II

(Non-legislative acts)

DECISIONS

COMMISSION IMPLEMENTING DECISION (EU) 2015/1554

of 11 September 2015

laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods

(notified under document C(2015) 6188)

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals [1], and in particular Articles 49(3), 50(4), 57(b) and 61(3) thereof,

Whereas:

(1) Directive 2006/88/EC lays down minimum preventive measures for the surveillance and early detection in aquatic animals of the listed diseases set out in Annex IV to that Directive ('the listed diseases') and the control measures to be applied in the event of the suspicion of or an outbreak of the listed diseases. It also lays down the requirements for the achievement of disease-free status for Member States or for zones or compartments thereof.

(2) The eradication of the listed diseases and the achievement of disease-free status for a Member State, zone or compartment should be based on the same principles and follow the same scientific approach throughout the Union. For that reason, it is necessary to lay down at Union level specific requirements for eradication and surveillance schemes, and the sampling and diagnostic methods to be used by the Member States to obtain disease-free status for the whole of a Member State or a zone or compartment thereof.

(3) The laboratory examinations to be carried out in the event of the suspicion or confirmation of the presence of the listed diseases, should be the same at Union level and should follow the same scientific standards and protocols. In accordance with Directive 2006/88/EC, it is necessary to establish specific diagnostic methods and procedures to be used by the laboratories designated for that purpose by the competent authority of the Member States.

(4) The Aquatic Animal Health Code adopted by the World Organisation for Animal Health (OIE) (the 'Aquatic Code') sets out standards for improving the health of aquatic animals and the welfare of farmed fish worldwide, including standards for safe international trade in aquatic animals and products thereof. A number of chapters of the Aquatic Code set out recommendations concerning the use of certain diagnostic tests. Such tests provided for by the OIE are laid down in the OIE Manual of Diagnostic Tests for Aquatic Animals (the 'Aquatic Manual'). In order to ensure that Union's requirements with regard to aquatic animal disease diagnostics are consistent with international standards, the rules laid down in this Decision should take into account the standards and recommendations of the Aquatic Code.

[1] OJ L 328, 24.11.2006, p. 14.

(5) In this respect, for many of the listed diseases, the Aquatic Manual lists several tests and procedures to be used for the purpose of laboratory examinations. In order to uniform the scientific basis for the diagnostic work for the listed diseases at Union level, it is necessary to choose among the diagnostic tests and procedures recommended by the OIE and to specify which tests should be mandatory for the purpose of laboratory examination when carrying out surveillance programmes and to rule out the suspicion of or to confirm the presence of the listed diseases. While there also will be a need for having alternative methods and procedures available in certain cases, descriptions and some scientific explanations for when and how the alternative methods could be applied should be provided. This is in particular necessary for the more detailed diagnostic procedures.

(6) In order to produce precise and reproducible diagnostic results, it is important that the detailed procedures and protocols to be used are validated in accordance with the relevant quality standards referred to in Part I of Annex VI to Directive 2006/88/EC. For many of the diagnostic methods provided for in this Decision, the use of commercial test kits is a necessary part of the diagnostic protocols and those test kits have been validated in accredited tests by the European Reference laboratories (EURL) for the respective diseases. In the interest of legal certainty, the commercial names of those validated commercial test kits should be referred to in this Decision.

(7) It may be difficult for certain Member States to achieve disease-free status for the whole of the Member State or for a zone or compartment thereof with regard to one or more of the listed diseases. In such situations the Member State may wish not to obtain or regain disease-free status for those listed diseases. The minimum control measures to be applied in cases where the Member State concerned does not wish to obtain or regain disease-free status, should be the same at Union level and should follow the same criteria. It is therefore necessary in accordance with Directive 2006/88/EC to lay down detailed rules for the containment of those listed diseases and the minimum requirements for the lifting of those containment measures.

