100 bp	VWR PAG2101
Filter Tip Pipette Tips	VWR (various)
96 Well PCR Plates	Bio-Rad 2239441
Microseal Film	Bio-Rad MBB1001
PCR tube strips .2 ml (8 Well strip)	VWR 20170-002 or Bio-Rad TBS 0201
Mycoplasma PCR Detection kit	Sigma MP0025
Gloves	Cardinal Health JJ5740/sm, JJ5741/med
Microcentrifuge Tubes (DNA/RNA Free)	VWR
Distilled DNA free Water	VWR TX10977-015WAR / 10977-015

VII. Glossary of Terms

Amplicon- Double-stranded DNA "product" created during PCR.

Annealing - The binding of complementary DNA or RNA sequences via hydrogen bonding between bases.

Annealing temperature- The temperature at which a length of single-strand (heat denatured) DNA or RNA will anneal to a complementary strand. Lower temperatures may permit non-specific binding.

Bases- Adenine (A), cytosine (C), guanine (G) and thymine (T) are constitutive molecules of DNA. Bases in DNA form two pairs of complementary molecules, hydrogen bonds can link adenine to thymine and guanine to cytosine. If the sequence of bases matches a complementary sequence in a second strand of DNA the bonds can hold the two strands together; thus forming double-stranded DNA.

Baseline – Background signals that accumulate during the initial cycles of PCR that are used to determine the baseline fluorescence before there is significant accumulation of the target amplicon.

Base pair (bp)- A matching pair of bases e.g. adenine/thymine or guanine/cytosine.

Contamination- Extraneous DNA present in the sample or reaction that will be amplified and give a false positive result. Contamination problems in PCR most often arise from amplicons that were produced in previous PCR assays.

Ct – Cycle threshold. A statistically significant fluorescence detection level above the baseline.

Denaturation- Heat or chemical treatment to break the bonds between double-stranded nucleic acid molecules thus forming single strand molecules lacking secondary structure.

cDNA- DNA complementary to messenger RNA, synthesized from an RNA template by reverse transcriptase enzyme.

DNA Polymerase- An enzyme that synthesizes a double-stranded DNA molecule using single strand DNA and a primer as template, e.g. Taq.

Elongation or Extension- Phase of PCR cycle following annealing of primer during which the Taq polymerase manufactures a strand of DNA. The optimum temperature depends on the enzyme used but is usually between 68-72ºC.

Gel electrophoresis- Amplicons of differing lengths can be separated by their migration distance through an agarose gel while subjected to an electrical field. The negatively charged DNA can be visualized by staining with a fluorescent dye and viewing under UV light. The size of the amplicons is predicted by the positioning of the primers on the target DNA and a product or 'band' of the correct size is taken as a positive result. Results are often compared to a DNA ladder which has known size DNA segments.

Hybridization- Process of allowing complementary strands of nucleic acid to bind via hydrogen bonds between the bases to form a double-stranded molecule. If one of the strands has been labeled with a marker it will facilitate detection of the complementary strand.

Nested PCR- A second PCR is performed on the product of an earlier PCR using primers which are internal to the originals. This improves sensitivity without impairing specificity.

Oligonucleotides (Oligos)- Short sequences of nucleotides. They can be used as primers or probes.

They may be chemically 'labeled' during synthesis.

PCR- Polymerase chain reaction. Amplification of specific lengths of DNA by repeated 'thermal cycling' reactions using polymerase enzyme. The basic PCR process is covered by patents owned by Hoffman-La Roche Inc and Hoffman-La Roche Ltd.

Primer- Oligonucleotide that will bind to its target DNA and 'prime' the manufacture of a new strand by DNA polymerase. They are usually between 15 and 30 bases in length. Forward and reverse primers are used to flank the target sequence in a PCR reaction.

Probe- An oligonucleotide that has been labeled. When the probe binds or hybridizes to a complementary strand of DNA or RNA (its target), the molecule becomes labeled and can then be detected.

Primer-dimer- At room temperature temporary association of primers may take place allowing the Taq enzyme to polymerize small strands of DNA called primer-dimers. Can give a false positive result if the detection of amplicons is via labeled primers.

RT PCR- Reverse transcriptase PCR. Using reverse transcriptase enzymes cDNA is synthesized from the mRNA. The cDNA is then amplified by PCR.

Reverse transcriptase- An RNA-dependent DNA polymerase used to make cDNA from mRNA.

Target sequence- The specific piece of DNA or RNA to be amplified by the PCR and flanked by the primers.

Taq- Thermostable DNA polymerase, originally isolated from *Thermus aquaticus*, a bacteria that is found living in hot springs.

TaqMan- For simple and rapid detection of PCR products as they are produced. An oligonucleotide probe that is specific for the target to be amplified is labeled with a fluorescent tag and a quenching molecule. During the extension step of a PCR the Taq enzyme will disrupt probe bound to the target separating the fluorescent tag from its quencher molecule thus permitting fluorescence.

Thermal cycler- Although PCR may be performed using manual transfer between a series of water or oil baths, for reproducible results or diagnostic work a reliable programmable thermal cycling machine is essential. These machines allow rapid and exact changing of temperatures required in a PCR protocol.

VIII. Appendix – PCR data sheet examples

Single Round PCR Data Sheet – Gel Map

Assay Type, Date, Tech:_____

Primer Sets: _____
Initial Amplification: upstream _____ downstream _____

I. Sample Identification_____

First Comb Second Comb

Sample ID	Sample Number	Dilution	Results	**Sample ID**	Sample Number	Dilution	Results
1	Ladder			**21**	Ladder		
2				**22**			
3				**23**			
4				**24**			
5				**25**			
6				**26**			
7				**27**			
8				**28**			
9				**29**			
10				**30**			
11				**31**			
12				**32**			
13				**33**			
14				**34**			
15				**35**			
16				**36**			
17				**37**			
18				**38**			
19				**39**			
20				**40**			

II. Master Mix Preparation

PCR Reagent	Lot	Stock Concentration	Volume/Reaction Total =	Volume for ____reactions	Comments
DEPC water					
Buffer					
$MgCl_2$					
dNTP's					
Primer Upstream					
Primer Downstream					
Taq Polymerase					

III. Gel Preparation/Running

Gel Conc/# wells	Grams of Agarose	Volume of Buffer (ml)	Voltage	Time ran	Comments

IV. Staining

Stain/Conc.	Lot	Stain Time	Destain/Time	Comments

Results Summary

Date Data Entered:_____

Comments/Other:_____

Nested PCR Data Sheet – Gel Map

Assay Type, Date, Tech:_____

Primer Sets:

Initial Amplification: upstream _____ downstream _____

Nested Amplification: upstream _____ downstream _____

I Sample Identification_____

Sample ID	Sample Number	Dilution	Results	**Sample ID**	Sample Number	Dilution	Results
1	Ladder			**21**	Ladder		
2				**22**			
3				**23**			
4				**24**			
5				**25**			
6				**26**			
7				**27**			
8				**28**			
9				**29**			
10				**30**			
11				**31**			
12				**32**			
13				**33**			
14				**34**			
15				**35**			
16				**36**			
17				**37**			
18				**38**			
19				**39**			
20				**40**			

II. Master Mix Preparation

PCR Reagent	Lot	Stock Concentration	Volume/Reaction Total =	Volume for ____reactions	Comments
DEPC water					
Buffer					
$MgCl_2$					
dNTP's					
Upstream Primer 1					
Downstream Primer 1					
Taq Polymerase					
AMV RT					
RNasin					

PCR Round 2

PCR Reagent	Lot	Stock Concentration	Volume/Reaction Total =	Volume for ____reactions	Comments
DEPC water					
Buffer					
$MgCl_2$					
dNTP's					
Upstream Primer 2					
Downstream Primer 2					
Taq Polymerase					

III. Gel Preparation/Running

Gel Conc/# wells	Grams of Agarose	Volume of Buffer (ml)	Voltage	Time ran	Comments

IV. Staining

Stain/Conc.	Lot	Stain Time	Destain/Time	Comments

Results Summary

Date Data Entered:_____

Comments/Other:_____

QPCR Data Sheet- Plate Map Assay Date/Init:_____

Primer forward:_____ Primer reverse:_____

Probe: _____

I. Sample Information

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

II. Master Mix Preparation

PCR Reagent	Lot	Concentration	Volume/Reaction Total = µl	Volume for ____reactions	Comments
DEPC water					
ABI Taqman OR Sybergreen mix					
BSA					
Forward Primer					
Reverse Primer					
Taqman Probe					

METHOD:

- make up MM
- mix, spin
- aliquot _____µL
- add _____ µL sample DNA
- cover plate with adhesive film
- spin at 900 rpm for 1 min
- select _____as the detector (reporter=_____, quencher=_____)
- run _____cycles, _____µL volume

Date Data Entered:_____ Comments/Other:_____

CHAPTER 14

List of the Most Common Agents of Disease in Finfish and Shellfish within Alaska (not a complete catalogue)

Theodore R. Meyers

I. Finfish

A. Bacteria

1. *Renibacterium salmoninarum* - Bacterial Kidney Disease (BKD)
2. *Aeromonas salmonicida* (typical and atypical) - Furunculosis
3. *Aeromonas hydrophila/liquefaciens* - Motile Bacterial Septicemia
4. *Pseudomonas fluorescens* - Motile Bacterial Septicemia
5. *Pseudomonas sp.* - Motile Bacterial Septicemia
6. *Listonella anguillarum* - Vibriosis
7. *Yersinia ruckeri* (types 1 & 2) - Enteric Redmouth
8. *Serratia liquefaciens* - Bacterial Septicemia
9. *Flavobacterium psychrophilum* - Coldwater Disease (sequela myeloencephalitis)
10. Unidentified Flavobacteria - Superficial skin and gill infections

B. Fungi

1. *Saprolegnia sp.** - External egg and body fungus, internal systemic mycoses
2. *Phoma sp.* - Internal infections of air bladder and other organs

*Note: Based on genetic sequencing, water molds (oomycetes) have been tentatively re-classified as the class Oomycota in the phylum Heterokontophyta more closely related to photosynthetic brown algae and diatoms.

