FRED Reports

LABORATORY TECHNIQUES IN VIROLOGY

by

Marie P. Fried

Number 29

Alaska Department of Fish & Game
Division of Fisheries Rehabilitation,
Enhancement and Development

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GLOSSARY

I. Centrifuging of samples

A. Centrifuge at full speed to attain 2000 g for the following centrifuges:

Clinical centrifuge	12 tube head	4100 rpm	2120 g
HN-S centrifuge	24 tube head	3700 rpm	2000 g
HN-S centrifuge	12 tube head	4000 rpm	2000 g

B. Turn centrifuge on and set timer as instructed in procedure. Turn centrifuge off upon completion of time. Tubes must be balanced when centrifuging at 2000 g.

II. Cleaning glassware

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

III. Controls

A. Monolayer control: cells are grown in presence of growth medium (MEM-10 for closed systems, and MEM-10-TRIS for open systems). If CPE appears in monolayer control wells, test is invalidated and must be repeated.

B. Mock control (negative control): Cells are inoculated with diluent (MEM-0-TRIS). When using plaque assay, MEM-0-TRIS is allowed to adsorb onto cells for 1 h. Following adsorption, 1 x Methylcellulose (overlay) ia pipetted into each mock control well. If CPE appears in mock control wells, test is invalidated and must be repeated with different MEM-0-TRIS or overlay.

IV. Dilutions (making dilutions with dilution blanks)

Discard pipet tip into bleach bucket after every dilution. Mix contents of each dilution blank with Vortex mixer. Do not use MLA pipettor to mix contents of dilution blank. Blue MLA pipettor is calibrated to

deliver 0.1 ml (100 lambda) and orange MLA pipettor is calibrated to deliver 0.01 ml (10 lambda).

When using 100 lambda MLA pipettor to make 10^{-1} dilutions, pipet 0.1 ml sample into 0.9 ml MEM-0-TRIS dilution blank. When using 10 lambda MLA pipettor to make 10^{-2} dilutions, pipet 0.01 ml sample into 1 ml MEM-0-TRIS dilution blank.

V. Dilutions listed in virus assays are suggested dilutions. Actual dilutions used for an assay will depend on sample type (routine survey or pre-release inspection vs. suspected epizootic), past history of sample location, and fish species.

VI. Drying flat bottom 96 multi-well plates

Sterile Kim-Wipes, laid on plate, are used to dry plate surface. Used tissues are lifted straight up off plate and placed in autoclave bag. If ethanol gauze/swab is used on plate surface, do not allow ethanol or fumes to get into wells. Ethanol is toxic to tissue culture cells

VII. Medium volume needed per flask to grow tissue culture cells

Medium volume	Flask size
10 ml	25 cm^2
30 ml	75 cm^2
60 ml	150 cm^2

VIII. Replicates

Replicates of each dilution per sample are used throughout research experiments. Number of replicates will depend on statistical analyses to be performed on experimental results.

When using replicates, make 1 set of dilutions per sample as instructed in procedure. Plate replicates from each dilution blank. Do not make replicate dilution blanks.

IX. Serum neutralization

A. Antibody molecules in serum (antiserum) neutralize the antigenic receptor sites on the virion, preventing virion attachment to and subsequent penetration of host tissue culture cells. Neutralization of viruses by antibodies is usually specific.

Serum neutralization assay is performed by mixing undiluted or slightly diluted antiserum with dilutions of virus (antigen). Sufficient dilutions of virus are needed to ensure a positive result (no CPE observed) that might otherwise be missed if antigen is too concentrated.

B. Test antiserum to find lowest dilution not toxic to tissue culture cells using following dilutions: undiluted, 1:10, 1:50, and 1:100. Label tube with appropriate dilution for subsequent serum neutralization assays and freeze at -80°C.

X. Tissue culture grade water and reagents

It is important to use tissue culture grade water and reagents for growing tissue culture cells. Non-tissue culture grade water and reagents may be toxic to tissue culture cells.

XI. Trypsin is a proteolytic enzyme used to disaggregate cells. It is neutralized by serum proteins. When using trypsin to disaggregate cells, decant trypsin prior to trituration of cell suspension. Cell suspension is triturated in presence of growth medium (MEM-10 for closed systems, and MEM-10-TRIS for open systems).

XII. Versene is a chelating agent which binds divalent cations causing cells to round and release from flask surface.

MAINTENANCE OF STOCK CELL LINES: PASSAGE OF CONFLUENT CELL MONOLAYER

I. Aseptically decant medium into beaker containing diluted bleach.

II. Rinse cell monolayer with 3-10 ml versene-PBS, depending on flask size, then decant versene-PBS.

III. Add 3-10 ml versene-trypsin or versene-PBS to flask using pipettor. Leave on cells until cells appear rounded when examined with an inverted light microscope. Cell monolayer will become milky colored. Immediately decant versene-trypsin or versene-PBS.

IV. Dislodge cells by striking flask on palm of hand.

V. Add 5-15 ml MEM-10 to flask and triturate cell suspension (20+ times) with pipettor until cells are in groups of one's, two's, or three's when examined with an inverted light microscope.

VI. Label flask(s) with date, cell line initials, passage number, and operator initials as follows:

EPC_{86}
10-17-83 MPF

EPC are the cell line initials, 86 is the passage number, 10-17-83 is the passage date, and MPF are the operator's initials.

VII. Dilute cells as follows:

A. Method I

Add MEM-10 to flask to make appropriate dilution. When diluting 75 cm^2 flask of EPC cells 1:4 (1 flask into 4 flasks) 120 ml MEM-10 need to be added to the flask. Decant or pipet similar aliquots of cell suspension into pre-labeled flask(s).

B. Method II

Following trituration, pipet similar aliquots of cell suspension into pre-labeled flask(s). Add appropriate aliquot of MEM-10 to each flask.

VIII. Incubate cells and allow to grow and form cell monolayer without changing medium.

INCUBATING CELL LINES

I. Non-infected cells

Cell Line	Optimum Growth Temperature	Growth Temperature Range
EPC	23-27°C	N/A
CHSE 214	16-23°C	12-23°C
FHM	25-30°C	14-34°C
RTG 2	15-20°C	4-26°C

Incubate all cell lines at 4°C for long term maintenance.

