

## **CHAPTER 12**

# **Corroborative Testing of Viral Isolates**

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## I. Introduction

Serological testing is an important step in viral identification and confirmation. Several viruses such as IHNV and VHSV can produce similar cytopathic effects on cell cultures making it difficult to determine the identification of the viral agent based on cell culture results alone. Serological tests are highly specific allowing accurate and rapid identification of viruses based on unique antigenic characteristics. The serum neutralization test has long been the standard test for corroboration of viral isolates for these reasons. However, advances in molecular techniques such as DNA probes and Polymerase Chain Reaction (PCR) allow identification of viral isolates based on their nucleotide sequences. These sequences are used to develop primers specific to the viral strain, or group of viruses. These molecular tools provide an increased sensitivity and unsurpassed specificity that allows rapid and accurate identification of viral isolates.

## II. 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<sub>10</sub> 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 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 - can be used to confirm the identity of suspected IHNV isolates from possible viral epizootics or from fish species. Flat bottom 24-well plates are used for this serum neutralization assay.

### 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 them to form a monolayer 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  $1 \times 10^5$  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 unused 24-well plate appropriately, as in the example.
2. Aseptically pipet 200  $\mu$ L of diluted antiserum into appropriate wells.
3. Aseptically pipet 200  $\mu$ L of diluted normal serum into appropriate wells.
4. Aseptically pipet 200  $\mu$ L of MEM into appropriate wells. Add 400  $\mu$ L of MEM into tissue control well (MEM only).
5. Aseptically pipet 200  $\mu$ L of each known and unknown virus into appropriate wells.
6. Incubate for one hour at room temperature on a 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	+
Normal Serum	KV + NS	KV + NS	UV + NS	UV + NS	MEM	+
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 duplicate 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.

- 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.
- Overlay EPC cells with 100  $\mu$ L of 7% PEG for a few minutes prior to inoculations. PEG solution should be made up in MEM-10.
- Pipet 100  $\mu$ L of each dilution into appropriate well of PEG-treated EPC cells.
- Incubate for 30 minutes at room temperature to allow virus adsorption.
- Overlay wells with 1 mL of methylcellulose overlay medium.
- Incubate at 15°C for 7 days in a sealed plastic bag or plastic container.
- 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.
- Pour off stain, rinse monolayers with water and allow plates to air dry.
- Count and record numbers of plaques.

#### E. INTERPRETATION

- 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.
- 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.
- 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.
  - Calculating pfu/mL: Viral titer for each sample is expressed as mean plaque-forming units (pfu) per mL of test tissue 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.
  - 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 =  $1/0.1 \text{ mL} = 1/10^{-1} = 10^1$   
 $20 \text{ plaques} \times 10^4 \times 10^1 = 2.0 \times 10^6 \text{ pfu/mL ovarian fluid}$

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 un-neutralized virus in MEM. Example:

<u>Antisera</u>	<u>Log<sub>10</sub> pfu/mL Remaining Virus</u>			
	IHNV	NI	VHSV	NI
IHNV	2.0	<b>2.8</b>	4.4	<b>0.4</b>
VHSV	4.8	<b>0</b>	<1.0	<b>&gt;3.8</b>
MEM	4.8	<b>0</b>	4.8	<b>0</b>

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.

### III. Immunoblot

Dot blot is a relatively simple and quick assay to differentiate virus detected in cell culture; however, it should be employed as an additional test for viral identification. Serum neutralization or PCR is still suggested for confirmation.

Run controls for each assay using known virus as positive controls; cell cultures and PBS as negative controls. Follow good laboratory techniques by handling all suspect samples as positive for virus. Discard capillary tubes, wash solutions, and all supplies coming in contact with samples into an appropriate biohazard container or a chlorine solution.

NOTE: Heat-inactivated IHN and VHS viruses (60°C for 2 hours) work well in dot blot and eliminate the need for biohazard precautions.

#### A. BLOTTING PROCEDURES

1. Don't touch the nitrocellulose membrane (paper) with hands. Use forceps and handle the paper by the edges.
2. Draw a grid of 1 cm squares on the paper using a permanent alcohol/waterproof marker.

Cut into strips; one strip will be used for each antisera.

3. Pour PBS 1X into a glass staining dish. Slide one edge of strip just under the surface of the PBS and slowly submerge paper until uniformly wet. After soaking for a few minutes, remove strips and air dry on bibulous paper for 5 minutes.
4. Make a map of your sample placement. With microcapillary tubes, slowly spot 10 $\mu$ L of each sample on a square of the paper. Air dry until paper appears completely dry (approximately 5-10 minutes). If using two antisera, repeat this process, in the same order, on the second antisera strip.
5. In a glass staining dish, immerse the strips for 20 minutes in the 3% gelatin solution. Keep at 37°C to prevent gelatin from solidifying.
6. Remove each strip and quickly rinse in PBS 1X to remove excess gelatin. Then place each strip in a plastic Ziploc bag or "seal-a-meal" pouch and add 3-5 mL of the antisera of choice (IHN, IPN, or VHS). Seal and incubate at 37°C for 60 minutes. Agitate (by palpating pouch) every 15 minutes.
7. The primary antibody is saved for reuse. Open sack and carefully remove all antisera, making sure antisera is returned to its appropriate container.
8. Place the paper in a glass dish with Tween 20 solution (TPBS) and wash for 30 minutes, changing the TPBS every 10 minutes. The TPBS should be approximately 3-4 cm deep for each wash. Use a separate dish for each antisera used.
9. Put paper strips in a plastic Ziploc bag or "seal-a-meal" pouch and add 5-10 mL of the secondary antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP)]. Incubate for 60 minutes at 37° C. Agitate every 15 minutes.
10. Open sack and discard the second antibody solution. Wash strips as in step 8 above.

## **B. COLOR DEVELOPMENT**

1. Prepare solutions A and B at room temperature during the final wash. Mix the solutions immediately prior to use.
2. Put paper in a glass dish, add the color developer solution, and place in the dark. Allow to develop 7-10 minutes.
3. Remove strips from the developer and wash in running water for 15 minutes. Positive reactions will appear as dark blue spots where samples were blotted. Place strips between two 96-well plate sealers if you wish to keep results as reference or records.

### C. FORMULAS FOR DOT BLOT REAGENTS

PBS 10X (Calcium & Magnesium Free) - Indefinite shelf life @ room temperature

NaCl	80.0 gm
KCl	2.0 gm
Na <sub>2</sub> HPO <sub>4</sub>	11.5 gm
KH <sub>2</sub> PO <sub>4</sub>	1.0 gm
Thimerosal (0.01%)	0.1 gm
qs to 1000 mL with cell culture water	

PBS 1X - Shelf life at least 1 month @ room temperature

Cell culture water	900.0 mL
PBS 10X (Ca <sup>++</sup> , Mg <sup>++</sup> free)	100.0 mL

3% Gelatin - Shelf life stable for at least 1 month @ 4°C

Gelatin [Enzyme Immunoassay (EIA) Grade]	3.0 gm
PBS 1X	100.0 mL
Heat or microwave to dissolve gelatin.	

Tween 20 Solution (TPBS) - Shelf life at least 1 month @ room temperature

PBS 1X	500.0 mL
Tween 20	0.1 mL (5 drops)

Primary Antibody – Recommended dilution for the primary antibody (i.e., rabbit antisera against IHN, IPN, or VHS) is 1:1000.

Secondary Antibody - Goat anti-Rabbit Horseradish Peroxidase Conjugate (GAR-HRP)

	1:2000	1:3000
GAR-HRP	10.0 µL	10.0 µL
PBS 1X	20.0 mL	30.0 mL

Color Development Solution (CDS)

Solution A - HRP Color Development Reagent	30.0 mg (0.03 gm)
Cold methanol (reagent grade)	10.0 mL
Solution B - Cold hydrogen peroxide (30% stabilized reagent grade) (Recommend J. T. Baker, cat. #JT-2186-1, 500 mL.)	30.0 µL
PBS 1X	50.0 mL

Prepare A and B solutions during final wash period, prior to color development. Mix A and B solutions together immediately prior to use to make color development solution.

