

II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DECISION

of 22 February 2001

laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases and repealing Decision 92/532/EEC

(notified under document number C(2001) 426)

(Text with EEA relevance)

(2001/183/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 91/67/EEC of 28 January 1991 concerning the animal health conditions governing the placing on the market of aquaculture animals and products ⁽¹⁾, as last amended by Directive 98/45/EC ⁽²⁾, and in particular Article 15 thereof,

Whereas:

- (1) The sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases have been laid down in Commission Decision 92/532/EEC ⁽³⁾, as amended by Commission Decision 96/240/EC ⁽⁴⁾.
- (2) Since the time of adoption of Decision 92/532/EEC, new practical and scientific developments have taken place and Directive 91/67/EEC has been amended. This requires the sampling plans and diagnostic methods to be updated.
- (3) Such updating relates to the examination and identification of viruses causing viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) and to changes in accordance with the last amendments of Directive 91/67/EEC.
- (4) The Community reference laboratory for fish diseases, established by Council Directive 93/53/EEC ⁽⁵⁾, has been consulted.
- (5) The sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases intro-

duced by Decision 92/532/EEC must be repealed for the sake of clarity.

- (6) The measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

The sampling plans and diagnostic methods for the detection and confirmation of viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) are laid down in the Annex.

Article 2

The present Decision shall repeal Decision 92/532/EEC.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 22 February 2001.

For the Commission

David BYRNE

Member of the Commission

⁽¹⁾ OJ L 46, 19.2.1991, p. 1.

⁽²⁾ OJ L 189, 3.7.1998, p. 12.

⁽³⁾ OJ L 337, 21.11.1992, p. 18.

⁽⁴⁾ OJ L 79, 29.3.1996, p. 19.

⁽⁵⁾ OJ L 175, 19.7.1993, p. 23.

ANNEX

SAMPLING PLANS AND DIAGNOSTIC METHODS FOR THE DETECTION AND CONFIRMATION OF VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS) AND INFECTIOUS HAEMATOPOIETIC NECROSIS (IHN)

INTRODUCTION

This Annex:

- (a) provides for guidelines and minimum requirements on sampling plans and diagnostic methods for the detection and confirmation of the presence of viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN);
- (b) integrates the provisions of Annexes B and C to Directive 91/67/EEC for the approval of and the maintenance of status of zones and of farms in non-approved zones;
- (c) sets out provisions aiming at the proper diagnosis of VHS and IHN and at the official recognition of the status of zones and of farms in non-approved zones in accordance with Articles 5 and 6 of Directive 91/67/EEC;
- (d) is directed towards both the authorities responsible for the control of VHS and IHN and the laboratory personnel performing the tests with regards to these diseases. Accordingly, emphasis is put on the sampling procedures, principles and applications of laboratory tests and evaluation of their results, as well as on detailed laboratory techniques. However, when appropriate the laboratories may apply modifications to the tests described in this Annex or use different tests, provided that an equal sensitivity and specificity can be demonstrated.

Part I includes sampling plans and diagnostic methods for the surveillance of VHS and IHN in order to obtain and maintain approved status of a zone or a farm in a non-approved zone.

Part II describes the diagnostic procedures for the confirmation of VHS and IHN in the event of a suspicion.

Part III sets out the criteria and guidelines for an official health inspection programme, documenting historical freedom from VHS and/or IHN.

Part IV gives recommendations on the procedure for VHS and IHN virus titration to verify the susceptibility of the cell cultures to infection.

Acronyms and abbreviations are listed in Part V.