(8) Commission Decision 2001/183/EC ([1]) lays down the requirements concerning sampling plans and diagnostic methods for the detection and confirmation of the listed diseases infectious haematopoietic necrosis and viral haemorrhagic septicaemia. Commission Decision 2003/466/EC ([2]) lays down requirements concerning sampling plans and diagnostic methods for the detection of infectious salmon anaemia, as well as criteria for zoning and official surveillance following suspicion and confirmations of the presence of that disease. Commission Decision 2002/878/EC ([3]) lays down the requirements concerning the sampling plans and the diagnostic methods for the detection and confirmation of the mollusc diseases Bonamiosis and Marteiliosis. In order to update the requirements, those three Decisions should be replaced by this Decision. Accordingly, Decision 2001/183/EC, Decision 2002/878/EC and Decision 2003/466/EC should be repealed.

(9) As certain Member States require time to update their national reference laboratories in order to comply with the requirements laid down in this Decision, it should apply from 1 April 2016.

(10) The measures laid down in this Decision are in accordance with the opinion of the Standing Committee on Plants, Animals, Food and Feed,

HAS ADOPTED THIS DECISION:

Article 1

Subject matter

This Decision lays down rules for the following:

(a) the surveillance, buffer zones, sampling and diagnostic methods to be used by Member States in connection with the disease status of the Member States or zones or compartments thereof for the non-exotic aquatic animal diseases listed in Part II to Annex IV to Directive 2006/88/EC (the 'listed diseases');

([1]) Commission Decision 2001/183/EC 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 (OJ L 67, 9.3.2001, p. 65).

([2]) Commission Decision 2003/466/EC of 13 June 2003 establishing criteria for zoning and official surveillance following suspicion or confirmation of the presence of infectious salmon anaemia (ISA) (OJ L 156, 25.6.2003, p. 61).

([3]) Commission Decision 2002/878/EC of 6 November 2002 establishing the sampling plans and diagnostic methods for the detection and confirmation of the presence of the mollusc diseases Bonamiosis (*Bonamia ostreae*) and Marteiliosis (*Marteilia refringens*) (OJ L 305, 7.11.2002, p. 57).

(b) the diagnostic methods to be used for laboratory examinations in the case of the suspicion or confirmation of the presence of listed diseases; and

(c) the minimum control measures to be applied in the event of the suspicion or confirmation of a listed disease in a Member State, zone or compartment not declared free of that listed disease.

Article 2

Definitions

For the purpose of this Decision, the following definitions shall apply:

(a) 'viral haemorrhagic septicaemia' ('VHS') means a disease caused by the viral haemorrhagic septicaemia virus (VHSV), also known as the Egtved virus, a virus belonging to the genus *Novirhabdovirus*, within the family *Rhabdoviridae*;

(b) 'infectious haematopoietic necrosis' ('IHN') means a disease caused by infectious hematopoietic necrosis virus (IHNV), a virus belonging to the genus *Novirhabdovirus*, within the family *Rhabdoviridae*;

(c) 'koi herpesvirus disease' ('KHVD') means a disease caused by koi herpesvirus (KHV), which belongs to the family of *Alloherpesviridae*. The scientific name is cyprinid herpesvirus 3 (CyHV-3);

(d) 'infectious salmon anaemia' ('ISA') means a disease caused by infection with highly polymorphic region (HPR)-deleted salmon anaemia virus (ISAV), a virus belonging to the genus *Isavirus*, within the family *Orthomyxoviridae*;

(e) 'infection with *Marteilia refringens*' means a disease caused by an infection with the paramyxean protozoan *Marteilia refringens*;

(f) 'infection with *Bonamia ostreae*' means a disease caused by an infection with the haplosporidian protozoan *Bonamia ostreae*;

(g) 'white spot disease' ('WSD') means a disease caused by white spot syndrome virus (WSSV), which is a double stranded DNA virus of the genus *Whispovirus*, in the family *Nimaviridae*.

