

## 4.6 Identification of Viruses

Methods used for confirmation must have high specificity for the agents they are used to identify but high sensitivity is not required since the cell culture screening method amplifies the virus. The serum neutralization and indirect fluorescent antibody test (IFAT) have long been used for viral identification, Alkaline Phosphatase immunocytochemical staining has been useful in the identification of IHNV in archived samples and historical studies, and polymerase chain reaction (PCR) procedures have now been developed for many of the listed viruses. With the exception of WSHV, procedures for one or more of these three confirmation methods have been included for each virus. The identification of IHNV, IPNV, SVCV, and VHSV may be confirmed using serum neutralization. The identification of IHNV, IPNV, ISAV, and VHSV may be confirmed using IFAT. The identification of IHNV, IPNV, ISAV, LMBV, OMV, SVCV, and VHSV may be confirmed using PCR. The identification of IHNV may be confirmed using APIC stain. WSHV suspect cultures and all replicating agents not identified with these procedures will be sent to an appropriate reference laboratory for identification.

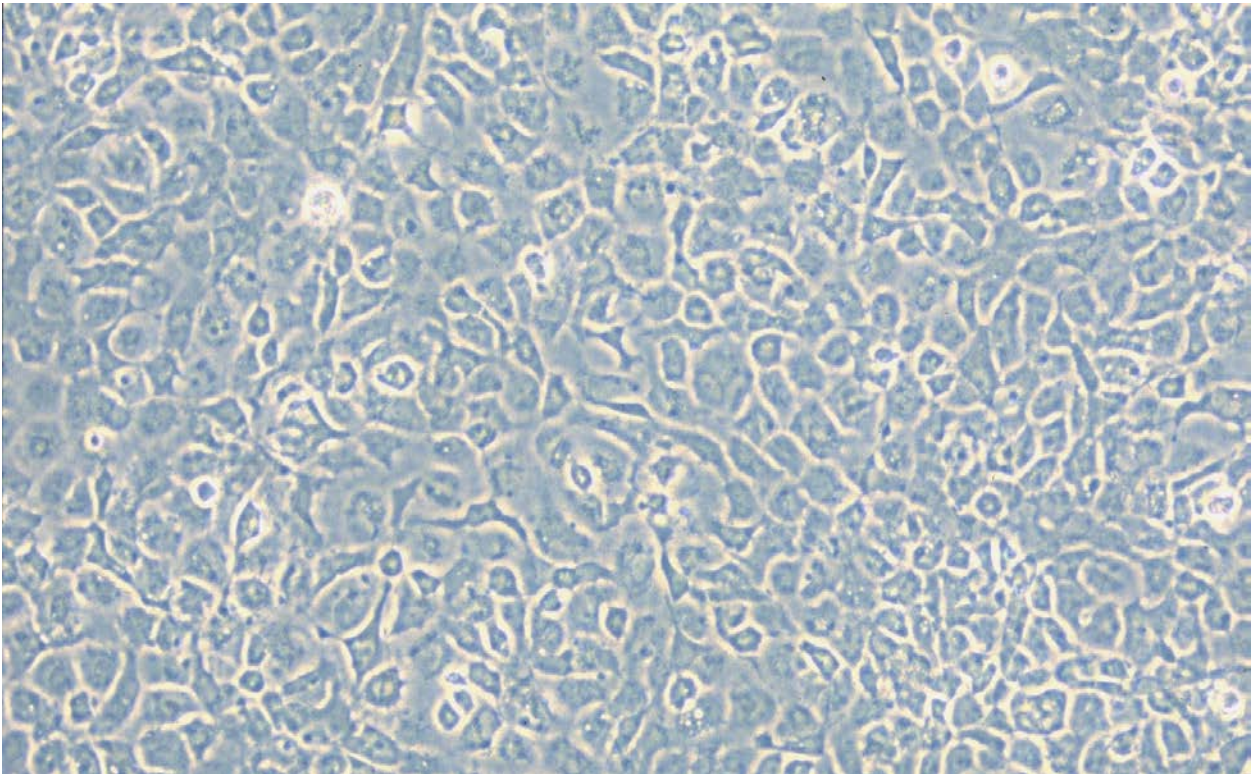
### A. Infectious Hematopoietic Necrosis Virus (IHNV)

Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae*. IHNV has a wide geographic range that includes North America, Europe, and the Far East. The virus is primarily found in salmonids with rainbow trout fry being highly susceptible to disease. Older fish are more resistant to infection but may become carriers. Transmission is primarily horizontal but cases of egg associated transmission have been recorded as well as transmission by fomites. The virus may be shed in ovarian fluid and excretory products such as feces and may also be isolated from the kidney, spleen, encephalon, and digestive tract of clinically ill fish. Under natural conditions, most clinical disease from IHNV is seen in fry when water temperature is between 8 to 15°C with fish exhibiting darkening of the skin, ascites, exophthalmia, and petechial hemorrhages internally and externally. Degeneration and necrosis of the hematopoietic tissue in the kidney is thought to be the actual cause of mortality (Egusa 1991; OIE 2006; Wolf 1988).

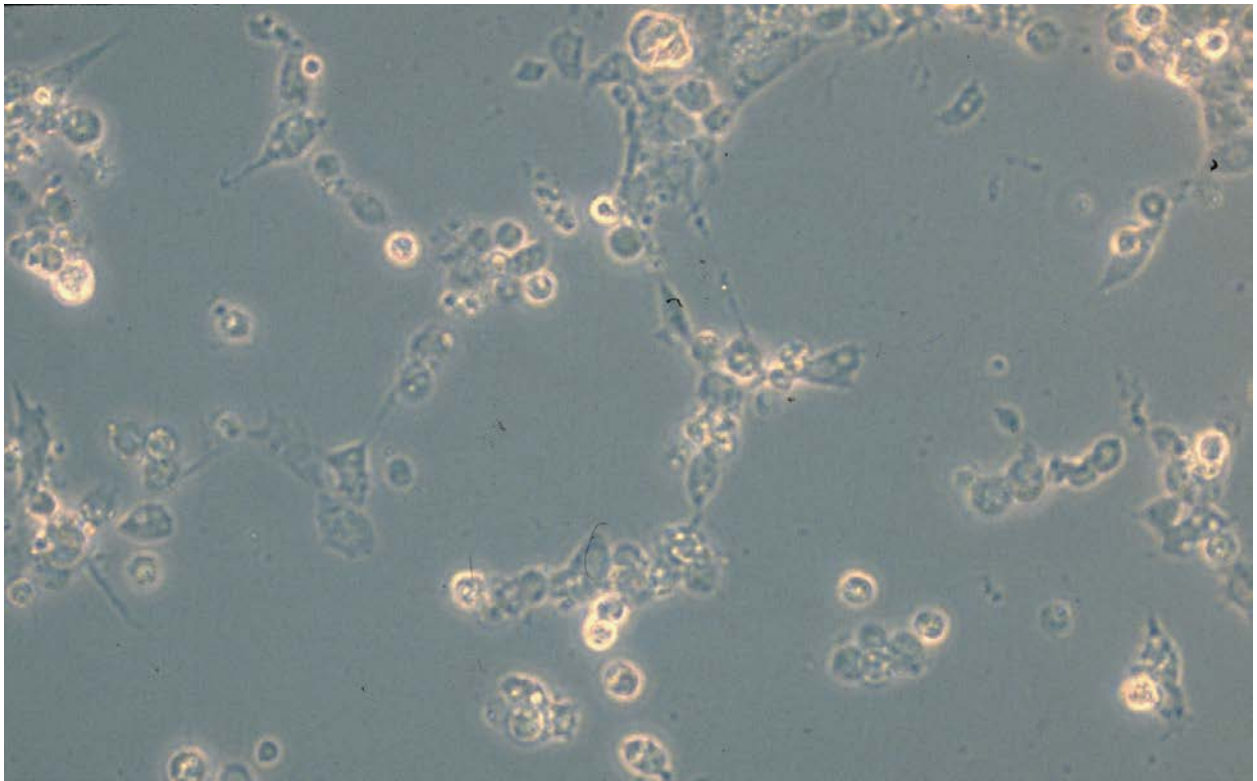
1. Screening Method
  - a. Cell culture on EPC cell line incubated at 15°C.
  - b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
  - c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IHNV.**
  - d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.

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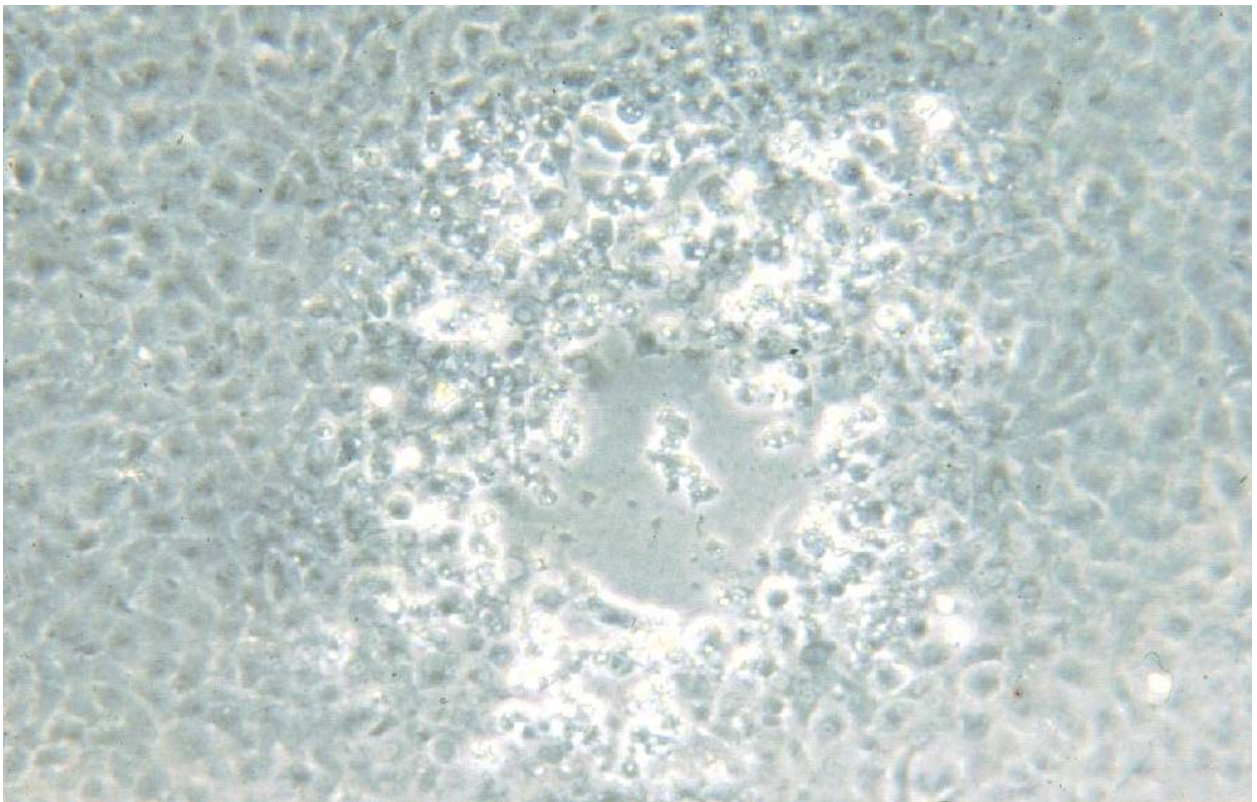
- i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. Polyethylene glycol (PEG) has been used to enhance plaque formation but is not necessary to detect IHNV in open systems without an overlay (Batts and Winton 1989) see Figures 4.1 to 4.3.
- ii. The Polymerase Chain Reaction (PCR), serum neutralization, or Indirect Fluorescent Antibody Test (IFAT) methods may be used to confirm the cause of the CPE is due to the presence of IHNV.



