EURL FOR FISH DISEASES

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF IHN AND VHS

PART 1

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF IHN AND VHS

I. **Diagnostic methods and procedures for the surveillance of VHS and IHN**

When sampling and laboratory examination for the purpose of obtaining or maintaining disease-free health status with regard to IHN or VHS as set out in Section I of Part 1 of Annex I are carried out, using the diagnostic methods set out in points II.1 and II.2 of Part 1 of that Annex, the detailed diagnostic methods and procedures set out in following points I.1 to I.6 shall apply.

I.1. Preparation and shipment of samples from fish

I.1.1. Tissues for virological examination on cell culture

Before shipment or transfer to the laboratory, pieces of the organs to be examined shall be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing transport medium.

The quantity of fish material suitable for virological examination on cell culture and by RT-qPCR is dependent on fish size. Thus, whole alevin (body length < 4 cm), viscera including kidney (4 cm < body length < 6 cm) or, for larger size fish, kidney, spleen, heart and/or encephalon, and ovarian fluid from brood fish at the time of spawning shall be the tissues to be sampled.

Ovarian or seminal fluid or organ pieces from a maximum of 10 fish may be collected in one sterile tube containing at least 4 ml transport medium and represent one pooled sample. The tissue in each sample shall weigh a minimum of 0,5 gram (g).

The virological examination on cell culture shall be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.

I.1.2. Samples for reversed transcriptase polymerase chain reaction (RT-PCR or RT-qPCR) analysis

Samples shall be taken from the fish in accordance with the procedure described in point I.1.1 using a sterile instrument and transferred to a sterile plastic tube containing transport medium. Tissue from 10 fish may be collected in one tube and shall represent one pooled sample. However, in case the amount of inoculum is small, tissue from up to five fish may be used. Alternatively, samples may be pooled in RNA stabilization reagents, such as 0,2 g tissue/ml reagent according to the recommendation from the manufacturers, although each fish shall be processed individually and shall not be pooled in the samples because of the small amount of material to be used for extraction.

Whole fish may also be sent to the laboratory.

I.2. Shipment of samples for fish

Tubes containing fish tissues in transport medium for cell cultivation or RT-PCR/RT-qPCR analysis shall be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples shall be avoided. The temperature of a sample during transit must never exceed 10 °C and ice must still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen.

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish shall be wrapped up in paper with absorptive capacity and shall finally be shipped in a plastic bag. Live fish may also be shipped.

I.3. Collection of supplementary diagnostic material

When approved by the diagnostic laboratory, other fish tissues may be collected as well and prepared for supplementary examinations.

I.4. Preparation of samples for cell culture examination and RT-qPCR

I.4.1. Freezing in exceptional cases

Where practical difficulties arise, which make it impossible to process the samples within 48 hours after the collection of the fish tissues, it may be acceptable to freeze the tissue specimens in transport medium at – 20 °C or below and to carry out virological examination within 14 days. However, the fish tissue shall only be frozen and thawed once before examination. Records shall be kept with details on the reason for each freezing of fish tissue samples.

I.4.2. Homogenisation of organs

In the laboratory, the fish tissue in the tubes shall be completely homogenised, either by stomacher, blender or mortar and pestle with sterile sand, and subsequently suspended in the original transport medium.

If a sample consists of a whole fish less than 4 cm long, it shall be minced with sterile scissors or scalpel after removal of the body behind the gut opening. If a sample consists of a whole fish with body length between 4 cm and 6 cm, the viscera including kidney shall be collected. If a sample consists of a whole fish more than 6 cm long, the tissue specimens shall be collected as described in point I.1. The tissue specimens shall be minced with sterile scissors or scalpel and homogenised as described in the first paragraph of this point and suspended in transport medium.

The final ratio between tissue material and transport medium shall be adjusted in the laboratory to 1:10.

I.4.3. Centrifugation of homogenate

The homogenate shall be centrifuged in a refrigerated centrifuge at 2 °C to 5 °C at 2 000 to 4 000 × *g* for 15 minutes and the supernatant collected and may be treated for either four hours at 15 °C or overnight at 4 to 8 °C with antibiotics. If the sample has been shipped in a transport medium, the treatment of the supernatant with antibiotics may be omitted.