Article 3

Minimum requirements for eradication and surveillance programmes

Member States shall ensure that the rules on surveillance and eradication programmes, buffer zones, sampling and diagnostic methods set out in Annex I and the specific methods and detailed procedures set out in Annex II are complied with, when disease-free status is to be granted, withdrawn or restored for a Member State or for a zone or compartment thereof for one or more of the listed diseases.

Article 4

Minimum requirements for diagnostic methods and specific procedures

Member States shall ensure that the control methods set out in Annex I and the specific diagnostic methods and detailed procedures set out in Annex II are complied with, when laboratory examinations are carried out in order to confirm or rule out the presence of a listed disease.

Article 5

Minimum control measures for the containment of listed diseases and minimum requirements for the lifting of containment measures in Member States, zones or compartments not declared free of listed diseases

Member States shall ensure that the minimum control measures and minimum requirements for the lifting of containment measures set out in Annex I are complied with, when carrying out control measures and the lifting of containment measures for one or more of the listed diseases in a Member State or in a zone or compartment thereof not declared free of those listed diseases.

Article 6

Repeals

Decisions 2001/183/EC, 2002/878/EC and 2003/466/EC are repealed.

Article 7

Date of application

This Decision shall apply from 1 April 2016.

Article 8

Addresses

This Decision is addressed to the Member States.

Done at Brussels, 11 September 2015.

For the Commission
Vytenis ANDRIUKAITIS
Member of the Commission

ANNEX I

SURVEILLANCE AND CONTROL METHODS

I. **Introduction**

This Annex sets out:

(a) requirements for eradication and surveillance programmes, as provided for in Article 44 of Directive 2006/88/EC, and the sampling and diagnostic methods to be used to declare disease-free status for Member States or zones or compartments thereof as provided for in Chapter VII of that Directive;

(b) sampling and diagnostic methods to be used for laboratory examinations in the case of the suspicion of and to confirm the presence of the non-exotic diseases listed in Part II of Annex IV to Directive 2006/88/EC (the 'listed diseases') as provided for in Articles 28(a) and 57(b) of that Directive;

(c) the containment measures to be taken in the case of confirmation of the presence of a listed disease, as provided for in Article 39 of Directive 2006/88/EC, and the measures to be taken in order to obtain Category III health status for a Member State, zone or compartment that has Category V health status.

The requirements set out in this Annex cover the following listed diseases:

1.	Viral haemorrhagic septicaemia (VHS)	Part 1
2.	Infectious haematopoietic necrosis (IHN)	Part 1
3.	Koi herpesvirus (KHV) disease	Part 2
4.	Infectious salmon anaemia (ISA)	Part 3
5.	Infection with *Marteilia refringens*	Part 4
6.	Infection with *Bonamia ostreae*	Part 5
7.	White spot disease (WSD)	Part 6

II. **Definitions**

For the purpose of Annex I and II, the following definitions shall apply:

(a) 'continental compartment' means one or more farm(s) situated on the continental part of one or more Member State(s), being under a common biosecurity system and containing an aquatic animal population with a distinct health status with respect to a specific disease;

(b) 'continental farm' means a farm keeping aquaculture animals situated on the continental part of the territory of one Member State;

(c) 'continental zone' means a precise geographic area situated on the continental part of one or more Member State(s) with a homogenous hydrological system comprising parts of a water catchment area from the source(s) to a natural or artificial barrier that prevents the upward migration of aquatic animals from lower stretches of the water catchment area, an entire water catchment area from its source(s) to its estuary, or more than one water catchment area, including their estuaries, due to the epidemiological link between the catchment areas through the estuary;

(d) 'officially declared infected farm' means a farm keeping aquatic animals where one or more of the listed diseases has been confirmed by the competent authority in accordance Articles 28(a), Article 29 and Article 57(b) of Directive 2006/88/EC.

(e) 'contact farm' means a farm keeping aquatic animals which in any way is demonstrated or strongly suspected to have been contaminated by infectious material from an officially declared infected farm.