## **IV. Indirect Fluorescent Antibody Test (IFAT) for Viral Identification**

Immunofluorescence assay for IHNV is used as another serological test for confirming the identity of a viral isolate as IHNV (LaPatra et al. 1989). 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.

### **A. MATERIALS**

- cleaned FAT slides and coverslips (Freed, Inc.)
- disposable petri dishes
- gauze swabs
- airtight plastic container
- filtered (0.45  $\mu\text{m}$ ) distilled water
- MEM-10-3X (3x antibiotic)
- EPC cells
- MAB mouse monoclonal anti-IHNV antiserum (or other suitable antiserum)
- Goat anti-mouse IgG FITC conjugate (Cappel)
- 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 and incubate at 21°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 at 15°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 at 4°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.

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

Introduction: The DNA Probe detects and identifies isolates of IHNV, North American VHSV, and European VHSV, using a dot blot procedure from infected fish cell cultures.

Biotinylated DNA probes for IHNV and both European and North American strains of VHSV were developed by Deering et al.(1991) and 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 are extracted from tissue culture cells that have been infected for 24-48 hours. The probe for North American VHSV hybridizes specifically with a nearly unique 28-nucleotide sequence following the open reading frame of the N gene mRNA. The probe recognizing all strains of VHSV binds to a 29-nucleotide sequence near the center of the N gene common to both American and European strains. The IHNV-specific probe (Deering et al. 1991) recognizes a 30 base sequence unique only to IHNV.

#### **A. DNA PROBE SET UP**

Two days before running DNA Probe Test:

1. Prepare a 24-well microtiter plate with EPC or CHSE-214 cells so that it will be confluent by the next day.

2. 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.
3. Rinse needed glassware with DEPC treated water and let dry. Store on shelf in an area dedicated for this use.
4. Sterilize distilled water and make up solutions that need to be autoclaved (solutions f, g, l, m, n).

One day before running DNA probe test:

1. 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.
2. Prepare all other solutions needed to run the test. Adjust pH of final products carefully.
3. Fill both water baths and turn on. Adjust to 55°C and 65°C.
4. Get out rotator, Hybridot, and Seal-a-Meal 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:
    - i. Wet membrane in distilled water for 1 minute. Wet by capillary action at an angle.
    - ii. Pour water off.
    - iii. Soak for at least 5 minutes in 10X standard saline citrate (SSC).
  - i. For each probe used, heat approximately 140  $\mu$ L 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  $\mu$ L of product.
  - k. Warm prehybridization buffer to 55°C in water bath.
  - l. Add 170  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L of each RNA solution to wells of Hybridot which contain 200  $\mu$ L 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  $\mu$ L 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. Probes for IHNV and VHSV are synthesized from the sequences given in Derring et al. (1991) or Batts et al. (1993). They can be obtained from the USGS, Western Fisheries Research Center, Seattle, Washington.
  5. 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 Five Prime→Three Prime, Inc. (catalog #5302-336550)

(a) **PREHYBRIDIZATION BUFFER**

Distilled-deionized water	69.5 mL
10x Denhardt's solution	10 mL of 100x stock
2x SSC	10 mL of 20x stock
1% SDS	10 mL of 10% stock
0.1 mg/mL SSS DNA (Five Prime→Three Prime)	0.5 mL of 20 mg/mL stock

(b) **HYBRIDIZATION SOLUTION**

Prehybridization buffer	10 mL
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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
0.1% SDS	5 mL of 10% stock
distilled-deionized water	up to 500 mL

(d) **DENHARDT'S SOLUTION**

(Commercial product (Five Prime→Three Prime, Inc.) purchased at 100x stock concentration)  
(Catalog #5302-213502 for 100-mL size)

A 10x solution contains:

1% bovine serum albumin  
1% polyvinylpyrrolidone 360  
1% ficoll 400

(e) **SONICATED SALMON SPERM DNA (SSS DNA)**

(Commercial product (Five Prime→Three Prime, Inc.) purchased at 20 mg/mL)  
(Catalog #5302-754688 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)

(Option: may purchase product already prepared from Five Prime→Three Prime, Inc.)  
(Catalog #5302-227160)

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

NaCl (Sigma #S-3014, 3 M final concentration)	43.82 g
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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 (BRL #9543SA), 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 <sub>2</sub> (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 <sub>2</sub> (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
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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) **RNAzol B**

RNA isolation solvent, store at 2-8°C in dark. Cinna Biotech Laboratories, Inc. 6023 South Loop East, Houston, Texas 77033. 1-800-535-6286. Catalog #104B is 100-mL size. Contains guanidine thiocyanate, 2-mercaptoethanol, and phenol.

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

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

1. Dissolve AP color development buffer in 1 L volume of distilled-deionized water.
2. Filter-sterilize then store at 4°C until needed.
3. 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.

**RAPID DOT BLOT (7 h)**

CELLS INFECTED WITH VHSV OR IHNV

8:30

EXTRACT RNA (2 h)

10:30

BLOT RNA, GET READY TO  
PREHYBRIDIZE (0.5 h)

11:00

PREHYBRIDIZE MEMBRANE (1 h)

12:00

HYBRIDIZE TARGET RNA ON MEMBRANE  
IN PROBE SOLUTION (1 h)

1:00

COLOR DEVELOPMENT OF SPOTS  
(2.5 h)

3:30

## VI. Polymerase Chain Reaction (PCR) Methods for Detection of Fish Viruses

Personnel should be knowledgeable of the information in Appendix C - General Procedures for PCR and Appendix E - QA/QC for PCR before using these protocols for confirmation of viral fish pathogens.

### A. PCR FOR INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV)

Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of IHNV (Arakawa et al. 1990) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

#### 1. Extraction of RNA from cell culture fluid (Heat RNA release method):

Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

- a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 $\mu$ L fluid to 98 $\mu$ L water in microcentrifuge tubes.
- b. Heat tubes to 95°C for 2 min. in a heat block, or thermocycler.
- c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).
- d. Quantify RNA Template in the spectrophotometer (Appendix D). The optimum amount of RNA template should be around 100  $\mu$ g/mL (or 100 ng/ $\mu$ L). Generally, 1 $\mu$ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ $\mu$ L or use up to 5 $\mu$ L/reaction if reading falls below 50 ng/ $\mu$ L.

#### 2. Production of DNA by Reverse Transcription and Amplification by First Round PCR (refer to Appendix C for General Procedures for PCR).

- a. QA/QC (see Appendix E for QA/QC considerations for PCR).
- b. Using Worksheet 12.A.1. (Appendix A) Infectious Hematopoietic Necrosis Virus (IHNV) record case number and date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of MgCl<sub>2</sub> for this reaction should be determined when the

- procedure or any component of the reaction mixture is altered.
- c. First Round Primers for IHNV:  
Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'  
  
Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'
  - d. First Round Thermocycler Program for IHNV:
    - 1) Pre-dwell at 50°C for 15 minutes.
    - 2) Preheat or “Jumpstart” sample to 95°C for two minutes.
    - 3) 25 cycles as follows:
      - i. Denaturing at 95°C for 30 seconds.
      - ii. Annealing at 50°C for 30 seconds.
      - iii. Extending at 72°C for 60 seconds.
    - 4) Post dwell at 72°C for 7 minutes.
    - 5) Hold samples at 4°C after cycling is complete.
3. “Nested” Second Round PCR for IHNV:  
If the first round PCR provides insufficient amplified product a nested set of primers is used for additional DNA amplification.
- a. QA/QC (see Appendix E for QA/QC considerations for PCR)
  - b. Again use Worksheet 12.A.1 (Appendix A) for the Second Round (IHNV) to record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples and controls to be processed.
  - c. Second Round Primers for IHNV:  
Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'  
  
Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'
  - d. Second Round Thermocycler Program for IHNV
    - i. Preheat or “Jumpstart” sample to 95°C for two minutes.
    - ii. 25 cycles as follows:
      1. Denaturing at 95°C for 30 seconds.
      2. Annealing at 50°C for 30 seconds.
      3. Extending at 72°C for 60 seconds.

