2.2.8.1 Infectious Salmon Anemia Appendix 1

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Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for Infectious Salmon Anemia Virus (ISAV)

A. Principle/Purpose

To detect the presence of ISAV in kidney tissues and to confirm that cytopathic effect (CPE) observed in virology cell cultures is due to ISAV, through the amplification of cDNA fragments from the (-) sense ssRNA genome of ISAV using specific oligonucleotide primers complementary to the ISAV genome sequence.

B. Specimen

1. **Kidney tissue**
   A 30 to 50 mg size piece of middle kidney is excised from the sampled fish and blotted on a clean paper towel to remove excess blood. The scalpel should be flamed with 70 to 90% ethanol or dipped sequentially in a 10,000 ppm bleach solution and DI-H_2O between samples to prevent carryover contamination. The kidney is then submerged in five volumes (1 mL) of RNA Later™ in a 1.5 or 2.0 mL screw cap microfuge tube. The tissue can be stored in RNA Later™ for one day at 37ºC, one week at 25ºC, one month at 4ºC, or indefinitely at -20ºC without degradation. The tissue is considered compromised unless it is placed in RNA Later™ directly after sampling from the fish and stored below 37ºC before and during shipment to the laboratory.

2. **Supernatant**
   For cell culture plates that are planned to be maintained longer, the sample is harvested in a laminar flow hood by inserting a 1.0 mL sterile syringe with needle through the mylar film covering the cell culture plate. Some of the cells are scraped from the monolayer and pulled up with 0.25 mL or more of the supernatant and placed in a sterile 1.5 mL microfuge tube. If the cell culture plate is no longer needed or supernatant is being harvested from a flask, supernatant is harvested in a laminar flow hood using a sterile pipet.

C. Materials

RNA Later™ (Ambion)
1.5 or 2.0 mL screw cap microfuge tubes
Sterile 1 and 10 mL pipets
Sterile plugged nuclease free 10 µL, 100 µL, and 1000 µL pipet tips (Fisher, VWR)
Trizol LS® Reagent and Trizol® Reagent (Life Technologies)
Ethanol (Fisher Scientific)
Chloroform (Sigma)
Nuclease-free disposable pellet pestle (Fisher Scientific)
Isopropyl alcohol (Fisher Scientific)
Alcohol burner
1 mL sterile syringes with needle (Fisher Scientific)
Ready-to-Go™ RTPCR Beads 0.5mL or 0.2mL format (AmershamPharmacia Biotech)
0.5-10 µL, 10-100 µL, 200-1000 µL micro pipetors with sterile, nuclease free plugged tips (Eppendorf/Fisher Scientific)
Nuclease free water (Promega)
Tris-EDTA buffer (Sigma)
Rnasin (Promega)
ISAV specific upstream primer (1D) 5' GGC TAT CTA CCA TGA ACG AATC (Life Technologies or Sigma)
ISAV specific downstream primer (2) 5' TAG GGG CAT ACA TCT GCA TC (Life Technologies or Sigma)
1.5 mL sterile nuclease free tubes (Fisher Scientific)
0.5 mL sterile nuclease free tubes (Eppendorf)
100 base pair DNA ladder (Promega)
ϕx174 HAE III digested DNA size markers (Promega)
TAE buffer (see Section 1, 2.2.8.2 ISA Appendix 2)
Molecular biology grade agarose (Life Technologies)
Ethidium bromide
Electrophoresis equipment
UV transilluminator
Mastercycler gradient thermocycler (Eppendorf/Fisher Scientific)
Microcentifuge (Eppendorf and IEC)
Photographic equipment with UV filter

1. **Preparation**
   Primers are rehydrated in Tris-EDTA buffer (final stock concentration 500 pmol/µL) and aliquoted as needed in nuclease free water (working stock concentration 50pmol/µL).

2. **Storage requirements**
   Primers, enzymes, and DNA ladders are held at –20°C. Manufacturer's requirements are followed for reagent kits and remaining materials are stored at room temperature.

D. Procedure

Listed below are procedures for the various stages of sample preparation for kidney tissue and cell culture supernatant using Trizol and Trizol LS Reagents, followed by a RT-PCR protocol.