PART 1

SURVEILLANCE AND CONTROL METHODS FOR VIRAL HAEMORRHAGIC SEPTICAEMIA (VHS) AND INFECTIOUS HEMATOPOIETIC NECROSIS (IHN)

I. **Requirements for surveillance and eradication programmes to obtain and maintain disease free-health statuses with regard to VHS and IHN and containment measures for those listed diseases**

I.1. General requirements for health inspections and sampling for VHS and IHN:

(a) the health inspections and, where appropriate, the sampling shall be carried out during the period of the year when the water temperature is below 14 °C or whenever the water temperature is likely to reach its lowest annual points;

(b) when targeted surveillance in wild populations is required in accordance with the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the number and geographical distribution of sampling points shall be determined to obtain a reasonable coverage of the Member State, zone or compartment. The sampling points shall be representative of the different ecosystems where wild populations of susceptible species are located;

(c) when farms or wild populations are to be subject to health inspections or sampled more than once per year, the intervals between the health inspections and between the collection of samples shall be at least 4 months and as long as possible, taking into account the temperature requirements provided for in point (a);

(d) all production units, such as ponds, tanks and net cages, shall be subject to health inspections for the presence of dead, weak or abnormally behaving fish. Particular attention shall be paid to the water outlet area where weak fish tend to accumulate because of the water current;

(e) fish of susceptible species to be collected as samples shall be selected as follows:

(i) if rainbow trout are present, only fish of that species shall be selected for sampling, except where other susceptible species are present which show typical signs of VHS or IHN; if rainbow trout are not present, the sample must be representative of all other susceptible species which are present;

(ii) if weak, abnormally behaving or freshly dead but not decomposed fish are present, such fish shall be selected; if more than one water source is utilised for fish production, fish representing all water sources shall be included in the sample;

(iii) the fish selected shall include fish collected in such a way that all parts of the farm, as well as all year classes, are proportionally represented in the sample.

I.2. Specific requirements to obtain disease-free health status (category I) with regard to VHS and IHN

I.2.1. Surveillance programmes:

(a) a Member State, zone or compartment that has Category III health status as referred to in Part B of Annex III to Directive 2006/88/EC with regard to VHS or IHN or both, may achieve Category I health status with regard to those listed diseases provided that all farms keeping susceptible species listed in Part II of Annex IV to that Directive within that Member State, zone or compartment comply with the requirements laid down in Annex V to that Directive and all those farms and, when required by the second paragraph of point 2 of Part I of Annex V thereto, sampling points in wild populations selected in accordance with that Part, have been subject to one of the following surveillance programmes:

(i) model A — 2-year surveillance programme:

The farms or sampling points must have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 1.A set out in Section II.

During that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 must have produced negative results for either VHS or IHN or both, and any suspicion of either VHS or IHN or both must have been ruled out in accordance with the sampling and diagnostic methods set out in point II.3;

(ii) model B — 4-year surveillance programme with reduced sample size:

The farms or sampling points must have been subject to health inspections and sampled for a minimum period of four consecutive years as laid down in Table 1.B set out in Section II.

During that 4-year period, the testing of all samples using the diagnostic methods set out in point II.2 must have produced negative results for either VHS or IHN or both and any suspicion of either VHS or IHN or both must have been ruled out in accordance with the sampling and diagnostic methods set out in point II.3;

(b) if during the implementation of the surveillance programme referred to in point (a), infection with either VHS or IHN or both is confirmed in a farm included in that surveillance programme, and therefore the farm's Category II health status has been withdrawn, that farm may immediately regain its Category II health status and continue the implementation of the surveillance programme to obtain disease-free status without implementing an eradication programme as set out in point I.2.2 provided that the farm complies with the following conditions:

(i) it is a continental farm whose health status regarding either VHS or IHN or both is independent of the health status of aquatic animal populations in the surrounding natural waters as regards those listed diseases in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC;

(ii) it is emptied, cleansed, disinfected and fallowed; the duration of the fallowing period shall be at least 6 weeks;

(iii) it has been restocked with fish sourced from Member States, zones or compartments with a Category I health status with regard to either VHS or IHN or both.

