# CHAPTER 11

# Virology

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## I. Introduction

Detection of aquatic animal viruses historically has been by growth and isolation on living cell cultures appropriately researched and chosen for the propagation of target viruses and species of host. Viral detection can also include immunological and nucleotide testing procedures. The determination of a testing procedure is a complex decision involving factors of cost, timeliness, sensitivity, specificity, efficiency, and available host tissues and technology.

For the purposes of the Wild Fish Health Survey, the USFWS has chosen the use of cell culture for initial screening and corroboration of test results using appropriate nucleotide primers of specific viral pathogens in polymerase chain reaction (PCR) tests. Other corroborative tests may also be utilized, including serum neutralization, indirect fluorescent antibody techniques, biotinylated DNA probes, and immuno-dot blot tests (see Chapter 12 - Corroborative Testing of Viral Isolates). The following sections describe the procedures and methods for virology using standard cell culture techniques.

**Definitions**: Several terms are used routinely in virology and throughout this section. A full Glossary of terms can be found in Appendix A.

**Media Formulations:** See Appendix B: Media Used in Tissue Culture and Virology.

## II. Selection of Appropriate Cell Lines

All viral testing will utilize cell lines traceable to cell lines from the American Type Culture Collection (ATCC) when available. At the minimum, cell lines will be tested annually for viral sensitivity and mycoplasma infection: see section VI. Quality Control in Tissue Culture, in Chapter 10 -Tissue Culture of Fish Cell Lines. Two cell lines, primary and complimentary outlined in Table 2, will be used for each sample set. Additional cell lines may be used at the discretion of the testing laboratory (Table 2).

All viral testing of salmonids and herring will be on both chinook salmon embryo (CHSE-214) and *epithelioma papillosum cyprini* (EPC).

All viral testing of ictalurids will be on fat head minnow (FHM) or brown bullhead (BB) and chinook salmon embryo (CHSE-214).

All viral testing of acipenserids will be on either white sturgeon spleen (WSS-2) or white sturgeon skin 1 (WSSK-1) and chinook salmon embryo (CHSE-214).

Viral testing for other species will be on cells selected for detection of viruses important for the specific aquatic ecosystem where samples are derived. The testing laboratory will justify the choice of cell lines following the guidelines in Tables 1 and 2.

**Table 1 - Recommended cell lines to detect target viruses in various fish families.**

| Family Group | Primary Cell Line | Incubation Temp (°C) | Complimentary Cell Line | Incubation Temp (°C) | Virus Groups[a] |
|---|---|---|---|---|---|
| Acipenseridae (Sturgeon) | WSS-2, WSSK-1 | 15-20 | CHSE-214 | 15 | WSHV |
| Polyodontidae (Paddlefish) | WSS-2, WSSK-1 | 15 | CHSE-214 | 20 | IPNV |
| Percichthyidae (Temperate Basses) | FHM | 15 | CHSE-214 | 25 | IPNV, LMBV |
| Cyprinidae (Carp/minnows) | EPC, FHM | 15 | CHSE-214 | 25 | IPNV, SVCV |
| Catostomidae (Suckers) | FHM | 15 | CHSE-214 | 25 | IPNV |
| Centrarchidae (Sunfishes) | FHM, BF-2 | 15 | CHSE-214 | 25 | IPNV, LMBV |
| Percidae (Perch) | FHM | 15 | CHSE-214 | 25 | IPNV, LMBV |
| Salmonidae (Trout / Salmon) | CHSE-214 | 15 | EPC | 15 | IPNV, IHN, VHS,OMV |
| Clupeidae (Herring) | CHSE-214 | 15 | EPC | 15 | IPNV, IHN, VHS,OMV |
| Ictaluridae (Catfish) | BB, FHM | 15 | CHSE-214 | 25 | IPNV |
| Other | FHM | 15 | CHSE-21 | 25 | IPNV |

[a] Viruses: IPNV- Infectious Pancreatic Necrosis Virus and other related aquatic birnaviruses; WSHV - White Sturgeon Herpesvirus; IHNV- Infectious Hematopoietic Necrosis Virus; VHSV - Viral Hemorrhagic Septicemia Virus; OMV - *Oncorhynchus masou* Virus; LMBV – Largemouth Bass Virus; SVCV, Spring Viremia of Carp Virus. See Table 2 for detection of specific viruses, or viruses of regional interest such as ISAV and SVCV.