**Figure 4.1.** Normal EPC monolayer. Photo courtesy of Jim Winton, USGS.



**Figure 4.2.** CPE typical of a rhabdovirus on EPC monolayer. Photo courtesy of Jim Winton, USGS.



**Figure 4.3.** CPE plaque of IHNV on EPC monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

## 2. Confirmation Methods for IHNV

## a. Polymerase Chain Reaction (PCR) Method for Confirmation of IHNV

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.

## i. Extraction of RNA from Cell Cultures

Total RNA from infected cells is extracted using a phase-separation method (eg. phenol-chloroform or TRIZOL, Invitrogen) or by RNA affinity spin columns (eg. RNeasy Total RNA kit, Qiagen) according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separations methods should be used for extraction of RNA from cell culture fluids.

## ii. Production of DNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

3. Using Section 2, 4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus (IHNV), 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 four to the number of samples so that there is enough to run controls.)

## 4. Primers for IHNV\*

a. Forward: 5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3'

b. Reverse: 5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'

\*2010 edition note: The primer sequences for the IHNV RT-PCR assay have been modified from those in 2005 editions or earlier of this manual. The new primers target the central portion of the G gene, instead of the previously published primers which targeted the N gene. The G gene primer set has been tested extensively (Emmenegger et al., 2000, Kurath et al., 2003) and avoids using a series of six consecutive 'G's' that were less than optimal for PCR in the N gene forward primer.

## 5. Thermocycler Program for IHNV

a. Incubate at 50°C for 30 minutes for Reverse Transcriptase reaction.

b. Denature sample at 95°C for two minutes.

c. 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.
- d.* Final extension at 72°C for seven minutes.

**Hold samples at 4°C after cycling is complete. PCR Products can be refrigerated for one month or frozen at -20°C for long-term storage.**

- iii Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)
  - 1. Visualize the DNA
    - 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 PCR assays.
    - a.* **Bands occurring at the 693 bp location are confirmatory for IHNV and are reported as POSITIVE.**
    - b.* **If there is an absence of appropriate bands with no indication of problems with the assay, consider testing for other viruses or consult an appropriate reference laboratory.**
  - 2. Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)
    - Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)
- b. Serum Neutralization Method
  - See Section 2, 4.7 Serum Neutralization for the general procedure.
  - i.* Use the cell line on which the initial CPE was produced.
  - ii.* Incubate plates at 15°C.
- c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IHNV
  - See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.
  - i.* Use an EPC cell line.
  - ii.* Incubate virus inoculated monolayers at 15 to 18°C.
- d. Direct Alkaline Phosphatase Immunocytochemistry (APIC) Staining Method for the Confirmation of IHNV (modified from Drolet et. al, 1993) – The Alkaline Phosphatase Immunocytochemical (APIC) procedure is a method for confirming the identity of isolates

suspected to be Infectious Hematopoietic Necrosis Virus. This is a direct assay that can detect antigen in formalin fixed and stained tissue culture cells for up to one year. There are four basic steps for the APIC assay: preparing and fixing cell monolayers; infecting the cell monolayers with the virus; staining the plate; reading and interpreting the stained plate. The procedure outlined below uses reagent kits obtained from Vector Laboratories, Burlingame, CA and DiagXotics Inc., Wilton, CT but suitable stain kits and antibodies are also available from other sources.

### i. Preparation of Materials

1. Prepare the reagents according to the instructions provided by the supplier.
2. Appropriate QA/QC testing should be performed periodically on the antibodies, antisera, stains, and cell lines to ensure accurate results are obtained during this assay.

### ii. Preparation of Cell Monolayers in 96-well plates

1. At least four sets of plates are made for each assay to provide sufficient room for 6-8 replicates of each sample, positive control (reference) viruses, and negative control (uninoculated) cell lines. One set of plates is then fixed, stained and examined at approximately 8, 12, 24, and 48 hours as necessary for confirmation. Observing the cultures at multiple time points allows for visualization of the virus at the most appropriate stage of infection of the cell monolayer.
2. Working in a clean hood, seed sufficient wells of the 96-well plate with the appropriate volume of that cell suspension as in Section 2, 4.3.B "Seeding Procedures for Plates."
3. Incubate at 20 to 25°C for 18 to 24 hours or until the cells are 80 to 100% confluent as this minimizes excessive cell loss during fixation and staining.

### iii. Virus Sample Inoculation and Incubation

#### 1. Preparation of the Suspect and Known Virus Sample

- a. Using a pipette, stir and scrape the bottom of the suspect virus well or reference virus-infected flask (Section 2, 4.7.C.2 "Procedure for Producing Reference Viruses") to be subcultured to dislodge the cell layer.
- b. Aspirate the fluid and cell debris from the well or flask and place in a sterile tube for centrifugation. Samples may be diluted up to 1:100 in HBSS.
- c. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.
- d. Use supernatant from this tube for inoculation of the cell cultures in the plates.

#### 2. Inoculation of the Cell Cultures with the Suspect and Known Virus Samples

- a. Aspirate media from each well of the wells to be inoculated, leaving a small

amount to inhibit cell dehydration.

- b.* Inoculate 50 to 100  $\mu\text{L}$  of supernatant prepared in “Preparation of the Suspect and Known Virus Sample” (above) from each sample onto four replicate wells in each of four sets of plates.
- c.* Inoculate 50 to 100  $\mu\text{L}$  of reference virus onto replicate wells in each of four sets of plates, one set to be used at each time period.
- d.* At least one negative (uninoculated) control well is made on each plate set for each cell line used so that one will be stained and examined at each time period.
- e.* Absorb 30-60 minutes without rocking at 15 to 18°C, sample removal is not necessary.
- f.* Add 100  $\mu\text{L}$  MEM-5 (Section 2, 4.9.F “MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1)”) per well.

3. Incubate at 15 to 18°C.

iv. Plate Fixation

1. Working under the hood at room temperature, add 50  $\mu\text{L}$  10 % Neutral Buffered Formalin (NBF) (Section 2, 2.3.C.3 “10% Neutral Buffered Formalin (10% NBF)”) to each well without dumping the media.
2. Fix for five minutes.
3. Remove media and fixative and disinfect prior to disposal.
4. Add 200  $\mu\text{L}$ /well tap water and let stand for 1 minute.
5. Remove water and disinfect prior to disposal.
6. Allow plate to air dry.
7. Store plate at 5-25 °C until stained.

v. Staining (Modified from Vectastain kit instructions)

1. Pre-heat incubator to 37-45 °C. Buffer may be heated in the incubator for more rapid staining.
2. Add 200  $\mu\text{L}$  of wash buffer to each well and let soak for 1 minute. Remove buffer.
3. Repeat step 4.12.E.2 above to remove any remaining fixative.
4. Add 50  $\mu\text{L}$  of blocking reagent to each well.
5. Incubate at 37-45 °C for 30 minutes.

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6. Remove blocking reagent by turning the plate upside down and shaking. Do not wash.
7. Dilute DiaXotics 14D primary antibody 1:10 in buffer and add 50  $\mu$ L to each well.
8. Incubate at 37-45  $^{\circ}$ C for 15-30 minutes.
9. Wash by repeating step 4.12.E.2 three times.
10. Add 50  $\mu$ L of Vectastain Biotinylated Universal Antibody to each well.
11. Incubate at 37-45  $^{\circ}$ C for 15-30 minutes.
12. Wash by repeating step 4.12.E.2 three times.
13. Add 50  $\mu$ L of Vectastain ABC-AP Reagent to each well.
14. Incubate at 37-45  $^{\circ}$ C for 15-30 minutes.
15. Wash by repeating step 4.12.E.2 five times.
16. Add 50  $\mu$ L of Vector Red Alkaline Phosphatase Substrate to each well (should be used within 15 minutes of preparation).
17. Incubate in the dark at room temperature for 15-30 minutes.
18. Wash by repeating step 4.12.E.2 three times.
19. Dry and Store at 25  $^{\circ}$ C.
20. Examine at 400-1000x magnification.