C. Protozoa

1. *Trichodina sp.*- External gill and skin infections
2. *Capriniana sp. (Trichophrya)* - External gill infections (commensal)
3. *Ichthyobodo (Costia) necatrix* - External gill and skin infections
4. *Epistylis sp.* - External gill and skin infections
5. *Myxobolus sp.* - Skin and internal infections in both fresh and saltwater fish species
6. *Henneguya sp.* - Skin and internal infections in both fresh and saltwater fish species
7. *Ceratomyxa shasta* - Internal infections of salmonids
8. *Ichthyophonus* – internal protozoan parasitizing marine fish species

D. Viruses

1. Infectious Hematopoietic Necrosis Virus (IHNV) (sockeye salmon and rarely, chum and chinook salmon)
2. Viral Erythrocytic Necrosis Virus (VEN) (Pacific herring)

3. Viral Hemorrhagic Septicemia Virus Type IVa (VHSV) (Pacific herring, cod hake, pollock)
4. Aquareovirus (chinook salmon)
5. Paramyxovirus (chinook salmon)
6. Erythrocytic Necrosis Virus (ENV) (chinook salmon)

E. Non-infectious diseases or causes of mortality

1. Gas Bubble Disease (air entrapment; drop in barometric pressure; heating of very cold water)
2. Gill hyperplasia (feed or particulate abrasion; ammonia or formalin toxicity)
3. White Spot Disease (handling; soft water and/or aluminum toxicity)
4. Drop Out (too little yolk at swimup; sequela to white spot or not osmocompetent in seawater situations)
5. High egg or yolksac fry mortality (mechanical failure of incubator accompanied by ammonia toxicity and *Saprolegnia*; overloading, blank eggs or other developmental problem)
6. Excessive fat in body cavity and/or fatty liver (overfeeding during cold water temperatures)
7. Bloat (excessive feeding in seawater)

II. Shellfish - Bivalves

A. Bacteria

1. *Nocardia crassostreae* – Pacific oyster nocardiosis (PON) in vesicular connective tissues - not common (Pacific oyster)
2. Rickettsial intracellular organisms in vesicular connective tissue cells, digestive tubule cells (Pacific oyster), gill epithelium (weathervane scallop, blue mussel, clam species), and various other tissues.

B. Fungi – Systemic mycosis caused by unidentified fungus (basket cockle)

C. Protozoa

1. *Ancistrocoma*-like ciliates in the digestive tubules and gut (Pacific oyster)
2. Unidentified small eosinophilic thigmotrich ciliates on the gills (Pacific oyster)
3. *Sphenophyra*-like ciliates on the gills (Pacific oyster)
4. Unidentified gregarine-like organisms within vesicular connective tissue (Pacific oyster) and *Nematopsis sp.* (scallops, clams, mussels, cockles)
5. *Trichodina sp.* on gill and mantle epithelial surface (Pacific oyster, basket cockle, rock and weathervane scallops)
6. *Hexamita sp.* within the tissues as secondary invaders (Pacific oyster)
7. Coccidia-like organisms in connective tissue and kidney (native littleneck clam, basket cockle)
8. Microsporidia in ova, nervous tissue, connective tissue and muscle (clams, cockles)

D. Metazoa

1. Unidentified copepod on gills and in the digestive tubules, intestine and connective tissues (multiple locations and morphology suggest *Pseudomyicola sp*.) (Pacific oyster, basket cockle, blue mussel, rock scallop, native littleneck clam)
2. Unidentified trematode metacercariae and sporocysts in the connective tissues (blue mussel, razor clam, native littleneck clam, basket cockle)
3. Turbellaria – gill and gut (weathervane scallop, basket cockle, blue mussel, native littleneck clam)

E. Non-infectious anomalies

1. Pearls (blue mussel, Pacific oyster, weathervane scallop)
2. Hermaphroditism – potentially all bivalves; some are normally hermaphroditic
3. Summer Mortality - stress related due to prolonged near-mature condition of gonads in both sexes but primarily females (Pacific oyster)
4. Neoplasia – germinoma (Pacific oyster), mesenchymal tumor (blue mussel) secretory cell adenoma (geoduck clam)

F. Viruses

1. Viral gametogenic hypertrophy (Ovacystis) – papilloma or polyoma -like viruses in male and female gametocytes (Pacific oyster)
2. Intranuclear Cowdry-type A inclusions of digestive tubule cells and mantle epithelium, caused by herpes-like viruses (native littleneck clam, rock scallop, Pacific oyster)
3. Aquareovirus – fish virus probably bioaccumulated by vector bivalves (geoduck clam)
4. Aquabirnavirus – fish virus probably bioaccumulated by vector bivalves (native littleneck clam)
5. Disseminated neoplasia – leukemia caused by suspected retrovirus (blue mussel, native littleneck clam)

III. Shellfish - Crabs

A. Bacteria

1. Bacteremia, possibly from injury or stress (red, blue, golden king crabs; Dungeness crab; *bairdi* Tanner crab)
2. Rickettsial intracellular organisms in digestive gland epithelium (blue and golden king crabs)
3. Shell Disease – several Gram-negative bacterial species causing shell erosion (blue, red and golden king crab; Tanner crab, Dungeness crab)

B. Fungi – Black Mat Syndrome (Tanner and snow crabs)

C. Viruses

1. Herpes-like virus – bladder and antennal gland (red, blue, golden king crabs)
2. Aquabirnavirus – antennal gland (blue king crab)

D. Protozoa

1. Bitter Crab Dinoflagellate Syndrome -systemic (*bairdi* Tanner crab , *opilio* snow crab)
2. *Mesanophrys* ciliate – systemic (blue and golden king crabs, Tanner crab, Dungeness crab)
3. Haplosporidian-like organism - systemic (spot and pink shrimps)
4. Microsporidia including *Thelohania* – various tissues (red, blue, golden king crabs, coonstripe shrimp)

E, Metazoa

1. Rhizocephalan barnacle parasitism by *Briarosaccus callosus* - systemic (red, blue, golden king crabs)
2. *Carcinonemertes* nemertean worm egg predators (Dungeness, king, Tanner crabs)
3. Crab leech – external (all major commercial crab species)
4. Trematode metacercariae – various tissues (Dungeness crab)

IV. References

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Meyers, T.R. and T. Burton. 2009. Diseases of wild and cultured shellfish in Alaska. Alaska Department of Fish and Game, Anchorage. 130 pp.

CHAPTER 15

Detection of *Ichthyophonus* sp. in Fish Tissues

Theodore R. Meyers and Tamara Burton

I. Introduction

Ichthyophonus hoferi, the causative agent reported for the disease ichthyophoniasis, may actually comprise several different species yet to be identified. Although once considered a member of the fungi, *Ichthyophonus* was recently reclassified as a protozoan member of the class Mesomycetozoea, a highly diverse group of organisms having characteristics of both animals and fungi. The route of infection is probably through the intestinal tract. As with most diseases, the severity is dependent on the general stress and health of the fish host. Once within the body, *Ichthyophonus* is a systemic pathogen localizing in major organ systems including the heart.

II. Clinical Signs

The gross clinical signs of *Ichthyophonus* can be confused with other visually similar conditions. A strong inflammatory response against the parasite often results in visible granulomas encapsulating the macrospores of the organism. These granulomas contain host lymphocytes, macrophages, neutrophils, and fibrous connective tissue that appear as white, yellow or brown foci in infected tissues such as the spleen, liver, kidney, skeletal muscle and especially the heart.

III. Diagnostic Methods

A. Wet mount of tissue squash preparation
   1. Dissect with forceps and scissors 1-2 tissue foci of suspected *Ichthyophonus* granulomas.
   2. Place dissected tissues on a clean glass microscope slide with a drop of PBS and cover with a glass coverslip.
   3. At medium magnification on a microscope, examine the squash for various sized macrospores and germinating hyphae-like filaments typical of *Ichthyophonus*.

B. Histology
   1. Select tissues containing suspected *Ichthyophonus* granulomas and place in tissue cassettes in 10% buffered formalin or Bouin's fixative.
   2. Transfer to 70% alcohol after 48-72 hrs and store until needed.
   3. The tissues are processed for standard histological examination according to the methods outlined in Chapter 6 - Histology for Finfish and Shellfish.

