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 (7 days for LMBV) during which time samples exhibiting CPE are re-inoculated. At approximately 14 days (7 days for LMBV), blind passage of samples not exhibiting CPE is performed and monitored for a total combined incubation period (initial plus blind pass) of 28 days (14 days for LMBV). 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 2006; Bouchard et al., 1999; Plumb et al., 1999; Rovozzo and Burke 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.

d. It should not be assumed that one set of conditions is optimal for all viruses. Please refer to information on specific pathogens for detailed information on culture conditions.

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. Do not allow cell monolayer to dry out during incubation.

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

   b. Incubation temperature for WSHV is 20°C.

c. Incubation temperature for SVCV is 20 to 25°C.

d. Incubation temperature for LMBV is 25 to 30°C.

5. Dispense an adequate volume of the appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (Section 2, 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 (Section 2, 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 SVCV at 20 to 25°C.

   d. Incubate for LMBV at 25 to 30°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 (Section 2, 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 Section 2, 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 (14 days for LMBV) 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.