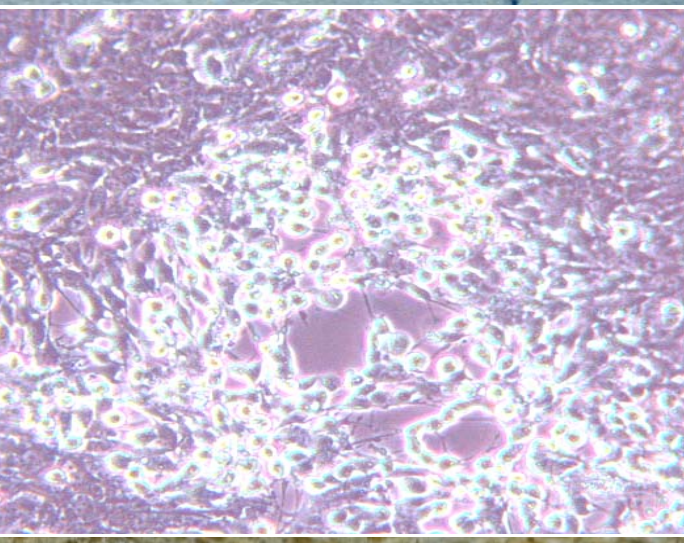
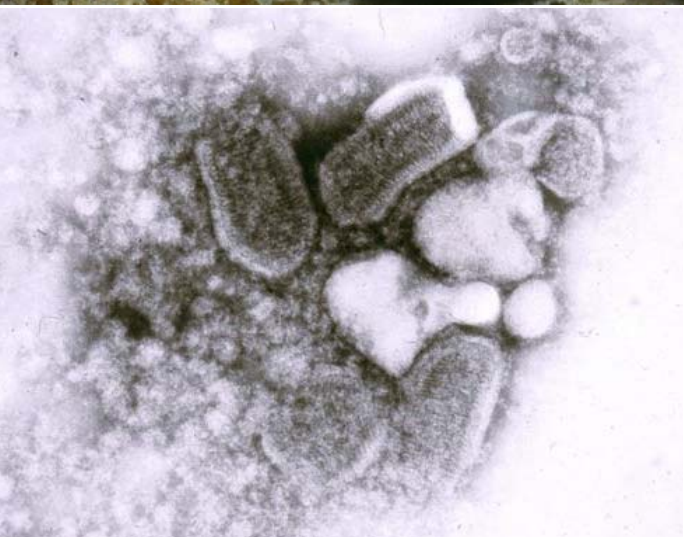




USFWS/AFS-FHS Standard Procedures for Aquatic Animal Health Inspections

Chapter 4 Virology

4.1 Virology Introduction

The procedures described in this chapter are specifically for the detection of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Largemouth Bass Virus (LMBV), *Oncorhynchus masou* Virus (OMV), Spring Viremia of Carp Virus (SVCV), Viral Hemorrhagic Septicemia Virus (VHSV), and White Sturgeon Herpesvirus (WSHV).

The initial detection method for all of these viruses is by observing cytopathic effect (CPE) in cell culture using virus isolation procedures. The presence of IHNV may be confirmed using serum neutralization, indirect fluorescent antibody test (IFAT), Alkaline Phosphatase Immunocytochemistry stain, or polymerase chain reaction (PCR) techniques. Confirmation of IHNV with antibodies enzymatically labeled with Alkaline Phosphatase instead of a fluorescent marker is new with this edition and enzymatic labels will probably be extended to other confirmatory tests in future additions. The presence of IPNV and VHSV may be confirmed using serum neutralization, indirect fluorescent antibody test (IFAT), or polymerase chain reaction (PCR) techniques. The presence of ISAV may be confirmed using IFAT or PCR techniques. The presence of LMBV and OMV are confirmed using PCR techniques. The presence of SVCV may be confirmed using serum neutralization or PCR techniques. WSHV suspect cultures will be sent to an appropriate laboratory for confirmation.

These procedures may also detect other replicating agents not listed here. When this occurs, every attempt will be made to complete the identification of the organism. Some of these viruses may occur in combination and the finding of one agent will not preclude following procedures that may identify other agents.

If one of these viruses or an unknown replicating agent is found, the proper parties and authorities will be notified in a timely manner and at least one representative sample of each isolate should be archived at -70°C to be used for future reference.

Blind passage of samples not exhibiting CPE after 14 days of primary incubation is included in these procedures to determine if it provides a significant increase in detection of viral agents. It is requested that laboratories using these procedures summarize their findings of primary and blind passage detections by virus and provide it to the Handbook Revision and Oversight Committee annually. If the data shows that blind passage of these samples is not providing a sufficient increase in viral detection, it will be removed from the procedures.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

4.2 Selection of Appropriate Cell Lines

Selection is based on the ability of the cell lines to detect the viruses of interest and, whenever possible, utilizing cell lines capable of detecting different viral agents to increase the efficiency of the laboratory procedures. At the minimum, one cell line specified for the virus of interest will be used and at least two cell lines should be used for all samples to maximize the detection of viral agents present in the samples (Bouchard 1999; OIE 2000; Plumb 1999b; Thoesen 1994; Wolf 1988).

A. General Considerations

1. All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available.

2. At the minimum, all cell lines should be tested annually for viral sensitivity and mycoplasma infection.

3. Work with only one cell line at a time.

4. Aseptic technique is required for cell culture work.

B. Cell Line Sensitivities

1. The EPC cell line provides high sensitivity for IHNV, SVCV, and VHSV.

2. The SHK-1 and ASK cell lines both provide high sensitivity for ISAV.

3. The FHM and BF-2 cell lines both provide high sensitivity for LMBV.

4. CHSE-214 cell line provides high sensitivity for IPNV and OMV.

5. The WSS-2 cell line provides high sensitivity for WSHV.

Table 4.1. Recommended cell lines to detect target viruses.

Virus[a]	Cell Line	Common Name	ATCC[b] Designation
IHNV	Epithelioma Papulosum Cyprini	EPC	
IPNV	Chinook Salmon Embryo	CHSE-214	CRL-1681
ISAV	Salmon Head Kidney	SHK-1	
	Atlantic Salmon Kidney	ASK	CRL-2747
LMBV	Fat Head Minnow	FHM	CCL-42
	or Bluegill Fry	BF-2	CCL-91
OMV	Chinook Salmon Embryo	CHSE-214	CRL-1681
SVCV	Epithelioma Papulosum Cyprini	EPC	
VHSV	Epithelioma Papulosum Cyprini	EPC	
WSHV	White Sturgeon Spleen	WSS-2	

[a] Viruses: IHNV- Infectious Hematopoietic Necrosis Virus; IPNV- Infectious Pancreatic Necrosis Virus and other related birnaviruses; ISAV – Infectious salmon anemia virus; LMBV – Largemouth Bass Virus and other related iridoviruses; OMV - *Oncorhynchus masou* Virus; SVCV – Spring viremia of carp; VHSV - Viral Hemorrhagic Septicemia Virus; WSHV - White Sturgeon Herpesvirus.

[b] American Type Culture Collection, Rockville, MD

4.3 Cell Culture

Standard animal cell culture techniques are used with adaptations for fish cell lines when necessary i.e., incubation temperature. Normal appearing cultures composed of rapidly dividing cells will be used for all assay work. Cells will be routinely subcultured to maintain healthy cells, approximately once every two weeks for most cell lines, or split weekly for seeding plates. Aseptic technique is required when working with any cell line. Only one cell line is worked with at a time (Freshney 1983; Jakoby 1979; Lannan 1994; Merchant, 1964; Rovozzo 1973; True 2000).

A. Subculture Procedures for Flasks

1. Suggested split ratios and seeding rates are given in Table 4.2.

2. Remove tissue culture medium by decanting fluid.

3. Slowly add trypsin-versene (EDTA) (4.9.E "Trypsin-Versene (EDTA)") solution and rock the flask gently for one minute. In a 75 cm^2 flask a volume of 3 to 4 mL is sufficient to coat cells.

4. Decant again.

5. Carefully observe the cell layer and repeat steps 3 and 4 as necessary until cells start detaching from flask. Dislodging of the cells may be completed by sharply striking the edge of the flask against the heel of a hand.

6. Add tissue culture medium to neutralize the trypsin. In a 75 cm^2 flask, 10 mL is sufficient.

7. Triturate to break up cell clumps and add an appropriate volume of fresh tissue culture medium for transferring to other flasks. Enumeration of cells in the suspension may be done at this time to determine the necessary volume to transfer (4.A2 Cell Enumeration Appendix 2)

8. Aspirate and dispense into new flasks. The sub-cultivation ratio is generally 1:4 to 1:6. Following manufacturers recommendations, bring total volume in each new flask up to the acceptable level with the appropriate tissue culture medium. For a 75 cm^2 flask, this will usually be about 20 mL. MEM-10/Hepes (4.9.G "MEM-10/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") works well in an open system for all cell lines listed in Table 4.2 except SHK-1 and ASK, which do best with L-15 (4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)").

9. Incubate flasks at room temperature (20 to 25°C) until they reach confluence and then incubate at the appropriate temperature for that cell line.

    a. SHK-1, ASK and CHSE-214 cells should be held at 15°C.

    b. WSS-2 cells should be held at 20°C.

c. FHM and BF-2 cells should be held at 25°C.

d. EPC cells may be held at 15 to 25°C.

Table 4.2. Seeding guidelines for the subculture of fish cell lines.

CELL LINE		Suggested Seeding Rate (per cm^2)	INCUBATION TEMP (°C)	
Common Name	Nominal split Ratio		Suggested	Range
BF-2	1:2 - 3	100,000	25 - 30	20 - 30
CHSE-214	1:3 - 6	100,000	15 - 20	4 - 27
EPC	1:3 - 6	250,000	15 - 25	15 - 30
FHM	1:4 - 6	250,000	25 - 30	0 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	1:2 - 3	250,000	15 - 20	15 - 20
ASK	1:2 - 3	250,000	15 - 20	15 - 20

B. Seeding Procedures for Plates

1. Monolayers on plates are prepared approximately 24 to 48 hours prior to inoculation with the sample.

2. A flask of visually healthy cells approximately 7 to 10 days old is selected and trypsinized as described previously. After neutralizing the trypsin with tissue culture medium, the total volume in the flask is adjusted to provide a cell concentration appropriate for the seeding rate listed in Table 4.2 and the area of the wells to be seeded. The appropriate volume of the cell suspension is then pipetted into each well of the plate.

   Example: When seeding EPC cells in a 24-well plate, 0.5 mL of a 1 X 10^6 cells per mL suspension is dispensed per well.

3. At a minimum, control wells are included in each plate set and should be included on each plate whenever possible. Control wells are made by dispensing tissue culture medium into plate wells that contain normal looking cell monolayers that have not been inoculated with a sample. These wells are observed during the incubation along with the sample wells for abnormalities that may arise due to media or cell problems. A plate set refers to the group of plates seeded from a single flask at the same time.

4. Incubate plates overnight at room temperature (approximately 20 to 25°C).

4.4 Sample Processing Procedures

Tissue processing for viral culture is described below. Ingredients and preparation procedures for buffers and other solutions/media are listed in 4.9 Reagents and Media (Amos 1985; OIE 2000; Rovozzo 1973; True 2000; Wolf 1988).

A. General Considerations

1. As during sampling and transport, care is taken to protect tissues and fluids from exposure to UV light, freezing, or high temperatures lethal to the viruses of interest.

2. During sample processing, dilution levels are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

3. Following inoculation of test media, remaining tissue/fluid products are kept at 4°C until all assays are completed. Subsequent to the completion of the assays, all material is decontaminated and discarded.

B. Processing of Kidney and Spleen Samples

1. If transport medium (3.C.2 “Hanks Balanced Salt Solution (HBSS)”) is used, it is poured off and disinfected before discarding. Tissue samples are weighed to the nearest 0.1 g and sterile sample dilution medium (4.9.A “Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)”) is added to make a dilution of 1:10 to 1:100 (w/v). Unless there is a high potential for cell toxicity from the sample, the 1:10 dilution will be used.

2. Tissues are homogenized and a measured amount of homogenate is pipetted into a sterile tube for centrifugation.

3. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

4. An aliquot of supernatant (for virologic evaluation) is transferred to a tube containing an equal amount of antibiotic incubation medium (anti-inc) (4.9.B “Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection” or 4.9.C “Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection”). Sample dilution is now 1:20 volume/volume.

   Tubes are vortexed and incubated for two hours at 15°C or 12 to 24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures (4.5.A “Plate Inoculation Procedures for Primary Culture”).

C. Processing of Coelomic (Ovarian) Fluid Samples

1. An equal volume from each ovarian fluid sample is pipetted into a sterile tube for centrifugation.

2. Centrifuge the tubes at 2000-3000 X g for 15 minutes at 4°C.

3. Undiluted ovarian fluid may be used to inoculate cell cultures as described in the inoculation procedures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts anti-inc) (4.9.B “Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection” or 4.9.C “Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection”) may be made.

4. If a dilution is made, tubes are vortexed and incubated for two hours at 15°C or 12 to 24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures.