Where practical difficulties arise, such as incubator breakdown or problems with cell cultures, which make it impossible to inoculate cells within 48 hours after the collection of the fish tissue samples, the supernatant may be frozen at – 80 °C and virological examination may be carried out within 14 days.

If the collected supernatant is stored at – 80 °C within 48 hours after the sampling, it may be reused only once for virological examination.

Prior to the inoculation of the cells, the supernatant shall be mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of Infectious pancreatic necrosis (IPN) virus and incubated with this for a minimum of one hour at 15 °C or a maximum of 18 hours at 4 °C. The titre of the antiserum shall be at least 1/2 000 in a 50 % plaque neutralisation test.

Treatment of all inocula with antiserum to IPN virus aims at preventing cytopathic effect (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 VHSV or IHNV.

When samples come from production units, which are considered free from IPN, the treatment of inocula with antiserum to IPN virus may be omitted.

I.4.4. Sample preparation for RT-PCR and RT-qPCR based surveillance programmes

If samples were collected in transport medium, the procedure set out in points I.4.2 and I.4.3 shall be carried out. After centrifugation, supernatant shall be collected and RNA extracted. If further examination is not to be undertaken directly after centrifugation, the samples shall be immediately frozen at – 20 °C or below.

For the analysis of fish tissues preserved in RNA stabilization reagent, subsequent work shall be carried out within the following time scales for samples stored at different temperatures:

samples stored at 37 °C: 1 day;

samples stored at 25 °C: 1 week;

samples stored at 4 °C: 1 month;

samples stored at – 20 °C: indefinitely

Pooled samples in RNA stabilization reagent shall be treated like single samples in RNA stabilization reagent. For samples pooled in RNA stabilization reagent, the sample amount shall not exceed that recommended by the manufacturer for extraction with RNA kits, such as RNeasy Mini kits (Qiagen) or similar. If larger samples are pooled, the extraction kits or methods must reflect this pooling.

Samples collected in RNA stabilization reagents shall not be used for cell cultivation.

I.4.5. Pooling of samples for RT-qPCR

As the RT-qPCR protocols given are of similar or higher sensitivity than the cell cultivation methods, it may be acceptable to use supernatant from homogenised fish tissue material of pooled organs from up to 10 fish in cell culture medium for PCR. However, due to the much smaller inoculum used for PCR compared to cell cultivation, all fish tissues shall be carefully homogenised before collating material for extraction.

The same principle shall also be applied if samples are collected in RNA stabilization reagents. However, in that case it is often difficult to collect representative material from up to 10 fish in one tube, and the number of fish per pool shall therefore be reduced to 2 to 5.

I.5. Virological examination on cell culture

I.5.1. Cell cultures and media

Bluegill fry cell line -2 (BF-2) or Rainbow trout gonad cell line - 2 (RTG-2) and either *Epithelioma papulosum cyprini* (EPC) or Fathead minnow (FHM) cells shall be grown at 20 to 30 °C in suitable medium, namely Eagle's Minimum essential medium (MEM) or modifications thereof, with a supplement of 10 % foetal bovine serum and antibiotics in standard concentrations.

When the cells are cultivated in closed vials, the medium shall be buffered with bicarbonate. The medium used for cultivation of cells in open units may be buffered with tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) (23 mM) and sodium bicarbonate (6 mM). The pH must be 7,6 ± 0,2.

The cell cultures to be used for inoculation with fish tissue material shall be young, normally 1 day old cell culture monolayers where possible; however, a range between 4 to 48 hours old may be accepted. The cells must be actively growing at inoculation.

I.5.2. Inoculation of cell cultures

Antibiotic-treated organ suspension shall be inoculated into cell cultures in two dilutions, namely the primary dilution and, in addition, a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1 000, respectively, in order to prevent homologous interference. At least two cell lines shall be inoculated as referred to in point I.5.1. The ratio between inoculum size and volume of cell culture medium shall be about 1:10.