**Table 2 - Recommended cell lines for detection of specific target viruses.**

| Virus | Primary Cell Line(s) | Complimentary Cell Line | Incubation Temp (°C) | Corroborative Test(s) [c] |
|---|---|---|---|---|
| IHNV | CHSE | EPC | 15 | PCR, SNA, IFAT, BDNAP |
| IPNV | CHSE | EPC | 15 | PCR, SNA |
| ISAV[a] | ASK, SHK-1 | CHSE | 15 | PCR |
| LMBV | FHM, BF-2 | CHSE | 20-25 | PCR |
| OMV | CHSE | EPC | 15 | PCR |
| SVCV[b] | EPC, FHM | CHSE | 20-25 | PCR |
| VHSV | EPC | CHSE | 15 | PCR, SNA, BDNAP |
| WSHV | WSS-2, WSSK | CHSE | 20 | PCR |

[a,b] PRI Viruses: ISAV- Infectious Salmon Anemia Virus; SVCV- Spring Viremia of Carp Virus.
[c] PCR- Polymerase Chain Reaction; SN- Serum Neutralization Assay, IFAT- Indirect Fluorescent Antibody Test; BDNAP- Biotinylated DNA Probe; (see Chapter 12).

## III. Target Tissues

For purposes of the Survey, most tissue collection will focus on organ samples from sub-adult animals. If sexually mature adults are available, the additional testing of coelomic fluid (ovarian fluid) from gravid females or seminal fluid from gravid males may also be done. Individual samples are encouraged, but samples may be pooled if necessary. No more than 5 individuals may be pooled into a single sample and samples from individuals must be approximately the same size. The following outline summarizes suggested sampling but the individual situation will dictate the best sample for viral testing.

### A. JUVENILE FISH ≤ 7.0 cm IN LENGTH

1. Fish 2.5 cm or less in length - use whole, cut off and discard any visible yolk sac
2. Fish 2.5 to 4.0 cm - cut off and discard heads and tails
3. Fish 4.0 to 7.0 cm - use viscera

### B. JUVENILE FISH > 7.0 cm IN LENGTH

1. Kidney and spleen

### C. ADULT FISH

1. Females - kidney and spleen, ovarian fluid from spawning or post-spawning fish.
2. Males - kidney and spleen, seminal fluid can be useful if organs are unavailable.

## IV. Tissue Collection Procedures

### A. WHOLE ALEVIN OR JUVENILE FISHES <7 cm IN LENGTH

1. Place 1 to 5 fish samples as described above into each Whirl-Pak® bag or sterile snap cap tube per pooled sample. Keep all samples cool during the collection procedure.

### B. TISSUE SAMPLES FROM FISH > 7 cm IN LENGTH

1. Aseptically, remove a piece of the kidney and spleen from each fish with forceps, scissors, scalpel and/or tongue depressor. For pooled samples, combine tissue samples from each fish into a single Whirl-Pak® bag or snap cap tube. The total sample for each bag or tube should be at least 0.5 g of tissue.

2. Seal Whirl-Pak® bag or tube. Keep samples cool while collecting remainder of samples.

3. Between each fish or pooled sample, clean instruments of any tissue with gauze sponges dipped in 70% ETOH, alcohol rinse, or wipe with iodophor followed by sterile water rinse.

### C. PROCEDURES FOR COLLECTING COELOMIC (OVARIAN) FLUID SAMPLES

1. Disinfect the abdomen of the fish with iodophor and wipe with a clean paper towel to remove any disinfectant or mucous which could drip into the sample.