PART I

Sampling plans and diagnostic methods for VHS and IHN surveillance in order to obtain and maintain approved status of a zone or a farm in a non-approved zone**I. Inspections and sampling**

1. *General provisions on clinical health inspections, collection and selection of samples for surveillance of zones or farms in non-approved zones in order to achieve or maintain approved status for VHS and/or IHN*

The clinical health inspections and the sampling of fish tissue and/or ovarian fluid to be carried out in zones or in farms in non-approved zones in order to achieve or maintain approved status for VHS and/or IHN in accordance with Annexes B and C to Directive 91/67/EEC are summarised in Tables 1A, 1B and 1C. Further details are laid down in Parts I.I.2 to I.I.4. Tables 1A and 1B shall not be applicable to new farms and farms which recommence their activities with fish, eggs or gametes from an approved zone or an approved farm in a non-approved zone, provided that they comply with the requirements laid down in Directive 91/67/EEC, Annex C, Part I.A.6(a) or I.A.6(b) or II.A.3(a) or II.A.3(b).

The clinical inspections must be carried out during the period October to June or whenever the water temperature is below 14 °C. When farms are to be clinically inspected twice per year, the intervals between the inspections must be at least four months. All production units (ponds, tanks, net-cages, etc.) must be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention has to be paid to the water outlet area where weak fish tend to accumulate because of the water current.

Fish to be sampled shall be selected as follows.

- If rainbow trout are present, only fish of that species shall be selected for sampling. If rainbow trout are not present, the sample has to be obtained from fish of all other species present whenever these species are susceptible to VHSV and/or IHNV (as listed in Annex A to Directive 91/67/EEC). The species have to be proportionally represented in the sample.
- If more than one water source is utilised for fish production, fish representing all water sources must be included for sampling.
- If weak, abnormally behaving or freshly dead (not decomposed) fish are present, primarily such fish shall be selected. If such fish are not present, the fish selected must include normally appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

2. *Specific provisions, including collection of samples, for surveillance of zones or farms in non-approved zones in order to achieve or maintain approved status for VHS and/or IHN*

1. A zone or a farm in a non-approved zone that is placed under the supervision of the official services can obtain an approved status for VHS and/or IHN subject to either:

(a) Model A — two-year surveillance programme

Following at least two years of absence of any clinical or other sign of VHS and/or IHN, all farms in the zone or any farm in a non-approved zone which are to be approved must be health-inspected twice per year for two years. During this two-year control period, which precedes achievement of approved status, the absence of clinical or other signs of VHS and/or IHN must have continued and samples must be collected for examination in accordance with Table 1A. Further, the samples must be selected, prepared and examined as described in Parts I.I to I.IV and the laboratory examinations must have produced negative results for VHS and/or IHN; or

(b) Model B — two-year surveillance programme with reduced sample size

Following an official health inspection programme documenting the historical freedom from VHS and/or IHN for at least four years, all farms in the zone or any farm in a non-approved zone which are to be approved must be health-inspected twice per year for two years. During this two-year control period, which precedes achievement of approved status, the absence of clinical or other signs of VHS and/or IHN must have continued and samples must be collected for examination in accordance with Table 1B. Further, the samples must be selected, prepared and examined as described in Parts I.I to I.IV and the laboratory examinations must have produced negative results for VHS and/or IHN. In order that a health inspection programme might be recognised by the official services for documentation of freedom with regards to VHS and/or IHN, it must fulfil the criteria and guidelines set out in Part III.

2. Special provisions for approval of new farms and farms which recommence their activities with fish, eggs or gametes from an approved zone or an approved farm in a non-approved zone

New farms and farms which recommence their activities with fish, eggs or gametes from an approved zone or an approved farm in a non-approved zone may obtain status in accordance with the requirements laid down in Directive 91/67/EEC, Annex C, I.A.6a/b or II.A.3.a/b. Accordingly, the sampling provisions laid down in Models A and B above (Parts I.I.2.1.a and I.I.2.1.b) shall not be applicable to such farms.

3. Surveillance programme for maintenance of approved status for VHS and/or IHN

In order to maintain approved status of a zone or a farm in a non-approved zone for VHS and/or IHN, inspections and sampling for examination must be carried out in accordance with Table 1C. The samples must be selected, prepared and examined as described in Parts I.I. to I.IV and the laboratory examinations must be negative as regards to the agents of VHS and/or IHN.

