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DISEASES**

Sampling and diagnostic manual for ISA



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Diagnostic procedures for the surveillance and confirmation of ISA

I.1. Preparation of samples from fish

Pooling of samples is not recommended. For surveillance purposes pooling may be accepted, however the number of fish to be pooled should be as low as possible.

Samples for RT-PCR analysis shall be taken from all of the fish sampled. A piece of *anterior or mid-kidney* shall be removed from the fish using a sterile instrument and transferred to a microfuge tube containing 1 ml RNA preservative solution of proven efficacy. Tissue from up to five fish may be collected in one tube of preservative solution and represent one pooled sample. The weight of tissue in one sample shall be approximately 0,5 g. When the fish are too small to obtain a sample of the required weight, pieces of kidney, heart, spleen, liver or pyloric caeca may be taken, in that order of preference, to make up 0,5 g.

Tissue for histological examination shall only be taken from freshly killed fish exhibiting clinical signs or post-mortem findings consistent with the presence of disease. Any external or internal lesions shall be sampled and in any case samples of liver, mid-kidney, heart, pancreas, gills and spleen shall be removed from individual fish using a scalpel and transferred to 8 to 10 % (vol/vol) buffered formol saline. The ratio of fixative to tissue must be at least 20:1 to ensure satisfactory preservation of the tissues.

Tissues for virological examination shall be taken from all of the fish sampled. Duplicate samples shall be taken for corroborative purposes. Pieces of the liver, anterior kidney, heart and spleen shall be removed from the fish using a sterile instrument and transferred to plastic tubes containing 9 ml transport solution, i.e. cell culture medium with antibiotics. A combination of 12,5 µg ml⁻¹ fungizone, 200 IU ml⁻¹ polymixin B, and 200 µg ml⁻¹ kanamycin is suitable but other combinations of proven efficiency may be used. Tissues from up to five fish may be collected in one tube containing transport solution and represent one pooled sample. The weight of tissue in one sample shall be 1,0 ± 0,5 g.

Kidney imprints shall be taken for IFAT examination from freshly killed fish only, i.e. within two hours of death. Due to low specificity and sensitivity of this assay its use is only recommended when clinical signs consistent with ISA are present or during an outbreak. A piece of mid-kidney shall be removed from the fish using sterile instruments. The tissue shall be blotted on absorbent paper to remove excess blood then repeatedly pressed against a poly-L-lysine-coated glass slide. The individual impressions shall be adjacent to each other, but not overlapping, to give a continuous single layer of cells. Blood and tissue fluid are not relevant material for this test. Leaving the kidney sample to 'drain' on the absorbent paper shall be avoided as this can lead to blood clotting causing large amounts of serum proteins to be deposited on the test slide. The imprints shall be air dried then kept cool and dry if they are not to be fixed immediately. Fixation of imprints shall be carried out within 72 hours of the fish being sampled. Alternatively, imprints may be frozen following air drying and stored for up to one month at – 20 °C prior to fixation.

Fish showing signs of anemia may be stunned and heparinised blood samples taken immediately for haematological examination, such as measurement of haematocrit.

I.2. Shipment of samples from fish

Whole fish may be transported to the laboratory if the temperature requirements during transportation, as described above can be fulfilled. Whole fish shall be wrapped in absorbent paper and shipped in a plastic bag, chilled as mentioned above.



Live fish may also be shipped but only under the supervision of the official service and when considering the additional disinfection and biosecurity issues when transporting live fish. .

Blood samples and tubes containing fish tissues for virological examination or RT-PCR analysis shall be placed in insulated containers (for instance thick-walled polystyrene boxes) together with sufficient ice or 'freeze blocks' to ensure chilling of the samples during transportation to the laboratory. Freezing must be avoided and ice shall still be present in the transport box at receipt or one or more of the 'freeze blocks' must still be partly or completely frozen. In exceptional circumstances RT-PCR samples and samples for virological examination may be snap-frozen and transported to the laboratory at -20°C or below.

For RT-PCR analysis of tissues preserved in *RNA later*, RNA extraction must be carried out within a certain time for samples stored at different temperatures. These times are given below:

- 37°C one day
- 25°C one week
- 4°C one month
- -20°C indefinitely

Slides for IFAT shall be shipped in slide holders with sufficient desiccant to keep the imprints dry and chilled.