II. Virus-infected cells

Virus	Optimum Growth Temperature	Growth Temperature Range
IHNV	13-18°C	4-20°C
IPNV	20°C	4-26°C

CYTOPATHIC EFFECT OF VIRUS INFECTION ON TISSUE CULTURE CELLS

Tissue culture cells have the ability to support the growth of viruses. Presence of virus is revealed by changes in the cells morphology and metabolism. Effect of virus infection on tissue culture cells is termed cytopathic effect (CPE).

I. IHNV induced CPE

A. Rounded and granular cells in grape-like clusters;

B. Margination of nuclear chromatin (Optical density of nucleoli increases and nuclear membranes appear thickened when examined with inverted phase light microscope.);

C. Plaques form in confluent cell monolayer. Infected cells accumulate at plaque margins.

II. IPNV induced CPE

A. Spindle-shaped cells;

B. Pyknosis of nuclei (Nuclei shrink in size and chromatin condense);

C. Plaques form in confluent cell monolayer. There will often be normal looking cells within a plaque.

III. Intensity of CPE

Confluent cell monolayers are examined with an inverted light microscope to determine intensity of CPE.

A. +/- Questionable CPE;

B. +1 Only one field observed contains CPE;

C. +2 Two or more fields observed contain CPE;

D. +3 All fields observed contain CPE;

E. +4 Cell monolayer is no longer attached to flask/plate. (It is possible for cell monolayer to separate naturally from edge of flask/plate. This is not CPE.)

IV. Cytopathic effect is observed in tissue culture cells 2-7 days following infection with IHNV, and 1-3 days following infection with IPNV.

ASSAYS USED IN VIROLOGY

Traditional assay is used in virology to isolate fish viruses. This assay is used to examine fish during suspected virus epizootics, fish from routine surveys, pre-release inspections, and new sample locations. Up to sixty fish samples are collected. Samples are pooled into five fish pools, except when examining fish from new sample locations. Ovarian fluid and/or tissue samples are collected. Ovarian fluid samples are preferred when examining adult fish for IHNV. Ninety-six well plates are used for traditional assay.

Plaque and $TCID_{50}$ assays are used in virology to titer fish viruses. Plaque assay is the preferred titration assay used in fish virology. This assay is used throughout all fish virus research experiments. Ovarian fluid and tissue samples are collected. Twenty-four well plates are used for plaque assay.

Serum neutralization assay is used in virology to identify fish viruses. This assay is used to identify viruses isolated in fish during suspected virus epizootics, and viruses isolated in fish species other than *O. nerka*.

COLLECTING OVARIAN FLUID, WHOLE FISH, AND TISSUE SAMPLES IN THE FIELD

I. Ovarian fluid samples

A. Field equipment

1. Ice chest and ice
2. Seventy 15 ml sterile screw cap plastic centrifuge tubes
3. Seventy clean paper cups
4. Two large plastic bags and tie labels
5. Two pairs of spawning gloves

B. Procedure for collecting samples

1. Partially strip ovarian fluid from one female fish into a clean paper cup. Avoid extrusion of blood, eggs, fecal material, and nematodes if possible.

2. Crimp edge of paper cup and pour 3-5 ml ovarian fluid sample from each fish into 1 sterile centrifuge tube per fish.

3. Tightly cap each tube and place in styrofoam rack. Retighten each cap prior to transport.

4. Place rack with centrifuge tubes in plastic bag. Label bag with number of samples, sample location, sample type, date, and accession number if available.

5. Transport samples in vertical position on ice in chest.

C. Storing samples in laboratory

1. Store samples at 4°C if samples will be processed within 4 days of collection.

2. Store samples at -80°C if samples will not be processed within 4 days of collection.

II. Whole fish and tissue samples

A. Field equipment

1. Whole fish - alevin or juvenile fish 10 cm or less in length

a. Ice chest and ice
b. Seventy Whirl Pac bags
c. Two large plastic bags and tie labels
d. Two pairs of spawning gloves

2. Tissue samples - fish greater than 10 cm in length

a. Ice chest and ice

b. Seventy 15 ml sterile screw cap plastic centrifuge tubes each containing 9 ml MEM-0-TRIS

c. Two large plastic bags and tie labels

d. Scissors

e. Seventy sterile tongue depressors

f. Scalpel and blades

g. Forceps

h. Seventy percent ethanol

i. Torch and striker or matches

j. Two pairs of spawning gloves

B. Procedure for collecting samples

1. Whole fish - alevin or juvenile fish 10 cm or less in length

a. Place each fish into 1 Whirl Pac bag per fish.

b. Place all Whirl Pac bags in plastic bag. Label bag with number of samples, sample location, sample type, date, and accession number if available.

c. Transport samples on ice in chest.

2. Tissue samples - fish greater than 10 cm in length

a. Aseptically remove from each fish a piece of spleen, anterior kidney, and liver with forceps, scissors, and tongue depressor. Combine tissue samples from each fish into 1 cm^3 tissue sample per fish.

b. Place tissue sample from each fish into 1 centrifuge tube containing MEM-0-TRIS per fish. [One gram (1 ml) of tissue and 9 ml MEM-0-TRIS is a 10^{-1} dilution of tissue to MEM-0-TRIS. Centrifuge tubes are marked from 1-15 ml on side of tube. Weight of tissue is approximated by observing number of ml tissue displaces on side of tube.]

c. Dip forceps and scissors in 70% ethanol and flame between each fish.

d. Tightly cap centrifuge tube and place in styrofoam rack. Retighten all caps prior to transport.

e. Place rack with centrifuge tubes in plastic bag.

Label bag with number of samples, sample location, sample type, date, and accession number if available.

f. Transport samples in vertical position on ice in chest.