3. *Preparation and shipment of samples from fish*

Before shipment or transfer to the laboratory pieces of the organs to be examined must be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10 % calf serum and antibiotics. The combination of 200 iu penicillin, 200 µg streptomycin, and 200 µg kanamycin per millilitre (ml) can be recommended, but other antibiotics of proven efficiency may be used as well. The tissue material to be examined is spleen, anterior kidney, and, in addition, either heart or cephalon. In some cases, ovarian fluid must be examined (Tables 1A to C).

Ovarian fluid or organ pieces from a maximum of 10 fish (Tables 1A to C) may be collected in one sterile tube containing at least 4 ml transport medium and represent one pooled sample. The tissue in each sample should weigh a minimum of 0,5 gram (g).

The tubes should 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. The temperature of a sample during transit should never exceed 10 °C and ice should still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen.

The virological examination must be started as soon as possible and not 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 transport medium and that the temperature requirements during transportation can be fulfilled (Part I.I.3, paragraph 3).

Whole fish may be sent to the laboratory if the temperature requirements during transportation can be fulfilled. Whole fish may be wrapped up in paper with absorptive capacity and must finally be shipped in a plastic bag, chilled as mentioned. Live fish can be shipped as well.

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

4. *Collection of supplementary diagnostic material*

According to agreement with the involved diagnostic laboratory, other fish tissues may be collected as well and prepared for supplementary examinations.

II. *Preparation of samples for virological examination*

1. *Freezing in exceptional cases*

Where practical difficulties arise (e.g. bad weather conditions, non-working days, laboratory problems, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples it is acceptable to freeze the tissue specimens in cell culture medium at - 20 °C or below and carry out virological examination within 14 days. The tissue, however, must be frozen and thawed only once before examination. Records must be kept with details on the reason for each freezing of tissue samples (such as storm, cell lines died, etc.)

2. *Homogenisation of organs*

In the laboratory the tissue in the tubes must 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 consisted of whole fish less than 4 cm long, these should be minced with sterile scissors or scalpel after removal of the body behind the gut opening. If a sample consisted of whole fish with body length between 4 cm and 6 cm, the viscera including kidney should be collected. If a sample consisted of whole fish more than 6 cm long, tissue specimens should be collected as described in Part I.I.3. The tissue specimens should be minced with sterile scissors or scalpel and homogenised as described above and suspended in transport medium.

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

3. *Centrifugation of homogenate*

The homogenate is 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 treated for either four hours at 15 °C or overnight at 4 °C with antibiotics, e.g. gentamicin 1 mg/ml may be useful at this stage.

If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted.

The antibiotic treatment aims at controlling bacterial contamination in the samples and makes filtration through membrane filters unnecessary.

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

(!) In exceptional cases e.g. when fish are collected in very remote areas with no possibility of daily mailing.

Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.) which make it impossible to inoculate cells within 48 hours after the collection of the tissue samples, it is acceptable to freeze the supernatant at -80°C and carry out virological examination within 14 days.

Prior to the inoculation of the cells the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of 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 must be at least 1/2000 in a 50 % plaque neutralisation test.

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 VHSV or IHNV.

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

III. *Virological examination*

1. *Cell cultures and media*

BF-2 or RTG-2 and either EPC or FHM cells are grown at 20°C to 30°C in suitable medium, e.g. Eagle's 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, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be $7,6 \pm 0,2$.

Cell cultures to be used for inoculation with tissue material should be young (4 to 48 hours old) and actively growing (not confluent) at inoculation.

2. *Inoculation of cell cultures*

Antibiotic-treated organ suspension is inoculated into cell cultures in two dilutions, i.e. 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 have to be inoculated (See Part I. III.1). The ratio between inoculum size and volume of cell culture medium should 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, has to be utilised. Use of cell culture trays is recommended, but other units of similar or bigger growth area are acceptable as well.

3. *Incubation of cell cultures*

Inoculated cell cultures are incubated at 15°C for 7 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 has to be performed to ensure cell susceptibility to virus infection.