- 1) Post dwell at 72°C for 7 minutes.
- 2) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

4. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
  - a. **Bands occurring at the 786 bp location in the First Round Assay and the 323 bp location in the Second Round Assay are confirmatory for IHNV and are reported as POSITIVE.**
  - b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IHNV.**
5. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, "Photodocumentation of the PCR Product Gel").

## B. PCR FOR INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV)

Infectious Pancreatic Necrosis Virus (IPNV) is a nonenveloped icosahedral shaped bi-segmented double-stranded RNA virus belonging to the *Aquabirnavirus* genus of the *Birnaviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of IPNV (Blake et al. 1995) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

### 1. Extraction of RNA from cell culture fluid (Heat RNA release method):

Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

- a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 $\mu$ L fluid to 98 $\mu$ L water in microcentrifuge tubes.
- b. Heat tubes to 100°C for 10 min in a heat block.
- c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).
- d. Quantify RNA Template in the spectrophotometer (Appendix D). The optimum amount of RNA template should be around 100  $\mu$ g/mL (or 100 ng/ $\mu$ L). Generally, 1 $\mu$ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ $\mu$ L or use up to 5 $\mu$ L/reaction if reading falls below 50 ng/ $\mu$ L.

### 2. Production of DNA by Reverse Transcription and Amplification by PCR (refer to Appendix C for General Procedures for PCR).

- a. QA/QC (see Appendix E for QA/QC considerations for PCR).
- b. Using Worksheet 12.A.2. (Appendix A) Infectious Pancreatic Necrosis Virus (IPNV), record case number and date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of MgCl<sub>2</sub> for this reaction should be determined when the procedure or any component of the reaction mixture is altered.
- c. Primers for IPNV:  
Forward: 5'- AAA GCC ATA GCC GCC CAT GAA C -3'  
  
Reverse: 5'- TCT CAT CAG CTG GCC CAG GTA C -3'

- d. Thermocycler Program for IPNV
  - 1) Pre-dwell at 50°C for 15 min.
  - 2) Preheat or “Jumpstart” sample to 95°C for 2 min.
  - 3) 35 cycles as follows:
    - i.* Denaturing at 95°C for 30 sec.
    - ii.* Annealing at 50°C for 30 sec.
    - iii.* Extending at 72°C for 60 sec.
  - 4) Post dwell at 72°C for 7 min.
  - 5) Hold samples at 4°C after cycling is complete.

PRC Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

3. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.
  - a. **A band occurring at the 174 bp location is confirmatory for IPNV and is reported as POSITIVE.**
  - b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IPNV.**
4. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, “Photodocumentation of the PCR Product Gel”).

## C. PCR FOR INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus belonging to the newly proposed *Isavirus* genus of the *Orthomyxoviridae*. The Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR) for confirmation of ISAV (Bouchard et al. 1999) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

### 1. Extraction of RNA from cell culture fluid (Heat RNA release method):

Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low. See Chapter 14 Non-Lethal Methodology for Detection of Fish Pathogens, ISAV section, for extraction method using the QIAGEN RNeasy® kit and amplification using Invitrogen® One Step RT-PCR System.

- a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2µL fluid to 98µL water in microcentrifuge tubes.
  - b. Heat tubes to 95°C for 2 min. in a heat block or thermocycler.
  - c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).
  - d. Quantify RNA Template in the spectrophotometer (Appendix D). The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5µL/reaction if reading falls below 50 ng/µL.
- ### 2. Production of DNA by Reverse Transcription and Amplification by PCR (refer to Appendix C for General Procedures for PCR).
- a. QA/QC (see Appendix E for QA/QC considerations for PCR).
  - b. Using Worksheet 12.A.3. (Appendix A) Infectious Salmon Anemia Virus (ISAV), record case number and date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of MgCl<sub>2</sub> for this reaction should be determined when the procedure or any component of the reaction mixture is altered.
  - c. Primers for ISAV:  
Forward: 5’- GGC TAT CTA CCA TGA ACG AAT C - 3’

Reverse: 5'- TAG GGG CAT ACA TCT GCA TC - 3'

d. Thermocycler Program for ISAV

- 1) Pre-dwell at 42°C for 15 min.
- 2) Preheat or “Jumpstart” sample to 94°C for 5 min.
- 3) 40 cycles as follows:
  - i.* Denaturing at 94°C for 45 sec.
  - ii.* Annealing at 59°C for 45 sec.
  - iii.* Extending at 72°C for 105 sec.
- 4) Post dwell at 72°C for 7 min.
- 5) Hold samples at 4°C after cycling is complete.

PRC Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

3. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). The use of 0.5X TAE buffer is recommended for gel preparation and electrophoresis running buffer (Appendix E). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

**a. A band occurring at the 493 bp location is confirmatory for ISAV and is reported as POSITIVE.**

**b. The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for ISAV.**

4. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, “Photodocumentation of the PCR Product Gel”).

## D. PCR FOR LARGEMOUTH BASS VIRUS (LMBV)

Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the *Ranavirus* genus of the *Iridoviridae* family. Protocols for confirmation of Largemouth Bass Virus by PCR were developed by Grizzle et al. (2003). DNA extraction procedures by BuccalAmp kit is a modification provided by J. Woodland (USFWS, Pinetop Fish Health Center). LMBV is a DNA containing virus so DNA is extracted from cell culture fluid, amplified with forward and reverse primers, and the products are then visualized by agarose gel electrophoresis. Note the PCR master mix contains Uracil-DNA glycosylase and the deoxynucleotide dUTP is used in place of dTTP.

1. Extraction of DNA from cell culture fluid - DNA extraction may be completed with one of the following methods.
  - a. Extract DNA following the procedures outlined in the BuccalAmp DNA extraction kit (Epicentre®). Collect sample by rotating the swab onto infected cells still attached to the well of cell culture plate. To obtain sufficient DNA, swab 1-2 wells (24 well plate) or 3-4 wells (48 well plate) containing infected cells. Follow remaining steps provided with the kit.
  - b. Extract DNA following procedures provided for QIAGEN DNeasy® Tissue kit.
    - i. Supernatant and cells from suspect sample wells are removed and centrifuged for 5 minutes at 300 Xg.
    - ii. Resuspend pellet in 200µL PBS.
    - iii. Add 20µL proteinase K solution and 200µL Buffer AL to the sample, mix thoroughly by vortexing.
    - iv. Incubate for 10 minutes at 70°C.
    - v. Add 200µL of 100% ethanol to the sample, mix thoroughly by vortexing.
    - vi. Pipet the mixture, including any precipitate into the DNeasy spin column sitting in the 2 mL collection tube provided.
    - vii. Centrifuge for 1 minute at 6,000 Xg . Discard flow-through and collection tube.
    - viii. Place the DNeasy spin column in a new 2 mL collection tube, add 500µL Buffer AW1, and centrifuge for 1 minute at 6,000 Xg. Discard the flow-through and collection tube.