C. Explant culture of fish tissues
   1. Disinfect the outside of the fish with 100 ppm Iodophor solution and dry with a paper towel.
   2. The tissue type and tissue size to be sampled will vary depending on the fish species

but generally for survey purposes the heart is the tissue of choice. If obvious tissue granulomas are present these may be sampled as well. For large adult chinook salmon, usually ½ of the heart or about 1 gm or 1cm^2 piece is taken. For juvenile or small fish, generally the whole heart is taken. In the case of Pacific herring surveys, the heart may be cut longitudinally in half, ½ placed into explant culture medium and the remaining ½ fixed in 10% buffered formalin. Only the fixed tissues from explant-positive samples would be processed for histology to determine intensity of infection.

Note: The number on each tissue cassette must correspond to the same fish sample placed into the culture tube. For example, fish # 1 has ½ heart in a tissue cassette labeled #1 and ½ heart in a culture tube labeled #1.

3. Aseptically remove the selected tissue using sterile forceps and scissors. Be careful not to introduce contaminating organisms (fungi and bacteria) from the outside of the fish or intestinal tract.
4. Aseptically drop each tissue into a plastic screw cap culture tube containing sterile MEM-5 with antibiotics (Do not use antimycotics which will inhibit growth of the organism) and close caps tightly. Do not stick forceps into the tube. Tubes of medium must be opened just before adding tissues and closed promptly to avoid introducing contaminating organisms into the cultures. The cap to each culture tube should be wiped with an alcohol swab before opening and both the cap and the mouth of the tube should not contact anything that is not sterile.
5. To avoid cross-contamination, disinfect sampling utensils between fish by removing organic material, soaking in 100 ppm iodophor solution, and rinsing with clean water before reuse. Alcohol and flaming may also be used to sterilize instruments between samples. Alternatively, disposable utensils may be used (sterile single edge razor blades and sterile pointed wooden sticks) and then discarded after a single sample is collected.
6. Incubate cultures at 14-15˚C (most standard refrigerators can be adjusted to this temperature) but DO NOT FREEZE. Cultures must be kept cold and sent immediately to the laboratory for incubation and reading.
7. Another option would be to collect tissues aseptically in individual Whirl-Pak® bags, maintain the samples on ice but do not freeze and send to the laboratory within 72 hours for explant culture.
8. Explant tube cultures are incubated at 14-15^{o}C for 14 days. Cultures are read on an inverted microscope at 7 and 14 days. Most positive samples will be obvious by day 7.
9. Typical *Ichthyophonus*-positive cultures often have a pronounced acid pH (yellow), have various sized macrospores loose in the medium and generally exhibit vegetative hyphae growing from the explant tissue.
10. Fungal contamination can occur but generally is rare if adequate aseptic technique is followed. Contaminated cultures can often be read effectively at 7 days before the fungus overwhelms the culture.

D. Polymerase Chain Reaction (PCR) – See molecular techniques chapter #13 for confirmatory procedures for *Ichthyophonus* using PCR.

IV. Comparison of diagnostic methodologies

A. A wet mount is a quick method requiring a minimum of skill and equipment that is useful for confirmation that a gross lesion is caused by *Ichthyophonus*. This method is commonly used for diagnostic cases.

B. Histological methods are useful for determining the intensity of infection within a tissue and provide material that can be permanently archived for future reference. Although highly specific when visualizing the organism, random histological examination of tissues is expensive, time consuming and is the least sensitive method of detection. Therefore, histology is not a good screening tool.

C. Explant culture is the gold standard used by most investigators as the screening tool for determining prevalence of *Ichthyophonus*. Explant culture is very sensitive and able to detect subclinical infections, is relatively inexpensive regarding materials and labor, requires little skill except for good aseptic technique and provides viable organisms that can be easily identified and archived for later studies. When used with histology of positive tissue samples the infection intensity may also be determined.

D. PCR is also highly sensitive and specific but slightly less sensitive than explant culture since *Ichthyophonus* granulomas can be very localized in tissues. When samples are tested by both PCR and explant culture, PCR occasionally provides false negative results due to the inherent sampling error introduced by the need to take very small portions of tissue for DNA extraction. In this case target DNA can be missed. PCR is expensive regarding materials and is labor intensive regarding the tissue extraction of DNA. Extreme care in the field and in the lab must be exercised to prevent cross-contamination. Because the test detects DNA of both living and dead parasites the status of infection cannot be determined with certainty. For these reasons PCR is not a good screening tool but is better used on selected known positive samples for further confirmation and/or genetic studies.

V. Subculturing, freezing and retrieving *Ichthyophonus*

A. Subculture two randomly selected uncontaminated *Ichthyophonus*- positive samples by first letting the spores settle to the bottom of the tube.

B. Using a sterile 1 ml pipette transfer about 0.1 ml of spores into a tube containing MEM 5 pH 3.5, 1% glucose medium.

C. Incubate at 14°C until a pellet forms in about 4-7 days.

D. Decant or pipette medium from the pellet into bleach solution and add freeze media (MEM 5 pH 3.5, 1% glucose, 20% glycerol) to the pellet.

E. Vortex and triturate with pipette to break up the pellet and transfer to cryovials and freeze at -70°C.

F. Samples are retrieved from the freezer by thawing a cryovial in lukewarm water and removing just before totally unthawed.

G. Centrifuge and pour off the freeze medium and re-suspend with enrichment medium and incubate at 14°C until pellet forms.

VI. Media and tubes

A. *Ichthyophonus* explant medium – Do Not include any antifungal chemicals which will inhibit parasite growth.

450 ml MEM
25 ml FBS
5 ml L-Glutamine (100mM)
1 ml Gentamicin (50mg/ml)
5 ml Penn.\Strep. Solution (Penn. 10,000 units/ml and Strep. 10,000 ug/ml)
7.5 ml Tris buffer (1M)
4 ml 7.5% Sodium bicarbonate

Add approximately 5-7 ml of medium to each sterile plastic screw capped round bottomed tube for explant culture and store refrigerated until needed.

B. *Ichthyophonus* enrichment medium

200 ml MEM 5
2 g Glucose
Adjust pH to 3.5
Filter sterilize

C. *Ichthyophonus* freeze medium

80 ml of enrichment medium
20 ml Glycerol
Filter sterilize

D. Screw capped polystyrene tubes used for explant culture - 16 X 125 mm (Falcon) VWR# 62404-949

VII. References

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Whipps C, Burton T, Watral V, St-Hilaire S and Kent M. 2006. Assessing the accuracy of a polymerase chain reaction test for *Ichthyophonus hoferi* in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Diseases of Aquatic Organisms 68:141-147.

CHAPTER 16

Fish Pathology Laboratory Hazard Communication and Chemical Hygiene Program

Tamara Burton

Introduction

The Alaska Department of Fish and Game, Fish Pathology Laboratory staff have developed a Hazard Communication and Chemical Hygiene Program to enhance our employees' health and safety. We intend to provide information about chemical and physical agent hazards and the control of hazards via our comprehensive program which includes container labeling, Material Safety Data Sheets (MSDS), Physical Agent Data Sheets (PADS), and training.

In the Anchorage lab the written Hazard Communication and Chemical Hygiene Program is kept in a binder in the middle lab under the fire extinguisher. A list of all chemicals and media in the laboratory is located on a hook on the back of the fume exhaust hood. In the Juneau lab this information is located in the large lab in the right end of the overhead cupboard above the embedding station. They are available for review by any employee at any time. All Fish Pathology Laboratory employees are required to read and sign this manual.

I. General Laboratory Guidelines

A. Each individual has the prime responsibility for his/her own safety. Everyone is required to read the Hazardous Communication and Chemical Hygiene Program and sign off on the designated sheet.

B. No smoking, eating or drinking is permitted in the laboratory.

C. Mouth pipetting is forbidden. Bulb pipetting devices and automatic pipettors are available and should be used at all times. There are no exceptions to this rule.

D. Visitors to the laboratory should be accompanied by a laboratory staff member. Visitors who will be spending time in the lab or using the facility should have a safety orientation by the Safety Officer.

E. Appropriate personal protection devices should be worn at all times when working with potentially hazardous materials.

F. Stickers and signs are displayed warning lab personnel of existing hazards. All potentially hazardous substances are labeled with health, flammability and reactivity data and personal protection recommendations.

G. Everyone should know the location of the Material Safety Data Sheets (MSDS) and how to use them.

H. Equipment, supplies, etc. should not be stored in corridors, in stairways, in exits, or in high traffic areas. Use designated storage areas.

I. Food should not be stored in the laboratory. Everything put into refrigerators/freezers should be properly labeled.

J. Work surfaces should be cleaned immediately if a spill occurs and at the end of each work session.

K. All activity that could result in release of hazardous vapors (solvents, etc.) should be done under a fume exhaust hood.

L. Contact lenses are not recommended for use in the laboratory. Some lenses can absorb certain solvents, offer no protection in a splash and actually concentrate caustic materials against the eye. If you choose to wear contact lenses, it is strongly advised to wear safety glasses when performing laboratory procedures.

M. All new employees should have a full indoctrination to the safety practices in the lab.

N. All accidents should be reported and an accident report filled out. The cause of each accident should be determined and action taken to eliminate reoccurrence.