If fish tissues are transported in fixative for histological examination they shall be shipped in leak proof tubes in impact-resistant containers, such as thick-walled polystyrene boxes.

Unless samples have been frozen, the virological examination must be started as soon as possible and not later than 72 hours after the collection of the samples. The sample for corroborative analysis shall be stored at -20°C or below on arrival at the laboratory.

All packaging and labeling must be performed in accordance with present national and international transport regulations as appropriate.

1.3. Collection of supplementary diagnostic material

With the agreement of the diagnostic laboratory, other fish tissues may be collected and prepared for supplementary examination.

II. Agent detection and identification methods

II.1. Examination of samples by RT-PCR

The recommended screening method for ISAV is RT-PCR or real-time RT-PCR. This section describes the procedures required for PCR amplification of part of the ISAV genome that may be performed on fish tissue or ISAV in culture

The PCR can vary depending on the conditions under which it is performed, e.g. depending on the thermal cycler or PCR buffer in use. Furthermore, false-positive results can occur because of e.g. false primer annealing or cross contamination. It is therefore important to include adequate positive and negative controls and sequence amplicons if there should be any doubts.

II.1.1. Total RNA extraction

All work with RNA should be performed on ice, using gloves.



Total RNA is extracted using e.g. RNA affinity spin columns, e.g. RNeasy Total RNA kit (Qiagen, Germany) according to the manufacturer's instructions. Purified RNA must be resuspended in distilled RNase-free water (e.g. water treated with 0.1% diethyl pyrocarbonate). The concentration and purity of the extracted RNA can be estimated by measuring the optical density at 260 nm and at 280 nm. An alternative approach can be to include internal controls targeted against the host genome, see II.1.3.

II.1.2. RT-PCR for ISAV detection

Today several RT-PCR methods can be used for ISAV genome amplification. A two-step RT-PCR can be performed whereby the RT and the PCR reactions steps are run in two separate tubes. However, a one-step reaction, where the two reactions are run in one tube, can also be performed. We recommend the one-step method (e.g. One Step RT-PCR kit, Qiagen) because the one tube assay minimise the risk of cross-contamination as no transfer of content have to be made- and furthermore it seems as sensitive as the two-step method.

Several specific ISAV primer pairs have been reported. We recommend using one of the assays described in the OIE diagnostic manual for ISA e.g. the ILA1/ILA2 primer pair that target segment 8 and have been found suitable for detection of ISAV in outbreaks and in carrier fish. The ILA2 reverse primer does not match isolates from North America and alternative primer set be used in these cases. Such alternative primer set could be the FA3/RA3 primer set described in the OIE manual. For a description of primers and assay, see table 1

Primer name	Sequence	Conditions (one step RT-PCR)	Product size	Reference
ILA1	5'-GGCTATCTACCATGAACGAATC-3'	1 cycle: 50 °C 30 minutes (RT) , 1 cycle: 94 °C 15 minutes , 36 cycles: of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 sec. 1 cycle: 72 °C for 5 min	155 bp	Mjaaland et al. 1997
ILA2	5'-GCCAAGTGTAAGTAGCACTCC-3'			

Table 1. Primers and conditions for RT-PCR assay targeting ISAV segment 8. Conditions may vary depending on e.g. buffer and enzymes.

RT-PCR assays with similar sensitivities and specificities to the described assays may also be used.

II.1.3. real-time RT-PCR for ISAV detection

The use of real-time RT-PCR may increase specificity and probably also sensitivity. The method can be performed more rapidly as no gel electrophoresis step is required and reduces the risk of cross contamination as it is possible to estimate the amount of viral genomic RNA within the sample tube. A drawback of the real-time RT-PCR assay is that it is often not possible to sequence amplified products. However, if there is doubt on the specificity of the amplified product, it is recommended to run another ISAV specific assay to verify the result.