- ix. Place the DNeasy spin column in a new 2 mL collection tube, add 500 $\mu$ L Buffer AW2, and centrifuge for 3 minutes at full speed to dry the membrane.
  - x. Place the DNeasy spin column in a clean 1.5 or 2 mL microcentrifuge tube and pipet 200 $\mu$ L Buffer AE directly onto the DNeasy membrane.
  - xi. Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 6,000 Xg to elute.
  - xii. Repeat steps x and xi.
  - xiii. Discard spin column and store DNA solution at -20 to -70°C until used for amplification.
- c. Quantify DNA Template in the spectrophotometer (Appendix D). The optimum amount of DNA template should be around 10 - 100  $\mu$ g/mL (10 - 100 ng/ $\mu$ L). Dilute template if more than 350 ng/ $\mu$ L or use up to 5 $\mu$ L/reaction if reading falls below 10 ng/ $\mu$ L.

2. Amplification of DNA by PCR (refer to Appendix C for General Procedures for PCR).

- a. QA/QC (see Appendix E for QA/QC considerations for PCR).
- b. An internal control may be performed using the highly conserved, 632 bp segment of the  $\beta$ -actin gene (GenBank accession L36342), see Worksheet 12.A.4 (Appendix A) for primer set. If  $\beta$ -actin is used an adjustment will be required to the master mix (less water). See also Grizzle et al. (2003).
- c. Using Worksheet 12.A.4. (Appendix A) Largemouth Bass Virus (LMBV), record case number and date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). Note this MM uses dUTP in place of dTTP to make a quasi DNA molecule. DNA is normally made of A, C, G, T nucleotides, however the replicated quasi DNA in this procedure makes DNA with uracil (U) nucleotide in place of thymine (T). The quasi DNA is degraded when placed in the MM and incubated at 20°C. In turn, the uracil-DNA glycosylase is inactivated at 95°C in the second step of the thermocycler program. Therefore this process allows for degrading of contaminant DNA containing U prior to amplification of new target DNA with U as a component. If running a large number of samples plan a negative control every 10 samples. The optimum concentration of MgCl<sub>2</sub> for this reaction should be determined when the procedure or any component of the reaction mixture is altered.
- d. Primers for LMBV:  
Forward: 5'- GCG GCC AAC CAG TTT AAC GCA A -3'

Reverse: 5'- AGG ACC CTA GCT CCT GCT TGA T -3'

e. Thermocycler Program for LMBV

- 1) UNG incubation at 20°C for 5 min.
- 2) UNG inactivation at 95°C for 3 min.
- 3) 15 cycles as follows:
  - i. Denaturing at 95°C for 45 sec.
  - ii. Annealing at 60°C for 45 sec.
  - iii. Extending at 72°C for 60 sec.
- 4) 20 cycles as follows:
  - i. Denaturing at 95°C for 45 sec.
  - ii. Annealing at 60°C for 45 sec.
  - iii. Extending at 72°C for 60 sec.; 5 seconds are added to each successive extension step beginning at the 16th cycle and proceeding through the 35th cycle (ie. 65, 70, 75....155 sec). If this routine can not be programmed into your thermocycler, set extension to 60 sec. for all 35 cycles.
- 5) Post dwell at 72°C for 7 min.
- 6) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated (4°C) for one month or frozen (-70°C) for long term storage.

3. Visualize the DNA by electrophoresis (1X TBE, ~75 volts for 1 – 1.5 hrs) of the product in 1.0% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay.

**a. A band occurring at the 248 bp location is confirmatory for LMBV and is reported as POSITIVE.**

**b. The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for LMBV.**

4. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, “Photodocumentation of the PCR Product Gel”).

## E. PCR FOR *ONCORHYNCHUS MASOU* VIRUS (OMV)

*Oncorhynchus Masou* Virus (OMV) is an enveloped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. OMV is considered an exotic pathogen in the United States and the maintenance of live virus for positive controls by serological methods may not be prudent in many laboratories. The Polymerase Chain Reaction (PCR) method may be used to confirm that the cause of the CPE is due to the presence of OMV or suspect samples may be sent to an appropriate laboratory for confirmation. A Polymerase Chain Reaction (PCR) procedure that does not require live positive control material has been developed for this virus (Aso et al. 2001). DNA is extracted from cell culture material, amplified with forward and reverse primers, and then products are visualized with agarose gel electrophoresis.

1. Extraction of DNA from Cell Culture Fluid:
  - a. Supernatant and cells from suspect sample wells are removed and a pellet is formed by centrifugation of this material at 19,000 Xg (14,800 rpm) for 15 minutes.
  - b. Wash the pellets twice with 1 mL PBS and mix with 200 µL of chelating resin (Sigma).
  - c. Incubate the mixture at 56°C for 20 minutes in a water bath, vortex, and then place in a boiling water bath for 8 minutes.
  - d. Vortex the samples and centrifuge at 8200Xg (10,000 rpm) for 90 seconds.
2. Amplification of DNA by PCR (refer to Appendix C for General Procedures for PCR).
  - a. QA/QC (see Appendix E for QA/QC considerations for PCR).
  - b. Using Worksheet 12.A.5. (Appendix A) *Oncorhynchus Masou* Virus (OMV), record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).
  - c. Primers for OMV:  
Forward: 5’-GTA-CCG-AAA-CTC-CCG-AGT-C-3’  
Reverse: 5’- AAC-TTG-AAC-TAC-TCC-GGG-G-3’
  - d. Thermocycler program for OMV:
    - 1) 30 cycles of the following regime:
      - i. Denaturing at 94°C for 30 seconds
      - ii. Annealing at 56°C for 30 seconds

*iii.* Extending at 72°C for 30 seconds

2) Hold samples at 4°C after cycling is complete.

Note: PCR Products can be refrigerated (4°C) for one month or frozen at -70 °C for long-term storage.

3. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C)-. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assay
  - a. **A band occurring at the 439 bp location is confirmatory for OMV and is reported as POSITIVE.**
  - b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for OMV.**
4. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, "Photodocumentation of the PCR Product Gel")
5. A laboratory capable of confirming the identity of OMV is the Laboratory of Microbiology, Hokkaido University, 3-1-1 Minato-cho, Hokodate, Hokkaido 041-0821, Japan, Phone/fax: (81.138) 40.88.10

## F. PCR FOR SPRING VIREMIA OF CARP VIRUS (SVCV)

Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Vesiculovirus* genus of the *Rhabdoviridae*. The Polymerase Chain Reaction (PCR) method may be used for confirmation of SVCV (Stone et al. 2003). The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

It should be noted that this PCR assay will also amplify Pike Fry Rhabdovirus (PFRV) and that further testing is required to distinguish isolates of the four genogroups described by Stone et al. (2003). Because several PFRV-like isolates cross-react in both the ELISA and immunofluorescent antibody test using anti-serum raised against SVCV, PCR products should be sequenced to identify the isolate.

### 1. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method):

Note: other methods for extraction of RNA are available including the Trizol Reagent™ method described by Strømme and Stone (1997). Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

- a. Dilute Cell Culture fluid (with some cell scrapings) 1:20 in molecular grade RNase free water by adding 5 $\mu$ L fluid to 95 $\mu$ L water in microcentrifuge tubes.
- b. Place tubes in heat block at 95°C for 2 min.
- c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).
- d. Quantify RNA Template in the spectrophotometer (Appendix D). The optimum amount of RNA template should be around 100  $\mu$ g/mL (or 100 ng/ $\mu$ L). Generally, 1 $\mu$ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ $\mu$ L or use up to 5 $\mu$ L/reaction if reading falls below 50 ng/ $\mu$ L.

### 2. Formation of cDNA by Reverse Transcription and Amplification by PCR (refer to Appendix C for General Procedures for PCR).