O. It is recommended that laboratory personnel have first aid and CPR training.

P. Everyone should be familiar with the locations and use of safety apparatus including fire extinguishers, eye washes, fire blanket, first aid kit, safety shower and fire alarm.

Q. Wash your hands after handling chemicals and hazardous substances.

R. If you have any safety concerns or suggestions, bring them to the attention of the other staff members and the Safety Committee so they can be discussed and improvements made.

II. Fire Precautions and Evacuation Procedures

A. Precautions - In order to minimize the possibility of fire in the laboratory the following guidelines should be followed:
   1. No smoking is allowed in the laboratory.
   2. Portable fire extinguishers should be located in conspicuous areas throughout the laboratory. Personnel should familiarize themselves with the locations and use of the extinguishers.
   3. Do not allow extinguishers to become blocked or hidden by equipment or supplies. The extinguishers should be inspected on a yearly basis by Fire Department personnel.
   4. Do not work with flammable substances around heat sources or anything that could spark, for example hot plates and electric stirrers.
   5. All personnel should be familiar with exits and fire evacuation plans for their work area.
   6. Post emergency telephone numbers by or on each telephone.
   7. Keep flammable liquids in designated cabinet and use safety cans if possible. Do not overstock flammable materials.

8. Have a fire blanket available to use for smothering fires on clothing or smothering ignited spills.
9. Use insulated gloves to handle hot or very cold objects.
10. Store compressed gas cylinders out of the mainstream of traffic and make sure they are secured properly.

B. Fire evacuation procedure
   1. In case of a fire the building fire alarm should sound and everyone should evacuate the building as quickly and orderly as possible through the nearest exit and meet other laboratory members at the designated meeting area. The designated area for both the Anchorage lab and Juneau lab is the front parking lot. Do not return to the building for any reason until fire department personnel or the building supervisor give the okay.
   2. Doors and windows should remain closed.
   3. Turn off gas outlets if you can do so easily.
   4. If the fire is small and isolated in the laboratory area, the individual discovering the fire should assess the situation. A fire extinguisher can be used by trained personnel.
   5. If a fire is beyond control, evacuate the area. The fire department should be notified of any fire that occurs in the building.

C. Use of fire extinguishers - fire extinguishers can be used by trained personnel based on the following guidelines:
   1. Flammable liquid fires - do not use water since it will spread the fire. A dry chemical extinguisher is best. Always aim the extinguisher at the base of the fire and use a sweeping motion. CO_2 extinguishers can be effective if the fire is small.
   2. Electrical fires - CO_2 extinguishers work best but dry chemical extinguishers are safe and effective. It is important to shut off the circuit if possible. If the power is off, water can be used.
   3. Gas fires - CO_2 extinguishers are recommended for use. If at all possible shut off the gas source.
   4. Paper and wood fires - a dry chemical extinguisher works best. Water can also be used.
   5. The extinguishers located in the Fish Pathology Laboratories are of the ABC type. This designation indicates the extinguisher is good for all of the following:
      a. Trash, wood, paper
      b. Liquid, grease
      c. Electrical

III. Equipment Safety

A. General equipment safety
   1. New employees should be trained on equipment use before required to use the equipment independently. Equipment manuals are available for reference.
   2. When a new piece of equipment is brought into the lab a designated individual should learn safe operating procedures by referring to the manuals or through help from a company representative. The remainder of the staff should be taught the proper use of the new equipment through an in-service or training session held during the weekly staff meeting or other designated time.
   3. Do not work around mechanical equipment while wearing neckties, jewelry, long dangling sleeves, etc. Long hair should be tied back.

4. Regularly scheduled preventive maintenance should be followed for each piece of equipment.

B. Glassware
   1. Inspect all glassware before use. Do not use glassware if broken, cracked or chipped.
   2. Dispose of broken glassware in a impact-resistant container labeled "Broken Glass Only"!
   3. Do not force glass tubing or thermometers into rubber stoppers. Use a lubricant such as glycerin and a towel to protect your hands while gently inserting. It is better to cut off a rubber stopper around a thermometer then risk breaking the thermometer if trying to remove it.
   4. Use tongs or gloves to remove glassware from heat (stir plate or autoclave). Hot glass can cause severe burns.
   5. Hold beakers, bottles and flasks by sides and bottoms rather than by the tops. The rims of beakers or necks of bottles and flasks may break if used as lifting points.
   6. To avoid breakage when clamping glassware, use coated clamps to prevent glass to metal contact and do not use excessive force to tighten clamps.
   7. Do not look down into a container being heated or containing chemicals. A reaction might cause the contents to be ejected causing injury.
   8. Cool all glassware slowly to prevent breakage. Only use autoclave-proof glassware (Pyrex etc.) for autoclaving.
   9. Do not use laboratory glassware for food or drinks.

C. Needles, syringes and scalpel blades
   1. Needles should not be bent or recapped following use unless you are using a needle-recapping device. A destruclip hypodermic safety device is available to cut needles and cut plastic syringe barrels.
   2. Used needles and syringes should be placed in a puncture-resistant container (sharps container) and decontaminated by incineration or autoclaving before discarding.
   3. Never discard needles into trash containers where they may puncture someone.
   4. Always dispose of scalpel blades in the sharps container. To remove blades from handles use the blade-away device to avoid lacerations.

D. Centrifuges
   1. Centrifuge tubes should be inspected before use. Never use broken, cracked or chipped centrifuge tubes.
   2. Do not centrifuge uncovered tubes of infectious materials or flammable liquids.
   3. Before starting centrifuge, make sure the rotor is symmetrically loaded, tube caps are correctly seated, and swinging buckets are symmetrically placed. Use blanks as appropriate to balance tubes.
   4. Do not operate centrifuge unless lid is closed. If a noise or vibration develops, stop centrifuge immediately and re-balance.
   5. Never put hands or fingers inside centrifuge while it is still in motion. Use the brake.
   6. If there is tube breakage in the centrifuge while it is operating, immediately turn off the centrifuge. Do not attempt cleanup for at least 60 minutes so that aerosols will be minimized.

E. Autoclaves

1. Use only type 1 borosilicate glass (Pyrex or equivalent) for sterilization of liquids. Do not use ordinary glass containers not designed for sterilization.
2. Do not attempt to open the door of an autoclave before the pressure gauge reads zero. Never force the door open.
3. It is best to wait until the temperature in the chamber cools down before opening. Always open the door slowly and wear heat-resistant gloves. It is advisable to crack the door seal first and wait until the chamber reaches room temperature before opening the door completely.
4. Bottles should be cool to the touch before moving them from the sterilizer. Never move boiling or bubbling bottles. Sudden complete opening of the autoclave door following a sterilization cycle can cause liquids to boil over or containers to burst.

F. Liquid nitrogen

1. Liquid nitrogen is colorless, odorless and non-toxic. Due to its extreme low temperature it can cause severe burns.
2. Always wear a full face shield, insulated gloves, apron and boots when working with liquid nitrogen and when adding or taking anything out of the liquid nitrogen storage container.
3. Do not use plastic containers even for temporary storage of liquid nitrogen. Use only containers designed for low temperature liquids.
4. Always transport containers of liquid nitrogen in an upright position and secured in place. Do not place units in closed vehicles where the nitrogen gas that is vented from the unit can accumulate.
5. When removing ampules that have been stored in liquid nitrogen, loosen the caps as soon as possible after taking them out. As a precaution also wrap the ampules in towels. The gas trapped in the ampules expands rapidly at room temperature and could burst the ampules.
6. Do not cover or plug the opening of any liquid nitrogen container. Use only the loose-fitting neck tube provided with the liquid nitrogen-approved container. Check it often to make sure venting is not restricted by accumulated ice or frost.
7. Use proper transfer equipment such as a funnel to prevent splashing and spilling when transferring liquid nitrogen between containers.
8. Do not overfill liquid nitrogen containers. Filling above the specified maximum level can result in overflow or spillage when the neck tube is placed in the opening.
9. Never use hollow rods or tubes as dipsticks to measure liquid nitrogen levels. When a warm tube is inserted into liquid nitrogen, liquid will spout from the top of the tube. Wooden or solid metal dipsticks are recommended.
10. Store and use liquid nitrogen in a well ventilated place. As the liquid evaporates, the resulting gas displaces normal air from the area. In closed areas, excessive amounts of nitrogen gas reduce the concentration of oxygen and can result in asphyxiation.
11. Never dispose of liquid nitrogen in confined areas or places where others may enter. Disposal should be done outdoors in a safe place by pouring the liquid nitrogen slowly on gravel or bare earth where it can evaporate without causing damage. Do not pour liquid nitrogen on pavement.
12. Rough handling can cause serious damage to liquid nitrogen containers. Dropping containers, allowing it to fall over, or subjecting it to impact or severe vibration can result in partial or complete loss of vacuum. To protect vacuum-insulation system, handle containers carefully. Do not roll or drag containers across the floor. Use a dolly or hand cart when moving containers.

13. Keep liquid nitrogen containers clean and dry. Do not store in wet, dirty areas. Use water or mild detergent for cleaning and dry the surface thoroughly.
14. Check the liquid nitrogen level in container frequently, at least once each week, and refill the unit as required to maintain protection of stored materials.