C. Storing samples in laboratory

1. Store samples at 4°C if samples will be processed within 4 days of collection.

2. Store samples at -80°C if samples will not be processed within 4 days of collection.

PROCESSING OVARIAN FLUID, WHOLE FISH, AND TISSUE SAMPLES FOR TRADITIONAL ASSAY

I. Preparing antibiotic cocktail

A. Aseptically pipet 0.02 ml gentamycin (50 mg/ml) and 0.2 ml fungizone (250 mg/ml) into sterile 5 ml test tube with snap cap. Twelve tubes of antibiotic cocktail are needed for twelve-5 pooled samples (60 fish).

B. Label tubes and freeze at -20°C in vertical position.

II. Processing ovarian fluid samples

A. Aseptically pipet 1 ml from each of 5 ovarian fluid samples into 1 sterile 15 ml centrifuge tube.

B. Centrifuge pooled ovarian fluid samples at 2000 g for 20 min.

C. Aseptically pipet 1.8 ml from each pooled ovarian fluid sample into 1 tube containing frozen antibiotic cocktail per pooled ovarian sample. Label tubes.

D. Incubate at 23-25°C (room temperature) for 2-6 h or at 4°C overnight. Pooled ovarian fluid samples (diluted 10^0) are ready for inoculation onto tissue culture cells for virus assay.

III. Processing tissue samples

A. Homogenize samples using Virtis or Contorque grinder.

B. Aseptically pipet 1 ml from each of 5 tissue samples into 1 sterile 15 ml centrifuge tube.

C. Centrifuge pooled tissue samples at 2000 g for 20 min.

D. Without disturbing pellet, aseptically pipet 1.8 ml from each pooled tissue sample into 1 tube containing frozen antibiotic cocktail per pooled tissue sample. Label tubes.

E. Incubate at 23-25°C (room temperature) for 2-6 h or at 4°C overnight. Pooled tissue samples (diluted 10^{-1}) are ready for inoculation onto tissue culture cells for virus assay.

IV. Processing alevin or juvenile fish

A. Alevin and juvenile fish up to 4 cm in length are processed whole. Aseptically remove spleen, anterior kidney, and liver from fish

greater than 4 cm in length.

B. Weigh 1 g samples, if possible, on flamed aluminum foil. Place tissue sample from each fish into one 15 ml sterile centrifuge tube per fish. (Five alevin fish may be pooled into one 15 ml sterile centrifuge tube prior to homogenization).

C. Pipet MEM-0-TRIS into tube to make a 10^{-1} dilution of tissue to MEM-0-TRIS. (i.e. One g of tissue and 9 ml MEM-0-TRIS is a 10^{-1} dilution of tissue to MEM-0-TRIS.)

D. Continue processing using method described in "Processing ovarian fluid, whole fish, and tissue samples for traditional assay: processing tissue samples".

TRADITIONAL ASSAY

I. Seeding flat bottom 96 multi-well plates

A. Determine number of plates needed for assay. Twelve-5 pooled samples (60 fish) can be assayed using 1 plate. Six wells are needed for each pooled sample along with 2 control wells.

B. Remove confluent cell monolayer using method described in "Maintenance of stock cell lines: passage of confluent cell monolayer". One confluent cell monolayer in a 25 cm^2 flask can make 3 plates if 30 ml MEM-10-TRIS are added to flask.

C. Pipet 0.1 ml cell suspension into each well per plate.

D. Sterile Kim-Wipes are used to dry plate surface. Cover each plate with lid or seal plate with adhesive mylar sheet.

E. Label each plate with date, cell line initials, passage number, and operator initials. Place plate(s) in plastic bag on top of moistened paper towel and seal bag with tape.

F. Incubate and allow to grow and form a 90% cell monolayer without changing medium.

II. Inoculating cells with ovarian fluid and tissue samples

A. Materials needed for assay

1. Appropriate number of 96-well plates with 90% cell monolayer (Do not use plates in which cells have been monolayered more than 4 days. Twelve-5 pooled samples can be assayed using 1 plate);

2. Pooled ovarian fluid and/or tissue samples in antibiotic cocktail;

3. MEM-0-TRIS dilution blanks (0.9 ml dilution blanks);

4. MEM-10-TRIS;

5. One hundred lambda MLA pipettor;

6. Plastic bag and tape;

7. Pipet tips.

B. Inoculating cells with samples

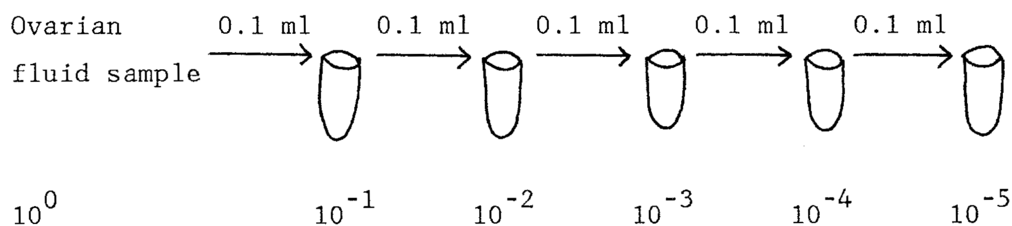
1. Method I

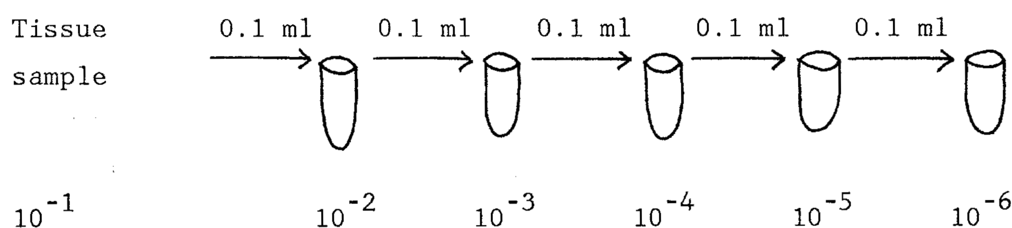
a. Dilute 0.1 ml of each pooled ovarian fluid sample (diluted 10^{0}) 10^{-1} through 10^{-5}, and 0.1 ml of each pooled tissue sample (diluted 10^{-1}) 10^{-2}through 10^{-6}. Dilutions are made immediately before

addition to monolayered cells, using 0.9 ml MEM-0-TRIS dilution blanks and 100 lambda MLA pipettor. Mix contents of dilution blank with Vortex mixer. Do not use MLA pipettor to mix contents of dilution blank.