Several different real-time RT-PCR assays (different combinations of buffers, probes and primer) for detection of ISAV are described. We recommend using one of the assays described in the OIE diagnostic manual by Snow et al. 2006 targeting segment 8 – see table 2. We recommend the one-step method (e.g. from Quantitech probe RT-PCR kit, Qiagen) because the one tube assay minimise the risk of cross-contamination.

Primer/probe	Sequence	Conditions (one step RT-PCR)	Reference
ISAV forward primer	5'- CTACACAGCAGGATGCAGATGT -3'	1 cycle: 50 °C 30 minutes (RT) , 1 cycle: 95 °C 15 minutes , 40 cycles: 94 °C for 15 sec, 60 °C for 60 sec	Snow et al. 2006
ISAV reverse primer	5'- CAGGATGCCGGAAGTCGAT -3'		
ISAV probe	5'-FAM- CATCGTCGCTGCAGTTC -3'		
ELF forward primer	5'- CCCCTCCAGGACGTTTACAAA -3'	1 cycle: 50 °C 30 minutes (RT) , 1 cycle: 95 °C 15 minutes , 40 cycles: 94 °C for 15 sec, 60 °C for 60 sec	Snow et al. 2006
ELF reverse primer	5'- CACACGGCCCCACAGGTACA -3'		
ELF probe	5'-FAM- ATCGGTGGTATTGGAAC -3'		

Table 2. Primers and conditions real time RT-PCR assays targeting ISAV segment 8 and salmon Elongation factor 1a (ELF) respectively. Conditions may vary depending on e.g. buffer and enzymes.

In order to assure quality of purified RNA it is recommended to include a control by performing a real-time RT-PCR targeting an endogenous fish mRNA. In table 2 are listed primer, probe and conditions for running a real time RT-PCR targeting the ELF salmon mRNA.

RT-PCR assays with similar sensitivities and specificities to the described assays may also be used.

II.1.4. Sequencing of amplified PCR products

Currently, it is believed that the HPR0 ISAV strain does not cause ISA whereas a strain carrying deletions in the HPR region may cause ISA (Christiansen et al. 2010). The HPR status of an ISAV strain can be determined by sequencing of the corresponding gene region. Recommended primers and conditions for RT-amplification of the HPR region is shown in table 3.

Primer name	Sequence	Conditions (one step RT-PCR)	Product size	Reference
ILAs6-3F	5'-ATGAGGGAGGTAGCATTGCA -3'	1 cycle: 50 °C 30 minutes (RT) , 1 cycle: 94 °C 15 minutes , 40 cycles: 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 60 sec. 1 cycle: 72 °C for 5 min	Variable	Christiansen et al. 2010
ILAs6-2R	5'-CATGCTTTCCAACCTGCTAGGA -3'			

Table 3. Primers and conditions for RT-PCR assays for sequencing of the HPR region.

RT-PCR assays with similar sensitivities and specificities to the described assays may also be used.

The purity of the amplified RT-PCR product should be checked by gel electrophoresis before sequencing. If only one pure fragment appear, it can be purified directly from the PCR reaction. If multiple amplified fragments are present, the fragment of interest should be purified by gel electrophoresis. Purification of PCR fragments from solutions or agarose gels can be made using PCR fragment affinity spin columns, e.g. RNeasy Total RNA kit (Qiagen, Germany) according to the manufacturer's instructions.

Sequencing can be performed using amplification primers at external specialised sequencing companies. Sequencing results can be analysed by "Blasting" sequences to other known sequences in e.g. the NCBI nucleotide database <http://www.ncbi.nlm.nih.gov/guide/>.

Furthermore, sequencing can eliminate any doubt on the specificity of an amplified RT-PCR product.

II.2. ISAV isolation on cell cultures

II.2.1. Preparation of samples

It is not recommended to freeze samples. However, where practical difficulties arise which make it impossible to inoculate cells within 72 hours after collection of the tissue samples, it is acceptable to freeze the tissue at – 80 °C for up to 28 days. The tissue must be frozen and thawed only once before examination.