I.2.2. Eradication programmes

I.2.2.1. General requirements

A Member State, zone or a compartment with Category V health status with regard to either VHS or IHN or both may achieve Category I health status with regard to those listed diseases, provided that all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within that Member State, zone or compartment have been subject to an eradication programme that complies with points (a) to (e):

(a) the minimum control measures laid down in Section 4 of Chapter V of Directive 2006/88/EC must have been effectively applied and a containment area, as referred to in Article 32(b) of that Directive comprising a protection zone and surveillance zone, must have been established in the vicinity of the farm(s) officially declared infected with either VHS or IHN or both those listed diseases.

The containment area must have been defined on a case-by-case basis taking into account factors influencing the risks for the spread of the listed disease to farmed and wild fish, such as: the number, rate and distribution of the mortalities of fish on the farm infected with either VHS or IHN or both; the distance and density of neighbouring farms; the proximity to slaughterhouses; contact farms; species present at the farms; the farming practices applied in the affected farms and in the neighbouring farms to the affected farms; the hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply as regards the geographical demarcation of those zones:

(i) a protection zone shall be established in the immediate vicinity of a farm officially declared infected with either VHS or IHN or both those listed disease and shall correspond to:

(1) in coastal areas: an area included in a circle with a radius of at least one tidal excursion or at least 5 km, whichever is larger, centred on the farm officially declared infected with either VHS or IHN or both, or an equivalent area determined according to appropriate hydrodynamic or epidemiological data;

(2) in inland areas: the entire water catchment area of the farm officially declared infected with VHS or IHN or both; the competent authority may limit the extension of the zone to parts of the water catchment area, or the area of the farm, provided that the prevention of the spread of either VHS or IHN or both is not compromised;

(ii) a surveillance zone shall be established by the competent authority outside the protection zone and shall correspond to:

(1) in coastal areas: an area, surrounding the protection zone, of overlapping tidal excursion zones; or an area, surrounding the protection zone, and included in a circle of radius 10 km from the centre of the protection zone; or an equivalent area determined according to appropriate hydrodynamic or epidemiological data;

(2) in inland areas: as an extended area outside the established protection zone;

(b) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with either VHS or IHN or both shall be subject to an official investigation comprising at least the following elements:

(i) the collection of samples for testing of 10 fish, when clinical signs or *post-mortem* signs consistent with infection with either VHS or IHN or both are observed or minimum 30 fish, when clinical or *post-mortem* signs are not observed;

(ii) one health inspection: in those farms where the tests referred to in (i) have produced negative results; health inspections shall continue once per month during the period when the water temperature is below 14 °C, except when fish ponds or net cages are covered with ice, until the protection zone is withdrawn in accordance with point I.2.2.1(c);

(c) all farms officially declared infected with either VHS or IHN or both shall be emptied, cleansed, disinfected and fallowed. The duration of the fallowing period shall be at least 6 weeks. When all farms officially declared infected within the same protection zone are emptied, at least 3 weeks of synchronised fallowing shall be carried out. This paragraph also applies to new farms officially declared infected during the implementation of the eradication programme.

When fallowing of the officially declared infected farms is carried out, the protection zones shall be converted into surveillance zones.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms within the established protection and surveillance zones. The length of the fallowing period for those farms shall be determined by the competent authority following a case-by-case risk evaluation;

(d) all farms officially declared infected with either VHS or IHN or both those listed diseases and all other farms fallowed within the established protection and surveillance zones as referred to in point (c), shall be restocked with fish sourced from Member States, zones or compartments with a disease-free health status (Category I) with regard to either VHS or IHN or both.

Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2.1(c);

(e) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme and when surveillance in wild populations is required, sampling points selected in accordance with point I.1, shall subsequently be subject to the surveillance scheme laid down in point I.2.1.