- a. QA/QC (see Appendix E for QA/QC considerations for PCR)
- b. Using Worksheet 12.A.5. (Appendix A) Spring Viremia of Carp Virus (SVCV), record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

- c. First Round Primers for SVCV:  
Forward (SVC F1): 5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'  
  
Reverse (SVC R2):5'-AGA TGG TAT GGA CCC CAA TAC ATH ACN CAY-3'

d. Thermocycler Program for SVCV:

- 1) Pre-dwell at 50°C for 20 minutes (cDNA synthesis)
- 2) Preheat or “Jumpstart” sample to 95°C for 2 minutes.
- 3) 30 cycles as follows:
  - i.* Denaturing at 95°C for 30 seconds.
  - ii.* Annealing at 50°C for 30 seconds.
  - iii.* Extending at 72°C for 60 seconds.
- 4) Post dwell at 72°C for 7 minutes.
- 5) Hold samples at 4°C after cycling is complete.

3. “Semi-Nested” Second Round PCR for SVCV:

If the first round PCR provides insufficient amplified product a semi-nested assay is used for additional DNA amplification. The semi-nested assay uses the same forward primer used in the first round of amplification.

- a. QA/QC (see Appendix E for QA/QC considerations for PCR)
- b. Again use Worksheet 12.A.5 (Appendix A) for the Second Round (SVCV) to record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples and controls to be processed.
- c. Second Round Primers for SVCV:  
Forward (SVC F1): 5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'  
  
Reverse (SVC R4): 5'- CTG GGG TTT CCN CCT CAA AGY TGY -3'

d. Second Round Thermocycler Program for SVCV

- 1) Preheat or “Jumpstart” sample to 95°C for two minutes.
- 2) 30 cycles as follows:
  - i.* Denaturing at 95°C for 30 seconds.
  - ii.* Annealing at 50°C for 30 seconds.
  - iii.* Extending at 72°C for 60 seconds.

- e. Post dwell at 72°C for 7 minutes.
- f. Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

- 2. Visualize the DNA by electrophoresis of the product in 1.5% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.

- a. **Bands occurring at the 714 bp location in the First Round Assay and the 606 bp location in the Second Round Assay are confirmatory for SVCVV and are reported as POSITIVE.**
- b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for SVCV.**

PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

- 6. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, “Photodocumentation of the PCR Product Gel”)

## G. PCR FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHSV)

Viral Hemorrhagic Septicemia Virus (VHSV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods or the biotinylated DNA probe method presented in this chapter (Section 12.V). Polymerase Chain Reaction (PCR) method for confirmation of VHSV (Einer-Jensen et al. 1995) is accomplished by extraction of total RNA from cell culture supernatant, subject to reverse transcription for production of appropriate complementary DNA (cDNA), which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.

### 1. Extraction of RNA from cell culture fluid (Heat RNA release method):

Note: other methods for extraction of RNA are available. Several suppliers of molecular reagents have developed extraction kits which may result in high yields of target RNA and may be useful in situations where virus titer is low.

- a. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 $\mu$ L fluid to 98 $\mu$ L water in microcentrifuge tubes.
  - b. Heat tubes to 95°C for 2 min. in a heat block, or thermocycler.
  - c. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).
  - d. Quantify RNA Template in the spectrophotometer (Appendix D). The optimum amount of RNA template should be around 100  $\mu$ g/mL (or 100 ng/ $\mu$ L). Generally, 1 $\mu$ L of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/ $\mu$ L or use up to 5 $\mu$ L/reaction if reading falls below 50 ng/ $\mu$ L.
- ### 2. Production of DNA by Reverse Transcription and Amplification by First Round PCR (refer to Appendix C for General Procedures for PCR).
- a. QA/QC (see Appendix E for QA/QC considerations for PCR)
  - b. Using Worksheet 12.A.7. (Appendix A) Viral Hemorrhagic Septicemia Virus (VHSV), record case number and date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls). The optimum concentration of MgCl<sub>2</sub> for this reaction should be determined when the procedure or any component of the reaction mixture is altered.
  - c. First Round Primers for VHSV:  
Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'

Reverse: 5'-TTC CGG TGG AGC TCC TGA AG-3'

d. First Round Thermocycler Program for VHSV:

- 1) Pre-dwell at 50°C for 15 minutes.
- 2) Preheat or “Jumpstart” sample to 95°C for 2 min.
- 3) 25 cycles as follows:
  - i. Denaturing at 95°C for 30 sec.
  - ii. Annealing at 50°C for 30 sec.
  - iii. Extending at 72°C for 60 sec.
- 4) Post dwell at 72°C for 7 minutes.
- 5) Hold samples at 4°C after cycling is complete.

3. “Nested” Second Round PCR for VHSV:

- a. QA/QC (see Appendix E for QA/QC considerations for PCR)
- b. Again use Worksheet 12.A.7 (Appendix A) for the Second Round VHSV to record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according the number of samples and controls to be processed.
- c. Second Round Primers for VHSV:

Forward: 5'-ATG GGC TTC AAG GTG ACA C-3'

Reverse: 5'-GTA TCG CTC TTG GAT GGA C-3'

d. Second Round Thermocycler Program for VHSV:

- 1) Preheat or “Jumpstart” sample to 95°C for two minutes.
- 2) 25 cycles as follows:
  - i. Denaturing at 95°C for 30 seconds.
  - ii. Annealing at 50°C for 30 seconds.
  - iii. Extending at 72°C for 60 seconds.
- 3) Post dwell at 72°C for 7 minutes.
- 4) Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated (4°C) for one month or frozen at -70 ° C for long-term storage.

1. Visualize the DNA by electrophoresis of the product in 1.2% agarose gel stained with ethidium bromide and observe using UV transillumination (Appendix C). Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
  - c. **Bands occurring at the 950 bp location in the First Round Assay and the 558 bp location in the Second Round Assay are confirmatory for VHSV and are reported as POSITIVE.**
  - d. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for VHSV.**
2. Photograph the gel - Photo document all gels and attach the photo to the case history information. (Appendix B, "Photodocumentation of the PCR Product Gel").

## H. PCR FOR WHITE STURGEON HERPESVIRUS (WSHV)

White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. A PCR method has been developed for this virus, however, the necessary sequences are not available at this time. Therefore, suspect samples must be sent to a reference laboratory for confirmation.

1. A laboratory capable of confirming the identity of WSHV is the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, Phone: 530-752-3411.

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## **Appendix 12.A – PCR Worksheets: Amplification of Nucleic Acid for the Corroboration of Viral Fish Pathogens**

- 12.A.1 Infectious Hematopoietic Necrosis Virus (IHNV)
- 12.A.2 Infectious Pancreatic Necrosis Virus (IPNV)
- 12.A.3 Infectious Salmon Anemia Virus (ISAV)
- 12.A.4 Largemouth Bass Virus (LMBV)
- 12.A.5 Oncorhynchus Masou Virus (OMV)
- 12.A.6 Spring Viremia of Carp Virus (SVCV)
- 12.A.7 Viral Hemorrhagic Septicemia Virus (VHSV)

## Worksheet 12.A.1: Infectious Hematopoietic Necrosis Virus (IHNV)

Case Number \_\_\_\_\_ Date \_\_\_\_\_

### Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ___ samples
d-H <sub>2</sub> O*		-	-	23.75 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 U/Rx <sup>†</sup>	9 U/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template <sup>a</sup>		-	-	5.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>†</sup> Rx = Reaction

<sup>a</sup>Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H<sub>2</sub>O equal to the volume of template subtracted.

### Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ___ samples
d-H <sub>2</sub> O*		-	-	27.5 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

**Primer Sets for IHN**

	<b>Forward</b>	<b>Reverse</b>
1 <sup>st</sup> round	5'-TCA AGG GGG GAG TCC TCG A-3'	5'-CAC CGT ACT TTG CTG CTA C-3'
2 <sup>nd</sup> round	5'-TTC GCA GAT CCC AAC AAC AA-3'	5'-GCG CAC AGT GCC TTG GCT-3'

**Control Information**

	<b>POSITIVE CONTROLS</b>		<b>NEGATIVE CONTROLS</b>	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				
2 <sup>nd</sup> round				

**Amplification (Thermocycle Process)**

<b>Round Number (Date &amp; time)</b>	<b>Program #</b>	<b>NOTES</b>

**Gel Preparation**

<b>Gel concentration</b>	<b>Apparatus and Gel Size</b>	<b>Weight of agarose (g)</b>	<b>Volume of Buffer (mL)</b>

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>
<b>16</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>	<b>26</b>	<b>27</b>	<b>28</b>	<b>29</b>	<b>30</b>

## Worksheet 12.A.2: Infectious Pancreatic Necrosis Virus (IPNV)

Case Number \_\_\_\_\_ Date \_\_\_\_\_

### Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for ___ samples
d-H <sub>2</sub> O*		-	-	23.75 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
Reverse Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template <sup>a</sup>		-	-	5.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>a</sup>Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H<sub>2</sub>O equal to the volume of template subtracted.

### Primer Sets for IPNV

	Forward	Reverse
1 <sup>st</sup> round	5'-AAAGCCATAGCCGCCCATGAAC-3'	5'- TCTCATCAGCTGGCCCAGGTAC-3'

### Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				

**Amplification (Thermocycle Process)**

Round Number (Date & time)	Program #	NOTES

**Gel Preparation**

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

## Worksheet 12.A.3: Infectious Salmon Anemia Virus (ISAV)

Case Number \_\_\_\_\_ Date \_\_\_\_\_

### Master Mix for Initial or First Round Reaction

PCR Reagent <sup>a</sup>	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 50µL)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	26.75 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
Forward Primer		50 pmoles/Rx	50 pmoles/µL	1.0 µL	
Reverse Primer		50 pmoles/Rx	50 pmoles/µL	1.0 µL	
Taq		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template <sup>b</sup>		-	-	5.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>a</sup>A MM protocol using Superscript One-Step RT-PCR System® (Invitrogen) is also acceptable. See ISAV section in Chapter 14 Non-Lethal Methodologies for Detection of Fish Pathogens.

<sup>b</sup>Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H<sub>2</sub>O equal to the volume of template subtracted.

### Primer Sets for ISAV

	Forward	Reverse
1 <sup>st</sup> round	5'-GGC TAT CTA CCA TGA ACG AAT C-3'	5'-TAG GGG CAT ACA TCT GCA TC-3'

### Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				

**Amplification (Thermocycle Process)**

Round Number (Date & time)	Program #	NOTES

**Gel Preparation**

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

## Worksheet 12.A.4: Largemouth Bass Virus (LMBV)

Case Number \_\_\_\_\_ Date \_\_\_\_\_

### Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (to total 35µL)	Volume for ___ samples
d-H <sub>2</sub> O <sup>a</sup>		-	-	Add to 35 µL total Rx volume	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	3.5 µL	
MgCl <sub>2</sub>		2.0 mM	25 mM	2.8 µL	
dNTP's <sup>b</sup>			<sup>b</sup>	0.7 µL	
GC-rich solution <sup>c</sup>		1X	5X	7.0 µL	
Uracil-DNA glycosylase		0.5 U/µL	1.0 U	0.5 µL	
Forward Primer		15 pmol/Rx	15 pmol/µL	1.0 µL	
Reverse Primer		15 pmol/Rx	15 pmol/µL	1.0 µL	
FastStart Taq		1.2 U/Rx	5 U/µL	0.24 µL	
DNA Template		-	-	Adjust to meet target quantity	-

<sup>a</sup> Add nuclease free water to Master Mix first, Taq last. Add to total of 35 µL per reaction.

<sup>b</sup> dNTP mixture contains 10µL of 100 mM dATP, 10µL of 100 mM dCTP, 10µL of 100 mM dGTP, 30µL of 100 mM dUTP, and 40µL d-H<sub>2</sub>O. Can be aliquoted to convenient volumes and stored at -20°C.

<sup>c</sup> Use concentration recommended by supplier. MM table uses GC-rich solution supplied with FastTaq® polymerase from Roche Molecular Biochemicals (Indianapolis, IN).

### Primer Sets for LMBV

	Forward	Reverse
1 <sup>st</sup> round	5'- GCG GCC AAC CAG TTT AAC GCA A -3'	5'- AGG ACC CTA GCT CCT GCT TGA T -3'

### Internal Control Primer Set for 632 bp segment of the β-actin gene (GenBank L36342)

	Forward	Reverse
1 <sup>st</sup> round	5'-TGC GTG ACA TCA AGG AGA AG -3'	5'-AAT CCA CAT CTG CTG GAA GG-3'

**Control Information**

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				

**Amplification (Thermocycle Process)**

Round Number (Date & time)	Program #	NOTES

**Gel Preparation**

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)
1%			

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products</b> (Loaded LEFT to RIGHT)														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

## Worksheet 12.A.5: Oncorhynchus Masou Virus (OMV)

Case Number \_\_\_\_\_ Date \_\_\_\_\_

### Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	15.5 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		1.5 mM	25 mM	3.0 µL	
dNTP's		0.8 mM	10 mM	4.0 µL	
TMAC		40 µM	100 µM	20.0 µL	
(+)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
(-)Primer		50 pmoles/Rx	100 pmole/µL	0.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
DNA Template <sup>a</sup>		-	-	1.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>a</sup>If more template is needed (up to 5.0 µL) subtract equal volume of d-H<sub>2</sub>O.

### Primer Sets for OMV

	Forward	Reverse
1 <sup>st</sup> round	5'-GTA CCG AAA CTC CCG AGT C-3'	5'-AAC TTG AAC TAC TCC GGG G-3'

### Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				

**Amplification (Thermocycle Process)**

Round Number (Date & time)	Program #	NOTES

**Gel Preparation**

Gel concentration	Apparatus and Gel Size	Weight of agarose (g)	Volume of Buffer (mL)

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

**Worksheet 12.A.6: Spring Viremia of Carp Virus (SVCV)**

Case Number \_\_\_\_\_ Date \_\_\_\_\_

**Master Mix for Initial or First Round Reaction**

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	23.75 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.25 mM	2.5 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		10 Units/Rx	40 Units/µL	0.25 µL	
RNA Template <sup>a</sup>		-	-	5.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>a</sup>Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H<sub>2</sub>O equal to the volume of template subtracted.

**Master Mix for Semi-Nested or Second Round Reaction**

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	27.5 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.25 mM	2.5 mM	5.0 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

**Primer Sets for SVCV**

	<b>Forward</b>	<b>Reverse</b>
1 <sup>st</sup> round	5'- TCT TGG AGC CAA ATA GCT CAR RTC -3'	5'-AGA TGG TAT GGA CCC CAA TAC ATH ACN CAY-3'
2 <sup>nd</sup> round	5'- TCT TGG AGC CAA ATA GCT CAR RTC-3' (same as 1 <sup>st</sup> round)	5'-CTG GGG TTT CCN CCT CAA AGY TGY-3'

**Control Information**

	<b>POSITIVE CONTROLS</b>		<b>NEGATIVE CONTROLS</b>	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				
2 <sup>nd</sup> round				

**Amplification (Thermocycle Process)**

<b>Round Number (Date &amp; time)</b>	<b>Program #</b>	<b>NOTES</b>

**Gel Preparation**

<b>Gel concentration</b>	<b>Apparatus and Gel Size</b>	<b>Weight of agarose (g)</b>	<b>Volume of Buffer (mL)</b>
1.5%			

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

**Worksheet 12.A.7: Viral Hemorrhagic Septicemia Virus (VHSV)**

Case Number \_\_\_\_\_ Date \_\_\_\_\_

**Master Mix for Initial or First Round Reaction**

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (50µL total)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	23.75 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
RNasin		9.75 Units/Rx	39 Units/µL	0.25 µL	
RNA Template <sup>a</sup>		-	-	5.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

<sup>a</sup>Adjust quantity of template RNA according to concentration/dilution determined by UV spec.- if less than 5µL is used add a volume of d-H<sub>2</sub>O equal to the volume of template subtracted.