4.5 Screening Method for Viral Isolation

The initial detection or screening method used for all listed viral agents is the observation of CPE in cell culture. Standard viral propagation techniques include plate inoculation and initial incubation for approximately 14 days during which time samples exhibiting CPE are re-inoculated. At approximately 14 days, blind passage of samples not exhibiting CPE is performed and monitored for a total combined incubation period (initial plus blind pass) of 28 days. Blind passage is included to optimize detection of low titer and/or slow growing viruses. To further maximize detection of viral agents, samples should be inoculated on at least two different cell lines (Amos 1985; OIE 2000; Bouchard 1999; Plumb 1999b; Rovozzo 1973; True 2000; Wolf 1988).

A. Plate Inoculation Procedures for Primary Culture

1. **General Considerations**

   a. All cell monolayers to be inoculated are to be at least 80% confluent, approximately 24 hours old, and visually healthy.

   b. Tissue culture plates are identified by labeling with the cell line, date of inoculation, and sample information.

   c. Aseptic technique is required.

2. Tissue culture medium is decanted from plates.

3. Inoculate with replication at least 2 cm^2 of cell monolayer with a minimum of 100 µL from each sample.

   Example: If using 24-well plates (2 cm^2/well), 100µL of each sample would be inoculated onto each of two wells of the plate.

4. To allow for viral adsorption, incubate plates for 30 to 60 minutes with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.

   a. Incubation temperature for IPNV, IHNV, VHSV, ISAV, and OMV is 15°C.

   b. Incubation temperature for WSHV, SVCV, and LMBV is 20 to 25°C.

5. Dispense an adequate volume of the appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (4.9.F “MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)”) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with Leibovitz’s L-15 (4.9.H “Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)”). If using 24-well plates, 0.5 mL of medium is adequate.

6. Seal each plate.

7. Incubate plates at the appropriate temperature for a minimum of 14 days.

   a. Incubate for IHNV, IPNV, ISAV, OMV, and VHSV at 15°C.

   b. Incubate for WSHV at 20°C.

   c. Incubate for LMBV and SVCV at 20 to 25°C.

8. Monitor cells at least twice per week for cytopathic effect (CPE). CPE is defined as any morphological change that cells may demonstrate in response to viral or toxic agents. It may range from foaming of the cytoplasm to focal clumping or local destruction of cells. Examples of the appearance of normal cell line monolayers and the CPE typical of these viruses are shown in Figures 4.1 to 4.12.

9. Re-inoculations are made from representative wells exhibiting CPE on these primary inoculations and from at least one well of all samples not exhibiting CPE (blind passage) according to the procedure in "Re-Inoculation Procedure" below.

B. Re-Inoculation Procedure

1. General Considerations

   a. Re-inoculation of wells showing toxicity and to confirm the presence of virus with typical CPE may be performed on individual wells at any time during the primary incubation. These will be maintained as individual samples and plated with replication during the re-inoculation procedure.

   b. Blind passage from at least one well of all samples not exhibiting CPE will be performed after 10 to 14 days of incubation of the primary culture. These samples may be combined in up to a five-pool sample (representing up to 25 fish) and plated with replication during the re-inoculation procedure. It is suggested that the wells remaining on the initial plate be left intact and observed for at least another 7 to 10 days for a total initial incubation period of 21 days.

      Example: On a lot inspection using five-fish pools and 24-well plates, two of the 12 samples exhibit CPE at day five and re-inoculation is performed as described below. The remaining 10 samples (20 wells representing 50 fish) show no evidence of CPE after 14 days of primary incubation and re-inoculation is performed by combining one well from each of five samples and inoculating this pooled sample onto two wells of a 24-well plate. This is repeated with the other five samples using a total of four wells on the re-inoculation plate.

   c. As in the initial tissue processing, sample dilution levels for re-inoculation are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

   d. Aseptic technique is required.

2. Using a pipette, stir and scrape the bottom of the well to be subcultured to dislodge the cell layer.

3. Aspirate the fluid and cell debris from the well and place in a sterile tube for centrifugation.

4. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

5. Remove a measured amount of supernatant and place in a separate sterile tube for dilution.

6. Dilute samples.

    a. For wells exhibiting CPE, use the lowest dilution possible for re-inoculation not to exceed 1:100 using sample dilution medium (4.9.A "Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)").

    b. For blind passage samples, up to a 1:5 dilution may be made.

7. If bacterial or fungal contamination is present, the sample should be filtered through a 0.45 µ filter before inoculation onto the plate.

8. Use the appropriate amount of each of these solutions to inoculate a new plate as described above in 4.5.A "Plate Inoculation Procedures for Primary Culture."

9. Monitor these re-inoculation plates at least twice per week for CPE. Total incubation time for both the primary and re-inoculation or blind pass samples is 28 consecutive days.

    Example: If the blind pass is performed on day 14 of the primary incubation, the re-inoculation plate is observed for at least an additional 14 days. If the blind pass is performed on day 10, the re-inoculation plate is observed for at least 18 days.

10. Results

    a. **If no CPE is noted after the 28 day combined incubation period with no apparent problems in the assay, samples are reported as negative and may be discarded using the proper decontamination procedures.**

    b. If CPE occurs at any time during this assay, it is considered a **PRESUMPTIVE positive** result and the identification of the virus should be confirmed by the appropriate method.

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 2000; 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.

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 1989) see Figures 4.1 to 4.3.

ii. The serum neutralization, PCR, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of IHNV

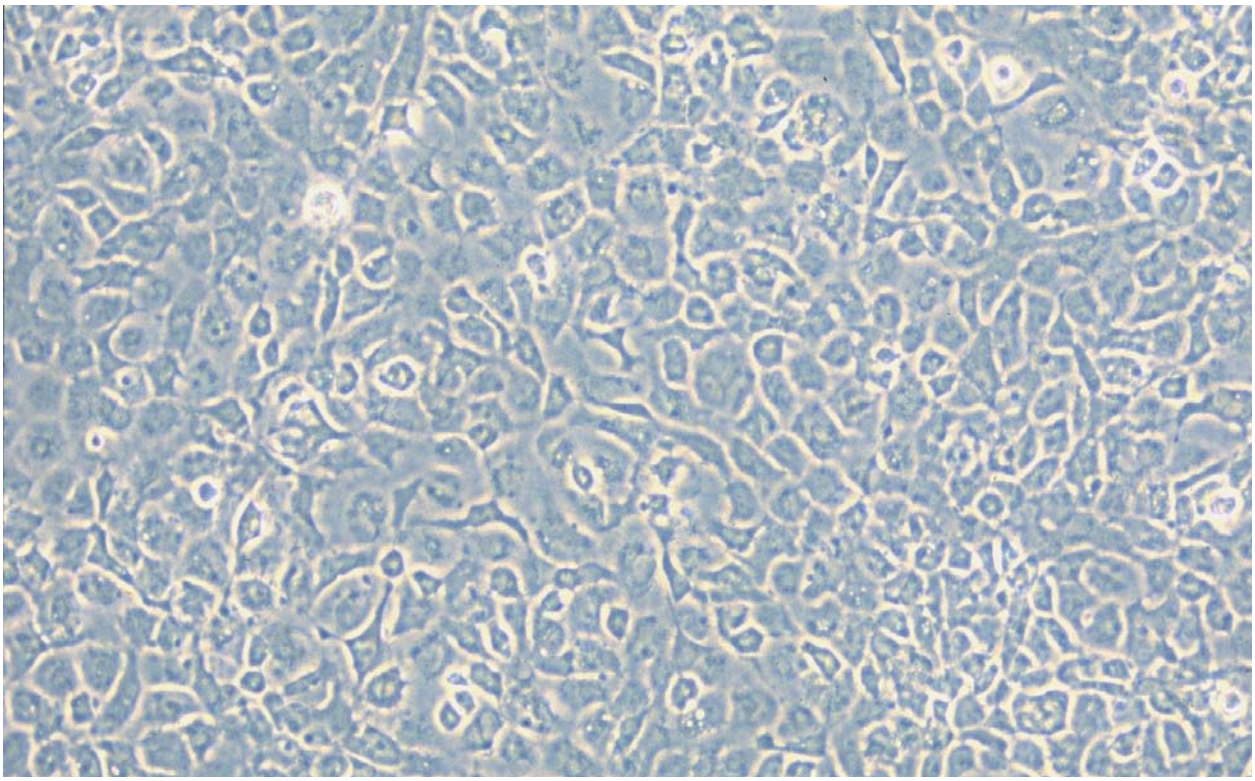


Figure 4.1. Normal EPC monolayer. Photo courtesy of Jim Winton, USGS.

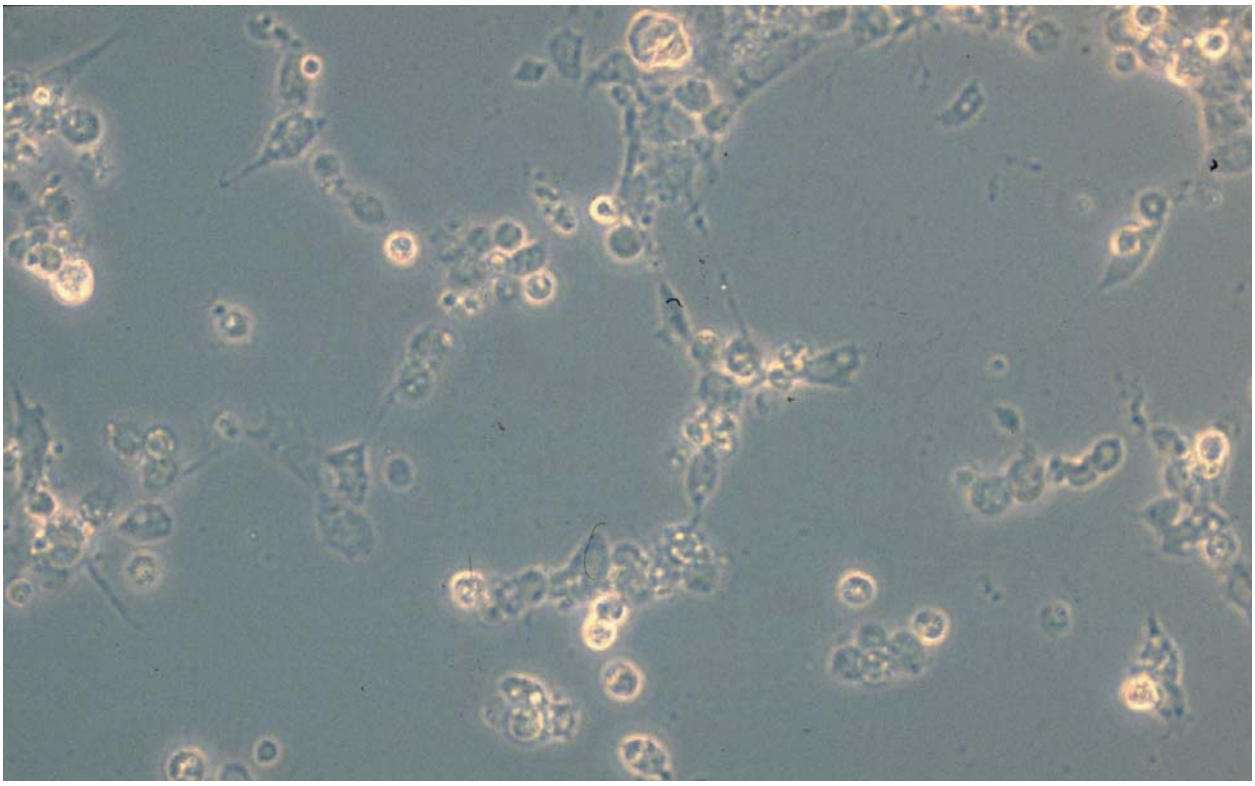


Figure 4.2. CPE typical of a rhabdovirus on EPC monolayer. Photo courtesy of Jim Winton, USGS.