2. Partially strip ovarian fluid from one female fish into a clean paper cup. If possible, avoid extrusion of blood, fecal material, and nematodes.

3. Crimp edge of paper cup to "strain out" any eggs present and pour 2-5 mL ovarian fluid from each fish into one tube per fish if samples are individual. Pour 1-2 mL per per fish if samples are pooled. Do not fill tube completely full. Do not palm or warm fluid, which could inactivate low levels of virus if present.

4. Tightly cap and place in tube rack. Keep all filled tubes in cooler chest on blue or wet ice while collecting remainder of samples.

**An Alternative Method** - Drawing ovarian fluid using an automatic pipettor if *in situ* contamination is a problem.

a. Install sterile tip on pipettor. Insert tip into the uro-genital opening of the fish while applying light pressure to the body.

b. Draw up a sample of ovarian fluid and place in one sterile tube per fish or pooled sample. Discard tips between samples.

### D. PROCEDURE FOR COLLECTING SEMINAL FLUID:

1. Express seminal fluid from gravid male into clean paper cup.

2. Pour 2-5 mL into tube for individual samples. Pour 1-2 mL into tube for pooled samples.

3. Tightly cap and place in tube rack.

4. Keep all filled tubes in cooler chest on blue or wet ice while collecting remainder of samples.

## V. Transport of Tissue Samples

### A. PACKING AND SHIPPING SAMPLES

1. Check seals on bags or tubes to ensure closure.

2. Place Whirl-Pak® bags into large plastic bag. Label bag with number of samples, location sample taken, sample type, date, life stage, species of fish and enclose a completed sample Submission Form (Chapter 2 – Appendix B).

3. Place tubes in proper rack, enclose in large plastic bag and label with appropriate information according to submission form.

4. Keep samples cool by transporting them in a suitable container with blue ice or bagged wet ice. Do not allow samples to freeze or expose them to UV light.

5. Check with receiving laboratory on special shipping instructions if samples are not delivered in person.

## VI. Processing Tissue, Coelomic and Seminal Fluid Samples

### A. PROCESSING TISSUE SAMPLES

All samples for viral testing should be processed within 48 hours and inoculated onto cell lines within 72 hours of collection. As during sampling and transport, care is taken to protect samples from UV light, freezing, or high temperatures that are lethal to the viruses of interest. Aseptic technique is required.

1. If transport medium is used, it is poured off and disinfected before discarding.

2. Tare balance with an empty tube or bag and weigh sample to the nearest 0.1 g.

3. Add sample dilution medium (Appendix B) to equal a 1:10 dilution (w/v). If toxicity is likely or suspected, additional tissue dilutions from 1:20 to 1:100 can be made. Final dilution prior to inoculation of tissue samples onto cell cultures must not exceed 1:100 (v/v).

4. Homogenize samples using a Stomacher® (Virtis® or Contorque® grinders require

considerable disinfection of containers between samples). Pour or pipet ~ 3-4 mL into a 12x75mm snap-cap tube.

5. Centrifuge tissue samples at 2000-3000 X g for 20 minutes.

6. Without disturbing the pellet, aseptically pipette 1 mL supernatant from each sample into its respective tube containing 1 mL of antibiotic incubation medium (Appendix B) and vortex. Sample dilution is now 1:20 v/v. Depending on tissue type, fatty material may accumulate near the surface of the tube. Be sure to collect supernatant from below the floating material. Label tubes.

7. Incubate for 2 hours at 15°C or 12-24 hours at 4°C.

8. Samples are re-centrifuged at 2000-3000 X g for 15 minutes and supernatant is inoculated onto tissue cultures as described in inoculation procedures (Section VIII).

**B. PROCESSING COELOMIC (OVARIAN) OR SEMINAL FLUID**

1. Centrifuge ovarian or seminal fluid samples at 2000x g for 20 min if using polypropylene tubes. It is optimal to process in a refrigerated centrifuge at 4°C to prevent warming of the sample.