**Master Mix for Nested or Second Round Reaction**

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (50µL total)	Volume for _____ samples
d-H <sub>2</sub> O*		-	-	27.5 µL	
PCR Buffer (no MgCl <sub>2</sub> )		1X	10X	5.0 µL	
MgCl <sub>2</sub>		2.5 mM	25 mM	5.0 µL	
dNTP's		0.2 mM	2 mM	5.0 µL	
(+)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
(-)Primer		50 pmoles/Rx	20 pmoles/µL	2.5 µL	
TAQ		2.5 Units/Rx	5 Units/µL	0.5 µL	
Round 1 Product		-	-	2.0 µL	-

\*Add nuclease free water to Master Mix first, TAQ last.

**Primer Sets for VHSV**

	<b>Forward</b>	<b>Reverse</b>
1 <sup>st</sup> round	5'-TCT CTC CTA TGT ACT CCA AG-3'	5'-TTC CGG TGG AGC TCC TGA AG-3'
2 <sup>nd</sup> round	5'-ATG GGC TTC AAG GTG ACA C-3'	5'-GTA TCG CTC TTG GAT GGA C-3'

**Control Information**

	<b>POSITIVE CONTROLS</b>		<b>NEGATIVE CONTROLS</b>	
	Extraction	PCR	Extraction	PCR
1 <sup>st</sup> round				
2 <sup>nd</sup> round				

**Amplification (Thermocycle Process)**

<b>Round Number (Date &amp; time)</b>	<b>Program #</b>	<b>NOTES</b>

**Gel Preparation**

<b>Gel concentration</b>	<b>Apparatus and Gel Size</b>	<b>Weight of agarose (g)</b>	<b>Volume of Buffer (mL)</b>

**Gel Template (Sample Placement Map)**

Ladder Brand / Lot # \_\_\_\_\_ Loading Buffer Brand / Lot # \_\_\_\_\_

Enter Sample ID below corresponding well number:

<b>PCR Products (Loaded LEFT to RIGHT)</b>														
<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>
<b>16</b>	<b>17</b>	<b>18</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>	<b>26</b>	<b>27</b>	<b>28</b>	<b>29</b>	<b>30</b>

## **Appendix 12.B – Photodocumentation of Agarose Gel Electrophoresis of PCR Products**

Case Number \_\_\_\_\_ Date: \_\_\_\_\_ Initials \_\_\_\_\_

Samples \_\_\_\_\_

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

## Appendix 12.C – General Procedures for PCR Protocols

### A. Preparation of Amplification Reaction Mixture

Specific amplification protocols may require one or two amplification reactions.

**Note:** samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures.

1. Under a UV cabinet, prepare “Master Mix” (MM) using pathogen-specific protocols in the worksheets of Appendix A. Calculate the amount of each reagent to go into the MM according to the number of samples to be processed. Add PCR reagents, **except for sample DNA**, in the order listed on the worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing and return them to freezer immediately after use. **Note:** Prepare enough MM for 2-3 more samples, including controls, than actually being tested to compensate for retention of solution in pipette tips and tube.
2. Place specified volume of MM into each PCR tube. Close caps tightly. Move PCR tubes to sample loading area.
3. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly.

### B. Running the PCR

All general considerations should be employed including the following:

1. Thermocycler should be programmed for the specific PCR condition used for each pathogen (see thermocycler programs in Section VI ).
2. Before loading into thermocycler, give tubes a “quick-spin” to ensure that all reagents and sample are drawn down from sides of tube.
3. Load the sample tubes into the wells following manufacturer’s recommendations.
4. Program thermocycler for appropriate cycle conditions and run reaction.
5. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes, perform a “quick-spin” to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.
6. PCR products can be refrigerated for up to a month following amplification (or for longer storage they may be frozen at -20°C).

### C. Detection of Product

Procedures for preparing the gel (refer to specific manufacturer's guidelines for preparation of gels and electrophoresis chambers):

1. Assemble the gel tray and position well comb in the tray according to manufacturer's recommendations. Note gel box combs are available in several sizes and will affect the quantity of DNA product that can be loaded. Verify that the comb you use will be sufficient for the amount of product you choose to separate by electrophoresis (generally 10 $\mu$ L).
2. Prepare buffer (Appendix F - Reagents) with distilled water to volume adequate for agarose gel and running buffer.

Note: either TAE or TBE buffer is acceptable. Generally a concentration of 1X is used however some protocols suggest a concentration of 0.5X. Use 1X unless specified in Visualization of PCR Product by Electrophoresis section for each pathogen.

3. Prepare agarose gel according to percent recommended (generally 1–2%) under pathogen section and the volume recommended for specific gel forms used.

- a. Weigh appropriate amount of agarose and add to proper volume of buffer.

Note: the same type of buffer and concentration must be used in both the agarose gel and running buffer.

- b. Heat solution to near boiling until agarose is completely dissolved.
  - c. Allow solution to cool to about 65°C, then pour agarose solution into gel tray. Avoid the formation of bubbles especially around the comb. Bubbles may be removed with a hot flame-sterilized needle.
  - d. Allow gel to cool completely for about 30 minutes and then carefully remove the comb.
  - e. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.
  - f. Slowly fill the chamber with the remaining buffer solution until the top of the gel surface is submerged.
4. Load samples into wells as indicated for each assay:
    - a. For each tube of PCR product to be visualized, mix 2  $\mu$ L of gel loading dye (Appendix F - Reagents) to every 10  $\mu$ L of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading. Parafilm® provides a good surface for performing this procedure.

- b. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight standard (one with bands at 100 bp increments in the 100 to 1,000 bp range) for base pair (bp) reference and positive and negative controls.
- c. Document gel lane assignments for each sample and control on the Amplification of Nucleic Acid by PCR worksheet (Appendix A) and allow for at least one lane for a DNA ladder reference (when possible one on each side of the gel is preferred).

#### **D. Electrophoresis**

Approximately 80 volts for 30 - 90 minutes or until tracking dye front approaches the edge of the gel. The rate of electrophoresis is dependent on gel width and size, so refer to manufacturer's recommendations. Generally small gel chambers can be run faster than larger gels.

Tracking dye mobility chart.

Percent agarose	Base pair size corresponding to dye in 1X TBE	
	XC	BPB
1.00	4100	400
1.25	2500	260
1.50	1800	200

#### **E. Staining the Gel**

Remove gel and tray and place in ethidium bromide (EtBr) solution (Appendix F - Reagents) for 15 to 20 minutes.

Note: EtBr solution can be reused and should be stored in a dark plastic tray container with a secure lid. EtBr is very toxic and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

#### **F. De-Staining the Gel**

In water for 5 to 60 minutes. De-stain water should be handled and disposed of appropriately (see Section E above "Staining the Gel").

#### **G. Visualize the DNA:**

1. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used (these are designated in each viral pathogen section).

Note: use UV protective goggles or face shield when working with UV light.

2. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.
3. Photo document all PCR gels and attach to Photodocumentation of PCR Product Gel worksheet (Appendix B), or provide reference for finding the photo documentation.