### vi. Results

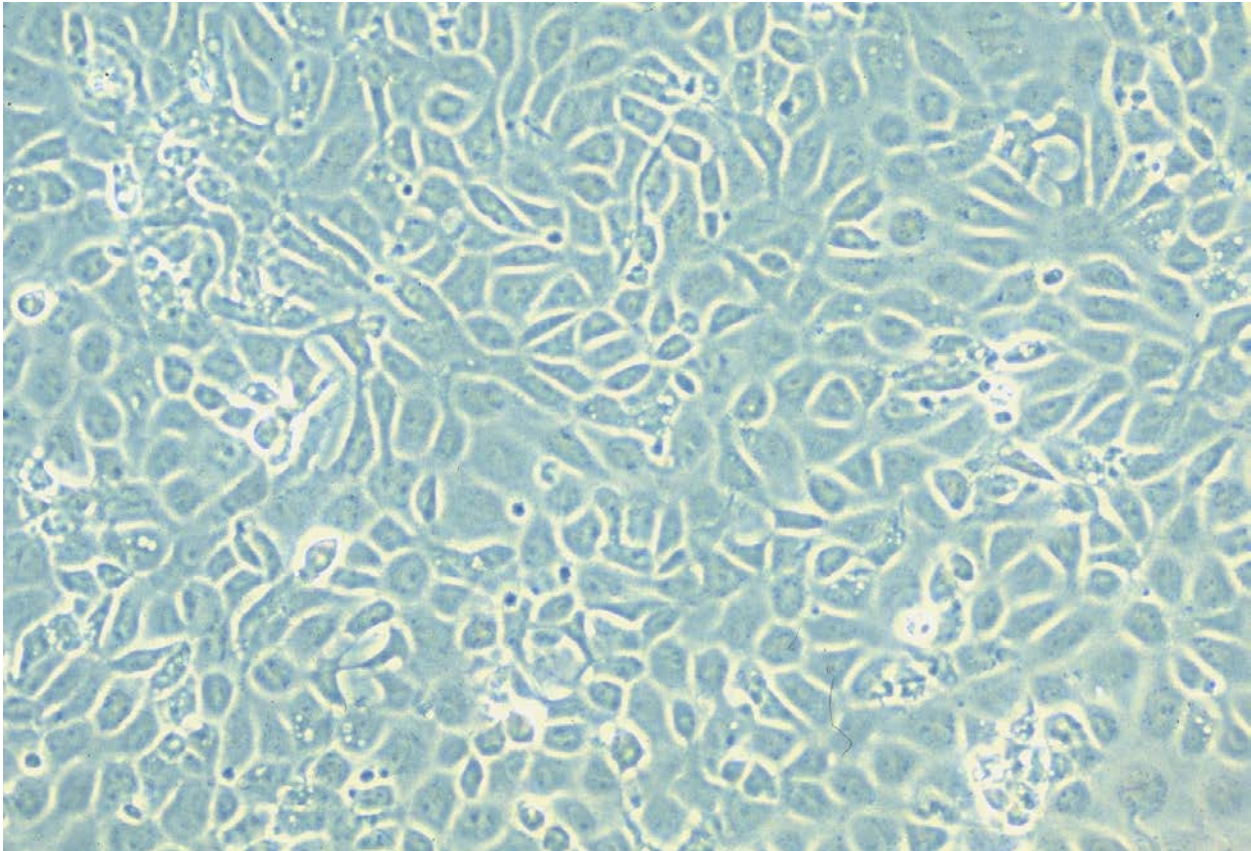
1. Positive controls should contain individual cells that appear red and early plaques will appear as clumps of several infected cells. No red should be seen on negative controls. If red "clouds" are seen, there may have been insufficient washing or a fold in the cell sheet.
2. With no problems identified in the assay and with red cells in the suspect sample which appear similar to the positive control at one of the time points, the suspect sample is considered **POSITIVE** for IHNV.
3. With no problems identified in the assay but without appropriate red color at any of the four time periods, the suspect sample is considered **NEGATIVE** for IHNV.

## B. 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*. There are many viruses in the *Aquabirnavirus* group, most of which have not been shown to cause disease in fish. If warranted, additional tests can be used to further identify the virus as IPNV. IPNV has a wide geographic range that includes North and South America, Asia, and Europe. It is very stable under a wide range of environmental conditions and is capable of surviving for several days in both fresh and saltwater. It is resistant to a wide range of chemical disinfectants including ether, chloroform, and quaternary ammonium compounds but is deactivated by 70% ethanol. Isolates display wide antigenic diversity and virulence. There are two sero-groups that do not cross-react in serum neutralization tests with the majority of strains belonging to sero-group A. IPNV has been isolated from several species of marine and freshwater fish and shellfish. Acute catarrhal enteritis has primarily been seen in salmonid fry and fingerlings with initial mortality occurring in the more robust individuals. Fish that survive the disease may become asymptomatic carriers and shed the virus through the feces and sex products. IPNV may be transmitted vertically as well as horizontally (Bruno and Poppe 1996; Egusa 1991; OIE 2000; Roberts 1982; Wolf 1988).

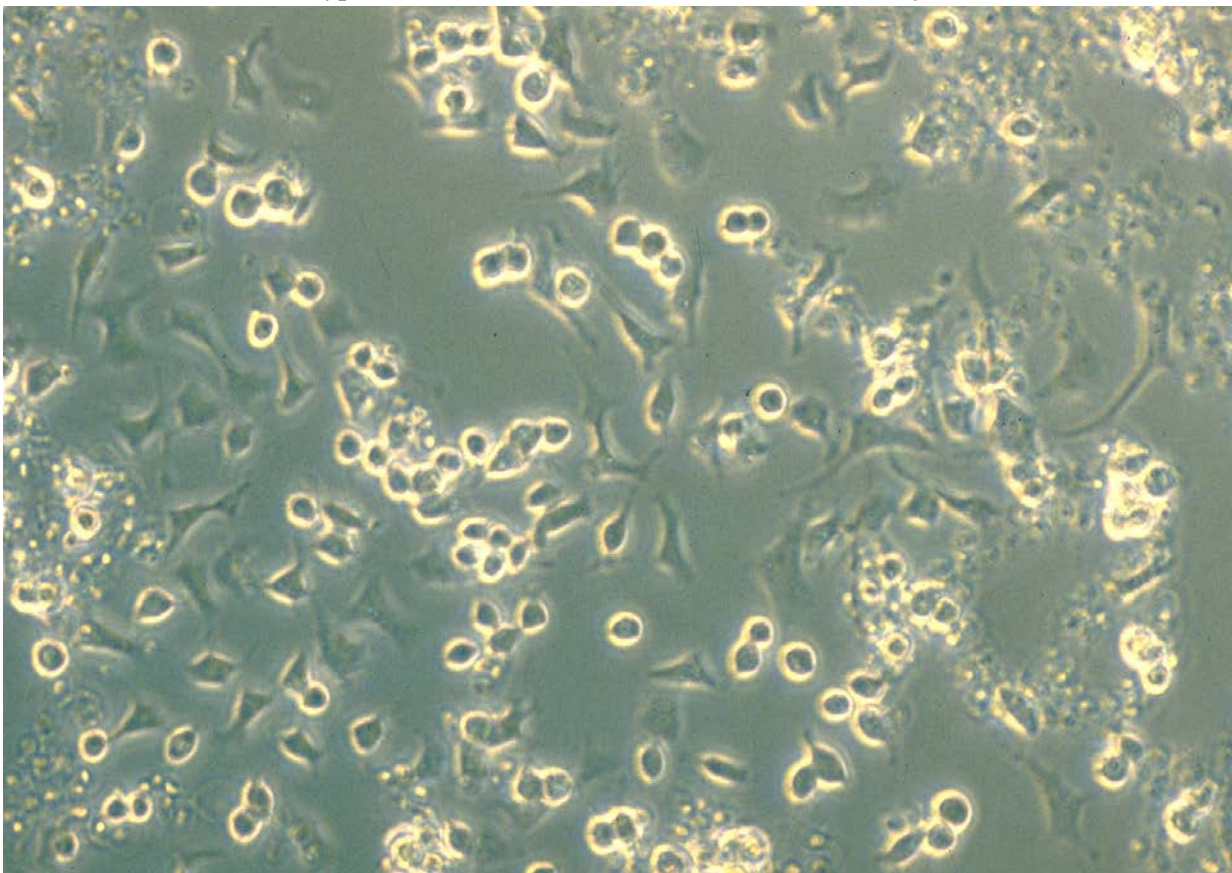
### Screening Method

- a. Cell culture on CHSE-214 cell line incubated at 15°C.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IPNV.**
- d. If CPE typical of IPNV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
  - i. The appearance of CPE typical of IPNV is described as stellate shaped plaques with spindle-shaped cells. Some of the cells within the plaque will exhibit nuclear pyknosis (nuclei shrink in size and chromatin condenses) with other cells appearing normal. See Figures 4.4 and 4.5. Typically, positive cultures result in rapidly lytic CPE but some cells may survive and reform a normal looking monolayer.
  - ii. The serum neutralization, PCR, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of IPNV.



**Figure 4.4.** Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

**Figure 4.5.** CPE typical of IPNV on CHSE-214 cells. Photo courtesy of Jim Winton, USGS.



Confirmation Methods for IPNV

- a. Serum Neutralization Method  
See Section 2, 4.7 Serum Neutralization for the general procedure.
  - i. Use the cell line on which the initial CPE was produced.
  - ii. Incubate plates at 15°C.
- b. Polymerase Chain Reaction (PCR) Method for Confirmation of IPNV (Modified from Blake et al., 1995): 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.
  - i. Extraction of RNA from Cell Culture Fluid (heat RNA Release Method)
    1. Dilute cell culture fluid 1:50 in molecular grade RNase free water by adding 2 µL fluid to 98 µL water in microcentrifuge tubes.
    2. Place tubes in heat block at 100°C for 10 minutes.
    3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself).
    4. Quantify RNA template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1 µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µL or use up to 5 µL/reaction if reading falls below 50 ng/µL.
  - ii. Formation of DNA by Reverse Transcription and Amplification by PCR
    1. QA/QC (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
    2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
    3. Using Section 2, 4.A1.B.2 Worksheet B.2 – Infectious Pancreatic Necrosis Virus (IPNV), 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 four to the number of samples so that there is enough to run controls in the assay.)
    4. Primers for IPNV
      - a. Forward: 5’ - AAA GCC ATA GCC GCC CAT GAA C -3’

- b. Reverse: 5'- TCT CAT CAG CTG GCC CAG GTA C -3'
- 5. Thermocycler Program for IPNV
  - a. Pre-dwell at 50°C for 15 minutes.
  - b. Preheat or “Jumpstart” sample to 95°C for two minutes.
  - c. 35 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.
  - d. Post dwell at 72°C for seven minutes.
  - e. 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.**
- iii. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)
  - 1. Visualize the DNA  
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 174 bp location are confirmatory for IPNV 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 IPNV.**
  - 2. Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)  
**Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)
- c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IPNV  
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.
  - i. Use a CHSE-214 cell line.
  - ii. Incubate virus inoculated monolayers at 15 to 18°C.