For each dilution and each cell line, a minimum of about 2 cm^2 cell area, corresponding to one well in a 24-well cell culture tray, shall be utilised. Cell culture trays shall be used where possible.

I.5.3. Incubation of cell cultures

The inoculated cell cultures shall be incubated at 15 °C for seven to 10 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution or equivalent substances shall be performed to ensure cell susceptibility to virus infection.

At least every 6 months or if decreased cell susceptibility is suspected, titration of frozen stocks of VHSV and IHNV shall be performed to verify the susceptibility of the cell cultures to infection. The procedure set out in Section III shall be used, if possible.

I.5.4. Microscopy

Inoculated cell cultures shall be inspected regularly, at least three times a week, for the occurrence of CPE at 40 to 150 × magnification. If obvious CPE is observed, virus identification procedures in accordance with point I.6 shall be initiated immediately.

I.5.5. Subcultivation

If no CPE has developed after the primary incubation for 7 to 10 days, subcultivation shall be performed to fresh cell cultures utilising a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures or wells constituting the primary culture shall be pooled according to cell line 7 to 10 days after inoculation. The pools shall then be inoculated into homologous cell cultures undiluted and diluted 1:10 (resulting in final dilutions of 1:10 and 1:100, respectively, of the supernatant) as described in point I.5.2. Alternatively, aliquots of 10 % of the medium constituting the primary culture shall be inoculated directly into a well with fresh cell culture (namely, well to well subcultivation). The inoculation may be preceded by pre-incubation of the dilutions with the antiserum to IPN virus at appropriate dilution as described in point I.4.3.

The inoculated cultures shall then be incubated for 7 to 10 days at 15 °C and inspected in accordance with point I.5.4.

If toxic CPE occurs within the first 3 days of incubation, subcultivation shall be performed at that stage, but the cells shall then be incubated for 7 days and sub cultivated again with further 7 days incubation. When toxic CPE develops after 3 days, the cells shall be passed once and incubated to achieve the total of 14 days from the primary inoculation. There must be no evidence of toxicity in the final 7 days of incubation.

If bacterial contamination occurs despite treatment with antibiotics, subcultivation shall be preceded by centrifugation at 2 000 to 4 000 × g for 15 to 30 minutes at 2 to 5 °C, or filtration of the supernatant through a 0,45 µm filter or both (low protein-binding membrane). In addition to this, subcultivation shall follow the same procedures as described for toxic CPE in the fourth paragraph of this point.

If no CPE occurs, the test may be declared negative.

I.6. Virus identification

If evidence of CPE has been observed in a cell culture, medium (supernatant) shall be collected and examined by one or more of the following techniques: Enzyme-linked immunosorbent essay (ELISA), Immunofluorescence (IF), neutralisation, RT-PCR or RT-qPCR. If these tests have not allowed definitive identification of the virus within 1 week, the supernatant shall be forwarded to the national reference laboratory or to the EU reference laboratory for fish diseases referred to in Annex VI to Directive 2006/88/EC for immediate identification.

I.6.1. ELISA

A double antibody sandwich ELISA shall be performed in order to identify the virus isolate. Microwell plates shall be coated with 50 µl/well (0,9 pg) of proven quality protein-A purified immunoglobulins(Ig) from rabbit antisera against IHNV or VSHV diluted in carbonate buffer (pH 9,6) containing 15 mM sodium azide and incubated from 18 hours to 2 weeks at 4 °C.

On a dilution plate, each sample containing 1 % Triton X-100 and the positive controls shall be diluted with buffer solution (namely, phosphate buffered saline (PBS)-T-BSA, 1 % BSA) in a 4-fold dilution: undiluted, 1:4, 1:16, 1:64. The ELISA plates shall be washed in PBS containing 0,05 % Tween-20 (PBS-T) and 50 µl of each dilution shall be transferred from the dilution plate to the washed and coated ELISA-plate.

ELISA plates shall then be incubated for 30 minutes at 37 °C. Subsequently plates shall be washed and incubated for 30 minutes at 37 °C with specific monoclonal antibodies (namely for VHSV identification MAb IP5B11 and for IHNV Hyb 136-3, respectively). 50 µl of horseradish-peroxidase (HRP) conjugated rabbit anti mouse antibodies diluted 1:1 000 in PBS-T-BSA shall be transferred to the ELISA plate.