At least every six months or if decreased cell susceptibility is suspected, titration of frozen stocks of VHSV and IHNV is performed to verify the susceptibility of the cell cultures to infection. A recommended procedure is presented in Part IV.

4. *Microscopy*

Inoculated cell cultures must be inspected regularly (at least three times a week) for the occurrence of CPE at 40 to 150 \times magnification. If obvious CPE is observed, virus identification procedures according to Part I.IV have to be initiated immediately.

5. *Subcultivation*

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

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to cell line 7 to 10 days after inoculation. The pools are then 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 Part I.III.2. Alternatively aliquots of 10 % of the medium constituting the primary culture is inoculated directly into a well with fresh cell culture (well to well subcultivation). The inoculation may be preceded by preincubation of the dilutions with the antiserum to IPN virus at appropriate dilution as described in Part I.II.3.

The inoculated cultures are then incubated for 7 to 10 days at 15 °C with observation as in Part I.III.4.

If toxic CPE occurs within the first three days of incubation, subcultivation may be performed at that stage, but the cells must then be incubated for seven days' and subcultivated again with a further seven days' incubation. When toxic CPE develops after three days, the cells may be passed once and incubated to achieve the total of 14 days from the primary inoculation. There should be no evidence of toxicity in the final seven days of incubation.

If bacterial contamination occurs despite treatment with antibiotics, subcultivation must be preceded by centrifugation at 2 000 to 4 000 × g for 15 to 30 min at 2 to 5 °C, and/or filtration of the supernatant through a 0,45 µm filter (low protein-binding membrane). In addition to this, subcultivation procedures are the same as for toxic CPE.

IV. *Virus identification*

1. *Virus identification tests*

If evidence of CPE has been observed in a cell culture, medium (supernatant) is collected and examined by one or more of the following techniques: neutralisation, IF, ELISA. 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.

2. *Neutralisation*

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

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

- serum containing group specific antibody against VHSV at a 1:50 (vol:vol) dilution ⁽¹⁾
- serum containing group specific antibody against IHNV at a 1:50 (vol:vol) dilution ⁽¹⁾
- pool of antisera against the indigenous serotypes of IPNV at a 1:50 (vol:vol) dilution ⁽¹⁾
- medium alone (positive control)

From each virus supernatant-serum mixture at least two cell cultures are inoculated with 50 µl each and then incubated at 15 °C. Development of CPE is checked as described in Part I.III.4.

Some VHSV strains do not react in neutralisation tests. Such isolates must be identified by IF or ELISA.

Other neutralisation tests of proven efficiency may be used alternatively.

3. *IF*

For each virus isolate to be identified, at least eight coverglasses or equivalent are seeded with cells at a density leading to about 60 % to 90 % confluence after 24 hours of cultivation. EPC cells are recommended 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.

When the cells have sedimented onto the glass surface (about one hour after seeding), or when the cultures have been incubated for up to 24 hours, the virus to be identified is inoculated. Four cultures are inoculated at a volume-to-volume ratio to 1:10, and four cultures at a ratio of 1:1 000. These are then incubated at 15 °C for 20 to 30 hours.

After incubation, the cultures are rinsed twice in Eagle's MEM without serum, fixed in 80 % ice cold acetone and then stained by means of a two-layer IFAT. The first reagent layer consists of polyclonal or monoclonal antibodies of reference quality. The second reagent layer is a fluorochrome-conjugated antiserum to the immunoglobulin used in the first layer. For each of the antisera tested at least one high-dose and one low-dose inoculated culture have to be stained. Proper negative and positive controls have to be included in the test. Fluorochromes such as FITC or TRITC are recommended.

Mount stained cultures using glycerol saline. Examine under incident ultraviolet (UV) light. Use 10 × or 12 × eyepieces and × 25 or × 40 objective lens with numerical apertures > 0,7 and > 1,3, respectively.

The above IF technique is given as an example. Other IF techniques (with regard to cell cultures, fixation and antibodies of reference quality) of proven efficiency may be used alternatively.

⁽¹⁾ Or as specified by the reference laboratory with regard to the possible cytotoxicity of the antisera.