2. Undiluted ovarian fluid may be used to inoculate cell cultures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts antibiotic incubation medium)(Appendix B) may be used. If a dilution is made, aseptically pipette supernatant without disturbing pellet from each sample into its respective tube containing antibiotic incubation medium and vortex. Label tubes.

3. Incubate for 2 hours at 15°C or 12-24 hours at 4°C.

4. Samples are re-centrifuged at 2000-3000 X g for 15 minutes and supernatant is inoculated onto tissue cultures as described in inoculation procedures (Section VIII).

## VII. Preparing Viral Test Plates

The quantal assay (also referred to as endpoint dilution) is used to examine fish when only the presence or absence of a virus needs to be verified which is the purpose of the Survey. Flat-bottomed 24 well plates are usually used for this assay but other cell culture plates may be used if applicable. For determination of the Tissue Culture Infective Dose - 50% endpoint ($TCID_{50}$) of a virus sample or isolate, replicate samples are necessary and 96 well plates become more useful. The $TCID_{50}$ assay is not routinely used in the Survey because the numbers of replicate dilutions required are often not practical. Thus, no methods will be included in this manual. Reed and Muench (1938) and Rovozzo and Burke (1973) describe the procedures for the TCID50 assay. The plaque assay is another quantification method that determines plaque forming units (PFU) or infectious particles (I.P.) of a sample. Flat-bottomed 24 well plates may be used for this, but several dilutions are necessary to accurately assess the titer of PFUs. The Survey has determined not to use this test for screening. Burke and Mulcahy (1980) describe the procedure for the plaque assay.

### A. SEEDING FLAT-BOTTOMED 24 (16mm) WELL PLATES

1. Determine number of plates needed for the assay. Remember that all samples will be inoculated onto at least two cell lines (i.e. EPC and CHSE-214). Sample inoculations will be done in duplicate on each cell line. Additionally, both monolayer and sham controls are needed, at least one set per accession number or more if several plates are used.

2. Remove confluent cell monolayer from a tissue flask using methods described in Chapter 10, Section III, Maintenance of Stock Cell Lines – Passage of Confluent Cell Monolayers.
3. Pipette 0.5 – 1.0mL cell suspension into each well of the plate(s). Mix the cell suspension frequently to keep the cells homogeneously suspended.

4. Add a few extra drops of MEM-10 to all corner wells to compensate for evaporation.

5. Any liquid spilled between wells may be aspirated off or dried by use of sterile gauze. Cover each plate with the accompanying lid.

6. Label each plate with the date, cell line initials, passage number, and operator initials. Seal lid to base with tape and place plate(s) in a plastic bag or into an airtight plastic container.

7. Incubate at a temperature appropriate for the particular cell line (Chapter 10 Table 2) until at least 80% confluent without changing the medium. Following seeding guidelines from Chapter 10 (Table 1), monolayers should be confluent within 24 hours. If necessary, plates can be made the same day as inoculation but they will have to be seeded with more cells. However, same day inoculation does not necessarily result in any earlier detection of virus.

## VIII. Inoculating Plates with Samples

### A. MATERIALS

1. Appropriate number of cell monolayers to be inoculated which are at least 80% confluent, approximately 24 hours old (do not use plates in which cells have been confluent more than 3 days), and are visually healthy. EPC cell monolayers may require thicker confluency to avoid retraction when placed at 15°C.

2. Ovarian fluid and/or tissue samples.

3. Dilution blanks containing MEM-5 with or without antibiotics (Appendix B).

4. Pipettor and sterile pipette tips.

5. Plate seal, plastic bags, or airtight plastic containers.

## B. INOCULATING CELLS WITH SAMPLES

1. Label each plate with the inoculation date and case number. The assay is usually done with the plate aligned with 6 columns across the top and 4 rows down. Label wells with sample numbers and identify controls.