G. Water baths
1. Water baths should be turned off when not in use.
2. Water levels should be maintained. If a water bath is left on without water in it, overheating is possible.
3. Distilled- or laboratory-grade filtered water should be used in water baths to prevent mineral deposits on heating elements.

H. Laboratory burners
1. Always locate burners away from flammables.
2. Do not reach over burner to turn it off or for any other reason. It is advisable to locate burners far back on the work surface to avoid accidental contact with the flame when reaching for supplies etc.
3. Turn burners off when not in use or when leaving the room, even if for a short while.
4. If flaming an instrument that has been dipped in alcohol, always let excess alcohol drip from the instrument before flaming. Hold instrument in a downward position until flame is burned out. Flaming alcohol can roll down an instrument onto hands if held in an upright position. Be sure the flame is out before dipping an instrument back into a container of alcohol to avoid igniting the alcohol.

I . Laboratory hoods
1. Hoods should be located away from high traffic areas and away from doors and windows.
2. A minimum face velocity of 60 ft/minute is recommended for general laboratory hoods.
3. Hoods used for highly toxic materials require face velocities of 100 ft/minute. This velocity is adequate to ensure that no contaminants escape into the room.
4. Hoods should be monitored monthly and more often if there is a problem.

IV. Compressed Gases

The Anchorage Fish Pathology Lab has several compressed-gas cylinders in the laboratory area. A small nitrogen tank is located by the hood in the back lab. A large oxygen tank is located in the back lab along the north wall. The Juneau Fish Pathology Laboratory has one small CO_2 tank next to the virology hood. The following precautions and guidelines should be followed when working with or around compressed-gas cylinders.

A. Compressed-gas cylinders should not be stored near stairwells or in high traffic areas where they could get knocked down.

B. Cylinders of oxygen should not be stored near (within 20 feet) cylinders containing flammable gases, any combustible materials or heat source.

C. Do not store any cylinder near heat sources or allow them to come into contact with electrical circuits.

D. Cylinders should be stored in an upright position and firmly secured so they cannot fall or be knocked over. Large cylinders should be secured in two locations.

E. Do not drop cylinders or allow them to strike each other violently.

F. Cylinders should be stored with valve safety covers in place unless a regulator is installed.

G. Only use cylinders that have contents properly identified.

H. Do not attempt to repair damaged cylinders or to force frozen cylinder valves.

I. Valve safety covers must be left in place until secured at the work site.

J. Do not use cylinders without regulators, or interchange regulators or other fittings. Needle valves and regulators are designed specifically for different families of gases.

K. Shut gas off when not in use.

L. Be certain employees who will be changing and operating cylinders are checked out on how to connect regulator, move and secure cylinders, etc.

M. Use of oil, grease, or lubricants on valves, regulators or fittings is prohibited.

V. Biohazards

Biohazards are materials containing microbiological agents or toxins that cause or may cause human disease. When working with biohazards, it is important to prevent possible accidents and exposures by using good microbiological techniques, proper safety equipment, and by being aware of what you are working with. Many of the agents worked with at the Fish Pathology Laboratories are not known to be pathogenic to people, but it is necessary to use biohazard precautions when working with any potentially infectious agent.

A. Never eat, drink, or smoke in laboratory areas and do not use laboratory refrigerators to store food or drinks.

B. Do not use laboratory glassware for food or drinks.

C. Wash hands after working with infectious agents. Use antibacterial soap.

D. Always wear disposable surgical gloves when performing necropsies.

E. Never mouth pipet for any reason.

F. Wear your lab coat to avoid contaminating your clothing and to protect your skin. Remove it when you leave the laboratory.

G. Keep your work area clean. Disinfect your work area before and after working with potentially infectious agents. Use appropriate disinfectants.

H. Beakers and pipet holders full of disinfectant should be available for discarding contaminated pipets and pipet tips.

I. Keep everything out of your mouth while in the laboratory. This includes your hands, pens and pencils, etc.

J. Aerosols act as a very efficient way of spreading infection. Some common sources of aerosols are pipetting, flaming loops, centrifuges, shaking machines, blenders, syringes and opening lyophilized ampules. Always minimize the generation of aerosols by using appropriate microbiological technique.

K. All potentially infectious materials should be decontaminated before being discarded. Use disinfectant solutions or the autoclave. If you are using the autoclave, run it often. Do not allow materials to stack up for more than one day in order to get a full load. No infectious waste should be dumped into the sewer or solid waste disposal system without being properly sterilized first. Items containing hazardous chemicals such as formalin, Bouin's fixative, or bleach should never be autoclaved.

VI. Hazardous Materials Identification System (HMIS)

A. Each potentially hazardous substance in the laboratory is labeled with a hazardous material identification system label. These labels give the users quick reference on health (blue), flammability (red), and reactivity (yellow) data and personal protection (white) recommendations.

B. Health, flammability and reactivity are rated based on the following hazard index:

4 severe hazard
3 serious hazard
2 moderate hazard
1 slight hazard
0 minimal hazard

C. Personal protection recommendations are listed based on the following personal protection index:

1. safety glasses
2. safety glasses, gloves
3. safety glasses, gloves, apron
4. face shield, gloves, apron
5. safety glasses, gloves, dust respirator
6. safety glasses, gloves, apron, dust respirator
7. safety glasses, gloves, vapor respirator
8. splash goggles, gloves, apron, vapor respirator
9. splash goggles, gloves, dust and vapor respirator
10. splash goggles, gloves, apron, dust and vapor respirator
11. air line hood or mask, gloves, full protective suit, boots

D. A poster outlining the hazard index and the personal protection index is located in both laboratories. Everyone should know the location of the poster in each lab and understand how to use it.

E. To further ensure that employees are aware of the chemical hazards of materials used in their work areas it is our policy to label all secondary containers.

F. In the Anchorage lab, responsibility for the labeling of new chemicals received has been assigned to the Laboratory Technician and is reviewed by the Safety Officer before being put into use in the lab. In Juneau, these functions are carried out by the Microbiologist I.

VII. Material Safety Data Sheets (MSDS) and Physical Agent Data Sheets (PADS)

MSDS and PADS are safety data sheets developed for every potentially hazardous substance and physical agent present in the laboratory. They are filed in alphabetical order for easy access. The MSDS contains information you will need for safely working with a specific product. Each provides details on chemical and physical dangers, safety procedures and emergency response techniques.

A. Each employee should know where in each lab the MSDS binder is located and how to use the contained information.

B. MSDS are generally organized as follows:
   1. Identification - identifies the manufacturer and the product. Emergency telephone numbers are usually listed.
   2. Hazardous ingredients - lists each product ingredient. Also lists the percentage of the ingredient, and its threshold limit value (TLV). A TLV measurement is the maximum amount of the hazardous substance in the air which is thought to be harmless to most workers exposed repeatedly to it.
   3. Physical data - lists selected chemical data which can be useful in deciding how dangerous a product is.
   4. Fire and explosion data - lists flash point, flammable limits, extinguishing methods, special fire fighting procedures and explosion hazards. A flash point near or below 100°F indicates a particularly unstable substance which might catch fire or explode as the result of static electricity or flame. The flammable limits refer to the range of vapor concentrations in air which will explode if heat is added. The lower the percentage of the LEL (lower explosive limit), the lesser the amount of a substance which has to be in the air before it is likely to explode.
   5. Health hazard data - lists possible health hazards associated with the substance. The effects of overexposure, first aid and emergency procedures are listed here. This section also covers signs and symptoms of exposure such as eye irritation, nausea, dizziness, skin rashes, headache, etc.
   6. Reactivity data gives information on conditions that could cause the product to react by burning, exploding, or releasing dangerous vapors.
   7. Spill or leak procedures - gives instructions for handling spills.
   8. Special protection information - lists information on the protective equipment and ventilation to be used with the product.
   9. Special precautions - any additional information is discussed here such as storage, handling and transportation guidelines.

C. Each company is responsible for generating their own MSDS information for the substances they sell. The above outline may vary with individual companies as the MSDS contents are not strictly regulated.

D. If you want additional information on substances you are working with, there are several sources. Those organizations or agencies that may be contacted for clarification or additional information include the Alaska Health Project, the federal Occupational Safety and Health Administration (OSHA), the State of Alaska Department of Labor Occupational Safety and Health Section (OSHS) and the National Institute for Occupational Safety and Health (NIOSH). If you would like to do some research on your own about any chemicals in the MSDS file, try the following publications. They usually can be found at the University of Alaska library or at the agencies and organizations outlined above:

Sax, N. I. 1979. Dangerous properties of industrial materials. 5th Edition. Van Nostrand Reinhold Co., New York (see pages 271-2 for key to toxicity codes).

Hamilton, A., and H. L. Hardy. 1974. Industrial toxicology, 3rd Edition. PSG Publishing Co., Acton, Massachusetts

Key, M., et. al., editors. 1977. Occupational diseases: A guide to their recognition. Government Printing Office, Washington DC. (An inexpensive paperback)

Mackison, F. W., and R. S. Stricoff, editors. 1982. Occupational health guidelines for chemical hazards. Government Printing Office NIOSH Publication No. 81-123, Washington DC.