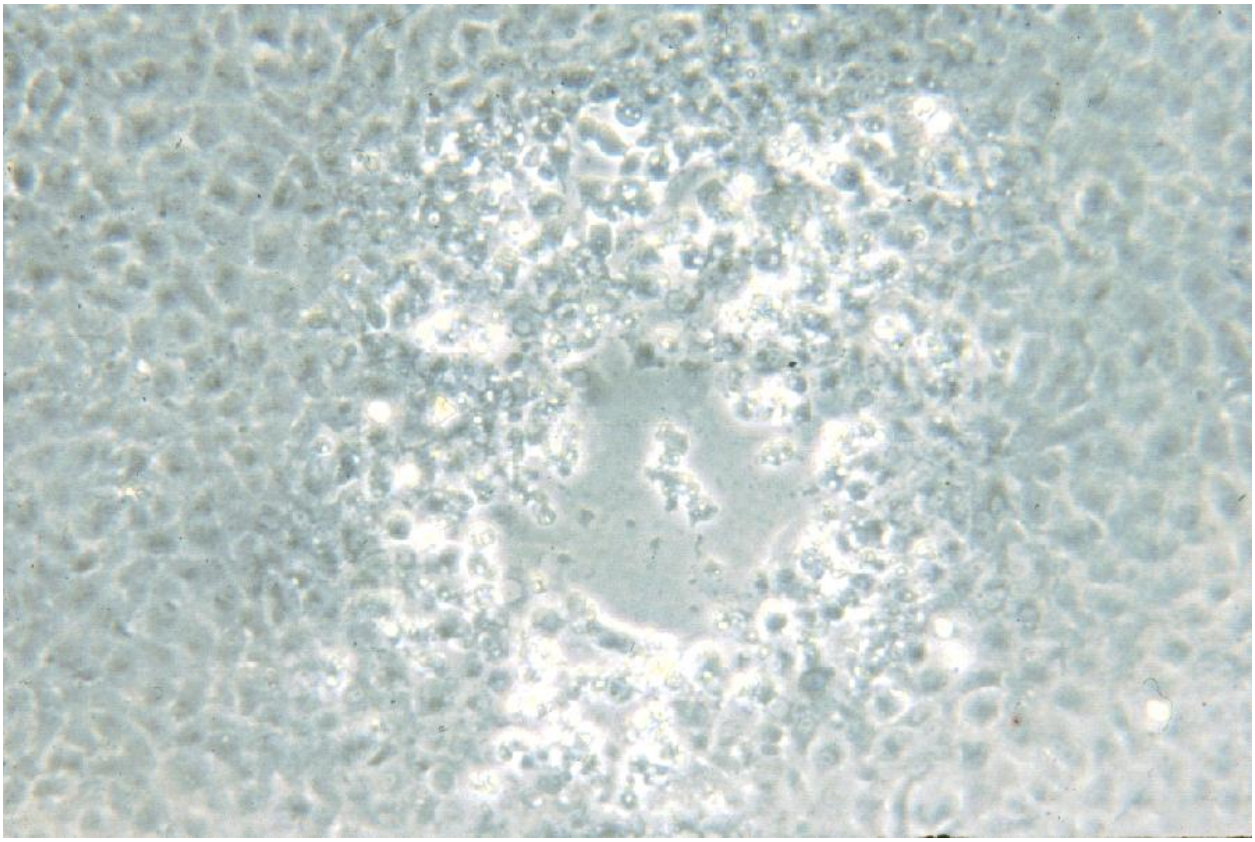


Figure 4.3. CPE plaque of IHNV on EPC monolayer. Photo courtesy of Scott LaPatra, Clear Springs. Foods, Inc.

2. Confirmation Methods for IHNV

a. Serum Neutralization Method
See 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 IHNV (Modified from Arakawa 1990): 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. Heat tubes to 95°C for two minutes in a water bath, heat block, 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 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. Production of DNA by Reverse Transcription and Amplification by First Round PCR

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

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

3. Using 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. First Round Primers for IHNV

a. Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'

b. Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'

5. First Round Thermocycler Program for IHNV

a. Pre-dwell at 50°C for 15 minutes.

b. Preheat or "Jumpstart" sample to 95°C for two minutes.

c. 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.

d. Post dwell at 72°C for seven minutes.

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

iii. "Nested" Second Round PCR for IHNV

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

2. Again use Section 2, 4.A1.B.1 Worksheet B.1 – Infectious Hematopoietic Necrosis Virus (IHNV) for the second round 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.

3. Second Round Primers for IHNV

a. Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'

b. Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'

4. Second Round Thermocycler Program for IHNV

a. Preheat or "Jumpstart" sample to 95°C for two minutes.

b. 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.

c. Post dwell at 72°C for seven minutes.

d. 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 (See 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 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.**

2. Photograph the Gel (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IHNV
See 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 be 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 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 (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 (4.9 A)

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 µL 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 µL 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 µL MEM-5 (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 µL 10 % Neutral Buffered Formalin (NBF) (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 µL/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 ^{o}C until stained.

v. Staining (Modified from Vectastain kit instructions)

1. Pre-heat incubator to 37-45 ^{o}C. Buffer may be heated in the incubator for more rapid staining.

2. Add 200 µL 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 µL of blocking reagent to each well.

5. Incubate at 37-45 ^{o}C for 30 minutes.

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 µL to each well.

8. Incubate at 37-45 ^{o}C for 15-30 minutes.

9. Wash by repeating step 4.12.E.2 three times.

10. Add 50 µL of Vectastain Biotinylated Universal Antibody to each well.

11. Incubate at 37-45 ^{o}C for 15-30 minutes.

12. Wash by repeating step 4.12.E.2 three times.

13. Add 50 µL of Vectastain ABC-AP Reagent to each well.

14. Incubate at 37-45 ^{o}C for 15-30 minutes.

15. Wash by repeating step 4.12.E.2 five times.

16. Add 50 µL 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 ^{o}C.

20. Examine at 400-1000x magnification.

vi. Results

1. Positive controls should contain individual cells that appear red and early plaques will appear a 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 1996; Egusa 1991; OIE 2000; Roberts 1982; 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 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.

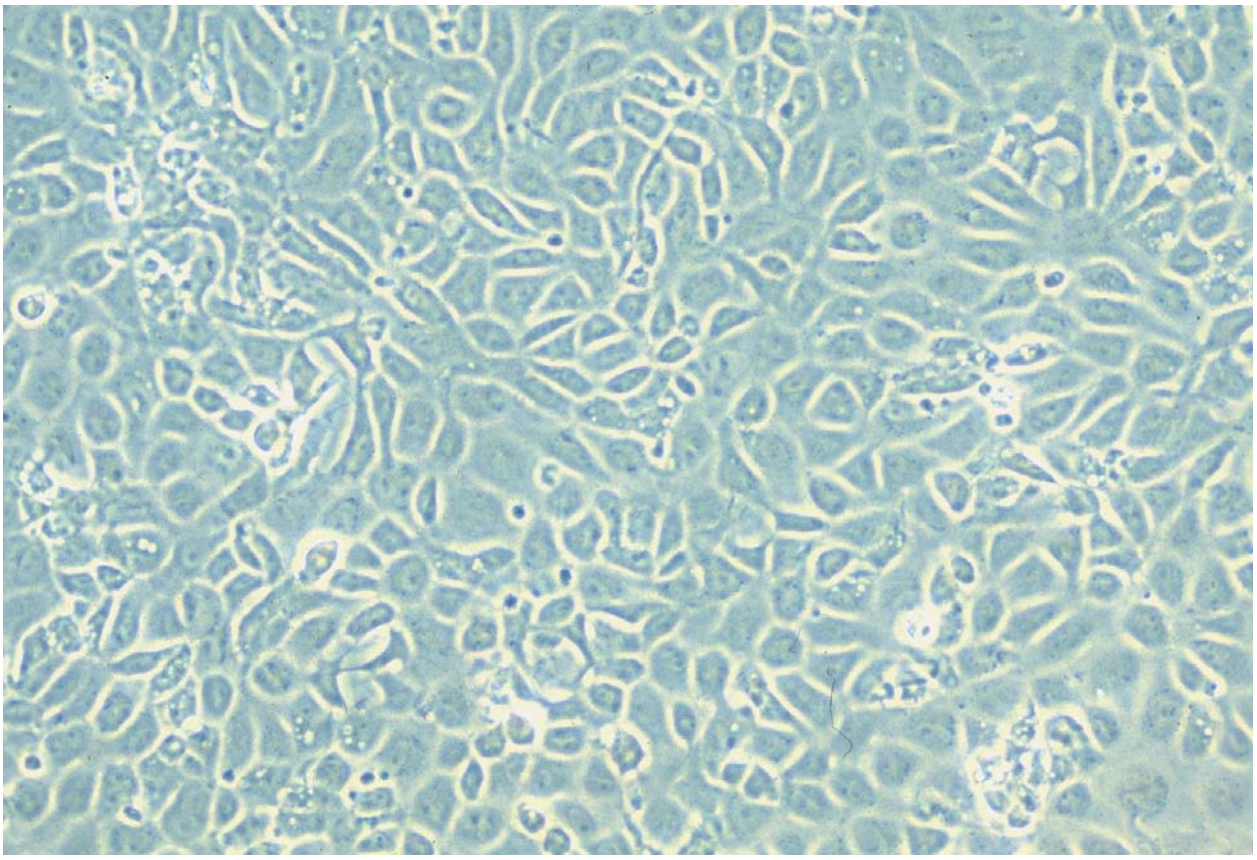


Figure 4.4. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

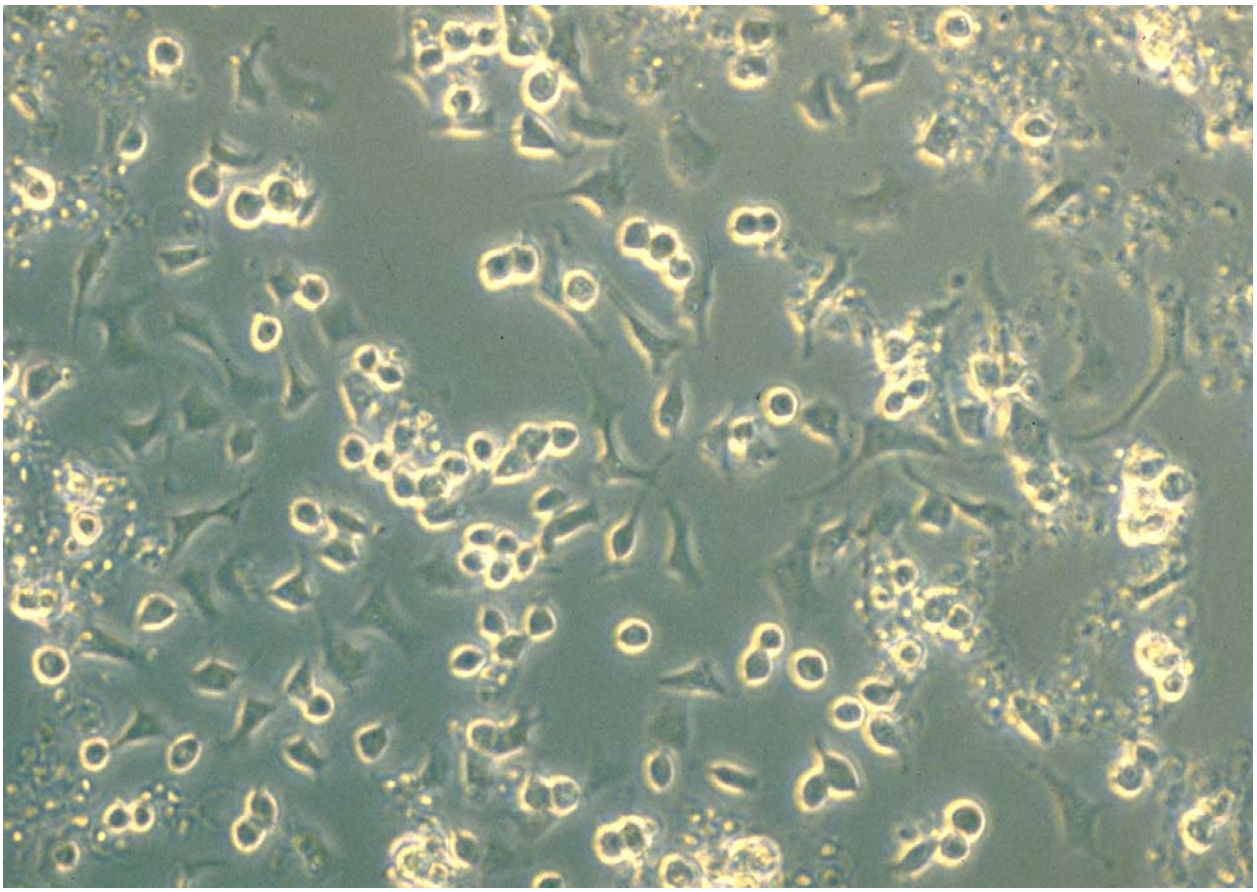


Figure 4.5. CPE typical of IPNV on CHSE-214 cells. Photo courtesy of Jim Winton, USGS.

2. **Confirmation Methods for IPNV**

   a. Serum Neutralization Method
   See 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 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 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

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

3. Using 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 (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 (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of IPNV
See 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 1996; OIE 2000).

1. **Screening Method**

a. Cell culture on SHK-1 cell line incubated at 15°C (Bouchard 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.