Finally, after renewed washing the reactions shall be developed adding 50 µl/well of ortho-phenylenediamine (OPD). The ELISA plates shall be incubated for 20 minutes at room temperature in the dark and the reaction shall be stopped by adding 100 µl/well 0,5 M H_2SO_4.

The absorbance shall be monitored at a wavelength of 492 and 620 nm in an ELISA reader. Samples shall be designated positive or negative after comparing the test results to the absorbance values for the positive and negative controls. In general, samples with combined absorbance (A) < 0,5 for undiluted material shall be considered negative, samples with A values between 0,5 and 1,0 shall be considered suspicious and samples with A values > 1,0 shall be considered positive.

Other ELISA versions with a proven similar efficacy may be used instead of those referred to in this point.

I.6.2. Immunofluorescence- IF

The identification of listed pathogens VHSV and IHNV shall be performed by infecting cells in 'Black' 96-well plates, conventional 24-well plates or cover slips into 24-well plates. When IHNV or VHSV or both are identified by infecting cells on cover slips, the following protocol shall apply:

(a) cover slips shall be seeded with cells at a density leading to between 60 % and 90 % confluence after 24 hours of cultivation. EPC cells shall be used where possible for this purpose because of their strong adherence to glass surfaces, but other cell lines such as BF-2, RTG-2 or FHM may be used as well. 150 µl cell culture supernatant in two different dilutions (1:10 and 1:1 000) shall be inoculated in duplicate onto 1-day-old monolayers and incubated at 15 °C for 24 hours;

(b) subsequently, cell culture medium shall be removed, and the infected cell monolayers fixed with 0,5 ml ice-cold, aqueous acetone solution (80 % vol:vol). Fixation shall take place in fume hood for 15 minutes at room temperature, then the acetone solution shall be removed and the cover glasses shall be air dried for at least 30 minutes. At this stage, the plates shall either be processed immediately or stored at – 20 °C for further use;

(c) specific monoclonal antibodies (namely for VHSV identification, MAb IP5B11 and for IHNV, Hyb 136-3 respectively) shall be diluted in 0,01 M PBST, pH 7,2 in the dilution recommended by the provider of the MAbs; 50 to 100 µl/well shall be added to the fixed monolayer and plates shall be incubated for one hour at 37 °C in a humid chamber;

(d) cover glasses shall be washed gently three times with PBS containing 0,05 % Tween-20 (PBS-T), and the buffer shall be removed completely after the last rinse. The cells shall subsequently be incubated for one hour at 37 °C with fluorescein isothiocyanate (FITC) — or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibodies against mouse immunoglobulin used as the primary antibody, diluted according to the supplier instructions, washed again in PBS-T, and dried. Stained cultures shall be mounted onto glass slides using glycerol saline and examined under incident ultraviolet (UV) light. Use 10 × or 12 × eyepieces and a × 25 or × 40 objective lens with numerical apertures > 0,7 and > 1,3 respectively.

Other IF techniques, with regard to cell cultures, fixation and antibodies of reference quality, of proven similar efficacy may be used instead.

I.6.3. Neutralisation

Cells from the collected supernatant shall be removed by centrifugation (2 000 to 4 000 × *g*) or membrane filtration (0,45 µm) with a low protein binding membrane and the supernatant shall be diluted 1:100 and 1:10 000 in cell culture medium.

Aliquots of a minimum of two supernatant dilutions shall be mixed and incubated for 60 minutes at 15 °C with equal parts of the following reagents separately:

(a) serum containing group specific antibody against VHSV at a 1:50 (vol:vol) dilution;

(b) serum containing group specific antibody against IHNV at a 1:50 (vol:vol) dilution;

(c) pool of antisera against the indigenous serotypes of IPNV at a 1:50 (vol:vol) dilution;

(d) medium alone (positive control).