Pipet 0.1 ml of each pooled ovarian fluid sample (diluted 10^{0}) and 0.1 ml of each pooled tissue sample (diluted 10^{-1}) into 1 of 6 wells allocated per sample. Also pipet 0.1 ml of each pooled ovarian fluid and tissue sample into one 0.9 ml MEM-0-TRIS dilution blank per sample. Discard pipet tip and mix contents of dilution blank with Vortex mixer.

Each pooled ovarian fluid sample is now diluted 10^{-1}, and each pooled tissue sample is now diluted 10^{-2}. Serially dilute each pooled ovarian fluid sample through 10^{-5} and each pooled tissue sample through 10^{-6}. Suggested dilution procedure:





b. Monolayered cells are inoculated with dilutions of 10^{0} through 10^{-5} per pooled ovarian fluid sample and with dilutions of 10^{-1} through 10^{-6} per pooled tissue sample. Aseptically pipet, using 100 lambda MLA pipettor, 0.1 ml of each dilution per sample into 1 well per dilution. Discard pipet tip into bleach bucket after each dilution. Six wells will be inoculated per sample. Two remaining wells in each column are control wells. Pipet, using 100 lambda MLA pipettor, 0.1 ml

MEM-10-TRIS into monolayer control well, and 0.1 ml MEM-0-TRIS into mock control well.

Always inoculate monolayered cells with 0.1 ml sample, MEM-10-TRIS (monolayer control), or MEM-0-TRIS (mock control). If dilution procedure used differs from suggested dilution procedure and requires use of the 10 lambda pipettor, the 100 lambda pipettor is still used to inoculate monolayered cells.

2. Method II

a. Dilute 0.01 ml of each pooled ovarian fluid sample (diluted 10^{0}) 10^{-1} through 10^{-6}, and 0.01 ml of each pooled tissue sample (diluted 10^{-1}) 10^{-2} through 10^{-7}. Dilutions are made using the 0.1 ml MEM-10-TRIS present in each of 6 wells allocated per sample and 10 lambda MLA pipettor. Discard pipet tip into bleach bucket after every dilution. Do not use MLA pipettor to mix contents of well.

Pipet, using 10 lambda MLA pipettor, 0.01 ml of each pooled ovarian fluid sample (diluted 10^{0}) and 0.01 ml of each pooled tissue sample into 1 of 6 wells allocated per sample. Discard pipet tip and mix contents of well by swirling plate on counter surface. Each pooled ovarian fluid sample is now diluted 10^{-1} and each pooled tissue sample is now diluted 10^{-2}. Serially dilute, using 10 lambda MLA pipettor, each pooled ovarian fluid sample through 10^{-6} and each pooled tissue sample through 10^{-7}.

b. Monolayered cells are inoculated with dilutions of 10^{-1} through 10^{-6} per pooled ovarian fluid sample and with dilutions of 10^{-2} through 10^{-7} per pooled tissue sample. Discard pipet tip into bleach bucket after each dilution. Six wells are inoculated per sample. Two remaining wells in each column are control wells. There are 2 monolayer control wells per pooled ovarian fluid sample, and 1 monolayer control well and 1 mock control well per pooled tissue sample. Pipet, using 10 lambda MLA pipettor, 0.01 ml MEM-10-TRIS into each monolayer control well and 0.01 ml MEM-0-TRIS into each mock control well.

Always inoculate monolayered cells with 0.01 ml sample, MEM-10-TRIS (monolayer control), or MEM-0-TRIS (mock control).

C. Sterile Kim-Wipes are used to dry plate surface. Cover each plate with lid or seal plate with adhesive mylar sheet.

D. Label each plate with accession number. Label wells with sample number, dilution number (10^{-1} through 10^{-6}). Also label mock and monolayer control wells. Place each plate in plastic bag on top of moistened paper towel and seal bag with tape.

E. Incubate plate(s) at 15°C for 14 days for IHNV.

F. Examine each well for CPE, using inverted light microscope, every 2-4 days following inoculation of monolayered cells. Record intensity of CPE.

Toxicity may be present in some samples at the lower dilutions. Review section entitled "Cytopathic effect of viruses on tissue culture cells" for description of IHNV induced CPE.

G. Incubate pooled ovarian fluid and tissue samples in antibiotic cocktail at 4°C until completion of assay. Discard used dilution blanks.

PROCESSING OVARIAN FLUID, WHOLE FISH, AND TISSUE SAMPLES FOR PLAQUE ASSAY

I. Processing ovarian fluid samples

A. Centrifuge ovarian fluid samples at 2000 g for 20 min. Label tubes.

B. Samples are ready for inoculation onto tissue culture cells for virus assay.

II. Processing tissue samples

A. Homogenize samples using Virtis or Contorque grinder.

B. Centrifuge tissue samples at 2000 g for 20 min. Label tubes.

C. Samples are ready for inoculation onto tissue culture cells for virus assay.

III. Processing alevin or juvenile fish

A. Alevin and juvenile fish up to 4 cm in length are processed whole. Aseptically remove spleen, anterior kidney, and liver from fish greater than 4 cm in length.

B. Weigh 1 g samples, if possible, on flamed aluminum foil. Place tissue sample from each fish into 1 sterile centrifuge tube per fish.

C. Pipet MEM-0-TRIS to make a 10^{-1} dilution of tissue to MEM-0-TRIS. (i.e. One g of tissue and 9 ml of MEM-0-TRIS is a 10^{-1} dilution of tissue to MEM-0-TRIS.)

D. Continue processing using method described in "Processing ovarian fluid, whole fish, and tissue samples for plaque assay: processing tissue samples".

PLAQUE ASSAY

I. Seeding 24 (16 mm) well plates

A. Determine number of plates needed for assay. Four wells are needed per sample along with control wells. Sixty samples can be assayed using 11 plates.