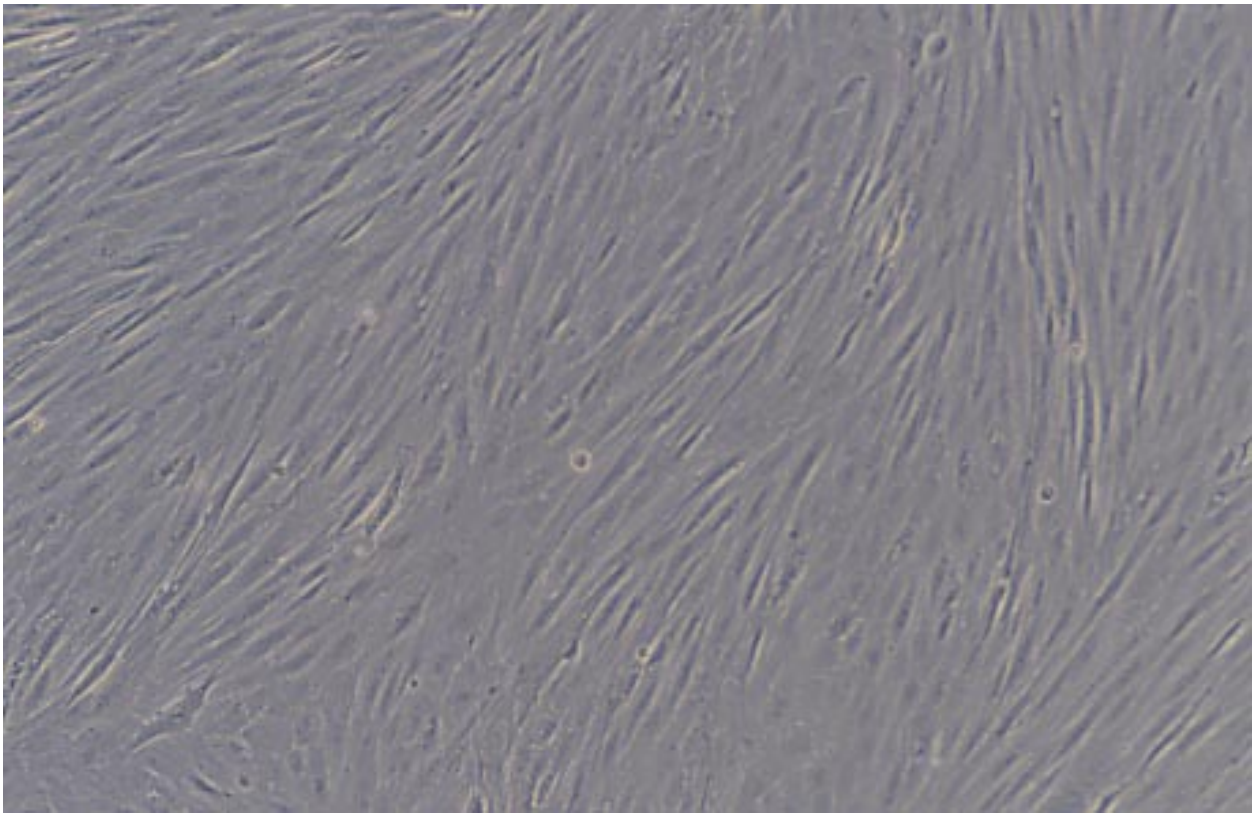


Figure 4.6. Normal SHK-1 monolayer. Photo courtesy of Jim Winton, USGS.

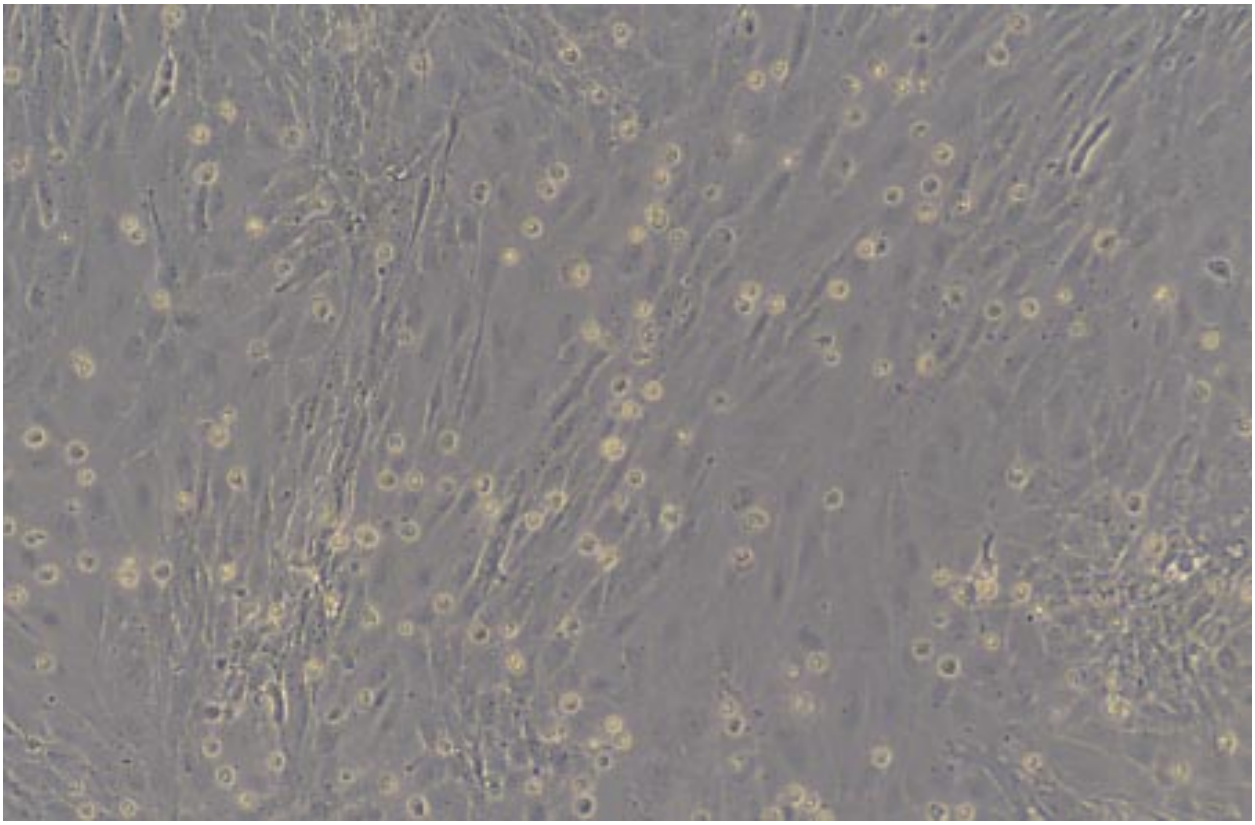


Figure 4.7. CPE typical of ISA on SHK-1 cells. Photo courtesy of Jim Winton, USGS.

2. Confirmation Methods for ISAV

a. Polymerase Chain Reaction (PCR) (Modified from Bouchard 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 µL fluid to 98 µL 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 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 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

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

3. Using 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 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 (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

b. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of ISAV
See 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 1999a). During an active infection, the virus may be isolated from several tissues including the kidney, spleen, and swim bladder.

1. Screening Method

a. Cell culture on FHM or BF-2 cell lines incubated at 20 to 25°C (Plumb 1999b).

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 inoculations 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 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.

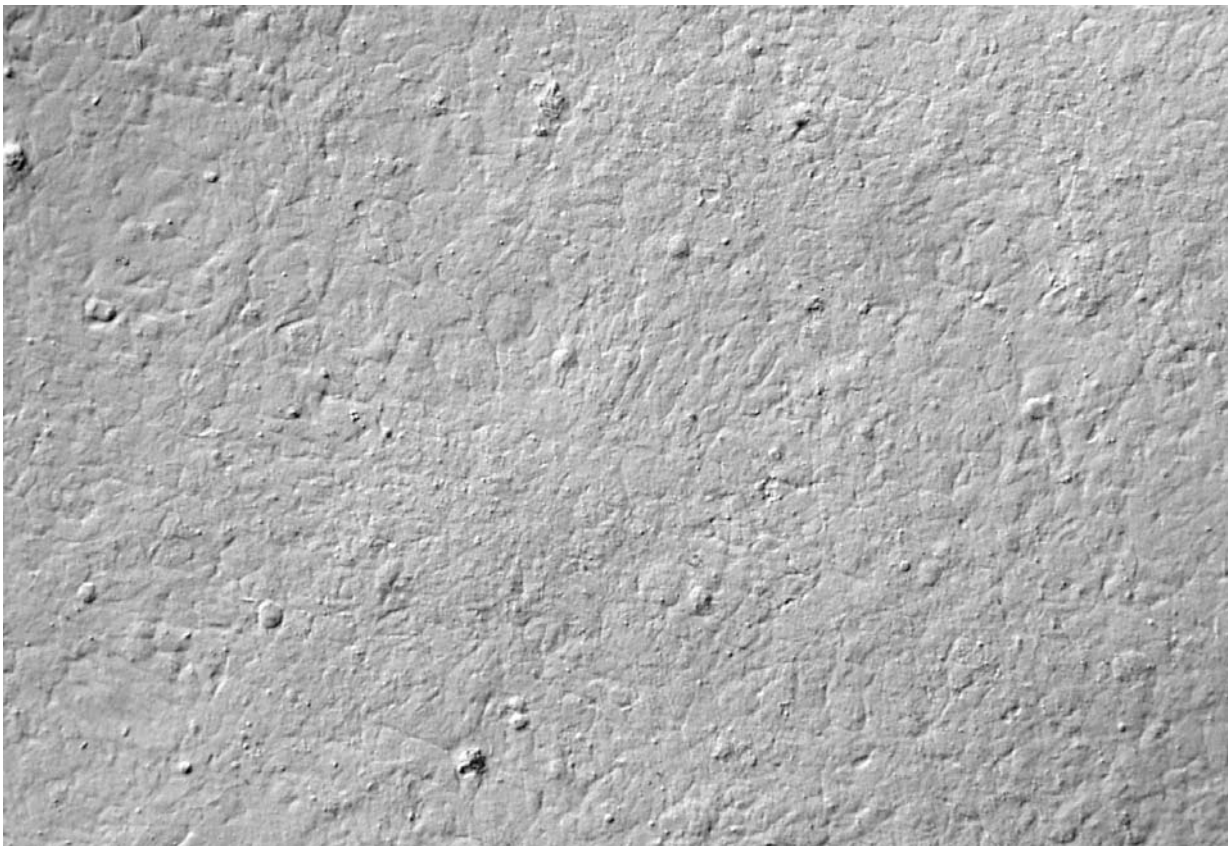


Figure 4.8. Normal FHM monolayer. Photo courtesy of John Grizzle, Auburn University.

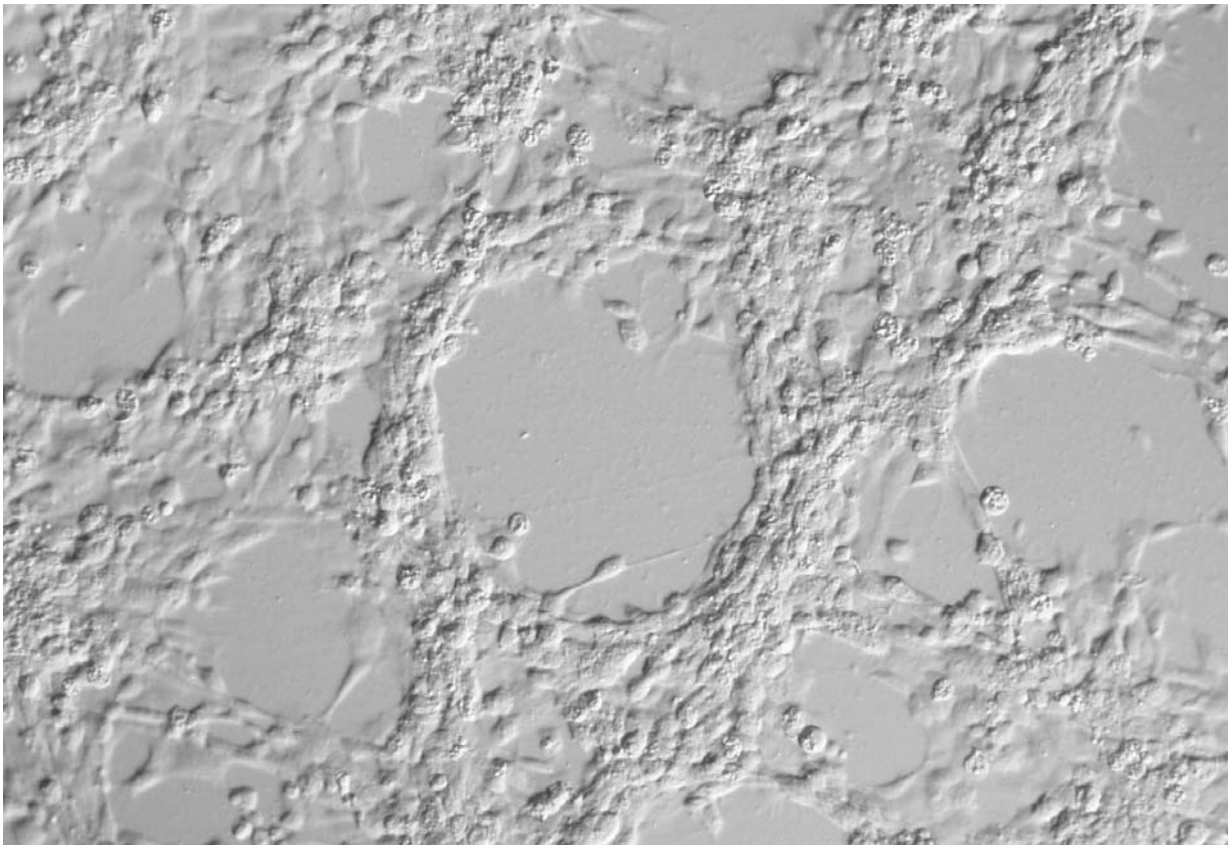


Figure 4.9. CPE typical of LMBV on FHM cells. Photo courtesy of John Grizzle, Auburn University.