1. Kidney Tissue Homogenization

a. Trizol Reagent

i. Remove kidney tissue to be extracted from RNA Later, place on a clean surface and trim a 20 to 30 mg piece using a scalpel flamed with 70 to 90% ethanol. Keep scalpel and forceps in 70 to 90% ethanol and flame between samples in order to prevent carryover to subsequent samples. Replace remaining kidney tissues in original microfuge tubes with RNA Later.

ii. Place trimmed tissue in a 1.5 mL microfuge tube containing 500 μL of Trizol.

iii. Grind tissue in the microfuge tube using a nuclease-free pellet pestle.

iv. Pulse-centrifuge microfuge tube for a few seconds in order to pull down any remaining large pieces of tissue.

v. Transfer 250 μL of the supernatant to another microfuge tube containing 750 μL of Trizol for a total of 1 mL.

2. RNA Extraction from Kidney Tissue

a. Trizol Reagent

i. Phase Separation
Incubate supernatant/Trizol mixture at room temperature for five minutes. Add 200 μL chloroform to each tube using a new pipet tip for each sample. Shake for 15 seconds and incubate at room temperature for five minutes. Centrifuge cold at 12000 X g for 15 minutes in order to separate phases.

ii. RNA Precipitation
Transfer clear aqueous phase to a new 1.5 mL microfuge tube (0.4 to 0.45mL). Avoid disturbing DNA in the interphase with the pipet tip. Add 500 μL of isopropyl alcohol. Incubate samples at room temperature for 10 minutes. Centrifuge cold at 12000 X g for 10 minutes.

iii. RNA Wash
Remove supernatant, leaving a small amount in the bottom of the tube so as not to disturb the pellet. Add 1 mL of 75% ethanol and vortex. Centrifuge cold at 7500 X g for five minutes.

iv. Redissolving RNA
Remove all of the supernatant. Add 20 to 40 μL Tris-EDTA buffer with Rnasin. Gently pipet up and down in order to dissolve RNA pellet.

3. RNA Extraction from Cell Culture Supernatant

Follow same procedures as described above for RNA Extraction from Kidney Tissue, except use Trizol LS instead of Trizol.

4. RT-PCR

a. Procedure for Ready-to-Go™ RT-PCR Beads

i. Prepare a master mix for the number of samples to be amplified (add one extra volume in order to ensure that the last tube to be filled is not short of the 50 µL reaction volume). Prepare master mix on ice according to manufacturer's instructions as follows:

Nuclease free water	46.8 µL
Pd $(N)_6$ random primers	1 µL
Upstream primer	50 pmol
Downstream primer	50 pmol

ii. Aliquot 49 µL of the master mix into each Ready-to-Go™ RT-PCR Beads tube.

iii. Add 1 µL of each RNA sample to a Ready to Go™ RT-PCR Beads tube.

iv. Place tubes in thermocycler and run RT-PCR59 program:
Lid 105ºC

1. 42ºC for 15 minutes.

2. 94ºC for 5 minutes.

3. 59ºC for 45 seconds.

4. 72ºC for 105 seconds.

5. 94ºC for 45 seconds.

6. 59ºC for 45 seconds.

7. 72ºC for 105 seconds.

Go to (*5*) for 36 cycles.

72ºC for 7 minutes.

Hold 4ºC.

v. Electrophorese PCR products along with ϕx174 HAE III digested DNA size markers or other appropriate DNA size markers on a 10 cm 2% agarose gel at 65V for 1 to 1.5 hours.

vi. Stain gel with ethidium bromide for 30-40 minutes. De-stain gel in DI-H_2O for 10 minutes.

vii. Photograph gel under UV illumination.

E. Reporting Results

Using the ISAV 1D/2 (Mjaaland et al. 1997) primer set, a 493 base pair fragment is amplified from ISAV-positive samples. Positive results are reported as an amplified band at the position where a 493 base pair fragment would be expected to migrate, based on the location of the positive control and appropriate DNA size marker bands.

References

Mjaaland, S., K. Rimstad, K. Falk, and B. H. Dannevig. 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. Journal of Virology 71:7681-7686.

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