B. Remove confluent cell monolayer using method described in "Maintenance of stock cell lines: passage of confluent cell monolayer". One confluent cell monolayer in a 75 cm^2 flask can make 4 plates if 100 ml of MEM-10-TRIS are added to flask.

Confluent monolayers in two-three 75 cm^2 flasks are needed to make 11 plates. When using more than 1 flask to make plates, combine cell suspensions from each flask into one 150 cm^2 flask following trypsinization and trituration in MEM-10-TRIS. If 11 plates are needed for assay, trypsinize cell monolayer in each flask and add 10-15 ml MEM-10-TRIS to each flask. Combine 2-3 resultant cell suspensions into one 150 cm^2 flask. Add MEM-10-TRIS to this flask to bring final volume in flask to 275-300 ml.

C. Pipet 1.0 ml cell suspension into each well per plate.

D. Cover each plate with lid.

E. Label each plate with date, cell line initials, passage number, and operator initials. Place plate(s) in plastic bag and seal bag with tape.

F. Incubate and allow to grow and form a 90-100% cell monolayer without changing medium.

II. Inoculating cells with ovarian fluid and tissue samples

A. Materials needed for assay

1. Appropriate number of 24 well plates with 90-100% cell monolayer. (Do not use plates in which cells have been monolayered more than 4 days.);

2. Ovarian fluid and/or tissue samples;

3. MEM-0-TRIS dilution blanks (0.9 and 1.0 ml);

4. MEM-10-TRIS;

5. One x methylcellulose overlay;

6. Ten lambda and 100 lambda MLA pipettors;
7. Plastic bag and tape;
8. Pipet tips.

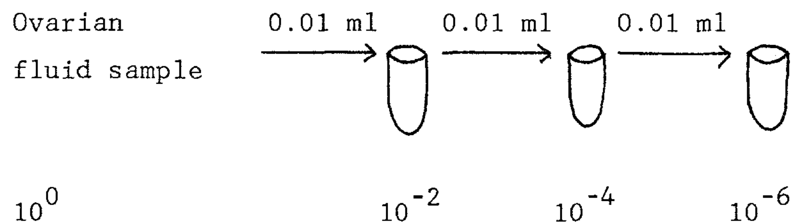
B. Inoculating cells with samples

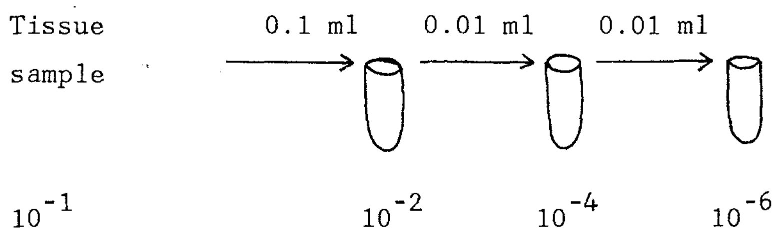
Dilute 0.01 ml of each ovarian fluid sample (diluted 10^{0}) 10^{-2}, 10^{-4}, and 10^{-6}, and dilute 0.1 ml of each tissue sample (diluted 10^{-1}) 10^{-2}, 10^{-4}, and 10^{-6}. Dilutions are made immediately before addition to monolayered cells, using 0.9 and 1.0 ml MEM-0-TRIS dilution blanks and 10 lambda and 100 lambda MLA pipettors. Mix contents of dilution blank with Vortex mixer. Do not use MLA pipettor to mix contents of dilution blank.

Pipet 0.1 ml of each ovarian fluid sample (diluted 10^{0}) and 0.1 ml of each tissue sample (diluted 10^{-1}) into 1 of 4 wells allocated per sample. Also pipet 0.01 ml of each ovarian fluid sample into one 1.0 ml MEM-0-TRIS dilution blank and 0.1 ml of each tissue sample into one 0.9 ml MEM-0-TRIS dilution blank per sample. Discard pipet tip and mix contents of dilution blank with Vortex mixer. Each ovarian fluid and tissue sample is now diluted 10^{-2}. Serially dilute each ovarian fluid and tissue sample 10^{-4} and 10^{-6}.

When using 10 lambda MLA pipettor to make 10^{-2} dilutions, pipet 0.01 ml sample into 1.0 ml MEM-0-TRIS dilution blank. When using 100 lambda MLA pipettor to make 10^{-1} dilutions, pipet 0.1 ml sample into 0.9 ml MEM-0-TRIS dilution blank.

Suggested dilution procedure:





C. Label plate(s) with accession number. Label wells with sample number, and dilution number (10^0, 10^{-2}, 10^{-4}, and 10^{-6} for each ovarian fluid sample and 10^{-1}, 10^{-2}, 10^{-4}, and 10^{-6} for each tissue sample). Also label mock and monolayer control wells.

D. Decant medium from wells by inverting plate over bleach bucket.

E. Monolayered cells are inoculated with dilutions of 10^0, 10^{-2}, 10^{-4}, and 10^{-6} per ovarian fluid sample and with dilutions of 10^{-1}, 10^{-2}, 10^{-4}, and 10^{-6} per tissue sample. Aseptically pipet, using 100 lambda pipettor, 0.1 ml of each dilution per sample into 1 well per dilution. Discard pipet tip into bleach bucket after each dilution.

F. When assaying 60 fish, plates 1, 3, 5, 7, 9, and 11 each have four control wells. Aseptically pipet, using 100 lambda MLA pipettor, 0.1 ml MEM-0-TRIS into each of 2 mock control wells per control plate. Pipet 1.0 ml MEM-10-TRIS into each of 2 monolayer control wells per control plate. Controls are made in duplicate.

G. Cover each plate with lid and place in plastic bag. Allow samples to adsorb onto cells for 1 h in hood. Minimize disturbance of plates.

H. Following adsorption, 1 ml of 1 x Methylcellulose (overlay) is carefully pipetted into each well except monolayer control wells. Overlay is added by either dripping overlay into center of each well, or by dripping overlay down side of well. Discard pipet between samples if pipet tip touches side of any well. When dripping overlay down side of well, add overlay to dilutions in following order: 10^{-6}, 10^{-4}, 10^{-2}, and 10^{-1} or 10^0 (highest to lowest dilution) to minimize carry-over of sample.