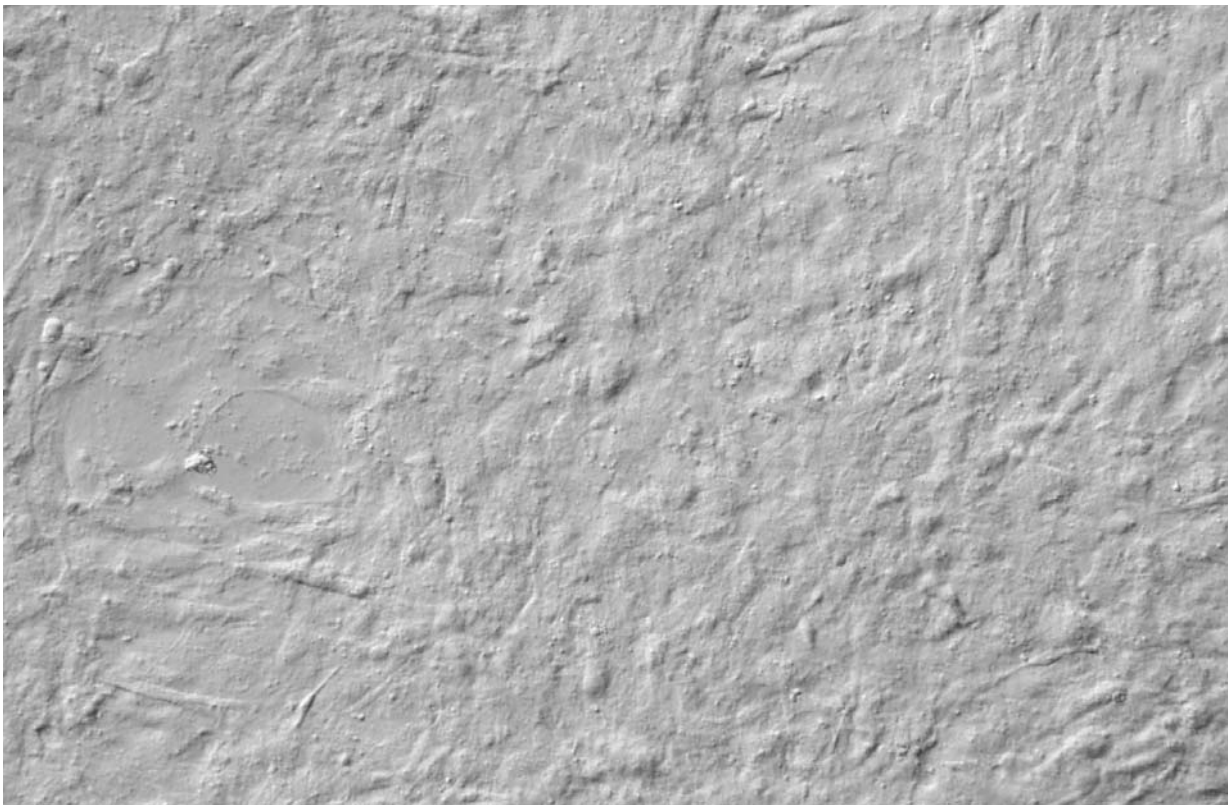


Figure 4.10. Normal BF-2 monolayer. Photo courtesy of John Grizzle, Auburn University.

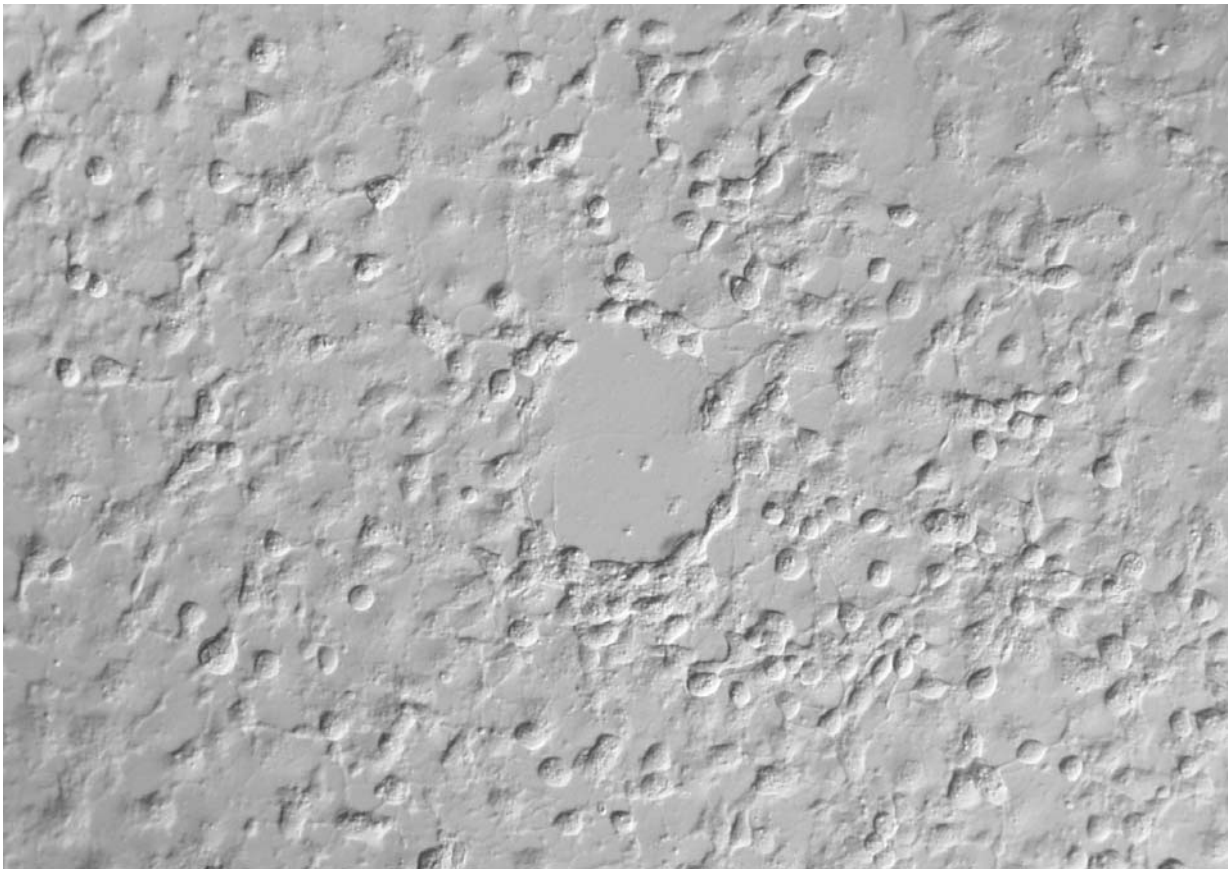


Figure 4.11. CPE typical of LMBV on BF-2 cells. Photo courtesy of John Grizzle, Auburn University.

2. Confirmation Method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb 1999b)

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.

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

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

Extract DNA follwing 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 (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 (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 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

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

iii. Using 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 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.

c. Visualization of PCR Product by Electrophoresis (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.**

d. Photograph the Gel (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

E. Oncorhynchus Masou Virus (OMV)

Oncorynchus 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.

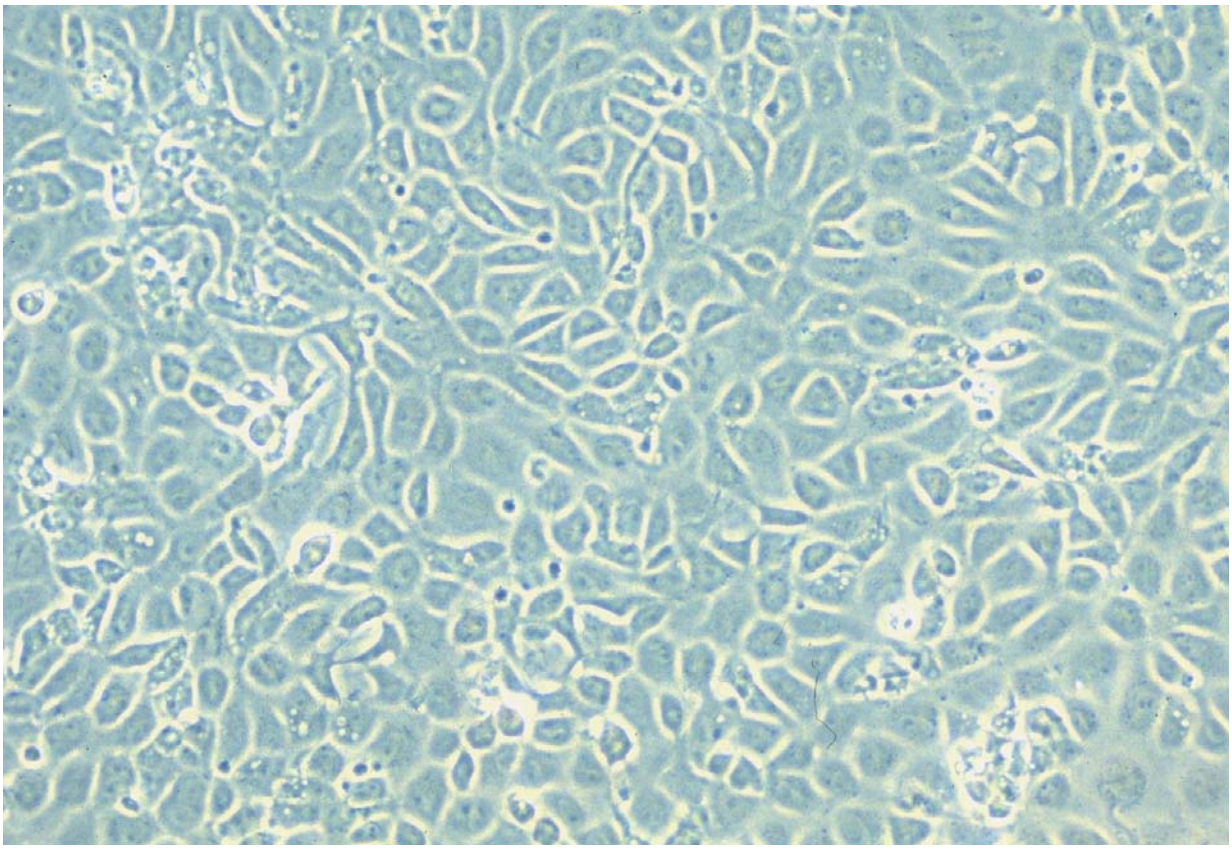


Figure 4.12. Normal CHSE-214 monolayer. Photo courtesy of Jim Winton, USGS.

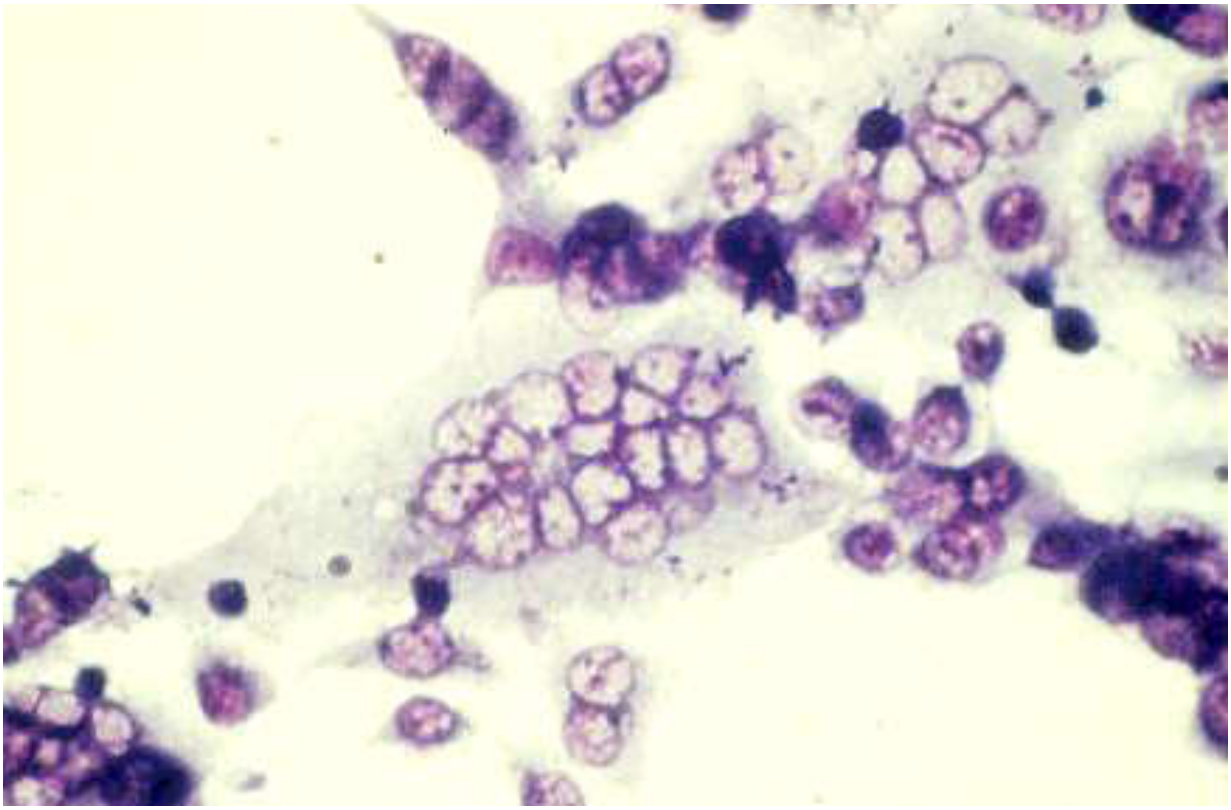


Figure 4.13. CPE typical of OMV on CHSE-214 cells. Photo courtesy of Mamoru Yoshimizu, Hokkaido University.