I.2.2.2. Requirements for regaining disease-free status for continental compartments comprising one single farm previously being declared free of either IHN or VHS or both

A continental compartment comprising one single farm previously declared free of either VHS or IHN or both of those listed diseases, whose health status with regard to those listed diseases is independent of the surrounding natural waters in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC, and whose Category I health status has been withdrawn in accordance with Article 53(3) of that Directive, may regain Category I health status immediately after the competent authority has confirmed that the following conditions have been complied with:

(a) the officially confirmed infected farm with either VHS or IHN or both must have been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period must be at least 6 weeks;

(b) the officially confirmed infected farm with either VHS or IHN or both has been restocked with fish sourced from Member States, zones or compartments with a category I health status with regard to either VHS or IHN or both.

I.3. Specific requirements for the maintenance of disease-free health status (Category I) with regard to either VHS or IHN or both

When targeted surveillance is required in order to maintain Category I health status, as provided in Article 52 of Directive 2006/88/EC, all farms keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment concerned shall be subject to health inspection and fish shall be sampled in accordance with Table 1.C set out in Section II of this Part, taking into account the risk level of the farm for the contraction of either VHS or IHN or both of those listed diseases.

When determining the health inspection frequency for Category I health status compartments with regard to either VHS or IHN or both, which are placed in continental areas and where the health status regarding VHS or IHN is dependent on the health status of the aquatic animal populations in surrounding natural waters in accordance with point 2 of Part II of Annex V to Directive 2006/88/EC, the risk for the contraction of either VHS or IHN or both shall be considered as high.

Disease-free status shall only be maintained as long as all samples tested, using the diagnostic methods set out in point II.2., produce negative results for either VHS or IHN or both of those listed diseases and any suspicion of either VHS or IHN or both are ruled out in accordance with the diagnostic methods set out in point II.3.

I.4. Requirements for the lifting of containment measures provided for in Article 39 of Directive 2006/88/EC, namely the change from Category V to Category III health status

A Member State, zone or a compartment which has Category V health status with regard to either VHS or IHN or both may achieve Category III health status with regard to those listed diseases provided that:

(a) the requirements set out in points I.2.2.1(a), (b) and (c) have been met. In case fallowing is not technically possible, the farms concerned shall be subject to an alternative measure which provides almost similar guarantee for extermination of either IHNV or VHSV or both from the environment of the farm;

(b) all farms officially declared infected and all other farms fallowed/been subject to alternative measures in accordance with (a) within the established protection and surveillance zones have been restocked with fish sourced from Member States, zones or compartments with a Category I, II or III health status with regard to either VHS or IHN or both;

(c) the restocking has only taken place after all farms officially declared infected, have been emptied, cleansed, disinfected and fallowed/been subject to alternative measures in accordance with (a).

II. **Diagnostic and sampling methods**

II.1. Organs to be sampled:

The tissue material to be examined is spleen, anterior kidney, and either heart or encephalon. When sampling broodstock ovarian or seminal fluid may also be examined.

In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney shall be collected.

Organ pieces from a maximum of 10 fish may be pooled.

II.2. Diagnostic methods to obtain and maintain disease-free status for either VHS or IHN or both

The diagnostic method, in accordance with the approved diagnostic methods and procedures set out in point I. of Part 1 of Annex II, to achieve or to maintain disease-free status for VHS or IHN or both shall be either:

(a) virus isolation in cell cultures followed by identification using enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), virus neutralisation test or real-time reverse transcriptase polymerase chain reaction (RT-qPCR); or

(b) RT-qPCR.