Stellman, J. M., and S. M. Daum. 1971. Work is dangerous to your health. Pantheon Books, New York.

E. If you are using a particular product for the first time or are not familiar with the properties of a product, review the MSDS before using the product.

F. MSDS are available to all employees for review during each workshift. If MSDS are not available or new chemicals in use do not have MSDS, please immediately contact the Safety Officer for your laboratory.

VIII. Personal Protection Equipment

The laboratory is equipped with many personnel protection devices that should be used when working with hazardous substances. These devices are designed to protect your clothing and body from injury, so get in the habit of using them.

A. Safety glasses, splash goggles or a full face shield should be used for eye and/or face protection.

B. Lightweight disposable gloves are available in three sizes and should be used to protect your hands. Heavyweight rubber gloves should be used if greater hand protection is warranted. Insulated gloves are available for use with autoclaves and ultralow freezers.

C. Laboratory coats should be worn by laboratory personnel and visitors while working in the laboratory. In general, laboratory coats should not be worn outside the lab, in the bathrooms, break room etc.

D. Eye washes, a fire blanket, a safety shower and a first aid kit are all located in the laboratory working area. Take time to familiarize yourself with the locations of each of these items and how to use them.

E. Aprons are available for use with liquid nitrogen and all employees should have adequate shoes for foot protection.

IX. Safety Equipment Listing

This is a listing of the safety equipment located in the Fish Pathology Laboratories in Anchorage and Juneau. New personnel should take a walk-through tour of either laboratory and locate all the items. Do not wait for an accident to happen before becoming familiar with the locations and use of the safety equipment.

A. Anchorage

1. Spill clean-up kit - It is located in the middle lab on the east wall. Kits for acids, caustics, solvents and mercury are located there along with an instruction manual.
2. Eyewashes - There are two eyewashes and they are both located in the middle lab. A continuous flow eyewash is located by the door on the north wall. A small bottle-type wash is located on the center island of the middle lab to the left of the hood.
3. Safety shower - A ceiling safety shower is in the middle lab in front of the north door.
4. First aid kit - First aid supplies are located in the middle lab in a drawer in the center island to the right of the hood.
5. Fire blanket - located on top of the freezer on the north wall in the middle lab.
6. Fire extinguishers - There are two extinguishers located in the lab. In the middle lab there is one mounted on the south wall and in the back lab there is one mounted on the west wall above the centrifuges. There is another extinguisher immediately outside the door to the front lab and one in the hallway outside the Fish Pathology offices.
7. MSDS binder in the middle lab under the fire extinguisher.
8. Emergency fire and rescue telephone numbers are posted on or by each telephone.
9. Fire Alarm - A fire alarm is located outside the front lab door by the outside exit.

B. Juneau

1. Spill clean-up pads and blankets - Absorbent pads for acid, caustic and solvent spills are located in the big lab in a rack mounted in a benchtop kickspace to the right of the door as you enter the lab.
2. Mercury spill kit - This kit is located in the big lab in the end overhead cabinet to the right of the door as you enter the lab.
3. Eyewash - The eyewash is located between the two labs to the left of the bathroom door.
4. Safety shower - A shower is located in the bathroom.
5. First aid kit - First aid supplies are located in the kitchen area in the upper left cabinet labeled with a red cross.
6. Fire blanket - The fire blanket is located in the big lab mounted in a benchtop kickspace to the right of the door as you enter the lab.
7. Fire extinguishers - There are three extinguishers in the building. One is just to the right of the front exit, another is mounted on the left wall in the mechanical room entryway and the third is mounted to the right of the back double doors.
8. Respirator - A respirator and extra cartridges is located in the big lab on the shelves to the left of the TV.
9. MSDS binder - The red MSDS binder is located in the big lab in the right end of the overhead cupboard above the embedding station.
10. Emergency phone numbers - Each office phone is labeled with the local emergency phone number. The phones located in the labs have a more extensive list of emergency numbers mounted on the wall next to each phone.
11. Generator - In the event of a power outage, much of the building can be supplied with emergency power by a portable generator located in a shed on the back deck.
12. A 24-hour security panel with outside alarm is located next to the front outside exit and monitors four smoke detectors in various areas of the lab.

X. List of Extremely Hazardous Chemicals

The following is a list of the extremely toxic and hazardous chemicals as defined by OSHA present in the Fish Pathology Laboratories. Further information on each noted chemical can be obtained by reviewing the MSDS and the section of this manual on special precautions for handling extremely hazardous and toxic substances.

A. Carcinogens: chloroform, formaldehyde, formalin, chromium trioxide.

B. Mutagens, teratogens: amethopterin, colchicine, vinblastine.

XI. Chemical Hygiene Plan

The following chemical hygiene plan was developed to help educate Pathology Laboratory employees on chemical safety and to ensure safe work practices in the laboratory.

A. General chemical safety

1. Always read the label on a chemical container before you move, handle, open or use it. If you do not understand something on the label, get more information before using it. Read the MSDS.
2. Know the location of all safety equipment and how to use the spill clean-up kits.
3. Store chemicals in their original containers.
4. When purchasing new chemicals, consider buying from companies that have safety packaging; e.g., plastic-coated bottles, safety cans. Only purchase limited quantities to reduce the amounts of extra chemicals sitting around the laboratory for long periods of time.
5. Make sure all containers and secondary containers are properly labeled.
6. Store hazardous wastes in labeled containers that are located in a secured safe area.
7. Never mix unknown chemicals or wastes.
8. Store incompatible chemicals or wastes in separate storage areas.
9. Wear the recommended protective clothing and safety equipment when handling chemicals.
10. Make sure the areas where chemicals are stored and used are well ventilated.
11. Clean up spills immediately. If for some reason this is not possible, secure the spill site so other laboratory personnel will not come into contact with it accidentally.
12. Keep chemical storage areas clean.
13. Wash your hands after working with chemicals.
14. Report all injuries and exposures to chemicals and fill out an accident report form.
15. Treat injuries/exposures promptly and get medical attention for eye contact with chemicals or ingestion of hazardous materials.
16. In Anchorage, all chemicals to be disposed of should be transported to the hazardous materials facility at the Hiland/Eagle River Municipality of Anchorage landfill site. In Juneau, the local sanitation department has periodic hazardous material drop-off days, once a month, usually on a Saturday. In both labs certain materials (alcohols, acids, formalin) may be allowed to be washed down the sink drain after adequate neutralization and/or dilution in accordance with guidelines from the EPA and the local sewage treatment facility.
17. Do not smell or taste chemicals.
18. Inspect gloves and test hoods before use.
19. Never pipet by mouth.
20. Avoid wearing contact lenses.

21. Do not eat, drink, chew gum, or apply cosmetics in the laboratory. Never store food or beverages in the laboratory refrigerators or freezers.

B. Chemical procurement
   1. Notify the laboratory Safety Officer when any hazardous chemical is ordered. Information on proper handling, storage and disposal should be communicated by the Safety Officer to those persons involved.
   2. Do not receive any chemicals that are not properly labeled.
   3. Chemicals should be received in the laboratory office and the Safety Officer should be notified upon arrival.
   4. All hazardous chemicals should have a MSDS provided by the manufacturer.
   5. All chemicals should be labeled with a hazardous material identification system (HMIS) sticker that has been properly filled out before being used in the lab. The responsibility of labeling new chemicals received has been assigned as indicated in section VI. F.
   6. All chemicals should be dated when they are opened or made. Reagents that are made up should be labeled with the reagent name, strength, date made and expiration date. All secondary containers should be labeled with a HMIS label.
   7. Store highly toxic substances in unbreakable secondary containers.

C. Chemical storage
   1. Always store chemicals in the designated storage area.
   2. Limit chemicals stored in the laboratory to the smallest practical quantity.
   3. Do not store old chemicals. Routinely check expiration dates of chemicals, and if outdated dispose of them.
   4. Return all reagents and chemicals to proper places when not using them. Be sure caps are secure.
   5. Never store chemicals on the floor where they could be knocked over.
   6. Chemicals should be inventoried on a regular basis and a list of these chemicals and their locations available for quick reference. In Anchorage, a listing of chemicals and media is located on a hook on the back of the fume exhaust hood. In Juneau, this list is in the right overhead cupboard above the embedding station in the big lab.
   7. Exposure to heat and sunlight should be avoided.

D. Special precautions for handling extremely hazardous and toxic substances
   1. There are several substances worked with at the Fish Pathology Laboratories that are considered extremely hazardous to health. These include: carcinogens, which are substances capable of causing cancer; mutagens which are substances capable of causing chromosomal damage; and teratogens, which can cause congenital malformations in a fetus. Extreme care must be used when handling these substances. If any exposure occurs, a medical consultation should follow. The protective measures discussed below should always be followed when working with these substances.
   2. Carcinogens
      a. Chloroform - used very infrequently for staining of histological tissues and as a solvent. Stored in the black poison cabinet in Anchorage and in cabinet below fume hood in Juneau.
      b. Formaldehyde/formalin - used routinely for fixation of histological tissues. Stored in the black poison cabinet in Anchorage and in cabinet below fume hood in Juneau.

c. Chromium trioxide - used very infrequently for histological staining. Stored on oxidizer shelf in Anchorage and in dry chemicals cabinet in Juneau.
d. Spurr's resin ingredients (used for TEM embedding) - None in Anchorage. Stored in dry chemicals cabinet in Juneau.