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 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.

a. 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.

b. Amplification of OMV DNA

i. General QA/QC Considerations (See 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

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

iii. Using 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.**

c. Visualization of PCR Product by Electrophoresis (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 (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

d. 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.

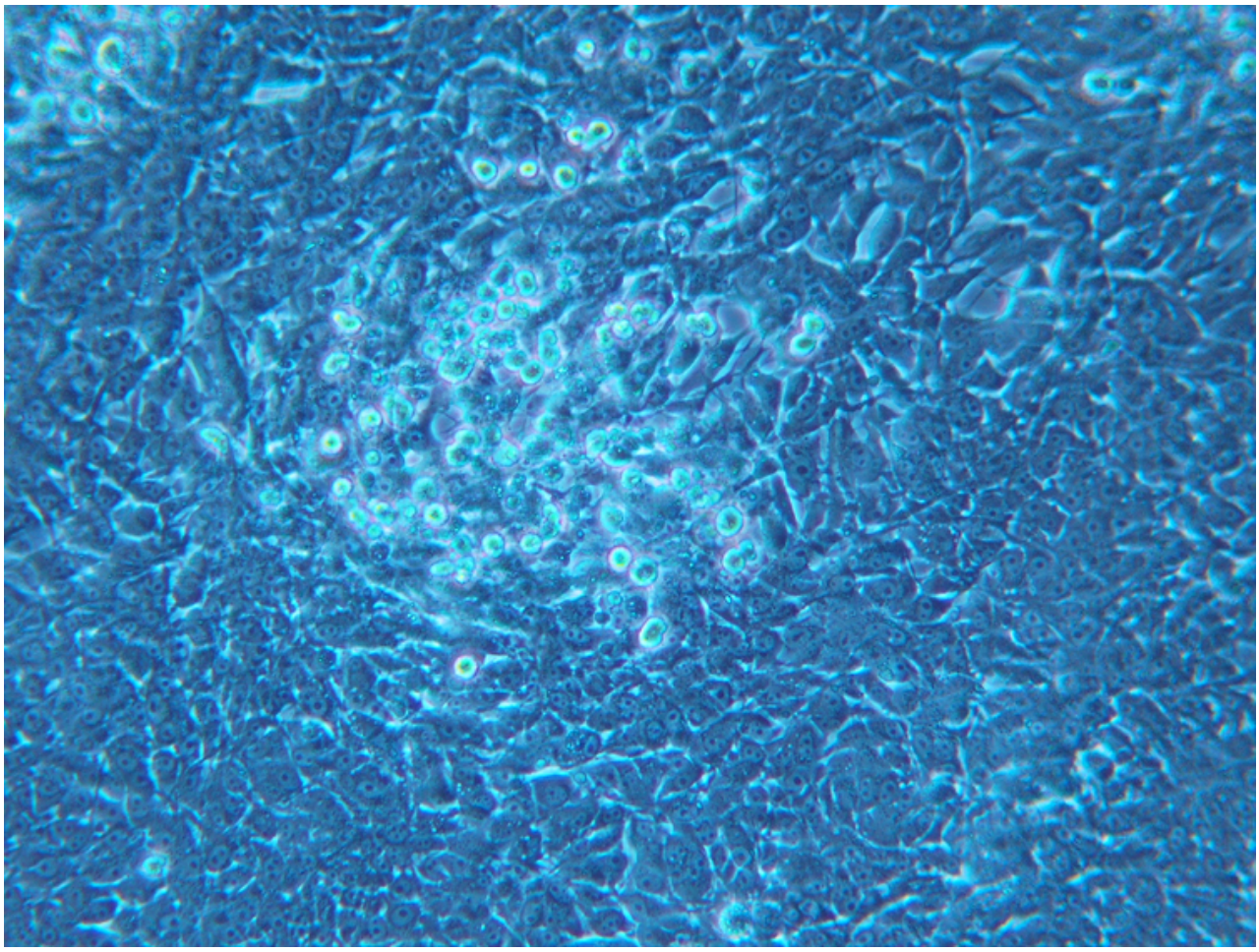


Figure 4.14. CPE plaque of SVCV on EPC monolayer. Photo courtesy of Andrew Goodwin, University of Arkansas.

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 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 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 Strømmen & 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 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.)

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

3. Using 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:

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 µl of PCRMM and add 2.5 µl of RT product for each RNA sample. Add 1 µl of each of the primers SVCV F1 & SVCV R2 (primer concentration 50 pM/µl)

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 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	Deoxyguanidine
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 in Continental Europe is mostly seen in trout, grayling, white fish, pike, and turbot and is characterized by edema and hemorrhage due to impairment of osmotic balance. Disease in North America is primarily seen in Pacific herring and pilchard but the virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning adult Coho and Chinook salmon. Fry are most susceptible to disease, which usually occurs at water temperatures between 4 to 14°C. A carrier state may develop with fish shedding virus in the feces, urine, and sexual fluids as well as being present in the internal organs. Although present in ovarian fluid, vertical transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods (OIE 2000; 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 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 serum neutralization, PCR, or IFAT methods may be used to confirm the cause of the CPE is due to the presence of VHSV.

2. **Confirmation Methods for VHSV**

   a. Serum Neutralization Method
   See 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 VHSV (Modified from Einer-Jensen 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 (with some cell scrapings) 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 95°C for two 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 First Round PCR

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

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

3. Using 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. First Round Primers for VHSV

a. Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'

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

5. Thermocycler Program for First Round VHSV

a. Pre-dwell at 50°C for 15 minutes.

b. Preheat or "Jumpstart" sample to 95°C for two minutes.

c. 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.

d. Post dwell at 72°C for seven minutes.

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

iii. "Nested" Second Round PCR for VHSV

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

2. Again use 4.A1.B.7 Worksheet B.7 – Viral Hemorrhagic Septicemia Virus (VHSV)

for the second round to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according to the number of samples and controls to be processed.

3. Second Round Primers for VHSV

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

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

4. Thermocycler Program for Second Round VHSV

a. Preheat or "Jumpstart" sample to 95°C for two minutes.

b. 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.

c. Post dwell at 72°C for seven minutes.

d. 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 "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 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.**

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

2. Photograph the Gel (6.3.G "Visualize the DNA")
Photo document all gels and attach the photo to the case history information. (4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel)

c. Indirect Fluorescent Antibody Test (IFAT) Method for the Confirmation of VHSV
See 4.8 Indirect Fluorescent Antibody Test (IFAT) Procedures for the general procedure.

i. Use an EPC cell line.

ii. Incubate virus inoculated monolayers at 15-18°C.

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 1999a).

1. **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.

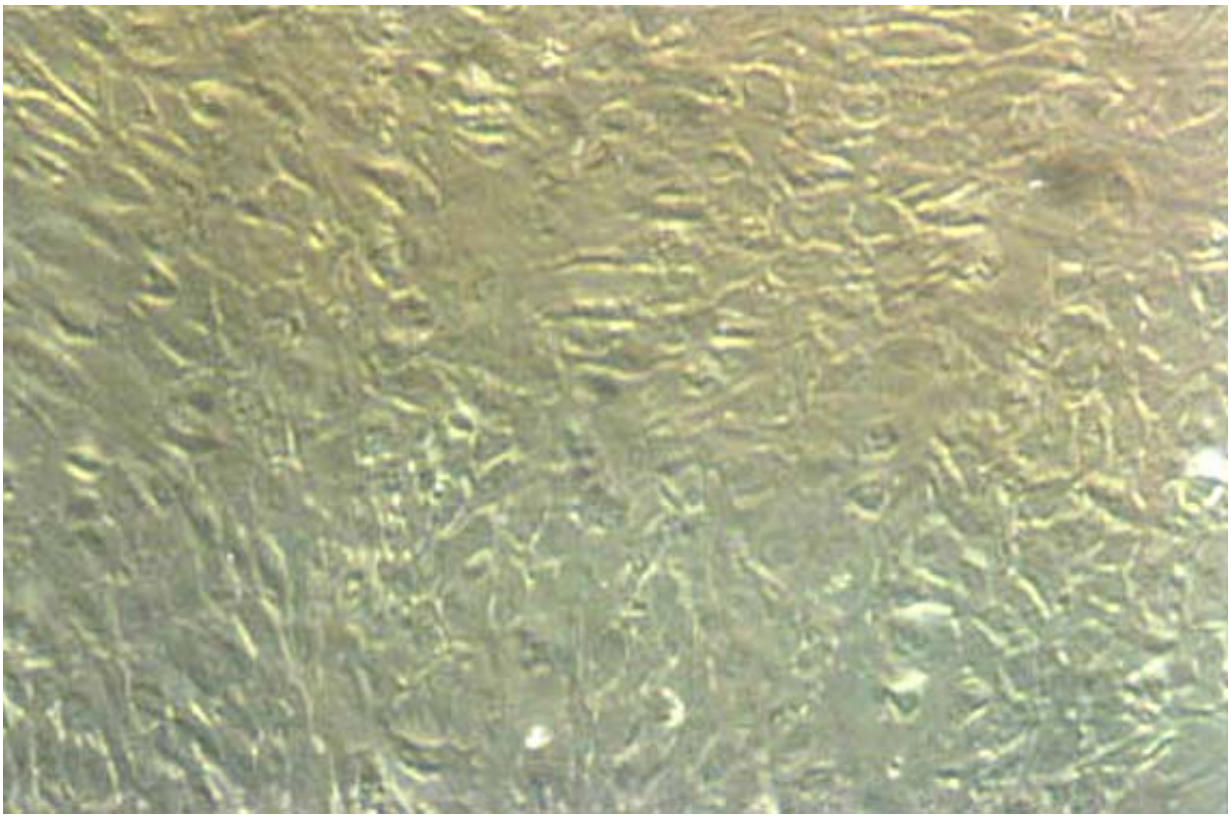


Figure 4.15. Normal WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

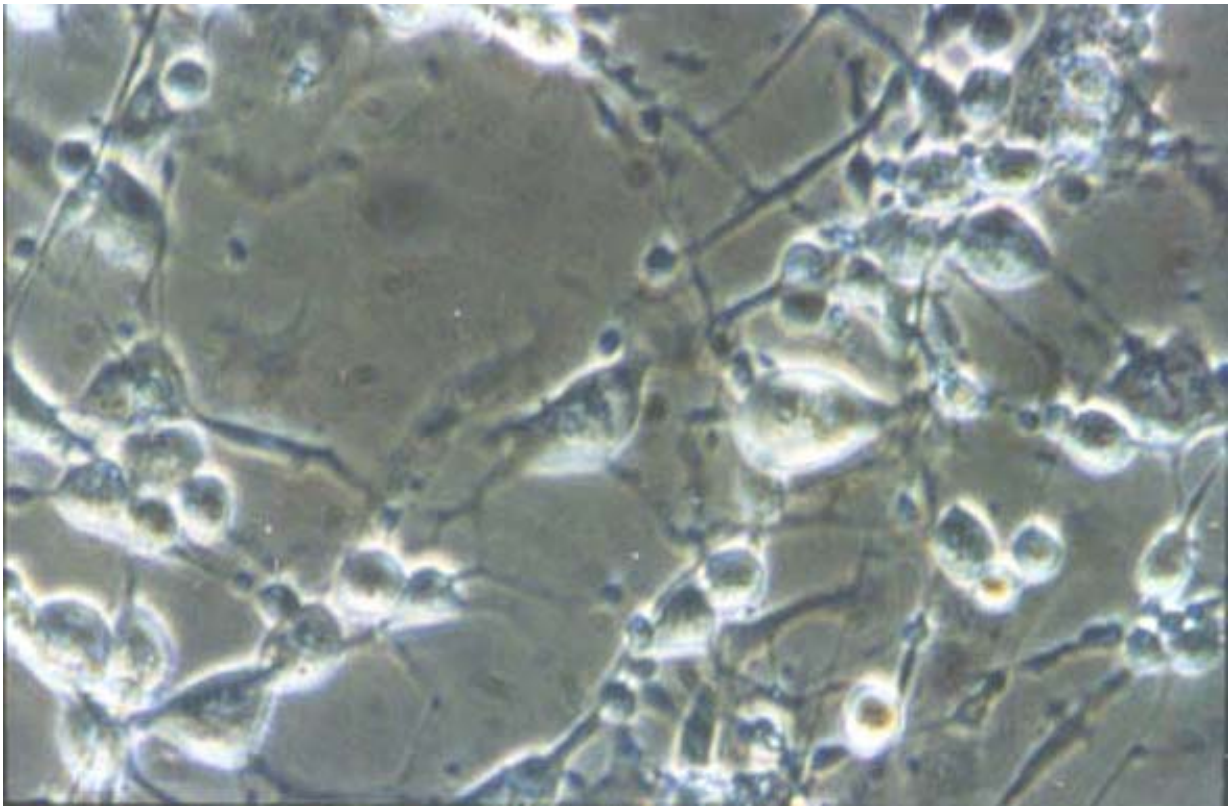


Figure 4.16. CPE typical of WSHV on WSS-2 monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

2. **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.