I. Cover each plate with lid. Place plate(s) in plastic bag and seal bag with tape.

J. Incubate at 15°C for 7 days for IHNV. Label bag with date plate is to be fixed and stained, day 7.

J. Incubate ovarian fluid and tissue samples at 4°C until completion of assay. Discard used dilution blanks.

K. On day 7, examine cell monolayers with inverted light microscope to determine whether cells within plaques have lifted off plate. If cells within plaques have lifted off plate(s), fix and stain plates for at least 1 h by carefully pipetting 0.5 ml of 0.5% crystal violet in 40% formalin to each well.

L. Cell monolayers are gently rinsed with tap water and allowed to air dry.

III. Counting plaques

A. Number of plaques per well are counted to determine virus titer. Virus titer for each sample is expressed as mean plaque forming units per ml of virus (pfu/ml). Wells with more than 200 plaques are not counted and are labeled too numerous to count (TNTC).

1. Following equation is used to express pfu/ml in 1 well:

plaques x 1/dilution x 1/# ml

i.e. The 10^{-5} dilution of sample 1 has 10 plaques in cell monolayer. One-tenth ml of the 10^{-5} dilution was pipetted onto well.

$$1/\text{dilution} = 1/10^{-5} = 10^{5}$$
$$1/\#\ \text{ml} = 1/0.1\ \text{ml} = 1/10^{-1} = 10^{1}$$

$$10\ \text{plaques} \times 10^{5} \times 10^{1} = 1 \times 10^{7}.$$

2. Determine mean pfu/ml for each sample as follows:

Sample #	# Plaques	Dilution #	pfu/ml	mean pfu/ml
1	20	10^{-5}	2.0×10^{7}	2.5×10^{7}
1	3	10^{-6}	3.0×10^{7}	

If replicates of each dilution were plated, determine mean pfu/ml using all countable wells.

B. Record mean pfu/ml

C. Examine a few plaques, using inverted light microscope, and note typical IHNV induced CPE as described in "Cytopathic effect of viruses on tissue culture cells".

SERUM NEUTRALIZATION ASSAY

I. Seeding 96 multi-well plates

A. Determine number of plates needed for assay. One virus sample can be assayed using 1 plate.

B. Prepare plate using method described in "Traditional assay - seeding flat bottom 96 multi-well plates".

C. Incubate and allow to grow and form a 90% cell monolayer without changing medium.

II. Preparing virus (known and unknown) and antiserum

A. In a test tube rack, make 1 row of 9 test tubes and 1 row of 8 test tubes. Use sterile 5 ml test tubes with snap caps. Label test tubes as follows:

unk + anti 10^{-1}	unk + anti 10^{-2}	unk + anti 10^{-3}	unk + anti 10^{-4}	known + anti 10^{-1}	known + anti 10^{-2}	known + anti 10^{-3}	known + anti 10^{-4}	anti
unk 10^{-1}	unk 10^{-2}	unk 10^{-3}	unk 10^{-4}	known 10^{-1}	known 10^{-2}	known 10^{-3}	known 10^{-4}	

B. Antiserum

1. Dilute antiserum to appropriate dilution (marked on label) with MEM-0-TRIS. Nine ml of diluted antiserum are needed for the assay.

2. Aseptically pipet 1.0 ml diluted antiserum into each of 8 tubes labeled virus + antiserum and into 1 tube labeled antiserum.

C. Known and unknown virus samples

1. Dilute 0.3 ml of each known and unknown virus sample 10^{-1} through 10^{-4} in tubes labeled virus. Dilutions are made immediately before addition to monolayered cells, using 2.7 ml MEM-0-TRIS dilution blanks. Discard pipet between each dilution. Mix contents of each dilution blank with Vortex mixer. Do <u>not</u> use pipet to mix contents of dilution blank.

D. Virus and antiserum

1. Aseptically pipet 1.0 ml of each virus dilution into corresponding tube labeled virus + antiserum. Pipet from 10^{-4} to 10^{-1} dilution per virus sample using 1 pipet per sample (highest to lowest dilution) to minimize carry-over of sample.

2. Aseptically pipet 1.0 ml MEM-0-TRIS into tube labeled antiserum.

3. Incubate tubes at 15°C for 1 h. Thoroughly mix contents of each tube with Vortex mixer every 15 min.

III. Inoculation of sample

A. Aseptically pipet, using 100 lambda MLA pipettor, 0.1 ml MEM-0-TRIS into each mock control well in columns 1 and 11, and 0.1 ml MEM-10-TRIS into each monolayer control well in column 6.

B. Diagram of flat bottom 96 multi-well plate

	1	2	3	4	5	6	7	8	9	10	11	12	
unk + anti	MOCK CONTROL					MONOLAYER CONTROL					MOCK CONTROL	ANTISERUM CONTROL	known + anti
unk													known
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		10^{-1}	10^{-2}	10^{-3}	10^{-4}			

C. Aseptically pipet, using 100 lambda MLA pipettor, 0.1 ml from tube labeled antiserum, into each antiserum control well in column 12.

D. Aseptically pipet, using 100 lambda MLA pipettor, 0.1 ml of each known and unknown virus and antiserum dilution and 0.1 ml of each known and unknown virus dilution into each of 4 wells (see diagram above). Pipet from 10^{-4} to 10^{-1} dilution per virus sample using 1 pipet tip per sample (highest to lowest dilution) to minimize carry-over of sample. Discard pipet tip between virus samples.

E. Sterile Kim-Wipes are used to dry plate surface.

F. Cover plate with lid or seal plate with adhesive mylar sheet. Label each plate with date, label each quadrant, and label control wells. Place plate in plastic bag and seal with tape.

G. Incubate plate(s) at 15°C for 6 days for IHNV.

H. Examine each well every 1-2 days.

1. First, examine the control wells. If CPE occurs in control wells, test is invalidated and must be repeated with either a different MEM-0-TRIS or a higher dilution of antiserum.