4. ELISA

Wells in microtitre plates are coated overnight with recommended dilutions of purified immunoglobulin fractions of antibodies of reference quality.

After rinsing of wells with PBS-Tween-20 buffer, the virus to be identified is added to the wells in two- or four-fold dilution steps and allowed to react with the coating antibody for 60 minutes at 37 °C. Following rinsing with PBS-Tween-20 buffer, biotinylated antibodies of a specificity corresponding to that of the coating antibodies are added and allowed to react for 60 minutes at 20 °C. Following another rinse as above, HRP-conjugated streptavidin is added and allowed to react for one hour at 20 °C. After a last rinse, bound enzyme is visualised using appropriate ELISA substrates (OPD or others).

The above biotin-avidin based ELISA version is given as an example. Other ELISA versions of proven efficiency may be used instead.

TABLE 1A

Inspection and sampling scheme for zones and for farms in non-approved zones for the two-year control period which precedes achievement of approved status for VHS and/or IHN

(in accordance with Directive 91/67/EEC, Annexes B and C and the provisions set out in Part I of this Annex)

	Number of clinical inspections per year (two years)	Number of laboratory examinations per year (two years)	Laboratory examination for presence of virus ⁽¹⁾	
			Number of growing fish (Organ material)	Number of broodstock fish (Ovarian fluid)
Continental zones and farms				
(a) Farms with broodstock	2	2	120 (first inspection) ⁽²⁾ 150 (second inspection)	30 (first inspection) ⁽³⁾ 0 (second inspection)
(b) Farms with broodstock only	2	1	0	150 (first or second inspection) ⁽³⁾
(c) Farms without broodstock	2	2	150 (first and second inspections)	0
Coastal zones and farms				
(a) Farms with broodstock	2	2	120 (first inspection) 150 (second inspection)	30 (first inspection) ⁽³⁾ 0 (second inspection)
(b) Salmonid farms without broodstock	2	2	30 (first and second inspections) ⁽⁴⁾	0
(c) Non-salmonid farms without broodstock	2	2	150 (first and second inspections)	0

Maximum number of fish per pool: 10

⁽¹⁾ Alternatively, a reduced sample size as listed in Table 1B may be used if requirements as described in the text Parts II.1, I.I.2.1.b and III are fulfilled.

⁽²⁾ Clinical inspections.

⁽³⁾ In exceptional circumstances, if it is impossible to obtain ovarian fluid, organs may be sampled instead.

⁽⁴⁾ The samples have to be collected no sooner than three weeks after transfer of fish from fresh to saltwater.

TABLE 1B

Inspection and sampling scheme for the two-year control period which precedes achievement of approved status for VHS and/or IHN in zones and farms in non-approved zones with an officially recognised documented history of freedom from these diseases

(in accordance with Directive 91/67/EEC, Annexes B and C and the provisions set out in Parts I and III of this Annex)

	Number of clinical inspections per year (two years)	Number of laboratory examinations per year (two years)	Laboratory examination for presence of virus	
			Number of growing fish (Organ material)	Number of broodstock fish (Ovarian fluid)
Continental zones and farms				
(a) Farms with broodstock	2	2	0 (first inspection) ⁽¹⁾ 30 (second inspection)	30 (first inspection) ⁽²⁾ 0 (second inspection)
(b) reine Brutanlagen	2	1	0	30 (first or second inspection) ⁽²⁾
(c) Farms without broodstock	2	2	30 (first and second inspections)	0
Coastal zones and farms				
(a) Farms with broodstock	2	2	0 (first inspection) 30 (second inspection)	30 (first inspection) ⁽²⁾ 0 (second inspection)
(b) Salmonid farms without broodstock	2	2	30 (first and second inspections) ⁽³⁾	0
(c) Non-salmonid farms without broodstock	2	2	30 (first and second inspections)	0

Maximum number of fish per pool: 10

⁽¹⁾ Clinical inspections.

⁽²⁾ In exceptional circumstances, if it is impossible to obtain ovarian fluid, organs may be sampled instead.