4.7 Serum Neutralization

Serum neutralization or plaque reduction assays are serological methods used to confirm the identity of an unknown viral isolate. The procedures involve the use of a known dilution of specific neutralizing antiserum mixed with multiple dilutions of the homologous and suspect virus and subsequent observation of the ability of those viruses to produce CPE when inoculated onto a sensitive cell line. Normal serum from the species of animal used to produce the antiserum (usually rabbit or goat) is used as the negative control to account for nonspecific inhibitors of the virus.

A. Plate Preparation

1. Seed plates (4.3.B "Seeding Procedures for Plates") with the appropriate cell line (4.2 Selection of Appropriate Cell Lines) 24 to 48 hours before inoculation with virus.

2. Monolayers should be visually healthy and at least 80% confluent at the time of inoculation.

B. Virus Sample Preparation

1. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^3 to 10^6 plaque-forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per mL of the homologous virus.

2. Dilute the suspect sample and positive control virus to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in sterile HBSS (4.9.A "Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)").

3. Combine equal volumes of each dilution of the suspect sample with the diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour with agitation at the appropriate temperature.

    a. For IHNV, VHSV, and IPNV, incubate at 15°C.

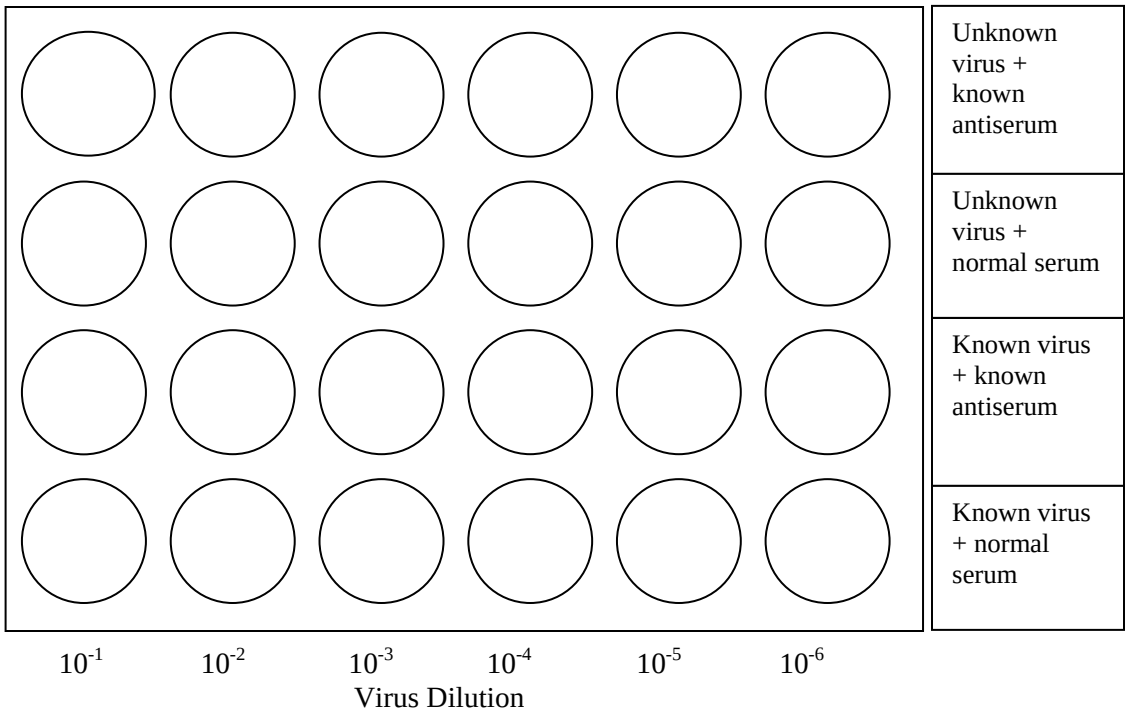
    b. For SVCV, incubate at 20 to 25°C.

4. Inoculate each of these mixtures onto the cell line in which the suspect virus was isolated as indicated in Diagram 1 below. Incubate at the above temperature for 14 days and observe plates for cytopathic effect (CPE) (see Figures 4.1 to 4.16).

    a. **Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification of the suspect virus.**

    b. Alternatively, in a sample mixed with antibody, a titer decrease of less than 2 $\log_{10}$ indicates no neutralization and the suspect sample is considered **negative** for that virus if no problems are identified with the assay.

Diagram 1. Diagram for serum neutralization assay using a 24-well plate.



C. Preparation of Reference Viruses

Where appropriate, positive controls are produced and frozen for use as needed in the serum neutralization assay.

1. Thawing of Frozen Viral Isolates

Thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.

2. Procedure for Producing Reference Viruses

a. Inoculate viral suspensions onto cell culture flasks containing visibly healthy monolayers of the appropriate cell line. This is done in a manner similar to the tissue inoculation described in 4.5.A "Plate Inoculation Procedures for Primary Culture" using an appropriate volume of inoculum for the flask size. If using a $25cm^2$ flask, 0.1 mL of viral inoculum is usually sufficient.

   i. Use EPC cell line for IHNV, SVCV, and VHSV isolates.

   ii. Use CHSE-214 cell line for IPNV isolates.

b. To allow for viral adsorption, incubate flasks at the appropriate temperature for one hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.

i. For IHNV, IPNV, and VHSV, incubate at 15°C.

ii. For SVCV, incubate at 20 to 25°C.

c. Aseptically dispense an appropriate amount of tissue culture media into the flask. For a 25 cm^2 flask this will be approximately 5 mL.

d. Incubate control sample flasks to allow replication of the viruses.

i. For IHNV, IPNV, and VHSV, incubate at 15°C until CPE occurs or for 14 days.

ii. For SVCV, incubate at 20 to 25°C until CPE occurs or for 14 days.

3. Procedure for Harvesting the Virus

a. Using aseptic technique, scrape the cell layer from the flask and triturate to break up.

b. Pour fluid and suspended cells into sterile tubes for centrifugation.

c. Centrifuge tubes at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

d. Use supernatant as positive control virus. Any fluid not needed for the assay may be aliquoted into vials and frozen at -70 C.

e. Any supernatant that is not frozen or used for the assay must be decontaminated before it is discarded.

4.8 Indirect Fluorescent Antibody (IFAT) Procedure

(Modified from LaPatra 1989)

The fluorescent antibody test (FAT) is a serological method for confirming the identity of viral isolates. The indirect FAT (IFAT) technique uses pathogen specific monoclonal anti-virus antibody (MAb) as the primary antibody (usually mouse IgM), and fluorescein isothiocyanate (FITC) conjugated antisera (usually goat or rabbit anti-mouse IgG) to the immunoglobulin used for the primary antibody. There are four basic steps for IFAT: preparing and fixing cell monolayers; infecting the cell monolayers with the virus; staining the slides with antibody reagents; reading and interpreting the slides.

A. Preparation of Materials

1. Sterilization

a. Place 12 to 18 mm diameter cover glasses in a heat resistant container and sterilize at 120 ^{o}C for 30 minutes.

b. Include dissecting forceps.

2. Dry completely in drying oven.

3. Prepare the antibody reagents according to the instructions provided by the supplier.

4. Appropriate QA/QC testing should be performed periodically on the antibodies, antisera, and cell lines to ensure accurate results are obtained during this assay.

B. Preparation of Cell Monolayers on Cover Glasses

1. Working in a clean hood, place one cover glass per well in a 24-well plate. At least four cover glasses are made for each of the unknown samples, positive control (reference) viruses, and negative control (uninoculated) cell lines. One cover glass from each of these groups is then 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. Using the cell line specified for the suspect virus, seed each well of the plate with the appropriate volume of that cell suspension as in 4.3.B “Seeding Procedures for Plates.”

3. Incubate at 20 to 25^{o}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.

C. 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 (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.

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 cover glass cell cultures.

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

a. Aspirate media from each well of the 24-well plate containing a cover glass, leaving a small amount to inhibit cell dehydration.

b. Inoculate 50 to 100 µL of supernatant prepared in "Preparation of the Suspect and Known Virus Sample" (above) from each sample onto four monolayered cover glasses in each of four labeled wells.

c. Inoculate 50 to 100 µL of reference virus onto each of four monolayered cover glasses, one of which will be used at each time period.

d. One negative (uninoculated) control monolayered cover glass is made for each cell line used, one of which will be stained and examined at each time period.

e. Absorb 1 hour without rocking at 15 to 18^{o}C, sample removal is not necessary.

f. Add 1 mL MEM-5 (Section 2, 4.9.F "MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)") or Leibovitz's L-15 (4.9.H "Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines") per well as appropriate for the cell lines being used.

3. Incubate at 15 to 18^{o}C.

D. Cover Glass Fixation and Mounting

1. Working under the hood at room temperature, aspirate media from each well to be examined at that time period (sample plus positive and negative control cover glasses). Disinfect the aspirated fluid prior to disposal.

2. Add 1 mL PBS (3.7.D.3 "Phosphate-Buffered Saline for FAT (PBS), pH 7.1") to each well and

let stand for five minutes.

3. Aspirate PBS and disinfect prior to disposal.

4. Repeat steps 2 and 3.

5. Add 1 mL cold (4^{o}C) methanol and let stand for five minutes.

6. Leave some or all of the methanol in the well. Tip the plate, without spilling fluid, and use sterile forceps to remove the cover glass from the well. Leaving some fluid in the well helps decrease the adherence of the cover glass to the bottom of the plate.

7. Allow cover glasses to air dry on a labeled absorbent surface such as lab paper.

8. If not stained immediately, store at 4^{o}C for short term (days to weeks) or at -20^{o}C for long term (weeks to months).

E. Staining and Viewing the Cover Glasses

1. Cover fixed cell cultures with 0.1 mL of mouse monoclonal anti-virus antiserum (MAb) specific for the suspect virus and incubate at room temperature for 30 minutes in a dark humidified chamber.

2. Flood the cells twice for five minutes each time with PBS.

3. Filter the goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated antibodies through a 0.45 µm membrane before use.

4. Cover fixed cell cultures with 0.1 mL of the second antibody (FITC conjugate) and incubate at room temperature for 30 minutes in a dark humidified chamber.

5. Flood the cells twice for five minutes each time with PBS.

6. Counter stain with a 0.01% solution of Evans blue (3.7.D.5.b “Evans Blue”) for one minute, rinse with PBS and air dry in the dark.

7. Place a drop of pH 9 fluorescent antibody (FA) mounting fluid (3.7.D.2 “FAT Mounting Fluid (pH 9.0)”) on the cells and place the cover glass cells down on a clean microscope slide.

8. Examine at 400 to 1000x magnification on a microscope equipped with epifluorescent illumination.

F. Results

1. Positive controls should contain focal clusters of cells exhibiting apple green cytoplasmic fluorescence (see Figure 4.17); no fluorescence should be observed in the negative controls.

2. With no problems identified in the assay and with fluorescence of the suspect sample which

appears similar to the positive control at one of the time points, the suspect sample is considered **POSITIVE** for the virus specific to those antibodies.

3. With no problems identified in the assay but without appropriate fluorescence at any of the four time periods, the suspect sample is considered **NEGATIVE** for the virus specific to those antibodies.

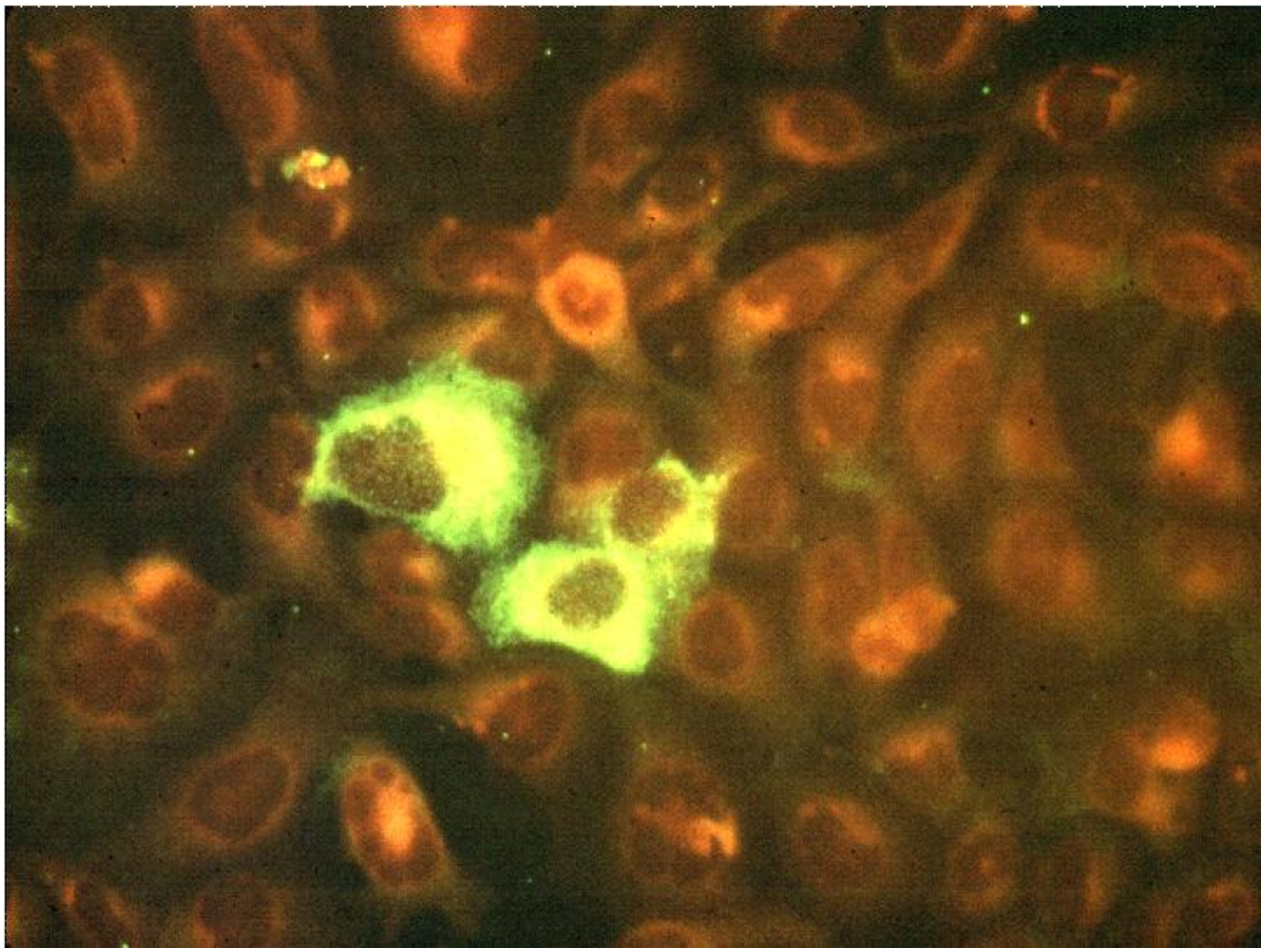


Figure 4.17. Typical fluorescence of positive IFAT for IHNV on EPC cell monolayer. Photo courtesy of Scott LaPatra, Clear Springs Foods, Inc.