3. Teratogens/Mutagens
   a. Amethopterin - used for fish stress experiment. In Anchorage, kept in gold virology freezer compartment. None in Juneau lab.
   b. Colchicine - used for chromosome counts. Kept in gold virology freezer compartment in Anchorage and in media refrigerator in Juneau.
   c. Vinblastine - used for chromosome counts. Kept in gold virology freezer compartment in Anchorage and in media refrigerator in Juneau.
4. Handling requirements for extremely hazardous and toxic substances
   a. These substances should be handled only under the fume exhaust hood.
   b. Avoid breathing vapors, contact with eyes, skin or clothing.
   c. Use protective rubber or neoprene gloves.
   d. Use safety glasses or face shield.
   e. Store in a cool area.

E. Prior approval - certain hazardous substances should not be used by the Laboratory Technician in the Anchorage lab without prior approval of his or her supervisor because of the hazards associated with them and the very limited usage. Currently they include:
   1. Amethopterin
   2. Colchicine
   3. Vinblastine

F. Environmental monitoring and safety equipment maintenance
   1. Initial monitoring for formaldehyde has been done in both labs and levels are below the permissible exposure limit (PEL) of 1 ppm as an 8-hour time weighted average. Initial monitoring will be repeated each time there is a change in procedure or equipment. Monitoring shall also be repeated at the request of a laboratory employee who has reason to believe formaldehyde exposure above the PEL has occurred.
   2. Fume hoods should be checked periodically with the flow meter to ensure proper functioning.
   3. Emergency equipment is checked on a monthly basis and recorded. Test eyewashes, safety showers, smoke alarms, safety lights and fume hoods for proper function. The first aid kit, spill clean-up kit and fire blanket are checked for completeness and new supplies are added as needed.
   4. Fire extinguishers are checked every year by fire department personnel.

G. Medical program
   1. All laboratory employees are encouraged to have a yearly physical.
   2. Whenever an injury or hazardous chemical exposure in the workplace occurs an employee must report it to their supervisor and seek medical attention.
   3. If signs or symptoms develop in an employee that may be associated with a hazardous chemical exposure in the workplace the employee must report it and seek medical attention.
   4. If exposure monitoring reveals an exposure level routinely above the PEL then the employee must seek a medical consultation by a qualified physician to determine if regular medical surveillance is desirable.

H. Characteristics of various chemical types

1. Reactives - reactives are chemicals that can react violently. Reactive chemicals include explosives, oxidizers and incompatible chemicals.
   a. Oxidizers, such as nitric acid, contain large percentages of oxygen. They should always be stored away from flammables since they could cause them to ignite. Oxidizers should also be kept away from materials that burn easily such as paper or wood.
   b. Incompatible chemicals may be stable on their own but react strongly when mixed together. They should always be stored in separate locations. See Table 1 in this chapter (page 16-19) for a partial list of incompatible chemicals.
   c. Picric acid is an explosive solid when dry and should be kept away from heat, sparks and flame. In the Municipality of Anchorage it must be kept in solution. Otherwise it should be stored wet with not less than half its own weight of water. Keep in a tightly closed container and check on a periodic basis for evaporation. Add a small amount of water when necessary. In the Juneau lab hydrated picric acid is stored in the storage cabinet on the outside deck near the rear exit.
   d. Keep fire, sparks, electrical and heat sources away from reactives.
2. Flammables - flammables are gases and liquids that burn, release vapors or explode at temperatures under 100°F.
   a. Keep flammables away from heat, fire sources, sparks and electrical equipment. They should also be stored away from sunlight.
   b. Flammables should be stored by themselves in a temperature-controlled, well-ventilated area.
   c. They should be stored in safety containers with vapor-tight caps. Most flammables are volatile which means they evaporate quickly.
   d. Always keep flammables away from reactives.
   e. Work with flammables in a well-ventilated area.
   f. When transferring liquids from bulk stock containers to smaller containers, use a fume hood.
   g. Do not use alcohol for cleaning purposes.
   h. Empty flammable containers should be rinsed thoroughly with water and disposed of with their caps off.
   i. Only heat flammables in a fume hood. Use electric plate for heating, not an open flame.
   j. In the case of an emergency or spill follow these guidelines:
      1). Immediately shut down all flames and sparking equipment.
      2). Ventilate the area by opening window, etc.
      3). If you are in any doubt as to the seriousness of the situation, evacuate the area as quickly as possible, let others know what has happened and close the doors behind you. Then, elicit help in assessing the severity of the situation.
      4). Spills should be cleaned up as soon as possible.
      5). If your clothing is contaminated, remove it immediately.
   k. Solvents are usually volatile, flammable liquids, that dissolve other substances. With volatile solvents, inhalation of vapors is a major hazard.
      1). Irritation or damage to skin, eyes, lungs and other organs can result if too much of a toxic solvent is absorbed.
      2). Solvent splashes in the eyes can result in acute symptoms including burning, watering, irritation and redness. Overexposure can eventually cause chronic symptoms like blurred vision, irritation and permanent vision damage.

3). Breathing in a solvent vapor can result in symptoms such as headache, nausea, sore throat, dizziness, fatigue or irregular heartbeat. Over time, liver, kidney or nervous system damage can occur.

4). Splashing solvents on the skin can cause dryness, rashes, burning or irritation. If a solvent enters the bloodstream, symptoms listed in 3 above can occur. Long-term skin exposure to solvents can cause chronic skin conditions which may include blistering, redness and general discomfort.

5). Never use paper towels, newspaper, etc. to wipe up solvent spills. Paper merely absorbs liquids but does nothing to eliminate the hazard. Follow directions in the cleanup kit specifically for solvent spills.

3. Corrosives - Corrosives are acids and bases with a pH of <2.0 or >12.5 and they are capable of destroying human tissue.
   a. Corrosives should be stored in an area by themselves.
   b. Acids and bases should be stored on separate shelves to keep them apart.
   c. When transferring corrosives, use a transfer carrier and pour them under a fume exhaust hood.
   d. Corrosives should be stored in tightly closed containers and separated from flammables.
   e. Many corrosives are incompatible with other chemicals. Review the incompatible chemical table on a regular basis.

I. Detection of hazardous chemicals

Toxic materials enter the body in one of four ways: inhalation, ingestion, adsorption through the skin or mucous membranes and entry through a wound. If proper protective equipment is utilized, exposure to chemicals above the PEL can be avoided. The following is a list of possible symptoms caused by exposure to hazardous substances. If you experience any of these symptoms, report it immediately.

1. Chemical smell.
2. Respiratory irritation which could be indicated by coughing, sore throat, difficulty in breathing, tightness in chest, irritation of nose or pharyngitis.
3. Irritation of eyes including watering, excessive blinking, burning, blurred vision or redness.
4. Headache, weakness, nausea or dizziness.
5. Itching or burning of skin, redness or rash.

XII. Spill Cleanup

A. A spill cleanup kit is located in the middle lab on the east wall in Anchorage and in Juneau it is mounted in a benchtop kickspace to the right of the entrance into the big lab. The kit enables laboratory personnel to clean up spills of acids, caustics, solvents and mercury.

B. A chemical spill is always a possibility when working in a laboratory. It is necessary that all personnel know how to handle a spill if it occurs.

C. Many of the most frequently used chemicals such as acids, bases and flammable solvents are also the most hazardous to handle. When spills occur, quick action is necessary to contain the spill and eliminate the hazardous situation. For effective spill cleanup, acids and caustics must be neutralized and solvent vapors must be smothered.

D. Read through the spill control products instruction manual. The manual gives information on spill cleanup capacity, hazards, general safety information, protective devices to wear and an outlined procedure.

E. One common accident in the laboratory is the breakage of thermometers causing a mercury spill.
   1. Mercury vapor is highly toxic so it is important to clean up mercury as thoroughly as possible, especially in confined areas.
   2. Gloves, eye protection and laboratory coat should be worn when cleaning up spilled mercury.
   3. The main bulk of the mercury should be physically collected using wooden spatulas and aspirator present in the cleanup kit.
   4. Use the elemental mercury absorbent material present in the cleanup kit to pick up any remaining mercury.

F. Material from a chemical spill is considered hazardous waste and should be disposed of accordingly.

G. Spill and disposal procedures and emergency first aid procedures are covered in the MSDS for individual chemicals. The following general guidelines should be followed immediately in the case of an accident or spill involving a chemical. As with all accidents, it should always be reported and medical attention sought if necessary.
   1. Eye contact - promptly flush eyes with water for a prolonged period (10-15 minutes) using the continuous flow eyewash.
   2. Ingestion - drink large amounts of water.
   3. Skin contact - flush infected area with water and remove any contaminated clothing.
   4. Inhalation - move to fresh air.

XIII. Accident Reporting

A. It is the responsibility of each employee to report all accidents to their supervisor.

B. An accident report should be filled out and routed to the appropriate people/offices.

C. Accidents should be discussed at the weekly laboratory staff meeting so causes can be determined and recurrences prevented. All accidents should be brought to the attention of the Safety Committee.