⁽³⁾ The samples have to be collected no sooner than three weeks after transfer of fish from fresh to saltwater.

TABLE 1C

Inspection and sampling scheme for zones and farms in non-approved zones in order to maintain approved status for VHS and/or IHN

(in accordance with Directive 91/67/EEC, Annexes B and C and the provisions set out in Part I of this Annex)

	Number of clinical inspections per year	Number of fish in sample group for laboratory examination ⁽¹⁾	
		Number of growing fish (Organ material)	Number of broodstock fish (Ovarian fluid)
Continental zones and farms			
(a) Farms with broodstock	2	20 (first or second inspection)	10 (first or second inspection) ⁽²⁾

	Number of clinical inspections per year	Number of fish in sample group for laboratory examination ⁽¹⁾	
		Number of growing fish (Organ material)	Number of broodstock fish (Ovarian fluid)
(b) Farms with broodstock only	2	0	30 (first or second inspection) ⁽²⁾
(c) Farms without broodstock	2	30	0
Coastal zones and farms			
(a) Farms with broodstock	2	20 (first or second inspection)	10 (first or second inspection) ⁽²⁾
(b) Farms without broodstock	1	30 ⁽³⁾	0

Maximum number of fish per pool: 10

⁽¹⁾ In approved zones samples only have to be collected by rotation in 50 % of the fish farms each year. In approved farms in non-approved zones samples have to be collected every year.

⁽²⁾ In exceptional circumstances, if it is impossible to obtain ovarian fluid, organs may be sampled instead.

⁽³⁾ The samples have to be collected no sooner than three weeks after transfer of fish from fresh to saltwater.

PART II

Diagnostic procedures for the confirmation of VHS and IHN in suspected outbreaks

One or more of the following techniques shall be carried out for the diagnosis of VHS and IHN:

- A. conventional virus isolation with subsequent serological virus identification,
- B. virus isolation with simultaneous serological virus identification,
- C. other diagnostic techniques (IFAT, ELISA).

The confirmation of the first case of VHS and/or IHN in farms in approved zones must not be based on method C alone. Either method A or B must also be used.

The tissue material meant for virological examination may in some cases have to be accompanied by supplementary material for bacteriological, parasitological, histological or other examination to allow for a differential diagnosis.

A. Conventional virus isolation with subsequent serological virus identification

I.1. Selection of samples

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

I.2. Preparation and shipment of samples from fish

As laid down in Part I.I.3

I.3. Collection of supplementary diagnostic material

As laid down in Part I.I.4

II. Preparation of samples for virological examination

As laid down in Part I.II

III. Virological examination

As laid down in Part I.III

IV. Virus identification

As laid down in Part I.IV

B. Virus isolation with simultaneous serological virus identification

I.1. Selection of samples

As laid down in Part II.A.I.1

I.2. Preparation and shipment of samples from fish

As laid down in Part I.I.3

I.3. Collection of supplementary diagnostic material

As laid down in Part I.I.4

II.1. Homogenisation of organs

As laid down in Part I.II.2

II.2. Centrifugation of homogenate

The homogenate is 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 treated for four hours at 15 °C with antibiotics, e.g. gentamicin 1 mg/ml, or membrane filtered (0,45 µm) through a low protein binding membrane.

II.3. Treatment of supernatant with diagnostic antisera

The antibiotic treated or membrane filtered organ suspension is diluted 1:10 and 1:1 000 in cell culture medium and aliquots mixed and incubated for 60 minutes at 15 °C with equal parts of the reagents listed in Part I.IV.2.

III.1. Cell cultures and media

As laid down in Part I.III.1

III.2. Inoculation of cell cultures

From each virus-serum mixture (prepared as laid down in Part II.B.II.3) at least two cell cultures per cell line are inoculated with 50 µl each.

III.3. Incubation of cell cultures

As laid down in Part I.III.I

III.4. Microscopy

Inoculated cell cultures are inspected daily for the occurrence of CPE at 40 to 150 × magnification. If CPE is prevented by one of the antisera used, the virus can be considered to be identified accordingly.