4.9 Reagents and Media

Media used in tissue culture must be sterile. This may be accomplished by mixing all the ingredients and filtering with a 0.2 µm filter, or, by mixing of the stable ingredients, autoclaving, and then aseptically adding the labile ingredients such as glutamine, serum, and antibiotics.

A. Sample Dilution Medium - Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 mL
Tissue culture grade water	895.3 mL
$NaHCO_3$ (7.5%)	4.7 mL

Mix and ensure sterility.

B. Antibiotic Incubation Medium (Anti-Inc) Made with HBSS for Sample Disinfection

10X HBSS	100.0 mL
Tissue culture grade water	575.0 mL
$NaHCO_3$ (7.5%)	5.0 mL
Penicillin/streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL
250 µg/mL amphotericin B	
205 µg/mL desoxycholate	

Mix and ensure sterility. Store at 4° C. Equal volumes of sample and anti-inc are mixed together for sample disinfection.

C. Antibiotic Incubation Medium (Anti-Inc) Made with Minimum Essential Medium (MEM-0) for Sample Disinfection

10X MEM (Eagles Modified Medium)	100.0 mL
Tissue culture grade water	540.0 mL
L-Glutamine (200 mM)	10.0 mL
$NaHCO_3$ (7.5%)	30.0 mL
Tryptose phosphate broth	100.0 mL
Penicillin/streptomycin	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 µg/mL)	
Fungizone	160.0 mL

250 µg/mL amphotericin B
205 µg/mL desoxycholate

Mix and ensure sterility. This may be stored frozen for approximately three months. Avoid freeze-thaw cycles; thaw tubes immediately prior to use. Equal volumes of sample and anti-inc are mixed together for sample disinfection.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
$KHPO_4$	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Disodium versenate (EDTA)	0.2 g
Phenol red (0.5% solution)	2.0 mL
Tissue culture grade water	to 1000 mL

Autoclave and store at room temperature.

E. Trypsin-Versene (EDTA)

Trypsin (2.5% solution)	20 mL
Versene (EDTA) (1:5000)	480 mL

Store at -20° C.

F. MEM-5/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)

Tissue culture grade water	810.0 mL
Fetal bovine serum	50.0 mL
Sodium bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes buffer (1M)	15.0 mL
NaOH (1M)	5.0 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

If antimicrobials are included, use 796.0 mL of water above instead of 810.0 and add:

Gentamicin (50 mg/mL)	4.0 mL
Fungizone	10.0 mL
250 µg/mL amphotericin B	
205 µg/mL desoxycholate	

Mix and ensure sterility. Store at 4° C.

G. MEM-10/Hepes (Tissue Culture Medium for all Cell Lines Except SHK-1 and ASK)

10X MEM	100.0 mL
Tissue culture grade water	760.0 mL
Fetal bovine serum	100.0 mL
Sodium bicarbonate (7.5% solution)	10.0 mL
L-Glutamine (200 mM)	10.0 mL
Hepes buffer (1M)	15.0 mL
NaOH (1M)	5.0 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

Mix and ensure sterility. Store at 4° C.

H. Leibovitz's L-15 (Tissue Culture Medium for SHK-1 and ASK Cell Lines)

1X L-15 with 0.3g/L L-glutamine	1000.0 mL
Fetal bovine serum (5%)	50.0 mL
Gentamicin (50 mg/mL)	1.0 mL
2-mercaptoethanol (0.055 M)	0.7 mL
NaOH or HCL - as needed to adjust pH to 7.2-7.6	

Mix and ensure sterility. Store at 4° C.

4.10 Glossary

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

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

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

Controls

1. **Monolayer control -** tissue culture cells are grown in presence of tissue culture medium. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

2. **Sham control -** diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, tissue culture medium is added. If CPE appears in sham control wells, test is invalidated and must be repeated.

Cytopathic effects (CPE) - changes in the morphology and metabolism of tissue culture cells. It may be due to viral or toxic agents and the appearance may range from simple foaming of the cytoplasm or focal clumping of cells to complete destruction of the cell monolayer.

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

FITC - fluorescein isothiocyanate, a reagent which is used as an antibody label for the fluorescent antibody test.

FITC-conjugated (antibody) - describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

Fomite - an inanimate object such as a net, brush, or clothing, on which a pathogenic microorganism may be transmitted from one animal to another.

Homologous virus - as used in the viral serum neutralization procedure, it is the positive control virus of the same identity used to make the neutralizing antibody.

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

Normal serum - as used in the viral serum neutralization procedure, it is serum from the same species of animal in which the neutralizing antibody is produced. It is used as a control for any nonspecific viral inhibition that may occur even with a non-homologous virus.

Open system - a system of incubating tissue culture cells that is open to the transfer of air, i.e., a plate. Requires a medium that is buffered against rising pH due to CO_2 loss. Common buffering systems are TRIS and HEPES.

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

Plate set - a group of plates seeded from a single flask at the same time.

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

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

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

Subculture - transfer of tissue culture cells from one container to another for the purpose of forming a new monolayer.

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

Tissue culture-grade water - high quality water (low in ions, minerals, and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells. De-ionization at greater than 18 mOhms is sufficient to achieve this quality.

Titer - the number of infectious units or plaque-forming units per unit of sample, i.e. per gram or mL.

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

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

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

Trypsin - a proteolytic enzyme used to disperse cells and causes their release from the culture surface. Serum proteins neutralize it and its action is slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - ethylene di-amine tetra-acetic acid is a chelating agent involved in causing cells to release from the culture surface. Versene and trypsin are frequently used together.

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4.A1.A Worksheet A – PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
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18.			
19.			
20.			

4.A1.B Worksheet B – Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens

4.A1.B.2 Worksheet B.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 (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 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		-	-	5 µL	-

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

Primer Sets for IPNV

	Forward	**Reverse**
1st round	5'-AAAGCCATAGCCGCCCATGAAC-3'	5'- TCTCATCAGCTGGCCCAGGTAC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.B.3 Worksheet B.3 – Infectious Salmon Anemia Virus (ISAV)

Case Number_____ **Date**_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	26.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 µL	
AMV reverse transcriptase		4.5 Units/Rx	9 Units/µL	0.5 µL	
(+)Primer		50 pmoles/Rx	50 pmoles/µL	1 µL	
(-)Primer		50 pmoles/Rx	50 pmoles/µL	1 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		-	-	5 µL	-

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

Primer Sets for ISAV

	Forward	**Reverse**
1st 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
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.B.4 Worksheet B.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 (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	15.5 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		1.5 mM	25 mM	3 µL	
dNTP's		0.8 mM	10 mM	4 µL	
TMAC		40 µM	100 µM	20 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		-	-	1 µL	-

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

Primer Sets for LMBV

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

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.B.5 Worksheet B.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 50 µL)	Volume for ____samples
d-H_2O*		-	-	15.5 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		1.5 mM	25 mM	3 µL	
dNTP's		0.8 mM	10 mM	4 µL	
TMAC		40 µM	100 µM	20 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		-	-	1 µL	-

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

Primer Sets for OMV

	Forward	**Reverse**
1st 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
1st round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.B.6 Worksheet B.6 – Spring Viremia of Carp Virus (SVCV)

Case Number_____ **Date**_____

Reverse Transcription Master Mix (RTMM)

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 20 µL)	Volume for ____samples
d-H_2O[1]		-	-	10 µL	
RT buffer[2]		1X	5X	4 µL	
dNTP's		1 mM	10 mM	2µL	
M-MLV reverse transcriptase		20 Units/Rx	10 Units/µL	2 µL	
Primer SVCV R2 (below)		100 pmoles/Rx	100 pmoles/µL	1 µL	

1. Add nuclease free water to Master Mix first, M-MLV reverse transcriptase last.
2. 5X RT Buffer is 250 mM tris pH 8.3, 375 mM KCl, 50 mM DTT, 15 mM $MgCL_2$

PCR Master Mix (PCRMM)

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL)	Volume for ____samples
d-H_2O[1]		-	-	36.75 µL	
PCR buffer (no $MgCl_2$)[2]		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	10 mM	1 µL	
TAQ		1.25 Units/Rx	5 Units/µL	0.25 µL	

1. Add nuclease free water to Master Mix first, TAQ last.
2. 10X PCR Buffer is 100 mM Tris HCl pH 9.0, 500 mM KCl, 1 % Triton X-100

Primer Sets for SVCV

	Forward	**Reverse**
RT Reaction	None	5'- AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-CAN-CAY -3' (SVCV R2)
1st round	5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)	5'- AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-ACN-CAY -3' (SVCV R2)

2nd round	5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1)	5'- CTG-GGG-TTT-CCN-CCT-CAA-AGY-TGY -3' (SVCV R4)

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round PCR				
2nd round PCR				

Amplification (Thermocycle Process)

Round Number (Date & Time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of Agarose (grams)	Volume of Buffer (mL)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.B.7 Worksheet B.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 (µL) (to total 50 µL)	Volume for ____samples
d-H_2O*		-	-	23.75 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	5 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		-	-	5 µL	-

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

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µL	Volume for ____samples
d-H_2O*		-	-	27.5 µL	
PCR buffer (no $MgCl_2$)		1X	10X	5 µL	
$MgCl_2$		2.5 mM	25 mM	5 µL	
dNTP's		0.2 mM	2 mM	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	
Round 1 product		-	-	2 µL	-

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

Primer Sets for VHSV

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

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & Time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	**Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)**	**Weight of Agarose (grams)**	**Volume of Buffer (mL)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter sample ID below corresponding well number:

PCR Products (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**

4.A1.C Worksheet C – Photodocumentation of the PCR Product Gel

Case Number _____ Date _____

Samples _____

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

AFFIX PHOTODOCUMENTATION

Notes:

4.A2 Cell Enumeration

Rarely are cells counted during routine propagation of cell lines; however, the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions (True 2000). The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 mL, the cell concentration/mL is the average count per square x 10^4. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

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

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

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 minutes at 500 rpm) and re-suspended in protein-free medium or Hanks salt solution prior to counting.

A. Materials

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipet
Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
Trypsin - EDTA
Pipets 1-mL, sterile, cotton plugged
22 x 22 mm cover-slips

B. Procedure

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface as described in 4.3.A "Subculture Procedures for Flasks."

2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20 to 50 cells/mm^2 (1×10^5 to 2×10^5 cells/mL). Dilutions vary depending on age of the cells, cell density, and cell aggregation.

3. Aseptically transfer 0.5 mL of the cell suspension into a dilution tube.

   a. Add 0.5 mL Trypan Blue stain (0.1%).

      Note: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

4. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipet to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipet tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.

5. Using a microscope with a 10x ocular and a 10x objective, count 10 squares (five from each chamber) as outlined above.

6. Calculate the number of cells/mL and the total number of cells as follows:

   a. Cells/mL = x (mean) count per square x 10^4 x Trypan Blue dilution factor.

   b. Total cells in flask = cells/mL x total volume of cell suspension.

      Example: Total number cells counted in 10 squares = 300 cells
      x count/square = 300 cells/10 squares = 30 cells

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

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

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

   a. mL medium needed = (actual cells/mL) (vol. of cell suspension)/ desired cells/mL

      Example: actual count = 6 x 10^6 cells/mL
      desired count = 1 x 10^6 cells/mL
      volume of cell suspension = 8 mL

mL medium needed = x

x = mL medium needed = 6×10^6 cells/mL x 8 mL / 1×10^6 cells/mL
mL medium needed = 48×10^6 mL / 1×10^6
= 48 mL

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