Each sample (tissue pool in transport solution) shall be completely homogenised using a validated homogeniser, centrifuged at 2 000 to 4 000 × g for 15 minutes at 0 to 6 °C, and the supernatant shall be filtered (0,45 µm) and incubated with an equal volume of a suitably diluted pool of antisera to the indigenous serotypes of IPNV. The titre of the antiserum must be at least 1:2000 in a 50 % plaque neutralisation test. The mixture shall be incubated for one hour at 15 °C. This represents the inoculum.

Treatment of all inocula with antiserum to IPN virus (a virus which in some parts of Europe occurs in 50 % of fish samples) aims at preventing CPE due to IPN virus from developing in inoculated cell cultures. This will reduce the duration of the virological examinations as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of ISAV. When samples come from production units which are considered free from IPN, treatment of inocula with antiserum to IPN virus may be omitted.

II.2.2. Inoculation on cell cultures

SHK-1 cells (pass 65 or lower), ASK or TO cells shall be grown in L-15 medium (SHK-1 and ASK) or in H-MEM (TO) containing 5 % foetal bovine serum, 2 % (v/v) 200 mM L-glutamine, and 0,08 % (v/v) 50 mM 2-mercaptoethanol in 12- or 24-well plates. Other cell lines of proven effectiveness and sensitivity in isolating ISAV may be used, taking into consideration strain variability and the ability of different strains to replicate in different cell lines. Antiserum-treated organ suspension shall be inoculated into young actively growing cell cultures to give a final dilution of tissue material to culture medium of 1:1 000. For each organ suspension 40 µl of inoculum shall be added to one well containing 2 ml of culture medium. To minimise the risk of cross-contamination it is recommended that separate 12- or 24-well plates shall be used for samples from different fish farm sites.

One plate shall be left uninoculated to serve as a negative control. A separate plate shall be inoculated with a reference isolate of ISAV as a positive control, as follows. One hundred µl of a stock preparation of ISAV (minimum titre 10^7 TCID₅₀ ml⁻¹) shall be inoculated into the first well and mixed well. A volume of this material shall be transferred from the first well to the second well to make a 1:10 dilution and mixed well. This shall be repeated across the plate to make six 10-fold dilutions. Stock ISAV may be stored at – 80 °C for at least two years but once thawed must be used within three days. Note: care must be taken to prevent



cross-contamination of test plates with positive control material. To avoid this risk, positive controls shall be set up and handled separately from test plates.

Samples shall be incubated at 14 ± 2 °C for up to 15 days. Using a microscope, cell cultures shall be examined for CPE twice, between five to seven and 12 to 14 days following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately (II.2). If no CPE is observed by day 14, a haemadsorption or RT-PCR (II.1) shall be performed.

II.2.3. Subcultivation or passage

Subcultivation shall be carried out between days 13 to 15. Two hundred and twenty-five µl of culture supernatant shall be added to wells containing fresh actively growing SHK-1 cells in 12-well plates and incubated at 14 ± 2 °C for up to 18 days. Using a microscope, cell cultures shall be examined for CPE twice, between days five to seven and 14 to 18 following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately (III.6). If no CPE is observed by days 14 to 18, a haemadsorption or an RT-PCR test shall be performed (II.1).

If cytotoxicity occurs within the first seven days of incubation, subcultivation shall be performed at that stage, and the cells must be incubated for 14 to 18 days and subcultivated again with further 14 to 18 days incubation. If cytotoxicity occurs after seven days, subcultivation shall be performed once and the cells shall be incubated to achieve the total of 28 to 36 days incubation from the primary inoculation.

If bacterial contamination occurs in the primary culture, the test must be set up again using the tissue homogenate stored at -80°C . Prior to inoculation the tissue homogenate is centrifuged at $4\ 000 \times g$ for 30 minutes at 0 to 6 °C and the supernatant is filtered at 0,22 µm. If bacterial contamination occurs during the subcultivation step the supernatant shall be filtered at 0,22 µm, inoculated onto fresh cells and incubated for a further 14 to 18 days.

II.2.5. Virus identification tests

If evidence of CPE is observed at any stage, or if a haemadsorption test is positive, virus identification shall be carried out. The methods of choice for identification of ISAV are RT-PCR (II.1) and IF (II.2.6). If it is considered that other viruses may be present it is recommended that supplementary virus identification tests are carried out. If these tests have not allowed definitive identification of the virus within one week, the supernatant must be forwarded to a national reference laboratory or to the EU reference laboratory for fish diseases for immediate identification.