From each virus supernatant-serum mixture, at least two cell cultures shall be inoculated with 50 µl each and then incubated at 15 °C. The development of CPE shall be checked as described in point I.5.4.

VHSV strains and isolates that do not react in neutralisation tests shall be identified by IF or ELISA.

Other neutralisation tests of proven similar efficacy may be used instead.

I.6.4. RT-PCR/RT-qPCR

I.6.4.1. Preparation of viral RNA

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

RNA shall be extracted using the phenol-chloroform method or by RNA affinity spin columns, according to the manufacturer's instructions. Commercially available RNA extraction kits that will produce high quality RNA suitable for use with the RT-PCR protocols detailed in the points below may be used.

RNA shall be re-suspended in distilled RNAse-free water, (namely water treated with 0,1 % diethyl pyrocarbonate) or an appropriate elution buffer.

I.6.4.2. RT-PCR

The following primers shall be used for detection of IHNV:

Forward Primer 5′-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3′;

Reverse Primer 5′-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3′.

The following cycles shall be used (one-step RT-PCR): 1 cycle: 50 °C for 30 minutes; 1 cycle: 95 °C for 2 minutes; 30 cycles: 95 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 60 seconds; 1 cycle: 72 °C for 7 minutes and soak at 4 °C.

The following primers shall be used for detection of VHSV:

VN For 5′-ATG-GAA-GGA-GGA-ATT-CGT-GAA-GCG-3′;

VN Rev 5′-GCG-GTG-AAG-TGC-TGC-AGT-TCC-C-3′.

The following cycles shall be used (one-step RT-PCR): 50 °C for 30 minutes, 95 °C for 15 minutes, 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 68 °C for 60 seconds. Subsequently, the reaction shall be held at 68 °C for 7 minutes.

Quantity and specificity of the RT-PCR reactions shall be evaluated by gel electrophoresis in 1,5 % agarose gel with ethidium bromide and observed using UV transillumination. A 693 bp PCR amplicon may be observed for IHNV. For VHSV, the size shall be 505 bp.

The results of the PCR may vary depending on the conditions under which it is performed, namely the thermal protocols might need optimisation, depending on the thermal cycler in use. Furthermore, false-positive results may occur because of false primer annealing or laboratory contamination. Adequate positive and negative controls and sequence amplicons shall therefore be included to avoid any doubts. For the VHSV primers, special care shall be taken when using BF-2 cells, as the primers may react with the cell line DNA/RNA producing false-positive results of similar size. When testing supernatant from BF-2 cells, any amplified PCR fragments shall be sequenced.

I.6.4.3. RT-qPCR for VHSV

For VHSV, amplification shall be performed using the following primers and probe:

Forward primer: 5′-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3′;

Reverse primer: 5′-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3′;

and probe: 5′-FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1.

One-step RT-qPCR:

Negative template controls and positive controls shall be included on each plate run. Cycling conditions: 50 °C for 30 minutes, 95 °C for 15 minutes, 40 cycles of 94 °C for 15 seconds, 60 °C for 40 seconds, 72 °C for 20 seconds; adjust if necessary. Other RT-PCR or RT-qPCR versions of proven similar efficacy may be used instead.

I.6.4.4. RT-qPCR for IHNV

For IHNV, amplification shall be performed using the following primers and probe:

Forward primer: 5′- AGA-GCC-AAG-GCA-CTG-TGC-G-3′;

Reverse primer: 5′- TTCTTTGCGGCTTGGTTGA - 3′;

and probe: 5′ 6FAM-TGAGACTGAGCGGGACA-NFQ/MGB.

Two-step RT-qPCR:

As the following assay depends on a two-step amplification, extra care shall be taken when handling the tubes from one reaction to the other in order to prevent contamination.

Cycling conditions (after RT-step): 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes; adjustments shall be made if necessary.

Other RT-PCR or RT-qPCR versions of proven similar efficacy may be used instead.

