2.1.1 General Procedures for Virology

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A. Tissue and Fluid Sampling and Handling

The selection of tissues for virus assays varies according to the size and life stage of fish. The following tissues are the minimum that should be taken for virus assays.

Size/maturity of fish	Tissue assayed
Under 4 cm	Entire fish (remove yolk sac if present)
4-6 cm	Entire viscera (includes kidney)
Over 6 cm	Kidney and spleen
Sexually mature	Ovarian fluid, kidney, and spleen

For fish 4 to 6 cm or over 6 cm, it is recommended (OIE 2000) that brain tissue also be included. The addition of gill filaments to the sample pool may also increase the sensitivity of detection for some viruses.

After tissues and fluid are removed from the fish, they can be pooled; however, no more than five fish should be in one pooled sample of tissue or fluid. Approximately equal volume or weight proportions should be maintained for each specimen in a pool.

1. Storage of Samples

a. The samples should be maintained between 4 and 10 °C according to the virus(es) suspected. Samples should not be frozen.

b. The samples should not be stored longer than 48 hours.

c. Tissues may be stored in a buffered solution that contains antibiotics, antifungals, or both. The pH should be maintained within 7.4 to 7.8 or within the range that the suspected virus(es) are stable.

B. Preparing Samples for Virus Assays

The preparation of samples involves the homogenization of tissues and bacterial and fungal decontamination of tissues and fluids.

Homogenization can be accomplished in several ways; however, sonication is not acceptable for tissues. After homogenization, cellular material should be removed by centrifugation.

Decontamination can be accomplished either with the use of antibiotics and antifungals or by filtration of the supernatant of centrifuged tissues samples.

1. The antibiotics and antifungals that are used should be wide spectrum in their activity, and their concentrations should be effective in decontamination but not adversely affect cell cultures.

   The following compounds and concentrations should not exceed:

Gentamicin	1000 µg/mL
Penicillin	800 IU/mL
Streptomycin	800 µg/mL
Fungizone®	40 IU/mL
Mycostatin®	400 IU/mL

2. The supernatant from centrifuged tissue samples can also be decontaminated by filtration through a 0.45 µm filter. Passing tissue culture medium supplemented with serum through the filter before the sample is passed is recommended to minimize virus adherence to the filter.

C. Inoculating the Samples

1. Selection of Cell Cultures

Each virus section should be consulted to determine the most sensitive cell line(s) for a given virus. The cells should be normal appearing, rapidly dividing, and mycoplasma-free. Stock cell cultures should be routinely tested for susceptibility to specific viruses and for the presence of mycoplasma (see 2.A1 Methods for Testing for Mycoplasma Appendix 1). Penicillin (100 IU/mL) streptomycin (100 µg/mL), and antifungal agents such as Mycostatin (50 IU/mL) can be used in media for cell culture and virus assay work.

2. Inoculation

The cell cultures used for sample inoculation should be 80-90% confluent and not older than 48 hours. A minimum of 50 µL of sample should be inoculated per l.0 $cm^2$of cell sheet. Uninoculated controls must be used. Dilution of original samples should not exceed 1:10 for fluids and 1:100 for tissue samples.

3. Duration of Assay

The cell cultures should be incubated at 15°C and observed for cytopathological changes for a minimum of 14 days but 28 days incubation is recommended. Cell culture medium should be buffered or cells incubated so that a pH between 7.4 and 7.8 is maintained. A blind pass of 14 days is also recommended. The duration of the assay may need to be longer depending on which

viruses are suspected. When cytopathogical changes occur, the cultures should be subcultured or analyzed by serum neutralization or other confirmatory tests.

D. Virus Identification

Presumptive identification is based on the disease history of the fish stock, clinical signs observed at sampling, and cell culture results (CPE). However, to definitively identify a particular virus, certain confirmatory methods must be used.

1. Serum Neutralization Test

a. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^2 to 10^6 plaque forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per mL of the homologous virus.

b. Dilute the suspect sample from 10^2 to 10^6 in a sterile balanced salts solution.

c. Combine equal volumes of each dilution of the suspect sample and diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour at 15°C with agitation.

d. Inoculate onto the cell line in which the suspect virus was isolated. Incubate at 15°C for 14 days and observe for cytopathic effect (CPE). Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification.

2. Other Confirmatory Procedures

Other procedures that are routinely used for confirmatory virus identity include the fluorescent antibody test (FAT), dot blot methods, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR). Consult each virus section for references on alternative confirmatory methods or Section 2, 2.1 Table 2.1 Confirmatory Technique.

References

OIE Diagnostic Manual for Aquatic Animal Diseases. 2000. 3rd Edition. Office International Des Epizooties. Paris France.