If CPE is not prevented by any of the antisera, virus identification procedures according to Part I.IV have to be performed.

III.5. Subcultivation

If no CPE has occurred after 7 to 10 days, subcultivation has to be performed from cultures inoculated with supernatant plus medium (Part II.B.II.3) according to Part I.III.5.

C. Other diagnostic techniques

Supernatant prepared as described in Part I.II.2 can be submitted to IFAT or ELISA according to Part I.IV.3 or Part I.IV.4 respectively. These rapid techniques have to be supplemented with a virological examination according to either A or 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 in an approved zone.

Tissue material may be subjected to other diagnostic techniques such as RT-PCR, IF on frozen sections immunohistochemistry on formaline fixed tissue material. These techniques must always be accompanied by inoculation of non-fixed tissue material on cell cultures.

PART III

Documented history of freedom from VHS and/or IHN in zones or farms in non-approved zones

Guidelines and criteria for an official health inspection programme

1. A health inspection programme can only be initiated either:
 - after an officially recognised eradication programme for VHSV and/or IHNV including removal of all fish in the farm, cleaning, disinfection and fallowing before restocking with fish from approved farms, or
 - on fish farms with no history of VHSV or IHNV infection.
2. The health inspection programme must be based on both clinical inspections and laboratory examinations.
3. The programme must include two annual clinical health inspections according to the guidelines given in Part I.

4. At least at one of the inspections carried out each year, 30 fish tissue and/or ovarian fluid samples shall be collected from each farm. The samples shall be selected, prepared and subjected for laboratory examination according to Parts I, II and IV.
5. The health inspection programme shall be conducted for at least four years in all farms in the zone or in the farm (in a non-approved zone) to be approved.
6. For the official recognition of the programme, no cases of VHS or IHN shall occur or be detected (no clinical infections or virus isolations).

PART IV

Procedure for titration to verify the susceptibility of the cell cultures to infection

Recommended procedures for titration referred to in Part LIII3 are given below.

At least two VHSV isolates and one isolate of IHNV should be used. The isolates should represent the major group of viruses within the EU, e.g. 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 Europe. Well-defined isolates from the Member States should be used. Reference isolates are available from the EU reference laboratory for fish diseases.

Batches of virus in low cell culture passage numbers are 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 should be used. Use low MOI for inoculation (< 1).

At total CPE, virus is harvested by centrifugation of cell culture supernatant at $2\,000 \times g$ for 15 minutes, filter sterilised through $0,45 \mu\text{m}$ membrane filter and distributed in labelled cryotubes. The virus is kept at $-80 \text{ }^\circ\text{C}$.

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

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

Titration by end point dilution should include at least six replicates at each dilution step. The titres are 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 should no longer be used for surveillance purposes.

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

Records should be kept for at least 10 years.

PART V

Acronyms and abbreviations

BF-2	Bluegill fry -2 (cell line)
CPE	Cytopathic effect
CRL	Community reference laboratory for fish diseases
ELISA	Enzyme-linked immunosorbent assay
EPC	<i>Epithelioma papulosum cyprini</i> (cell line)
FHM	Fathead minnow (cell line)
FITC	Fluorescein isothiocyanate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HRP	Horseradish peroxidase
IF	Immunofluorescence
IFAT	Indirect fluorescent antibody test
IHN(V)	Infectious haematopoietic necrosis (virus)
IPN	Infectious pancreatic necrosis (virus)
MEM	Minimum essential medium

MOI	Multiplicity of infection (ratio of number of infectious virus particles added to a known number of cells in a culture)
OPD	Ortho phenylene diamine
PBS	Phosphate buffered saline
RTG-2	Rainbow trout gonad (cell line)
RT-PCR	Reversed transcriptase polymerase chain reaction
Tris-HCl	Tris (hydroxymethyl) aminomethane — HCl
TRITC	Tetramethyl-rhodamine-isothiocyanate
VHS(V)	Viral haemorrhagic septicaemia (virus)