D. In the case of a serious accident, the employee should seek medical treatment immediately.

XIV. Training

A. All new employees will receive a safety orientation by their supervisor or the Safety Officer and fill out the safety orientation checklist.

B. All employees will be trained to understand the hazardous substances in their work area at the time of their initial assignment and whenever a new hazard is introduced. The training will be conducted by their immediate supervisor with the assistance of other personnel as needed. At the completion of the initial training, the training

documentation form should be completed for each employee and placed in a central safety training file. Dates on which safety training occurred should be documented.

C. Safety concerns are discussed as a regular part of the weekly staff meeting. Any accidents, concerns, or new items of business may be brought up at this time. Training may also be done at this time. Safety items discussed are documented in the safety section of the weekly staff report.

D. Employees are encouraged to take safety classes including lab safety, hazardous material handling, first aid/CPR, field safety and other related courses when available. These should all be documented on the training documentation list.

E. Periodically, employees are required to perform hazardous non-routine tasks. Prior to starting work on such projects, each affected employee will be given information by their supervisor about hazards to which they may be exposed during such an activity.

F. It is critically important that all employees understand the training. If you have any questions please contact your supervisor or Safety Officer.

G. If anyone becomes aware of new hazards relating to chemicals we have in use, all employees will be informed of the new information immediately. MSDS will be updated by the Safety Officer as needed.

H. Should employees of another employer (such as contractor employees) be potentially exposed to hazardous chemicals or physical agents while working at one of the labs, it is the responsibility of the Safety Officer or supervisor in charge to hold a meeting with the other employer(s). In this meeting we will provide information concerning location of MSDS and PADS for the chemicals their workers may be exposed to or that may be brought to the work site and inform them where safety equipment is located.

I. A light aircraft safety manual is available and is required reading for employees traveling in small planes.

XV. Safety Committee/Program Review

A. The Safety Committee will consist of the Fishery Scientist/Principal Fish Pathologist , the Fish Pathologist II with supervisory responsibility, and the senior microbiologist. The Microbiologist II in the Anchorage Lab is the Anchorage Safety Officer as is the Fishery Scientist in the Juneau lab. The committee will have the primary responsibility to ensure that the safety program is carried out, but all employees must take responsibility for using safe practices in the workplace and for bringing concerns to the attention of the committee.

B. The Hazard Communication and Chemical Hygiene Program will be reviewed by the Principal Pathologist on a yearly basis. Changes, additions and deletions will be made as necessary. Special attention will be taken to assure training activities are being accomplished and documented.

C. All employees are required to review the Hazardous Communication and Chemical Hygiene Program and the MSDS of hazardous chemicals they work with on an annual basis. This should be recorded on the training documentation form.

TABLE 1

INCOMPATIBLE CHEMICALS

The following is a partial list of chemicals which are incompatible with each other. Reactions can take place which may liberate poisonous or flammable gases, cause explosions by contact or by their reaction products, or may ignite spontaneously. An occasional review of this list will be a reminder of these hazards and may suggest other incompatibles which may be encountered.

Substances in the right-hand column should be stored and handled so they cannot accidentally contact corresponding substances in the left-hand column.

Alkaline and Alkaline Earth Metals such as Sodium, Potassium, Cesium, Lithium Magnesium, Calcium, Aluminum	Carbon dioxide, Carbon tetrachloride and other Chlorinated hydrocarbons. (Also prohibit water, foam, and dry chemical on fire involving these Metals)
Acetic Acid	Chromic acid, Nitric acid, Hydroxyl-containing compounds, Ethylene glycol, Perchloric acid, Peroxides, and Permanganates
Acetone	Concentrated Nitric and Sulfuric acid mixtures
Acetylene	Chlorine, Bromine, Copper, Silver, Fluorine, and Mercury
Ammonia (Anhyd.)	Mercury, Chlorine, Calcium hypochlorite, Iodine, Bromine, and Hydrogen fluoride
Ammonium Nitrate	Acids, Metal powders, flammable liquids, Chlorates, Nitrates, Sulfur, and combustibles
Aniline	Nitric acid, Hydrogen peroxide
Bromine	Ammonia, Acetylene, Butadiene, Butane and other petroleum gases, Hydrogen, Sodium carbide, Turpentine, Benzene
Calcium Carbide	Water (see also Acetylene)
Carbon, activated	Calcium hypochlorite
Copper	Acetylene, Hydrogen peroxide
Chlorates	Ammonium salts, acids, metal powders, Sulfur, and combustibles
Chromic Acid	Acetic acid, Naphthalene, Camphor, Glycerin, Turpentine, Alcohol, and other flammable liquids

INCOMPATIBLE CHEMICALS (Continued)

Chlorine	Ammonia, Acetylene, Butadiene, Butane, and other petroleum gases, Hydrogen, Sodium carbide, Turpentine, and Benzene
Chlorine Dioxide	Ammonia, Methane, Phosphine, and Hydrogen sulfide
Fluorine	Isolate from everything
Hydrocyanic Acid	Nitric acid, Alkalis
Hydrogen Peroxide	Copper, Chromium, Iron, most metals or their salts, any flammable liquid, combustible materials, Aniline, Nitromethane
Hydrofluoric Acid, Anhyd. (Hydrogen Fluoride)	Ammonia
Hydrogen Sulfide	Fuming Nitric acid, oxidizing gases
Hydrocarbons (Benzene, Butane, Propane, Gasoline, Turpentine, etc.)	Fluorine, Chlorine, Bromine, Chromic acid, Sodium peroxide
Iodine	Acetylene, Ammonia, Hydrogen
Mercury	Acetylene, Fulminic acid, Ammonia
Nitric Acid (conc)	Acetic acid, Aniline, Chromic acid, Hydrocyanic acid, Hydrogen sulfide, flammable liquids and flammable gases
Oxygen	Oils, grease, Hydrogen and flammable liquids, flammable solids and flammable gases
Oxalic Acid	Silver, Mercury
Perchloric Acid	Acetic anhydride, Bismuth and its alloys, Alcohol, paper, wood
Phosphorus (white)	Air, Oxygen
Potassium chlorate	Acids (see also Chlorates)
Potassium Perchlorates	Acids (see also Perchlorates)
Potassium Permanganate	Glycerin, Ethylene glycol, Benzaldehyde, Sulfuric acid

INCOMPATIBLE CHEMICALS (Continued)

Silver	Acetylene, Oxalic acid, Tartaric acid, Fulminic acid, Ammonium compounds
Sodium	See Alkaline metals (above)
Sodium Nitrite	Ammonium nitrate and other Ammonium salts
Sodium Peroxide	Any oxidizable substance, such as Ethanol, Methanol, Glacial acetic acid, Acetic anhydride, Benzaldehyde, Carbon disulfide, Glycerin, Ethylene glycol, Ethyl acetate, Methyl acetate
Sulfuric Acid	Chlorates, perchlorates, Permanganates
Toluene Di-isocyanate	Avoid contact with strong Alkalis, other Isocyantes such as Caustic soda, to prevent uncontrollable polymerizations
Trichlorethylene	Reacts with strong Alkalis to form gases which ignite spontaneously

TABLE 2

PROPERTIES OF VARIOUS CHEMICALS

UNSTABLE CHEMICALS

Azides
Perchloric acid
Picric acid
Acetylene
Peroxides in ethers

CORROSIVE CHEMICALS

LIQUIDS	SOLIDS	GASES
Acetic acid	Barium hydroxide	Ammonia
Formic acid	Potassium hydroxide	Hydrogen chloride
Nitric acid	Sodium hydroxide	Hydrogen bromide
Sulfuric acid	Phenol	Hydrogen fluoride
		Formaldehyde

ACUTELY TOXIC CHEMICALS

Potassium cyanide
Chloroform
Phenol
Formalin

CHRONICALLY TOXIC CHEMICALS

Benzene
Carbon tetrachloride
Toluene
Xylene

FLAMMABLE CHEMICALS

	Flash Point
Acetone	0°F
Benzene	12°F
Carbon disulfide	-22°F
Diethylether	-49°F
Dioxane	54°F
Hexane	-7°F
Methanol	54°F
Toluene	40°F
Xylene	81°F

SAFETY ORIENTATION CHECKLIST

Date/Initials

1. Read and sign the written Hazard Communication and Chemical Hygiene Program. _____
2. Knows location of MSDS and knows how to use them. _____
3. Knows location of hazardous chemical inventory. _____
4. Has read MSDS on all hazardous chemicals that employees will be working with. _____
5. Knows location of safety equipment and has been shown how to use it. _____
6. Knows location of hazardous materials identification system (HMIS) poster and how to use it. _____
7. Understands how to use HMIS labels present on hazardous chemicals. _____
8. Knows where hazardous chemicals are used and stored. _____
9. Knows the evacuation procedures. _____
10. Knows location of spill clean- up kit and demonstrates how to use it. _____
11. Knows proper personal hygiene procedures to be used when working with chemicals and possible biohazards. _____
12. Has taken a walk through tour of lab facility and was shown location of spill cleanup kit, fire blanket, eyewashes, safety shower, fire extinguishers, fire alarm, first aid kit, safety goggles, apron, gloves, telephones, MSDS, and hoods. _____

_____ _____
Employee Name Date

_____ _____
Instructor's Name Date