2. Decant medium from wells by inverting the plates over a bleach bucket or use a pipet to aspirate off, leaving a small amount of medium on the cells to prevent drying in the center.

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

4. Inoculate negative control wells with 100µL of antibiotic incubation medium diluted to the same concentration as the test samples.

5. To allow for viral adsorption, incubate plates for 1 hour 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 LMBV, SVCV, and WSHV is 20-25°C.

6. Dispense an adequate volume of appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (Appendix B.F) works well in an open system for all cell lines listed in Table 1 except ASK and SHK-1 which respond better to Leibovitz L-15 (Appendix B.H). If using 24 well plates, 0.5 mL of medium per well is generally adequate.

7. Seal each plate with plate film, or place in an airtight container.

8. Following inoculation of monolayers, remaining tissue or ovarian fluid products are kept at 4°C until completion of all assays. Subsequent to the completion of all assays, all material is decontaminated and discarded.

## C. MINIMUM LEVELS OF DETECTION (assuming replicate wells)

1. For tissues, it is 50 infectious particles (I.P.)/g pooled sample or 250 I.P/mL/fish.

2. For ovarian fluid, not pooled, it is 10 I.P./mL

3. For ovarian fluids, pooled, it is 10 I.P./mL pooled sample or 50 I.P./mL/fish for a 5 fish pool.

## IX. Viral Plate Observation

Following inoculation of plates, all wells will be monitored on the following day and every other day for the next 2 working weeks for signs of CPE or toxicity. Plates will be monitored twice during the following week. Total observation period will be 3 weeks (21 days).
If no CPE, toxicity or abnormalities are observed in the 21 days, the samples are discarded and recorded as negative.

**A. Re-Inoculation** - If toxicity, abnormal pH or CPE is observed, one of the replicate wells of that sample will be aseptically aspirated, diluted 1:10 with MEM-0, filtered through a 0.45μm filter and re-inoculated onto another 24-well test plate in duplicate and monitored for an additional 14 days for CPE. All observations will be documented and recorded by the observer and kept on file with the laboratory records. If no CPE is observed in 14 days after re-inoculation, the sample is discarded and recorded as negative.

**B. Cytopathic Effects (CPE) of Virus Infection in Tissue Culture Cells**
In addition to the descriptions of CPE given here, CPE descriptions including photographs can be found in Standard Procedures for Aquatic Animal Health Inspections (USFWS and AFS-FHS 2003).

**IHNV-induced CPE**

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

**IPNV-induced CPE**

1. Spindle-shaped or "balloon-on-a-stick"-shaped cells.
2. Pyknosis of nuclei (nuclei shrink in size and chromatin condenses).
3. Plaques are stellate in a confluent cell monolayer and contain not only live cells but also normal looking cells (these are persistently infected and will reform a normal monolayer that is virus positive).
4. Little cellular debris.

**Herpesvirus-induced CPE**

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

**VHSV-induced CPE**

1. The North American VHSV isolates plaque very similarly to IHNV in EPC cells forming rounded and granular cells in grape-like clusters.
2. The European VHSV isolates differ from IHNV on RTG-2 cells by having more regular

plaque margins with uniformly distributed granular debris within the plaques. Also, affected cells do not show margination of chromatin.

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

**LMBV-induced CPE**

1. CPE within 48 hours after inoculation
2. Initial CPE - few pyknotic cells, which develop to form circular, cell free areas, with rounded cells at the margins.
3. Advanced CPE - Pyknosis, rounding and detached cell sheet. Entire cell sheet affected.

| Virus | Days | |
|---|---|---|
| IHNV, VHSV | 2-10 | If blindpassages are needed, do at 10-14 days and examine for an additional 14 days. |
| IPNV | 2-10 | " " |
| LMBV | 2-10 | " " |
| Herpesvirus | 14-30 | Frequently does not appear on the initial culture and must be blind passed at 28 days. Examine for an additional 28 days. |

**Note:** Toxicity can sometimes mimic viral CPE. Observing the gradual development of plaques over several days is the best way to distinguish viral CPE from toxicity.