2. Second, examine known virus columns and known virus and antiserum columns. There should be CPE in known virus columns but not in known virus and antiserum columns. This indicates that known virus has been neutralized and that cells are sensitive to known virus. Higher dilutions of known virus may require several days to develop CPE. If CPE appears in known virus and antiserum columns, virus has not been neutralized. Test is invalidated and must be repeated with higher dilution of virus, lower dilution of antiserum, or different antiserum.

3. Third, examine unknown virus columns and unknown virus and antiserum columns. Begin with lowest dilution (10^{-1}). If there is CPE in unknown virus dilutions, but not in unknown virus and antiserum dilutions, unknown virus has been neutralized and identified by type of antiserum used. If there is CPE in both unknown virus, and unknown virus and antiserum, virus has not been neutralized. Therefore, test indicates unknown virus is a different type of virus, or a different serotype of the virus used to produce the antiserum.

MEDIA

I. MEM-TP (Incomplete cell culture medium)

10 l	tissue culture grade water
	powdered Autopow
29.5 g	tryptose phosphate

Reconstitute powdered Autopow and tryptose phosphate in tissue culture grade water. Dispense medium in 450 ml quantities into clean bottles and autoclave. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles MEM-TP and store at room temperature.

II. MEM-10 (Complete cell culture medium for closed systems)

450 ml	MEM-TP
50 ml	fetal bovine serum (FBS)
5 ml	l-glutamine
0.5 ml	gentamycin (50 mg/ml)
5-8 ml	7.5% $NaHCO_3$

III. MEM-10-TRIS (Complete cell culture medium for open systems)

450 ml	MEM-TP
50 ml	FBS
5 ml	l-glutamine
0.5 ml	gentamycin (50 mg/ml)
5 ml	fungizone (250 mcg/ml)
8 ml	1 M Tris buffer
2-4 ml	7.5 % $NaHCO_3$

IV. MEM-O-TRIS

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

V. 2 x MEM-TP

5 l	tissue culture grade water
	powdered Autopow
29.5 g	tryptose phosphate

Reconstitute powdered Autopow and tryptose phosphate in tissue culture grade water. Dispense in 250 and 500 ml quantities into clean bottles and autoclave. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles 2 x MEM-TP and store at room temperature.

VI. 2 x Methylcellulose

15 g	methylcellulose
985 ml	tissue culture grade water

Place ingredients in flask. Add stir bar. Cover loosely with lid and autoclave. Allow to cool. Use magnetic stirrer to dissolve (4-24 h). Aseptically dispense 250 ml into each of four clean, sterile bottles. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles 2 x methylcellulose and store at 4°C.

VII. 1 x Methylcellulose (Overlay)

250 ml	2 x MEM-TP
250 ml	2 x Methylcellulose
50 ml	FBS
5 ml	1-glutamine
1 ml	gentamycin (50 mg/ml)
5 ml	fungizone (250 mcg/ml)
8 ml	1 M Tris buffer
2-4 ml	7.5% $NaHCO_3$

VIII. Standard antibiotic concentrations

0.5 ml gentamycin (50 mg/ml) brought to 500 ml with MEM-10 = 50 mcg/ml
5 ml fungizone (250 mcg/ml) brought to 500 ml with MEM-10 = 2.5 mcg/ml
2.5 ml mycostatin (10000 IU/ml) brought to 500 ml with MEM-10 = 50 IU/ml
5 ml penicillin (10000 IU/ml) brought to 500 ml with MEM-10 = 100 IU/ml
5 ml streptomycin (10000 mcg/ml) brought to 500 ml with MEM-10 = 100 mcg/ml

IX. Antibiotic cocktail concentrations

0.02 ml gentamycin (50 mg/ml) brought to 2 ml with sample = 500 mcg/ml
0.2 ml fungizone (250 mcg/ml) brought to 2 ml with sample = 25 mcg/ml

X. Versene-PBS (diamino-ethane-tetra-acetic acid = EDTA)

0.2 g	Na versenate (or 1 ml of 100 g/ml versene liquid)
8 g	NaCl
0.2 g	KCl
1.15 g	Na_2HPO_4
0.2 g	KH_2PO_4

Combine ingredients in flask. Bring volume to 1 liter with tissue culture grade water. Use magnetic stirrer to dissolve. Dispense 100 ml quantities into clean bottles and autoclave. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles Versene-PBS and store at 4°C.

XI. Versene-Trypsin

Make versene-PBS using method described above. Upon cooling of versene-PBS, aseptically add 4 ml 2.5% trypsin into each of 5 sterile 100 ml versene-PBS. Final trypsin concentration is 0.1%. Sterility test 0.5 ml in 5 ml thioglycolate broth.

XII. 0.5% Crystal Violet in 40% Formalin

2.5 g	crystal violet
300 ml	single distilled water
200 ml	formalin

Combine ingredients in 500 ml bottle. Use magnetic stirrer to dissolve. Label bottle 0.5% Crystal Violet in 40% Formalin.

XIII. 7.5% $NaHCO_3$

75 g	$NaHCO_3$

Add $NaHCO_3$ to flask. Bring volume to 1 liter with tissue culture grade water. Use magnetic stirrer to dissolve. Dispense 50 ml quantities into clean bottles and autoclave. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles 7.5% $NaHCO_3$ and store at 4°C.

XIV. 1 M Tris buffer

106.4 g	tris-hydrochloride
39.4 g	tris

Combine ingredients in flask. Bring volume to 1 liter with tissue culture grade water. Use magnetic stirrer to dissolve. Dispense 50 ml quantities into clean bottles and autoclave. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles 1 M Tris and store at 4°C.

XV. l-glutamine

Aseptically pipet 5 ml into each of 10 sterile 5 ml test tubes with snap caps. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label tubes and freeze at -20°C in vertical position.

XVI. Fetal bovine serum (FBS)

Aseptically dispense 50 ml FBS into each of 10 clean, sterile bottles. Sterility test 0.5 ml in 5 ml thioglycolate broth. Label bottles FBS and freeze at -20°C.