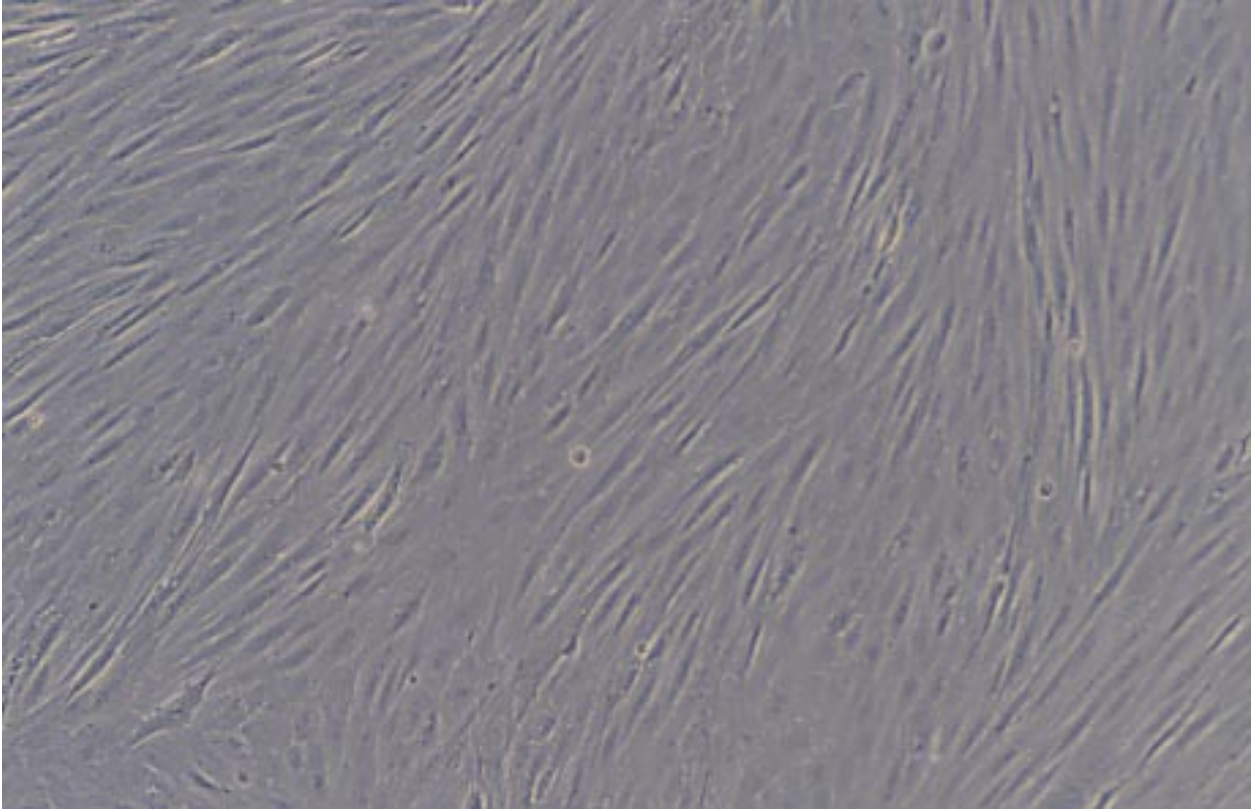
### C. Infectious Salmon Anemia Virus (ISAV)

Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus belonging to the newly proposed *Isavirus* genus of the *Orthomyxoviridae*. The disease is mostly seen in Atlantic salmon in salt water in the spring and fall associated with rapidly changing water temperature. Characteristics of the disease include anemia, ascites, petechial hemorrhages on the peritoneal surface and perivisceral fat, and congestion of the liver, spleen, kidney, and upper digestive tract. While Atlantic salmon are the only species known to suffer disease, the virus has been isolated from free ranging Atlantic salmon, Rainbow trout and Brown or sea trout; however, isolation of ISAV from carrier fish may be difficult. (Bruno and Poppe 1996; OIE 2000).

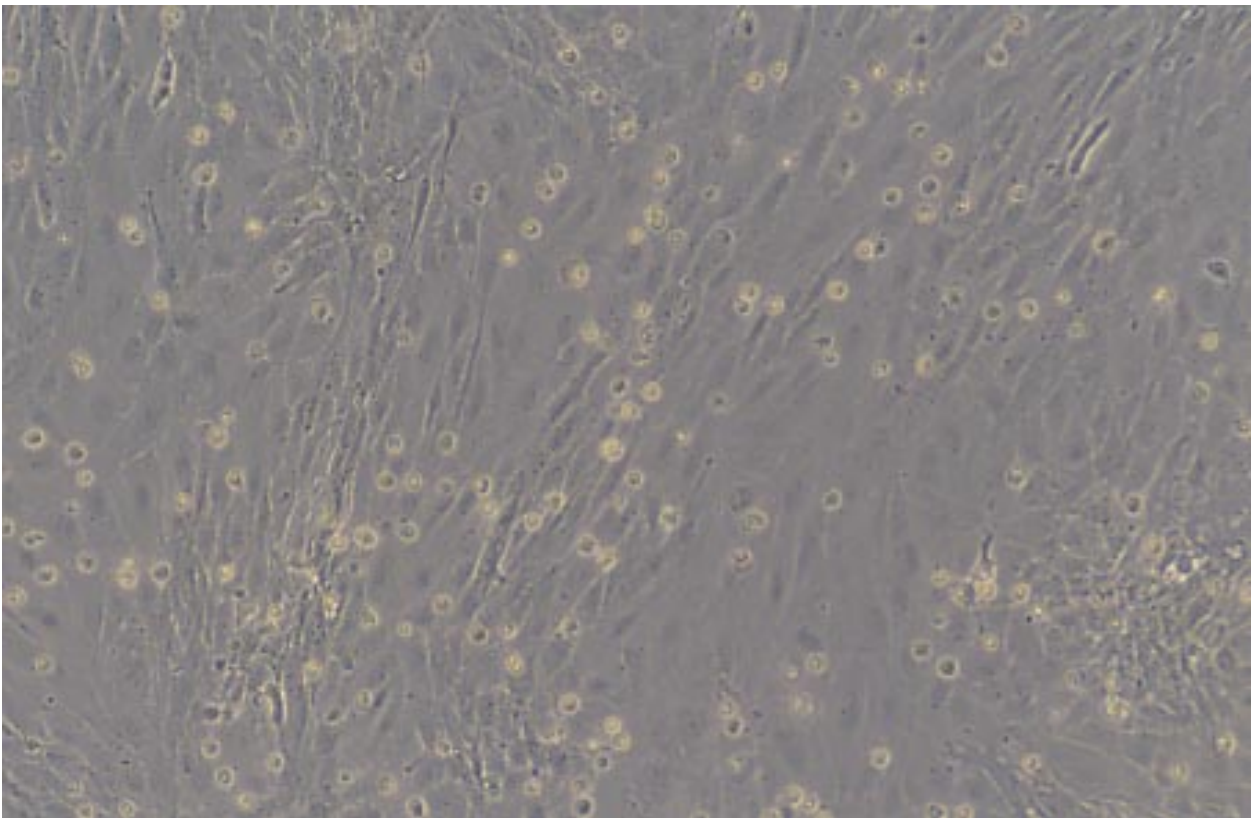
#### Screening Method

- a. Cell culture on SHK-1 cell line incubated at 15°C (Bouchard et al., 1999).
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for ISAV.**
- d. If CPE typical of ISAV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
  - i. The appearance of CPE typical of ISAV is described as plaques of vacuolated cells that round up and loosen from the growth surface. It may progress to involve the entire cell sheet with only small, rounded, refractile, and necrotic cells observable. See Figures 4.6 and 4.7.
  - ii. Polymerase chain reaction (PCR) or IFAT methods may be used to confirm the cause of the CPE is due to the presence of ISAV.

**Figure 4.6.** Normal SHK-1 monolayer. Photo courtesy of Jim Winton, USGS.



**Figure 4.7.** CPE typical of ISA on SHK-1 cells. Photo courtesy of Jim Winton, USGS.



## Confirmation Methods for ISAV

## a. Polymerase Chain Reaction (PCR) (Modified from Bouchard et al., 1999): 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.

## i. Extraction of RNA from Cell Culture Fluid (heat RNA Release Method)

1. Dilute cell culture fluid (with some cell scrapings) 1:50 in molecular grade RNAase free water by adding 2  $\mu$ L fluid to 98  $\mu$ L water in microcentrifuge tubes.
2. Heat to 95°C for two minutes in a heat block, water bath, or thermocycler.
3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself).
4. Quantify RNA template in the spectrophotometer. 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.

## ii. Formation of DNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
3. Using Section 2, 4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV), 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 four to the number of samples so that there is enough to run controls and empty slots in the assay.)

## a. Primers for ISAV

i. Forward: 5'-GGC TAT CTA CCA TGA ACG AAT C-3'

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

## b. Thermocycler Program for ISAV

- i. Pre-dwell at 42°C for 15 minutes.

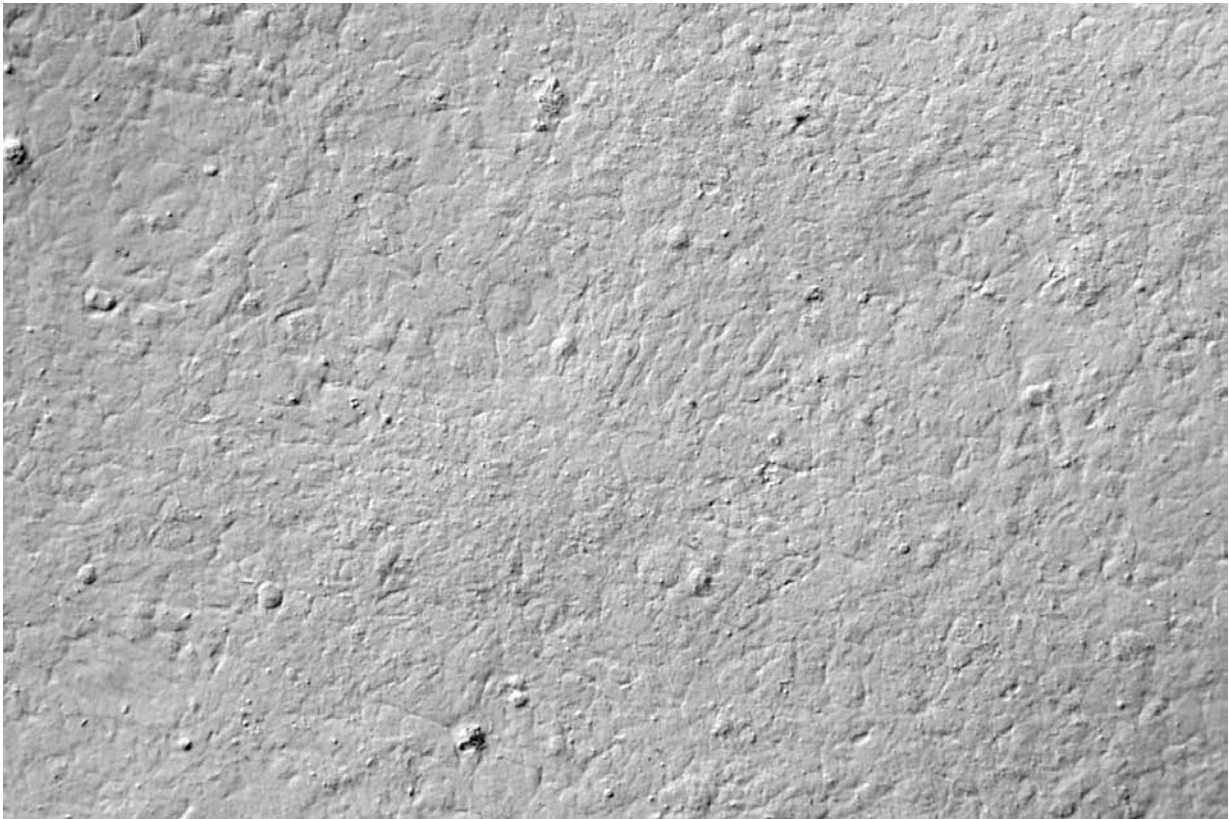
- ii. Preheat or “Jumpstart” sample to 94°C for five minutes.
  - iii. 40 cycles as follows:
    - 1) Denaturing at 94°C for 45 seconds.
    - 2) Annealing at 59°C for 45 seconds.
    - 3) Extending at 72°C for 105 seconds.
  - iv. Post dwell at 72°C for seven minutes.
  - v. Hold samples at 4°C after cycling is complete.  
**PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.**
- iii. Visualization of PCR Product by Electrophoresis (See Section 2, 6.3.C “Detection of Product.”)
- 1. Visualize the DNA  
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 assays.
    - a. **A band occurring at the 493 bp location is confirmatory for ISAV and the sample is reported as POSITIVE.**
    - b. **The lack of the appropriate band with no indication of problems with the assay are reported as NEGATIVE for ISAV.**
  - 2. Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)  
**Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)
- b. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of ISAV  
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.
- i. Use a SHK-1 cell line.
  - ii. Incubate virus inoculated monolayers at 15 to 18°C.