**C. Intensity of CPE**

Monolayers are examined with an inverted light microscope at low power (125X) to determine intensity of CPE. This general scoring scheme is used to record CPE intensity:

+1 = Only one field observed contains CPE
+2 = Two or more fields observed contain CPE
+3 = All fields observed contain CPE
+4 = CPE throughout entire monolayer or monolayer no longer attached to flask/plate. (Note: Separation or retraction of cell monolayer from flask/plate edge can be due to toxicity rather than viral CPE.)

**D. Corroborative Testing**

Appropriate PCR or immunological methods for corroboration and identification of virus will test CPE that is observed after re-inoculation. See Chapter 12 - Corroborative Testing of Viral Isolates, for specific protocols.

# X. Storing, Freezing and Thawing Viral Isolates

## A. PREPARATION OF VIRUS ISOLATES FOR FREEZING

1. Virus samples - suspected virus isolates from all fish species are frozen after completion of viral assays. At least 2 viral isolates (if 2 or more samples produce CPE) are frozen per date, location and species, preferably from wells having 4+ CPE.

2. Aseptically pipette 1.5-2 mL of tissue culture fluid and cell debris from the wells representing each isolate into four freezer vials. Seal tightly and label.

## B. FREEZING VIRUS ISOLATES

1. Freeze vials at -80°C. Virus should not be frozen in the liquid nitrogen dewer that contains the stock cell lines unless a herpesvirus is strongly suspected (i.e., the virus in whole cells could be more easily lost at -80°C).

2. Label each freezer vial per isolate with the case number, isolate number, number of passages through which cell line, fish stock and species, original sample type (ovarian fluid or tissue sample) and date frozen.

3. Log each isolate in the freezer notebook.

## C. THAWING VIRUS SAMPLES

1. One vial of the virus should be thawed and tested for viability before freezing all samples if the identity and stability of the isolate is unknown.*

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

3. Decant MEM-10 from the required number of 1 to 2-day-old monolayers in 25-$cm^2$ flasks. Pipet 0.1-mL virus sample onto each cell monolayer.

4. Allow virus to adsorb for 30 minutes at 15°C.

5. Add 5 mL MEM-10 to each flask and incubate at the appropriate temperature until all cells lift off each flask (4+ CPE).

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

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## Appendix 11.A - Glossary of Terms used in Tissue Culture & Virology

**BCS -** bovine calf serum taken from newborn calves.

**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 flask.

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

**Controls**

A. Monolayer control: tissue culture cells are grown in presence of growth medium MEM-10. If CPE appears in monolayer control wells, test is invalidated and must be repeated.
B. Sham control: diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, an overlay medium or MEM-10 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 due to suspected viral infection.

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

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

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

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

**Overlay -** a medium used in the plaque assay that is placed over a virus-inoculated cell monolayer to prevent physical spreading of viral particles except by cell-to-cell release of infectious particles. The overlay contains a semisolid medium such as methylcellulose or gum tragacanth.

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

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

**Serum neutralization -** antibody molecules in the antiserum neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the 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 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.

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

**Tissue Culture-Grade Water -** High quality water (low in ions, minerals and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells.

**Titer -** the number of infectious units or plaque-forming units (pfu) 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 (1 day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

**NOTE:** Inoculation of very high-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 5-7 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 that binds divalent cations active in forming cell cement (hyaluronic acid) causing cells to round and release from the culture surface.

## Appendix 11.B – Reagents and Media Used in Cell Culture and Virology

All chemicals should be reagent or tissue culture grade. Use only glassware which is dedicated to tissue culture, is new, or has been acid washed. Media used in tissue culture must be sterile. This may be accomplished by mixing all the ingredients and filtering with a 0.2µm filter, or, by mixing of the stable ingredients, autoclaving, and then aseptically adding the labile ingredients such as L-glutamine, serum, and antibiotics.