## Appendix 12.D – Analysis of Extracted DNA using a Gene Quant UV Spectrophotometer

- A. Turn on Gene Quant spectrophotometer before using (no need to let it warm up).
- B. Dilute QIAGEN DNA isolation 1:100 in order to make a 1:100 dilution (add 5 $\mu$ l of isolate to 495 $\mu$ l of sterile water).
- C. Take Reference Measurement first\*.
  - 1.) Add 100 $\mu$ l of sterile water to cuvette. Check that there are no bubbles or meniscus visible in the optical path window. Wipe sides of cuvette with lens paper.
  - 2.) Hit [ Set Ref ] button. Screen should say " Please wait ".
  - 3.) Wait for tone. Tone sounds, screen will display " Insert reference ". Quickly insert cuvette into slot.
  - 4.) Wait for the second tone and remove cuvette when it sounds and screen displays "Remove reference".
  - 5.) Once removed, display should show: "Absorbance": 260nm 0.000AU
  - 6.) Empty cuvette onto paper towel by gently knocking it upside down. Dry cuvette with canned air (keep air can level to prevent spraying liquid into cuvette).

\*Note: Once reference is set, there is no need to set it again (i.e. between samples).

### D. Sample Measurement

- 1.) Add 100  $\mu$ l of dilution sample to cuvette; check that there are no bubbles or meniscus in optical path, wipe sides with Kim-wipe.
- 2.) Press [ Sample ] button. Screen will display: " Please wait."
- 3.) When tone sounds, insert sample. Screen will display: "Insert sample."
- 4.) When second tone sounds, remove cuvette. Screen will read: "Remove sample."
- 5.) Screen will automatically show Absorbance at 260nm.
- 6.) Press the [ RNA / DNA ] button. Screen will now show: "dsDNA conc 1"  
 $X$  is the sample conc. in  $\mu$ g/mL  $X$   $\mu$ g/mL  
**RECORD THIS NUMBER ON DATA SHEET!**
- 7.) Press the [ Select ] button. Screen should now read:  
"dsDNA conc 2"  
 $X$   $\mu$ g/ $\mu$ L  $X$  is the conc. in  $\mu$ g/ $\mu$ L.  
**RECORD THIS NUMBER ON DATA SHEET!**
- 8.) If reading multiple samples, rinse cuvette with sterile water at least 3 times, then dry with canned air.
- 9.) For next sample repeat from step one of sample measurement.

- E. Calculate the amount of QIAGEN isolate sample to add per PCR reaction tube.

### FIRST LOOK AT THE TWO NUMBERS FOR CONCENTRATIONS

The range for dsDNA conc. 1 is: 1-4,000 µg/mL  
The range for dsDNA conc. 2 is: 0.001-0.2 µg/µL

Decide which of your two readings best fits within its range, use that conc. number.

1.) Take your concentration “X” number and multiply it by the dilution factor used.

$$(X) \times \text{dil. factor} = \text{actual conc. in tube.}$$

\* **Note: If DNA sample is too dilute** to measure at 1:200, **make a less dilute solution** for spec analysis. For example - make a 1:50. Record this dilution factor on worksheet.

2.) **Use this formula for dsDNA conc. #2:**

$$\frac{300\text{ng}}{(\text{dil. factor}) \times X} \times \frac{1\mu\text{L}}{1000\text{ng}} = \# \text{ of } \mu\text{l of DNA isolate to add per rxn tube.}$$

3.) **Use this formula for dsDNA conc. #1:**

$$\frac{300\text{ng}}{(\text{dil. factor}) \times X} \times \frac{1\text{mL}}{1000\text{ng}} \times \frac{1000\mu\text{L}}{1\text{mL}} = \# \text{ of } \mu\text{l of DNA isolate to add per rxn tube.}$$

4.) **Remember if the sample is at a 1:200 dilution then the dilution factor is 200.**

**RECORD THE AMOUNT TO ADD PER RXN TUBE ON DATA SHEET!**

\* In case this amount to add is less than 0.5µL you can make small dilutions of the sample DNA with sterile water to get a number between 1µL - 10µL.

F.) Shutdown

- 1.) Clean cuvette with sterile water and dry it with canned air, place cuvette in storage case.
- 2.) Shut machine off.

\*Note: It is safe to keep the machine on all day. The deuterium lamp goes into standby mode which does **not** shorten its life. Reference reading taken earlier in the day is still good after the instrument has been left in standby mode.

## Appendix 12.E – Quality Assurance/Quality Control for PCR

### A. GENERAL CONSIDERATIONS

1. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions discussed in Chapter 2, Sample Collection and Submission, to avoid cross-contamination.
2. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like “DNA Away”) to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray/wipe pipettors and working areas with DNA or RNase -Away and turn UV on for at least 30 minutes after use (UV light damages DNA).
3. **Wear and change gloves often.** This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.
4. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.
5. Mix and aliquot pre-amplification ingredients under bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.
6. **One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents!** Therefore, three separate areas of lab space are necessary to reduce the risk of contamination.
  - a. Master Mix (MM) Area with UV Hood:  
For mixing and aliquoting master mix reagents. Supply area with dedicated pipettes, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**
  - b. Sample Loading Area with UV Hood and Dedicated Pipettor:  
For loading of extracted (template) DNA from samples.
  - c. Amplified DNA Area:  
Supplied with pipettes dedicated for **amplified PCR product ONLY**. Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with “DNA Away” type solutions after working with amplified DNA.
7. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

8. Controls:
  - a. Extraction controls.  
A known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.
  - b. PCR Controls  
Sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.
9. Primers: Newly received primer batches should first be tested on known positive and negative controls.
10. Dispose of trash containing amplified DNA products frequently.

## **B. SAMPLE PROCESSING**

1. Tissue samples should be collected on a clean bench top, which has been disinfected using a 10% chlorine (or “DNA Away”) solution if possible. If collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil etc.)
2. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.
3. Keep samples cold and freeze as soon as possible at or below -20°C until processing can be accomplished.
4. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT-PCR should be frozen immediately and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

## **C. EXTRACTION OF DNA OR RNA FROM SAMPLES**

Individual protocols will vary in specific steps for extraction of genetic material; however, the following general considerations should be employed:

1. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

2. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.
3. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

#### **D. QUANTIFICATION OF DNA**

If the protocol used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in Appendix D, Analysis of Extracted DNA using a Gene Quant UV Spectrophotometer.

#### **E. INTERPRETATION OF PCR RESULTS**

Use of the appropriate controls should allow you to assess the integrity of your PCR result.

1. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR, or human error (e.g. loading errors).
2. False-positive reactions may result from contamination either directly from the sample lot being tested or from previously amplified target DNA.
3. For further help in troubleshooting, see PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).

## Appendix 12.F – Reagents

### 1. Electrophoresis Buffers

TAE Buffer:	10X	Sigma T-4035. Comes in prepared packets, add DI water and qs to 1.0L. Label as 10X- Stock (store at RT).
	1X	Diluted 1:10 from 10X stock. Label as 1X Working Solution (store at RT).
TBE Buffer:	10X	121g Tris Base 61.7g Boric acid 7.44g EDTA qs d-H <sub>2</sub> O to 2.0L
	1X	Diluted 1:10 from 10X stock. Label as 1X Working Solution (store at RT).

### 2. PCR Loading Buffer:

6X Sigma P-7206  
Pre-made 6X concentrate, ready to use (store - 0°C).

-OR-

Loading Dye:	6X	
Prepare in-house (Sambrook et al.)		Bromophenol blue 0.25% Xylene cyanol 0.25% Glycerol 30.0 % store at 4°C

### 3. Ethidium Bromide - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

**Note: USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH “CHEMICAL CARCINOGEN.”**

EtBr Stock Solution:	10mg/mL	100 mg Ethidium Bromide DI water 10 mL Protect from light (store at RT)
EtBr Working Solution	4.0ug/mL*	Add 200 µL STOCK SOLUTION to 500mL water Protect from light (store at RT)

\*References may suggest weaker working solutions (0.5 µg/mL) and staining periods of 45 to 60 minutes.