## D. Largemouth Bass Virus (LMBV)

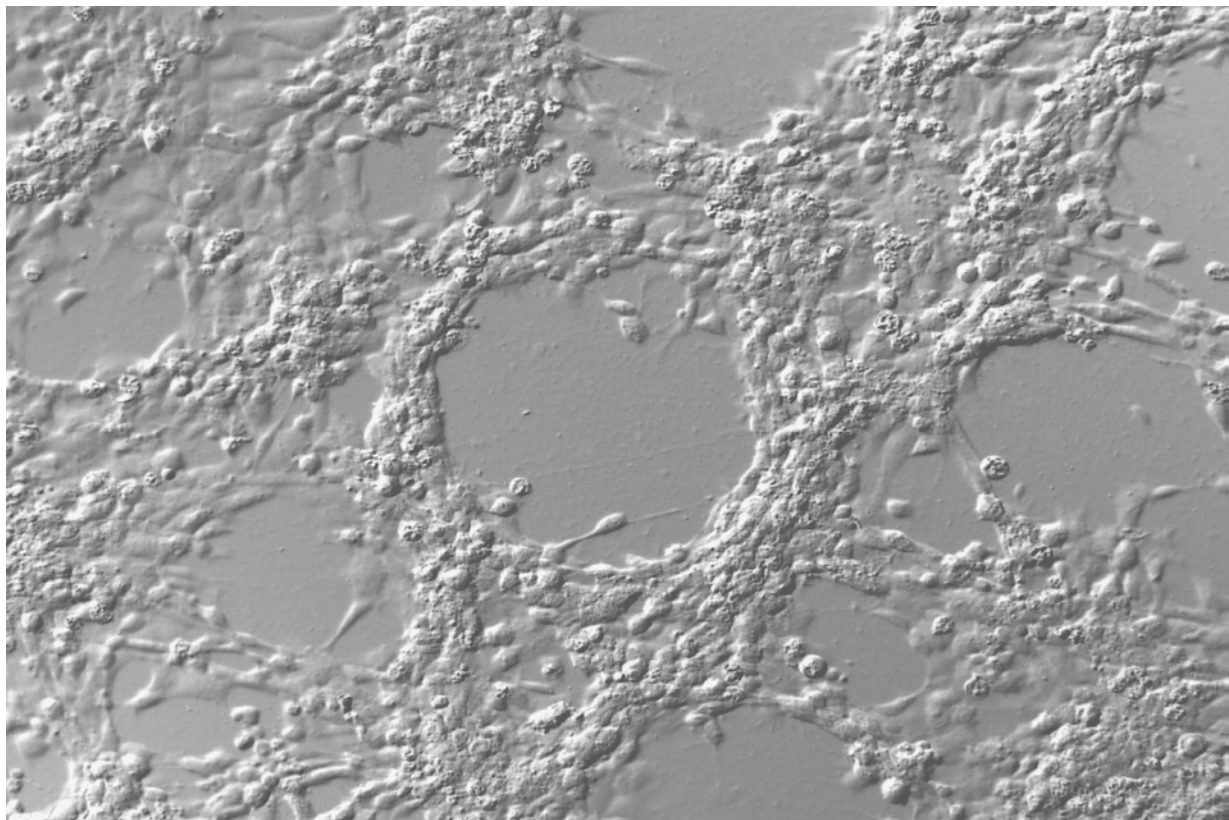
Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the ranavirus genus of the Iridoviridae family. LMBV infection has been found in centrarchid and esocid populations in the Mid-West and Southeastern United States and has been found experimentally to be associated with mortality in juvenile largemouth bass (Plumb 1999). During an active infection, the virus may be isolated from several tissues including the kidney, spleen, and swim bladder.

### Screening Method

- a. Cell culture on FHM or BF-2 cell lines incubated at 25 to 30<sup>0</sup>C ( Piaskoski et al. 1999; Grant et al. 2003; McClenahan et al. 2005)
- b. Monitor for CPE at least twice per week for an initial incubation period of 7 days. If CPE is not observed, make a blind pass and incubate for an additional 7 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial inoculations and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 7 days at 25-30 C. If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for LMBV.
- d. If CPE typical of LMBV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
  - i. The appearance of CPE typical of LMBV is described as circular cell free areas with rounded cells at the margins. See Figures 4.8 through 4.11.
  - ii. The polymerase chain reaction (PCR) method is used to confirm that the cause of the CPE is due to the presence of LMBV.

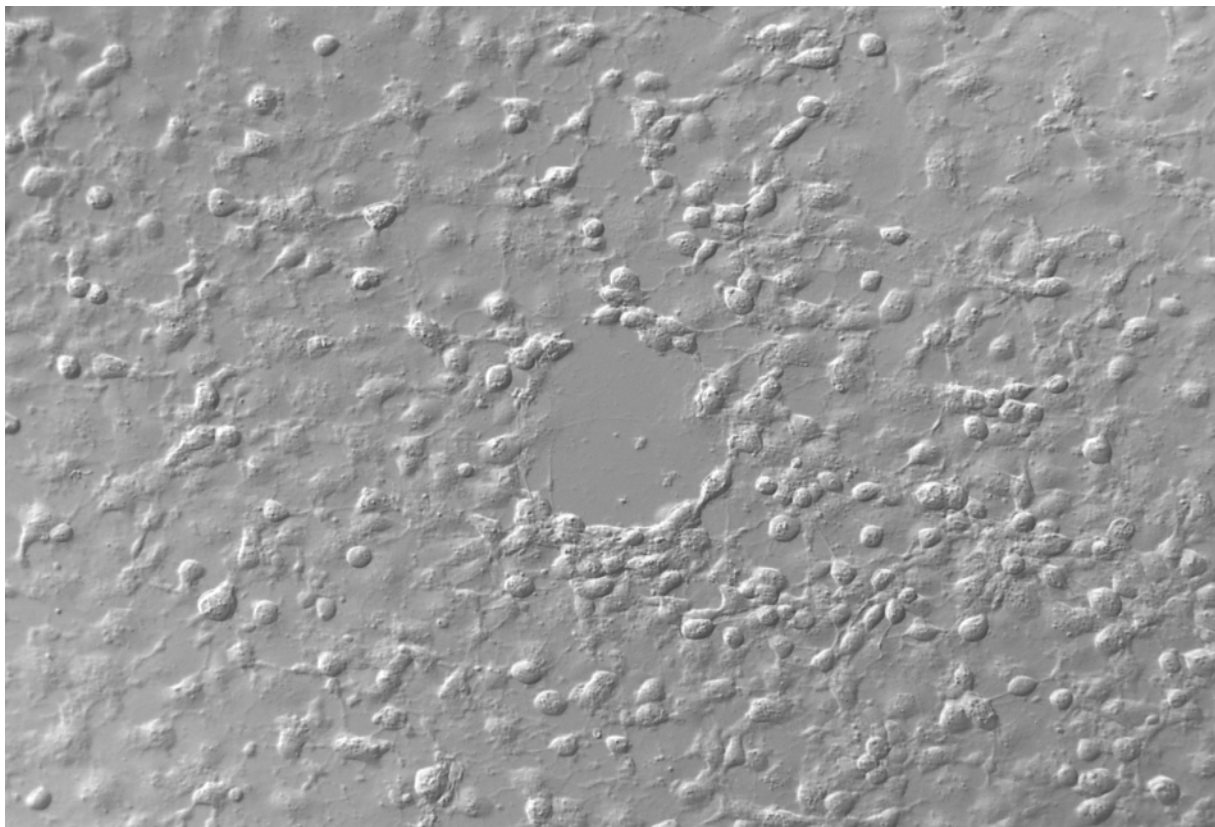


**Figure 4.8.** Normal FHM monolayer. Photo courtesy of John Grizzle, Auburn University



**Figure 4.9.** CPE typical of LMBV on FHM cells. Photo courtesy of John Grizzle, Auburn University.

**Figure 4.10.** Normal BF-2 monolayer. Photo courtesy of John Grizzle, Auburn University.



**Figure 4.11.** CPE typical of LMBV on BF-2 cells. Photo courtesy of John Grizzle, Auburn University.

**Confirmation Method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb et al., 1999)**

This is a DNA containing virus so DNA is extracted from cell culture fluid and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.

Extraction of DNA from Cell Culture-Use one of the following methods.

**BuccalAmp DNA Extraction (modified by J. Woodland, USFWS)**

Extract DNA following the procedures outlined in the Buccal Amp DNA extraction kit (Epicentre®). Collect sample by rotating the swab onto infected cells still attached to the well of the cell culture plate. To obtain sufficient DNA, swab 1-2 wells of a 24-well plate or 3-4 wells of a 48-well plate. Follow remaining steps provided with the kit.

**Qiagen DNA Extraction**

The following procedure employs an extraction kit available from Qiagen, Inc. (<http://www.qiagen.com>.) DNA Extraction kits of similar efficacy are available from many other sources and can be utilized as alternatives for extraction of DNA by following their protocol. These kits utilize “spin columns” for binding and elution of DNA from cell culture lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction.