II.2.4. Haemadsorption

Replication of ISAV in cell cultures does not always result in a CPE. Therefore, every well shall be subject to a RT-PCR test or a haemadsorption test as described below, or alternatively, every well shall be subject to an IF test as described in III.6.1.

Cell culture medium shall be removed from each well, including those of positive and negative controls, and placed in labelled sterile tubes. Five hundred µl of a 0,2 % (v/v) suspension of washed rabbit or horse red blood cells, or a 0,05 % (v/v) suspension of washed rainbow trout or Atlantic salmon red blood cells, shall be added to each well and incubated at room temperature for 45 minutes. The red blood cells shall be removed and each well shall be washed twice with L-15 medium. Each well shall be examined using a microscope.

The presence of clusters of red blood cells attaching to the surface of SHK-1, ASKS or TO cells shall be indicative of presumptive infection with an orthomyxovirus. If a haemadsorption test is positive a virus identification test shall be performed immediately (III.6).

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II.2. 6. Immunofluorescence (IF)

SHK-1 cells (pass 65 or lower), ASK or TO cells shall be grown in L-15 medium (SHK-1 and ASK) or in H-MEM (TO) containing 5 % foetal bovine serum, 2 % (v/v) 200 mM L-glutamine, and 0,08 % (v/v) 50 mM 2-mercaptoethanol in 12- or 24-well plates and used at greater than 50 % confluence. Other cell lines or growth medium of proven efficacy may also be used. Two hundred and twenty five μ l of putative virus-infected culture supernatant shall be added to each of two wells, mixed and 225 μ l transferred to two further wells, i.e. a 1:5 dilution. Two additional wells shall be left uninoculated to act as controls. Samples from each fish farm site shall be handled on separate plates, as shall the virus control. A virus control shall be established using a reference isolate of ISAV.

Plates shall be incubated at 14 ± 2 °C and examined microscopically for up to seven days. When early CPE is observed, or if no CPE is observed within seven days, the next step shall be fixation. Wells shall hereby be washed with PBS and fixed by incubation with 80 % acetone for 20 minutes at room temperature. Plates shall be air-dried and stained immediately or stored at 0 to 6 °C for no more than 24 hours prior to staining.

Replicate wells shall be stained with a mix of monoclonal antibody (MAb) 3H6F8 and 10C9F5 against ISAV, or other MAb of proven effectiveness and specificity, diluted in PBS and incubated at 37 ± 4 °C for 30 minutes. MAb shall be removed and plates washed three times with 0,05 % Tween 20 in PBS. Anti-mouse IgG FITC conjugate diluted in PBS shall be added to each well and incubated at 37 ± 4 °C for 30 minutes. Note: the dilutions of different batches of MAb and FITC conjugate shall be optimised in each laboratory. Antibody shall be removed and plates shall be washed three times with 0,05 % Tween 20 in PBS.

Wells shall be examined immediately using an inverted microscope set up for fluorescence microscopy with a suitable filter for excitation of FITC. A test shall be considered positive if fluorescent cells are observed. For a test to be valid the positive controls must score positive and the negative controls must score negative.

II.3. Examination of kidney imprints by IFAT

II.3.1. Preparation and staining of imprints

Slides shall be fixed in acetone or methanol/acetone (1:1) for three minutes and air-dried. Before staining each slide shall be examined and appropriate regions of the slide shall be circumscribed with ImmEdge™ pen, or similar, and allowed to air-dry. The slides shall be then placed in blocking solution (6% skimmed milk in PBS containing 0,2% Tween 20) and incubated with gentle agitation for 30 minutes at room temperature. Each slide shall be drained and placed horizontally in a slide box containing wet tissue paper to maintain a humid atmosphere.