DETECTING AND AVOIDING BACTERIAL AND FUNGAL CONTAMINANTS

I. Sterility test all tissue culture reagents using method described in "Media". Add 0.5 ml reagent or medium to be tested to 5 ml thioglycolate broth. Incubate at 23-25°C (room temperature) for 14 days. Check for growth on day 1, 2, and 14. If contamination is observed in thioglycolate broth autoclave and discard reagent.

II. Autoclave and discard any reagent or medium in which contamination is observed.

III. Autoclave and discard any flask or plate in which contamination is observed prior to inoculation of samples. Do not transfer cell monolayer that is contaminated.

IV. If contamination is observed in all dilutions of each sample, autoclave and discard plates. Centrifuge samples being held at 4°C. Pipet 1.9 ml of each sample into tube containing frozen antibiotic cocktail. Incubate at 23-27°C (room temperature) for 2 h. Filter sterilize using Gelman disposable filter and re-process as soon as possible.

WASHING GLASSWARE

I. Bottles and lids

Empty reagents and medium from bottles. Immediately fill bottle(s) with hot tap water. Replace lid. When enough bottles have accumulated to fill dishwasher, empty all bottles and place in dishwasher inverted. Do not accumulate bottles longer than 1 work week. Lids are placed in basket on top rack of dishwasher. Add Panasol to dishwasher and run dishwasher on normal cycle. Run single distilled water cycle 5 times to rinse bottles and lids.

Remove bottles and lids from dishwasher. Rinse each bottle and lid 5 times in tissue culture grade water. Drain bottles, replace lids and autoclave.

II. Contorques and Virtis grinders

Drain grinding instruments of bleach solution. Rinse with copious amounts of tap water. Clean each piece individually with Panasol and brush. Rinse again with copious amounts of tap water. Then rinse 5 times in single distilled water and 5 times in tissue culture grade water. Place into trays, cover with aluminum foil, label and autoclave.

STORING VIRUS SAMPLES

I. Viruses isolated in fish species other than *O. nerka* or during epizootics, are frozen upon completion of traditional, plaque, and serum neutralization assays. At least 2 virus samples are frozen per accession number. If samples were titered, freeze highest and lowest titered samples. (Viruses isolated from *O. nerka* are periodically frozen for research purposes.)

II. Label freezer vial with accession number, sample number, sample location, fish species, sample type (ovarian fluid or tissue sample), and date frozen as follows:

840084 #5
Hidden Lake
O. nerka O/F
10-14-83

III. Aseptically pipet 1.5-2 ml sample into each labeled freezer vial. Seal tightly. Autoclave and discard all remaining samples.

IV. Freeze at -80°C.

V. Thaw virus sample rapidly. Decant MEM-10 from required number of 75 cm^2 flasks. Pipet 0.1 ml virus sample onto each cell monolayer. Allow virus to adsorb for 1 h. Add 30 ml MEM-10 to each flask and incubate at 15°C until all cells lift off each flask (4+ CPE).

STORING VIRUS GROWN IN TISSUE CULTURE CELLS

I. Incubate virus and tissue culture cells until all cells lift off flask (4+ CPE). Pipet supernatant into sterile centrifuge tube(s) and centrifuge at 2000 g for 20 min.

II. Label 2 freezer vials per sample with accession number, sample number, number of passes through cell line, sample location, fish species, original sample type (ovarian fluid or tissue sample), and date frozen as follows:

840085 #5
P1 thru EPC (Pass 1 through EPC cells)
Hidden Lake
O. nerka O/F
10-14-83

III. Aseptically pipet 1.5-2 ml sample into each labeled freezer vials. Seal tightly.

IV. Freeze at -80°C.

V. Thaw virus sample rapidly. Decant MEM-10 from required number of 75 cm^2 flasks. Pipet 0.1 ml virus sample onto each cell monolayer. Allow virus to adsorb for 1 h. Add 30 ml MEM-10 to flask and incubate at 15°C until all cells lift off each flask (4+ CPE).

STORING TISSUE CULTURE CELLS

I. Remove confluent cell monolayers from two 75 cm^2 (or one 150 cm^2) flasks using method described in "Maintenance of stock cell lines: passage of confluent cell monolayer". Decant versene-trypsin and pipet 5 ml MEM-10 into each flask. Combine cell suspensions into 1 flask. Triturate cell suspension 2 times with pipettor. Pipet cell suspension into sterile centrifuge tube and centrifuge at 1000 rpm for 10 minutes. Resuspend cell pellet in 3 ml MEM-10.

II. In another sterile test tube, combine 2 ml MEM-10, 0.4 ml FBS, and 0.6 ml DMSO (MEM-20 with 20% DMSO).

III. Slowly add 3 ml MEM-20 with 20% DMSO to 3 ml cell suspension. Pipet six 1 ml quantities into labeled and cooled freezer vials. (Important to cool vials in ice water bath).

IV. Freeze all vials in foam block (for insulation) at -80°C for 24 h. Five vials are then placed at -150 to -180°C. Cells in sixth vial are tested for viability.

V. Thawing tissue culture cells

A. Rapidly thaw cell suspension in 1 vial.

B. Immediately decant cell suspension into one 25 cm^2 flask containing 10 ml MEM-10. Incubate flask for 24 h at proper temperature for cell line. Following 24 h incubation, decant medium and add 10 ml MEM-10.

CLEANING ELECTRONIC AIR PURIFIER FILTERS

I. Turn off switch #24 located in circuit box on east wall of sockeye wet lab.

II. Turn toggle switch above filter from "auto" to "off".

III. Turn off blower at outlet box on ceiling above blower.

IV. Clean inside blower chamber with disinfectant. Replace cleaned cover and tighten screws. Replace both filters in blower.

V. Remove electrostatic filter and pre-filter to wash container. Clean inside chamber with disinfecant.

VI. Fill beaker with 50 ml Panasol. Dissolve in hot tap water and soak filters for 15 min. Rinse filter 3-4 times and pre-filter 1-2 times until clean.

VII. Replace filters in air cleaner furnace. Turn toggle switch to "dry", turn on blower, and turn on switch #24. After 3-4 h turn toggle switch to "auto". (If arcing is heard after turning switch to "auto", filter is not dry. Return switch to "dry" until filter is completely dry.)

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