- i. Supernatant and cells from suspect sample wells are removed and centrifuged for five minutes at 300 X g.
- ii. Resuspend pellet in 200 µL PBS.
- iii. Add 20 µL proteinase K solution (Section 2, 5.6.E “Proteinase K”) and 200 µL buffer AL to the sample, mix thoroughly by vortexing.
- iv. Incubate for 10 minutes at 70°C.
- v. Add 200 µL 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 one 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 500 µL buffer AW1, and centrifuge for one 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 µL buffer AW2, and centrifuge for three 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 100 µL buffer AE directly onto the DNeasy membrane.
- xi. Incubate at room temperature for one minute, then centrifuge for one 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.
- xiv. Quantify the amount of DNA extracted with a spectrophotometer (Section 2, Chapter 6 Polymerase Chain Reaction (PCR)). The optimum amount of DNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1 µL of DNA template is sufficient per reaction. Dilute template if more than 300 ng/µL or use up to 5 µL/reaction if reading falls below 50 ng/µL.
- b. Amplification of LMBV DNA
- i. General QA/QC Considerations (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
  - ii. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
  - iii. Using Section 2, 4.A1.B.4 Worksheet B.4 - Largemouth Bass Virus (LMBV), 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 four to the number of samples so that there is enough to run controls in the assay.)
  - iv. Primers for LMBV (Grizzle et al., 2003)
    1. Forward: 5'-GCG GCC AAC CAG TTT AAC GCA A -3'
    2. Reverse: 5'- AGG ACC CTA GCT CCT GCT TGA T -3'
  - v. Thermocycler Program for LMBV
    1. Pre-dwell sample at 95°C for 3 min 15 sec.
    2. 35 cycles of the following regime:
      - a. Denaturing at 95°C for 45 sec.
      - b. Annealing at 60°C for 45 sec.
      - c. Extending at 72°C for 60 sec.
    3. Post-dwell at 72°C for 7 min.
    4. 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.**

Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)  
Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.

- i. **A band occurring at the 248 bp location is confirmatory for LMBV and the sample is reported as POSITIVE.**
- ii. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for LMBV.**

Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)

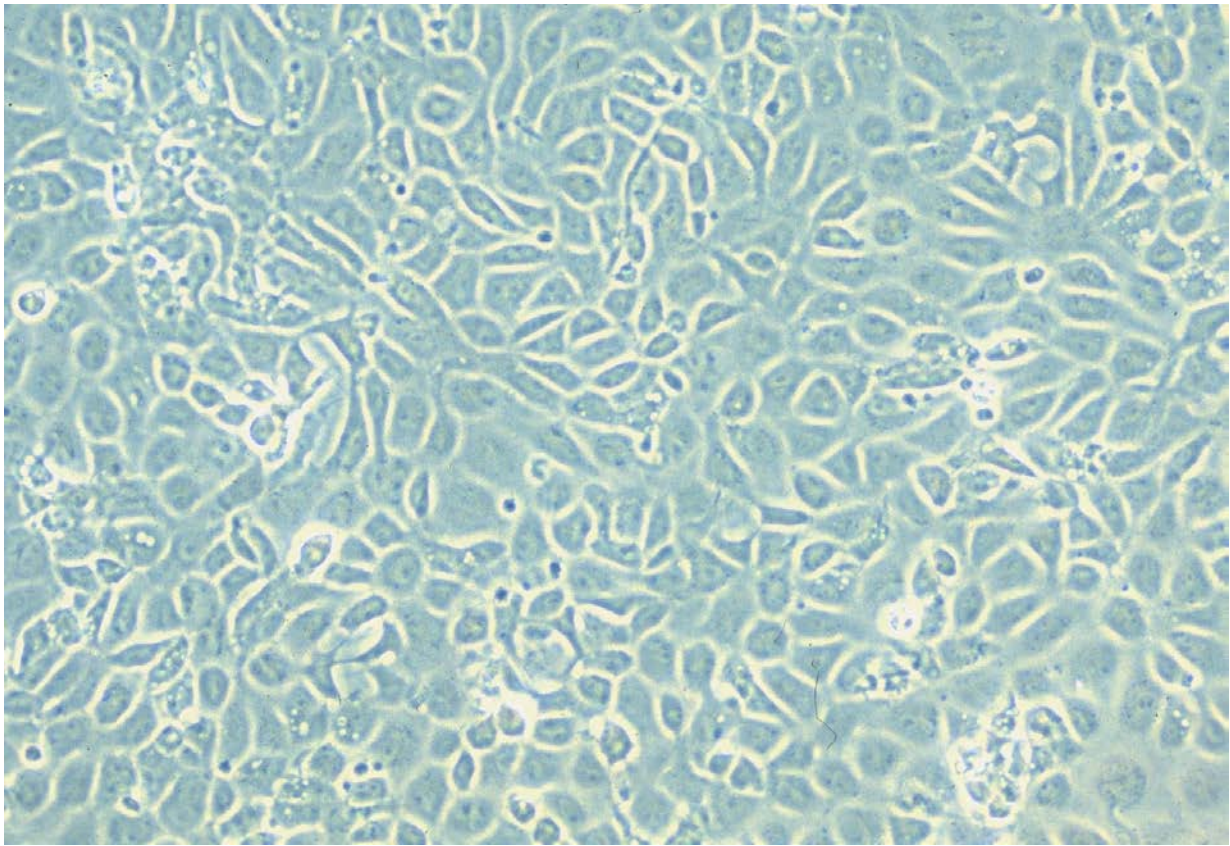
**Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

## E. Oncorhynchus Masou Virus (OMV)

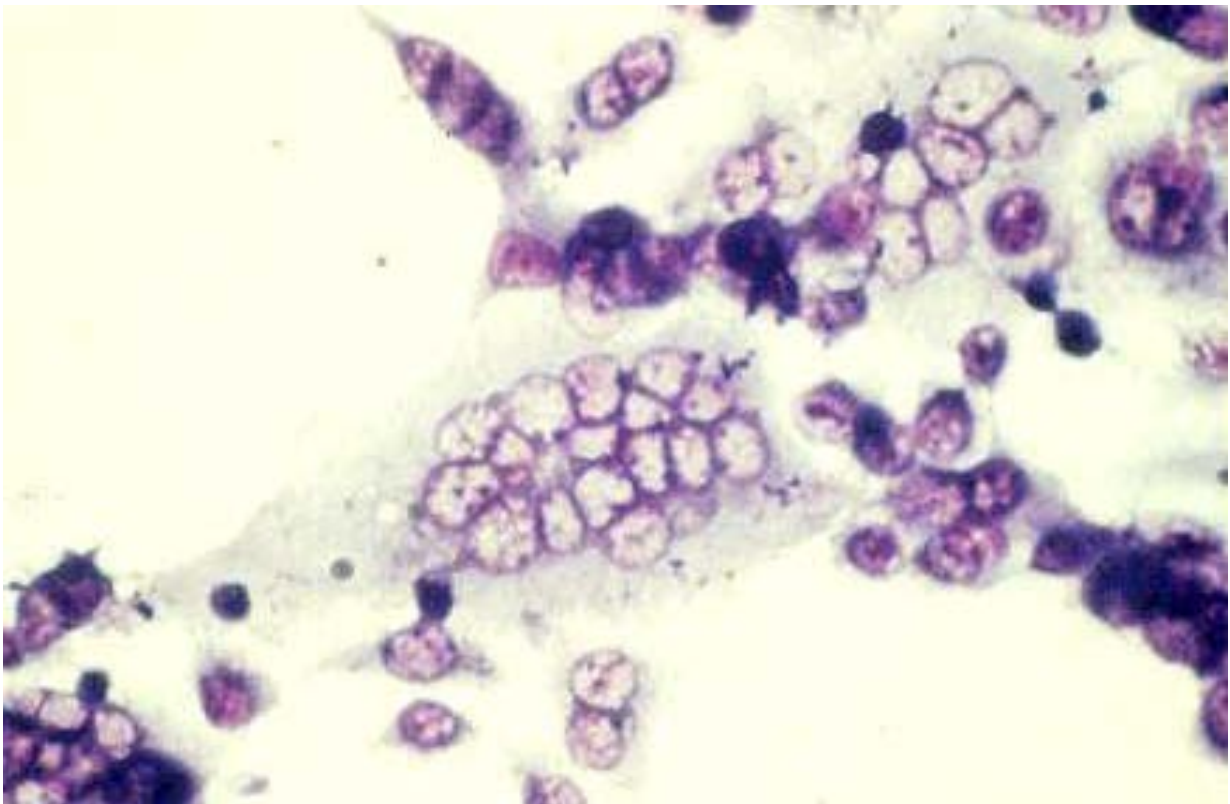
Oncorhynchus Masou Virus (OMV) is an enveloped double-stranded DNA virus belonging to the *Herpesvirus* genus of the *Herpesviridae*. Salmonids are the only fish known to be susceptible to infection with OMV with kokanee being the most susceptible. The geographic range has so far been limited to Japan and Eastern Asia. The initial disease is a septicemia that may cause edema and hemorrhage in fry during which time, the virus will be shed in the feces and urine and isolated from the liver, kidney, and spleen. Several months later, survivors may develop epithelial tumors around the mouth and fins with virus able to be isolated from these lesions. Most disease is seen in water temperatures below 14°C. Although OMV may be isolated from ovarian fluid at spawning, transmission is primarily by the horizontal route (OIE 2000; Wolf 1988).

### 1. Screening Method

- a. Cell culture on CHSE-214 cell line incubated at 15°C.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for OMV.**
- d. If CPE typical of OMV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
  - i. The appearance of CPE typical of OMV is described as the formation of rounded cells which progress to marked syncytia and eventual lysis of the entire cell sheet. See Figures 4.12 and 4.13.
  - ii. 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.



**Figure 4.12.** Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.



**Figure 4.13.** CPE typical of OMV on CHSE-214 cells. Photo courtesy of Mamoru Yoshimizu, Hokkaido University.

## 2. Confirmation Method for OMV

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. A polymerase chain reaction (PCR) procedure that does not require live positive control material has been developed for this virus (Modified from Aso et al., 2001). This is a DNA containing virus so DNA is extracted from cell culture material and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.

### Extraction of DNA from Cell Culture Fluid

- i. 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.
- ii. Wash the pellets twice with 1 mL PBS and mix with 200 µL of chelating resin (Sigma).
- iii. Incubate the mixture at 56°C for 20 minutes in a water bath, vortex, and then place in a boiling water bath for eight minutes.
- iv. Vortex the samples and centrifuge at 8200 Xg (10,000 rpm) for 90 seconds.

### Amplification of OMV DNA

- i. General QA/QC Considerations (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
- ii. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
- iii. Using Section 2, 4.A1.B.5 Worksheet B.5 – *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 four to the number of samples so that there is enough to run controls in the assay.)
- iv. Primers for OMV
  1. Forward: 5’-GTA-CCG-AAA-CTC-CCG-AGT-C-3’
  2. Reverse: 5’- AAC-TTG-AAC-TAC-TCC-GGG-G-3’
- v. Thermocycler program for OMV
- vi. 30 cycles of the following regime:
  1. Denaturing at 94°C for 30 seconds.
  2. Annealing at 56°C for 30 seconds.
  3. Extending at 72°C for 30 seconds.
- vii. Hold samples at 4°C after cycling is complete.