II. **Detailed diagnostic methods and procedures for the confirmation of or to rule out the suspicion of VHS or IHN or both in suspected outbreaks**

When a laboratory examination is required to confirm or rule out the presence of IHN or VHS or both in accordance with Article 57(b) of Directive 2006/88/EC using the diagnostic methods set out in point II.3 of Part 1 of Annex I, the following detailed diagnostic methods and procedures shall apply:

(a) conventional virus isolation with subsequent seroneutralisation, immune-chemical or molecular virus identification;

(b) virus detection by RT-PCR or RT-qPCR;

(c) other diagnostic techniques such as IFAT, ELISA, RT-PCR, IHC.

II.1. Conventional virus isolation with subsequent virus identification

II.1.1. Selection of samples

At least 10 fish showing typical signs of IHN or VHS shall be selected for examination.

II.1.2. Preparation and shipment of samples from fish

The preparation and shipment for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.2.

II.1.3. Collection of supplementary diagnostic material

The collection of supplementary material for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.3.

II.1.4. Preparation of samples for cell culture examination

The preparation of samples for cell culture examination for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.4.

II.1.5. Virological examination on cell culture

The virological examination for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.5.

II.1.6. Virus identification

The virus identification for the purpose of conventional virus isolation shall follow the methods and procedures laid down in point I.6.

II.2. Virus detection by RT-qPCR

II.2.1. Selection of samples

The selection of samples for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.1.2.

II.2.2. Preparation and shipment of samples from fish

The preparation and shipment for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.2.

II.2.3. Collection of supplementary diagnostic material

The collection of supplementary diagnostic material for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.3.

II.2.4. Sample preparation for RT-qPCR

The sample preparation for the purpose of virus detection by RT-qPCR shall follow the methods and procedures laid down in point I.6.4.1.

II.2.5. RT-qPCR

The virus detection by RT-qPCR shall follow the methods and procedures laid down in points I.6.4.1, I.6.4.3 and I.6.4.4.

II.3. Other diagnostic techniques

Supernatant prepared as described in point I.4.3 may be submitted to ELISA, Indirect fluorescent antibody test (IFAT), or RT-PCR in accordance with point I.6.1, point I.6.2 or point I.6.4 respectively. Tissue material may be subjected to other diagnostic techniques, such as IFAT on frozen sections, immunohistochemistry on formalin fixed tissue material. Those rapid techniques shall be supplemented with a virological examination in accordance with either point II(a) or point II(b) within 48 hours after sampling, if:

(a) a negative result is obtained; or

(b) a positive result is obtained with material representing the first case of IHN or VHS.

III. **Procedure for titration to verify the susceptibility of the cell cultures to infection**

When titration to verify the susceptibility of the cell cultures to infection as referred to in point I.5.3 is carried out, the procedures set out in the following paragraphs of this point shall be followed.

At least two VHSV isolates and one isolate of IHNV shall be used. The isolates shall represent the major group of viruses within the European Union, namely for VHSV one pathogenic isolate from rainbow trout in freshwater and one marine isolate pathogenic for turbot, and for IHNV one rainbow trout pathogenic strain from the European Union. Well-defined isolates from the Member States shall be used. Batches of virus in low cell culture passage numbers shall be propagated in cell culture flasks on BF-2 or RTG-2 cells for VHSV and on EPC or FHM cells for IHNV. Cell culture medium with at least 10 % serum shall be used. Low MOI for inoculation (< 1) shall be used.

At total CPE, the virus shall be harvested by centrifugation of cell culture supernatant at 2 000 × *g* for 15 minutes, filter sterilised through 0,45 µm membrane filter and distributed in labelled cryotubes. The virus shall be kept at – 80 °C.

1 week after freezing, three replicate vials with each virus shall be thawed under cold water and titrated on their respective cell lines. At least every 6 months, or if it is suspected that the susceptibility of a cell line has decreased, each virus isolate shall be thawed and titrated.

Titration procedures must be described in detail and the same procedure followed each time.

Titration by end point dilution shall include at least six replicates at each dilution step. The titres shall be compared with previously obtained titres. If the titre of any of the three virus isolates drops by a factor of 2 logs or more, compared with the initial titre, the cell line shall no longer be used for surveillance purposes.

If different cell lines are kept in the laboratory each line shall be examined separately.

Records shall be kept for a period of at least 10 years.