### A. Sample dilution medium – made with 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 | |
| NaOH or HCL | as needed to adjust pH to 7.2-7.6 |

Mix and ensure sterility. Store at 4° C.

### 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[a] | 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

| | |
|---|---|
| NaOH or HCL | as needed to adjust pH to 7.2-7.6 |

[a] optional, if not used, increase volume of TCG-water to 640.0 mL.

Mix and ensure sterility. This may be stored frozen for approximately 3 months. Avoid freeze-thaw cycles, thaw tubes immediately prior to use.

## 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 temp.

## 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 ASK and SHK-1)

| | |
|---|---|
| 10X MEM | 100.0 mL |
| Tissue Culture Grade Water | 815.0 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 or HCL | as needed to adjust pH to 7.2-7.6 |

If antimicrobials are included, use 801.0 mL of water above instead of 815.0 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 ASK and SHK-1)

| | |
|---|---|
| 10X MEM | 100.0 mL |
| Tissue Culture Grade Water | 765.0 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 or HCL | as needed to adjust pH to 7.2-7.6 |

Mix and ensure sterility. Store at 4° C.

## H. Leibovitz L-15 (enhanced growth formula[a])

**Media Components**:

| | | |
|---|---|---|
| Leibovitz L-15 Powder | BIO-Whittaker | 12-700F |
| L-Glutamine 200 mM | BIO-Whittaker | 17-605E |
| Gentamicin 50 mg/mL | BIO-Whittaker | 17-518Z |
| Foetal Calf Serum (FCS, Australian) | BIO-Whittaker | 14-506F |
| 2-mercaptoethanol (2-ME) 50 mM | GIBCO BRL | 31350-01D |

**Composition of Medium:**

| | |
|---|---|
| Leibovitz L-15 (reconstituted according to manufacture) | 500.0 mL |
| L-Glutamine (8.0 mM final concentration) | 20.0 mL |
| Gentamicin (final Conc. – 50 µg/mL) | 0.5 mL |
| FCS (final conc. – 15% v/v) | 75.0 mL |
| 2-ME (40 µM final concentration) | 0.4 mL |
| NaOH or HCL | as needed to adjust pH to 7.2-7.6 |

[a] ASK and SHK-1 cell lines typically experience slow growth. Growth performance has been shown to improve when Leibovitz L-15 medium is formulated with FCS from Australia and higher concentrations of L-glutamine (McAllister, P.E. 2003, Pers. comm.).

### Leibovitz's L-15 (Standard Formula)

| | |
|---|---|
| 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.

## I. Methyl cellulose (base for Overlay)

| | | |
|---|---|---|
| Methyl cellulose (4000 centipoises) | 8 | g |
| Tissue Culture Grade Water | 555 | mL |

1. Heat 225 mL d-$H_2O$ in a 1 L bottle with a stir bar to a near boil.
2. Add methyl cellulose and mix by swirling.
3. Mix on a stir plate and add 330 mL cold d-$H_2O$ washing down sides; stir until cool.
4. Stir at 4°C overnight.
5. Autoclave for 15 minutes at 121°C; will form an opaque solid.
6. Cool to room temperature and stir at 4°C until soluble; store at 4 °C

## J. Methyl cellulose Overlay

| | | |
|---|---|---|
| 10X MEM | 100 | mL |
| Tissue Culture Grade Water | 300 | mL |
| Fetal Bovine Serum | 50 | mL |
| L-glutamine (200 mM) | 10 | mL |
| Hepes buffer (1 M) | 15 | mL |
| $NaHCO_3$ (7.5%) | 10 | mL |
| NaOH (1 M) | 5 | mL |
| Fungizone (250ug/mL) | 10 | mL |
| Gentamicin (50mg/mL) | 4 | mL |
| Methyl cellulose base | 496 | mL |

Aseptically mix and store at 4° C.