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

- a. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)
  - i. Visualize the DNA
 

Carefully record location of bands on all positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.

    1. **A band occurring at the 439 bp location is confirmatory for OMV and the sample is reported as POSITIVE.**
    2. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for OMV.**
  - ii. Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)
 

**Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

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. Spring Viremia of Carp Virus (SVCV)

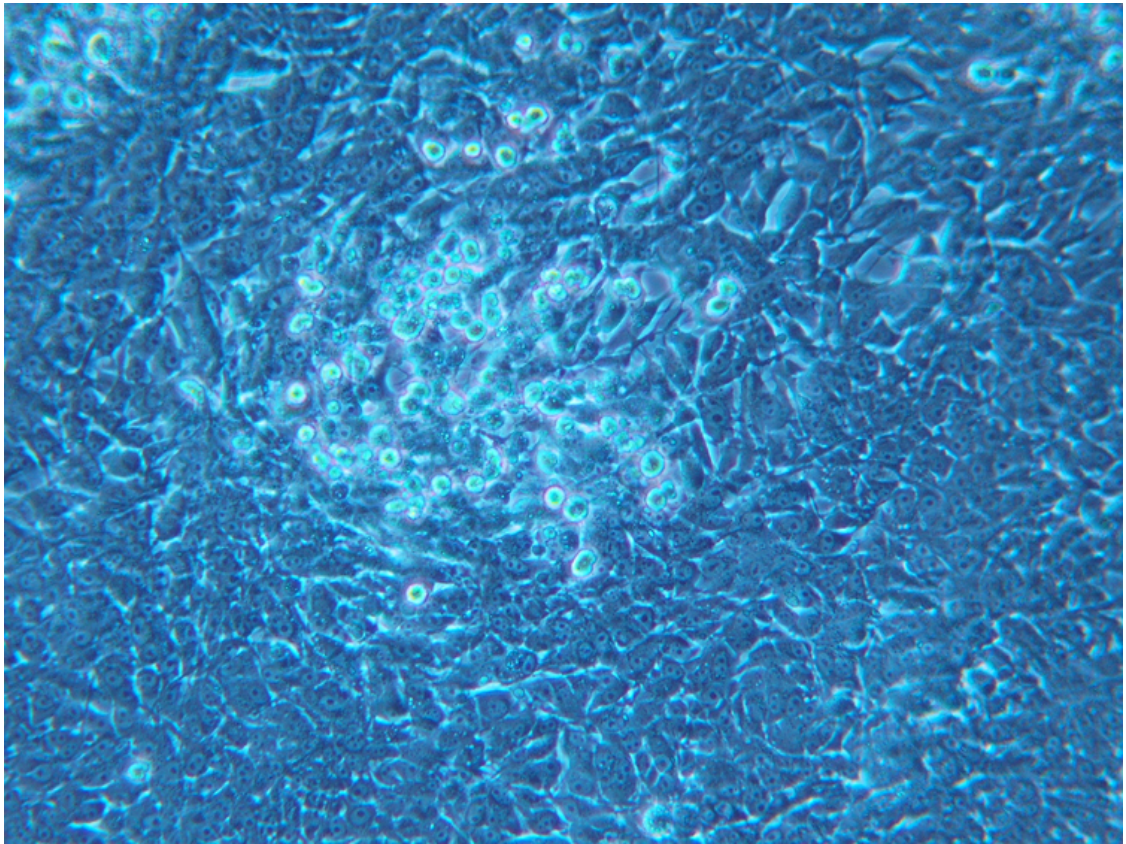
Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the *Vesiculovirus* genus of the *Rhabdoviridae*. The reported geographic range includes European countries that experience low water temperatures during the winter and cooler regions of the USA. The host range of SVCV includes Esocids and Cyprinids with the common carp being the principle host. Characteristic lesions include hemorrhages of the skin, gills, and viscera. Mortality is usually seasonal, often most severe in 1 or 2 year old fish during spring or early summer when water temperatures rise through the permissive range. The SVCV may rarely be found in ovarian fluid at spawning but transmission is primarily horizontal and may involve passive transfer by parasites such as the louse and leech (OIE 2000; Wolf 1988).

### 1. Screening Method

- a. Cell culture on EPC cell line incubated at 20 to 25°C.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for SVCV.**
- d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
  - i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear

chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above and figure 4.14 below.

- ii. The serum neutralization or polymerase chain reaction methods may be used to confirm that the CPE is due to SVCV.



**Figure 4.14.** CPE plaque of SVCV on EPC monolayer. Photo courtesy of Andrew Goodwin, University of Arkansas.

### 2. Confirmation Methods for SVCV

Spring Viremia of Carp Virus is one member of a larger group of closely related rhabdovirus-like fish pathogens. Due to the similarity between members of this group, confirmatory serum neutralization tests or PCR must be carefully controlled to prevent false positive results that identify a closely-related isolate as the highly regulated SVCV. As with all serum neutralization assays, neutralizing antibodies must be carefully tested for cross reactivity with non-SVCV isolates. When PCR is used for the confirmatory test, the OIE recommends sequencing the PCR product to confirm that the sequence represents a virus considered to be SVCV. The final identification of the virus rests on its position in a cladogram derived from a library of SVCV and related DNA sequences. Because of the taxonomic difficulties inherent in the proper classification of SVCV-like isolates, laboratories may wish to submit the virus to a reference laboratory for final confirmation. The Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in Weymouth, England is the OIE reference laboratory for SVCV. In the US, the National

## 4.6 Identification of Viruses - 27

Veterinary Services Laboratory in Ames, Iowa may also be able to provide this service. It is important to first contact the reference laboratory to confirm the availability of services and to discuss proper shipping methods.

- a. Serum Neutralization Method  
See Section 2, 4.7 Serum Neutralization for the general procedure
  - i. Use the cell line on which the initial CPE was produced.
  - ii. Incubate plates at 20 to 25°C.
- b. Polymerase Chain Reaction (PCR) method for confirmation of SVCV (OIE proposed 2003):. Total RNA is extracted from cell culture supernatant and subject to reverse transcription for production of appropriate cDNA which is then amplified with forward and reverse primers in a PCR reaction. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
  - i. Extraction of RNA from Cell Culture Fluid (method of Strømme & Stone, 1998)
    1. Total RNA is extracted from 100 µl of suspect viral tissue culture supernatant. The resulting RNA is dissolved in 40 µl molecular biology grade DNase- and RNase-free water. Extraction may be done using commercially available total RNA extraction kits that produce high quality RNA suitable for RT-PCR. Examples are Trizol Reagent(tm) (BRL, Life Technologies), SV Total RNA isolation system (Promega) and Nucleospin® RNA (BD Biosciences)..
    2. Quantify RNA template in the spectrophotometer. The optimum amount of RNA template should be around 100µg/ml (or 100ng/µl).
  - ii. Formation of cDNA by Reverse Transcription
    1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
    2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data Log Sheet, record appropriate data for each sample to be tested by PCR.
    3. Using Section 2, 4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV), record date of assay and then calculate the amount of each reagent to go into the “RT Master Mix” (RTMM) according to the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls in the assay.). About 19 µl of RTMM will be required per sample.
    4. For each sample to be tested, set up a tube with 19 µl of RTMM and 1µl of purified RNA template from above (Dilute template if more than 300ng/µl or use up to 5µl/reaction if reading falls below 50ng/µl. If more than 1 ul of template is used, reduce the water volume accordingly). Incubate the tubes at 37 C for 1 hour.
  - iii. First Round “Semi-Nested” PCR for SVCV:

## 4.6 Identification of Viruses - 28

1. Again use worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the PCR “Master Mix” (PCRMM) according to the number of samples and controls to be processed.
2. Prepare PCR tubes with 45.5  $\mu$ l of PCRMM and add 2.5  $\mu$ l of RT product for each RNA sample. Add 1  $\mu$ l of each of the primers SVCV F1 & SVCV R2 (primer concentration 50 pM/ $\mu$ l).

First Round Primers for SVCV:

a. Forward:

5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)

b. Reverse:

5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-ACN-CAY-3' (SVCV R2)

3. Place the tubes in a thermocycler and amplify using the following program.
  - a. 35 cycles as follows:
    - i. Denaturing at 95°C for 60 seconds
    - ii. Annealing at 55°C for 60 seconds
    - iii. Extending at 72°C for 60 seconds
  - b. Post-dwell at 72°C for 10 minutes.
  - c. 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.**

iv. Visualization of PCR Product by Electrophoresis (6.3.C)

1. Visualize the DNA – Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at the anticipated location.
  - a. **Bands occurring at the 714 bp location in the First Round are confirmatory for SVCV (see note on confirmation and SVCV taxonomy in F.2 above).**
  - b. Photograph the gel (6.3.G) – **Photo document all gels** and attach the photo to the case history information. (Worksheet C “Photodocumentation of the PCR Product Gel” Appendix 4.A1)
  - c. **If no appropriate band is seen**, proceed with Second Round “Semi-Nested” PCR for SVCV.

- v. Second Round “Semi-Nested” PCR for SVCV
  - 1. Prepare additional PCRMM and set up tubes for the second round of PCR exactly as for the first round, but, instead of the cDNA template (RT product), use 2.5 µl of the product from the first round of semi-nested PCR as the template and the primer set below.
  - 2. Semi-nested Second Round Primers for SVCV:
    - a. Forward:  
5’-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3’  
(SVCV F1)
    - b. Reverse:  
5’- CTG-GGG-TTT-CCN-CCT-CAA-AGY-TGY-3’  
(SVCV R4)
  - 3. Place the tubes in a thermocycler and amplify using the same program as for the first round (iii.4 above)
- vi. Visualization of PCR Product by Electrophoresis (6.3.C)
  - 1. Visualize the DNA – Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at the anticipated location.
    - a. **Bands occurring at the 606 bp location in the Second Round are confirmatory for SVCV (see note on confirmation and SVCV taxonomy in F.2 above).**
    - b. Photograph the gel (6.3.G) – **Photo document all gels** and attach the photo to the case history information. (Worksheet C “Photodocumentation of the PCR Product Gel” Appendix 4.A1)
    - c. **The lack of the appropriate bands with no indication of problems with the first or second round assay are reported as NEGATIVE for SVCV**

IUPAC code	Base Combination
A	Deoxyadenine
C	Deoxycytosine
G	Deoxyguanine
T	DeoxyThymidine
U	Deoxyuracil

I	Deoxyinosine
F	Phosphorothioate-A
O	Phosphorothioate-C
E	Phosphorothioate-G
Z	Phosphorothioate-T
V	A + C + G
D	A + T + G
B	T + C + G
H	A + T + C
W	A + T
S	C + G
K	T + G
M	A + C
Y	C + T
R	A + G
N	A + G + C + T

**Table 4.3** - International Union of Pure and Applied Chemistry (IUPAC) Nucleotide Sequence Codes

## G. 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*. Disease is characterized by edema and hemorrhage due to impairment of osmotic balance. In North America susceptible species include Pacific herring and pilchard on the West Coast, and significant mortality has been reported in muskellunge, freshwater drum, yellow perch, round goby, emerald shiners and gizzard shad in the Great Lakes. The virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning West Coast adult coho and Chinook salmon. For a more extensive list of susceptible species, please refer to Skall et al. (2005) or to Table 2.8.1 in Section 1 of the AFS/ FHS Blue Book (2007). Fry are most susceptible to disease, which usually occurs at water temperatures between 4 to 14°C, however naïve fish of all ages have been shown to be susceptible. Subclinical infections may develop, with fish shedding virus in the feces, urine, and reproductive fluids as well as being present in the internal organs. Although present in ovarian fluid, intraovum transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but can be separated by molecular methods (OIE 2006).