Each imprint shall be covered with a solution of a mix of monoclonal antibody (MAb) 3H6F8 and 10C9F5 against ISAV (or other antibody of proven specificity and efficacy) and the slide box shall be closed and incubated with agitation for 60 minutes at room temperature. The antibody shall normally be diluted 1:10 to 1:100 in 1 % skimmed milk but the actual dilution needs to be determined for each batch. Slides shall be washed three times for two minutes in PBS containing 0,1 % Tween 20. Each imprint shall be covered with a solution containing FITC goat anti mouse conjugate diluted 1:1 000 in 1 % skimmed milk and incubated in a humid environment for 60 minutes at room temperature. Slides shall be washed three times for two minutes in PBS containing 0,1 % Tween 20. Each slide shall be covered with CITIFLUORTM solution (500 μ l CITIFLUORTM mixed with 1,5 ml 0,1 % (v/v) Tween 20 in PBS) or other suitable mounting medium for 10 minutes. Slides shall be washed three times in PBS containing 0,1 % Tween 20. If a counter-stain is required, each imprint may be covered with propidium iodide (0,01 mg/ml) in PBS containing 0,1 % Tween 20 and incubated for three minutes at room temperature. Slides shall be washed three times for two minutes in

PBS containing 0,1 % Tween 20. Slides shall be drained and mounted in CITIFLUOR™ or other suitable mounting medium. Slides shall be stored in the dark at 4 °C prior to microscopic examination.

II.3.2 Examination using fluorescent microscopy

Each slide shall be examined on a microscope suitable for epi-fluorescent illumination, using a suitable filter that will excite FITC, causing it to emit characteristic green fluorescent. All fields within the regions defined by the ImmEdge™ pen shall be examined under ×10 and ×20 objectives and suspicious areas (those showing a green fluorescence) shall be further examined under a ×40 objective and phase/fluorescent illumination to ensure that the fluorescent staining is cell-associated. The stage coordinates for the suspicious regions shall be recorded for later confirmation of the nature of the fluorescence by a second examiner. Following examination by the primary reader slides that are positive or suspicious shall be re-examined by a second reader and the results confirmed.

II.3.3. Controls

Three types of control must be included with each batch of slides stained for IFAT:

- kidney imprint from uninfected Atlantic salmon (negative control),
- uninfected SHK-1 cell culture or other susceptible cell culture (negative control),
- ISAV-infected SHK-1 cell culture or other susceptible cell culture (positive control).

If available, a kidney imprint from an ISAV-infected Atlantic salmon is recommended as an additional positive control.

If a positive result is obtained with any negative controls the test is considered invalid for all slides in that batch. If all slides in a batch, including positive controls, are negative the test is considered invalid for all slides in that batch. In cases where failure of controls invalidates a batch of slides, those slides shall be destroyed and a re-test shall be carried out using the duplicate imprints.

II.3.4. Examination of other tissues

This technique can be applied to other fish tissues such as liver, spleen and heart providing a reasonable quantity of endothelial cells, leucocytes or lymphocytes can be deposited on the slide. The staining procedure remains the same for each tissue, although for some tissues it may be preferable to omit the propidium iodide staining relying on phase illumination to identify the cell types present in the imprint.

II.4. Histology

Paraffin-embedded sections shall be cut at 5 µm and stained using haematoxylin and eosin. Histological changes associated with ISA are described in the current edition of the OIE *Diagnostic manual for aquatic animal diseases*.

II.4.1. Immunohistochemistry (IHC)

IHC is a very useful tool for diagnosis of ISA. Procedures for preparation of tissue sections, staining, and interpretation is described in details in the [OIE Diagnostic manual for aquatic animal diseases](#).

III. Acronyms and abbreviations

ASK	Atlantic salmon kidney cells
SHK-1	Salmon head kidney cells
CPE	Cytopathic effect
DEPC	Diethylpyrocarbonate
ELF	Elongation factor 1 α
FITC	Fluorescein isothiocyanate

IF	Immunofluorescence
IFAT	Indirect fluorescent antibody test
IPN(V)	Infectious pancreatic necrosis (virus)
ISA(V)	Infectious salmon anaemia (virus)
mRNA	Messenger Ribonucleic acid
OIE	Office international des epizooties
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RT-(PCR)	Reverse transcriptase (polymerase chain reaction)
SHK-1	Salmon head kidney cells
TCID50	Tissue culture infective dose at the 50 % end point
TO	

IV. References

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