### Screening Method

See section 4.5 Screening Method for viral isolation

- a. Cell culture on EPC, FHM or BF-2 cell lines incubated at 15°C (Batts et al. 1991, Gagné et al. 2007). During incubation, it is critically important that the pH of the medium remain within the range of 7.4 - 7.8 because cytopathic effects (CPE) of VHSV will not develop in acidic cultures (Batts et al., 1991), probably due to pH-dependent conformational changes in the glycoprotein (Gaudin et al., 1999). This is especially problematic for cell lines derived from coolwater species that continue to metabolize efficiently at the incubation temperatures of the assay.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for VHSV.**
- d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
  - i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.
  - ii. The Polymerase Chain Reaction (PCR), serum neutralization, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of VHSV.

#### Confirmation Methods for VHSV

- a. Polymerase Chain Reaction (PCR) Method for Confirmation of VHSV (Modified from Einer-Jensen 1995): This Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers specific to the N gene of VHSV 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.
  - i. Extraction of RNA from Cell Cultures
 

Total RNA from infected cells is extracted using a phase-separation method (eg. phenol-chloroform or TRIZOL, Invitrogen) or by RNA affinity spin columns (eg. RNeasy Total RNA kit, Qiagen) according to the manufacturer's instructions. While all of these methods work well for drained cell monolayers or cell pellets, RNA binding to affinity columns can be affected by salts present in tissue culture media and phase-separations methods should be used for extraction of RNA from cell culture fluids.
  - ii. Formation of DNA by Reverse Transcription and Amplification by PCR
    1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)
    2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
    3. Using Section 2, 4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV), 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 four to the number of samples so that there is enough to run controls in the assay.)

4. Primers for VHSV\*
  - a. Forward: 5'-GGG-GAC-CCC-AGA-CTG-T-3'
  - b. Reverse: 5'-TCT-CTG-TCA-CCT-TGA-TCC-3'

\*2010 edition note: The primer sequences for VHSV have been modified compared to those provided in the 2005 and earlier editions of this manual. The new sequences now match the OIE (2006) VHSV primers.

5. Thermocycler Program for VHSV
  - a. Incubate at 50°C for 30 minutes for Reverse Transcriptase reaction.
  - b. Denature sample at 95°C for two minutes.
  - c. 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.
  - d. Final extension at 72°C for seven minutes.

**Hold samples at 4°C after cycling is complete.**

**PCR Products can be refrigerated for one month or frozen at -20°C for long-term storage.**

- iii Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)
  1. Visualize the DNA
 

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 PCR assays.

    - a. **Bands occurring at the 811 bp location are confirmatory for VHSV and are reported as POSITIVE.**
    - b. **If there is an absence of appropriate bands with no indication of problems with the assay, consider testing for other viruses or consult an appropriate reference laboratory.**
  2. Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)
 

**Photo document all gels** and attach the photo to the case history information. (Section 2, 4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

See Section 2, 4.7 Serum Neutralization for the general procedure\*.

- i. Use the cell line on which the initial CPE was produced.
  - ii. Incubate plates at 15°C.
- c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of VHSV  
See Section 2, 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure\*.
- i. Use the cell line on which the initial CPE was produced.
  - ii. Incubate virus inoculated monolayers at 15-18°C.

\*The Danish Institute for Food and Veterinary Research, Fish Disease Section is both the European and OIE reference lab for VHSV and should have anti-serum available. The contact is Dr Ellen Ariel. e-mail: [ear@dfvf.dk](mailto:ear@dfvf.dk).

## H. White Sturgeon Herpesvirus (WSHV)

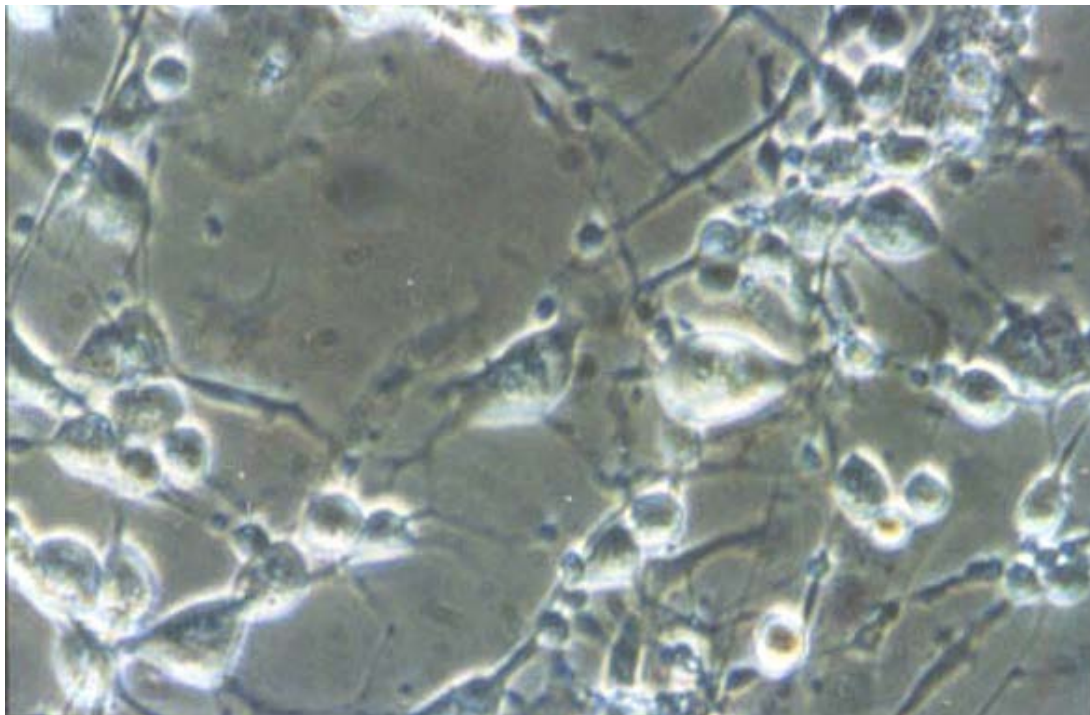
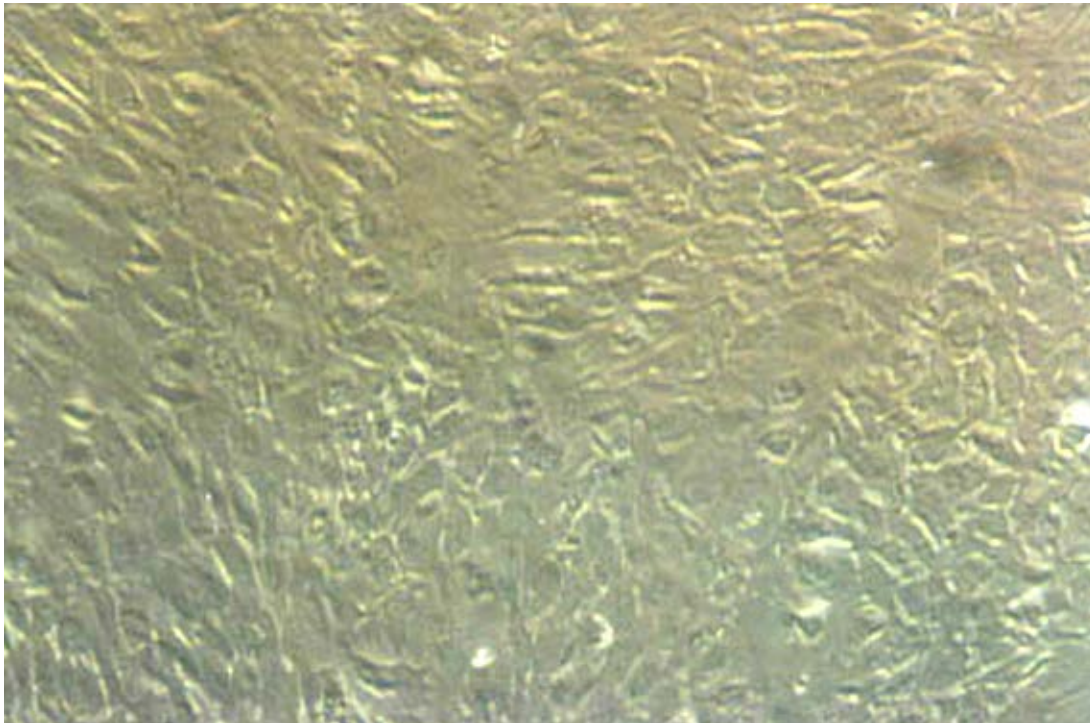
White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the Herpesvirus genus of the *Herpesviridae*. WSHV has been found in both feral and captive populations of sturgeon in California and Oregon. WSHV-1 has been found in juvenile cultured white sturgeon less than 10 cm. The susceptibility of other sturgeon species to WSHV-1 is not known at this time. WSHV-2 has been isolated from wild and cultured subadult and adult white sturgeon. A herpesvirus has also been isolated from shortnose sturgeon, although the relationship of this isolate to WSHV-1 or WSHV-2 has not been determined. Infected fish may present with lethargy, emaciation, excessive mucus production, fluid in the gastrointestinal tract, and focal skin lesions. Horizontal transmission has been demonstrated with both WSHV-1 and WSHV-2. WSHV-2 has been isolated from ovarian fluid but vertical transmission has not been demonstrated (LaPatra, personal communication 2002; Plumb 1999).

### Screening Method

- a. Cell culture on WSS-2 cell line incubated at 20°C.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for WSHV.**
- d. If CPE typical of a herpesvirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for WSHV.

- i. The appearance of CPE typical of WSHV and other herpesviruses includes the formation of syncytia. See Figures 4.15 and 4.16.
- ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of WSHV. **Figure 4.15.** Normal WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

**Figure 4.16.** CPE typical of WSHV on WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.



Confirmation Method for WSHV

- a. 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.
- b. 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.