II.3. Sampling and diagnostic methods to rule out or to confirm the presence of VHS or IHN

When a suspicion of either VHS or IHN or both is required to be confirmed or ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedures shall be complied with:

(a) the farm under suspicion shall be subject to at least one health inspection and one sampling of 10 fish, when clinical signs or *post-mortem* signs consistent with infection with either VHS or IHN or both are observed or minimum 30 fish, when clinical or *post-mortem* signs are not observed. Samples shall be tested using one or more of the diagnostic methods set out in points (i) and (ii) in accordance with the detailed diagnostic methods and procedures as set out in Section II of Part 1 of Annex II:

(i) conventional virus isolation in cell culture with subsequent immunochemical or molecular virus identification;

(ii) virus detection by RT-qPCR;

(iii) Other diagnostic techniques of proven similar efficacy such as Indirect fluorescent antibody test (IFAT), Enzyme-linked immuno-sorbent assay (ELISA), RT-PCR and Immunohistochemistry (IHC).

(b) the presence of VHS shall be considered as confirmed, if one or more of those diagnostic methods are positive for VHSV. The presence of IHN shall be considered as confirmed, if one or more of those diagnostic methods are positive for IHNV. The confirmation of the first case of VHS or IHN in Member States, zones or compartments previously not infected shall be based on conventional virus isolation in cell culture or RT-qPCR;

(c) Suspicion of either VHSV or IHNV or both may be ruled out, if cell cultivation or RT-qPCR tests reveal no further evidence of the presence of either VHSV or IHNV or both.

Table 1.A

Surveillance scheme for zones and compartments for the 2-year control period referred to in point I.2.1(a)(i) which precedes the achievement of disease-free status for VHS or IHN

Type of farm	Number of health inspections per year (2 years)	Number of samplings per year (2 years)	Number of fish in the sample (1)	
			Number of growing fish	Number of broodstock fish (2)
(a) Farms with broodstock	2	2	50 (first inspection) 75 (second inspection)	30 (first or second inspection) 0 (first or second inspection)
(b) Farms with broodstock only	2	1	0	75 (first or second inspection)
(c) Farms without broodstock	2	2	75 (3) (first and second inspection)	0

Maximum number of fish per pool: 10

(1) The samples must be collected no sooner than 3 weeks after the transfer of the fish from fresh to saltwater.
(2) Ovarian or seminal fluid of broodstock shall be collected at the time of maturation, in connection with stripping.
(3) Samples must be taken from the number of fish that will ensure the detection of VHSV or IHNV with a 95 % confidence if the design prevalence is 5 %.

Table 1.B

Surveillance scheme with a reduced sample size for the 4-year control period referred to in point I.2.1(a)(ii) which precedes the achievement of disease-free status for VHS or IHN

Type of farm	Number of health inspections per year	Number of samplings per year	Number of fish in the sample (1)	
			Number of growing fish	Number of broodstock fish (2)
First 2 years of the surveillance period				
(a) Farms with broodstock	2	1	0 (first inspection) 30 (second inspection)	0 (first inspection) 0 (second inspection)
(b) Farms with broodstock only	2	1	0	30 (first or second inspection)
(c) Farms without broodstock	2	1	30 (3) (first or second inspection)	0
Last 2 years of the surveillance period				
(a) Farms with broodstock	2	2	30 (first inspection) 0(second inspection)	0 (first inspection) 30 (second inspection)

Type of farm	Number of health inspections per year	Number of samplings per year	Number of fish in the sample (1)	
			Number of growing fish	Number of broodstock fish (2)
(b) Farms with broodstock only	2	2		30 (first and second inspection)
(c) Farms without broodstock	2	2	30 (3) (first and second inspection)	

Maximum number of fish per pool: 10

(1) The samples must be collected no sooner than 3 weeks after the transfer of the fish from fresh to saltwater.
(2) Ovarian or seminal fluid of broodstock shall be collected at the time of maturation, in connection with stripping.
(3) Samples must be taken from the number of fish that will ensure detection of VHSV or IHNV with a 95 % confidence if the design prevalence is 10 %.

Table 1.C

Surveillance schemes for zones or compartments to maintain disease-free status for VHS or IHN as referred to in point I.3

Risk level	Number of health inspections	Number of fish in the sample (3)
High	2 every year	30 (1) (2)
Medium	1 every year	30 (1)
Low	1 every 2 years	30 (1)

Maximum number of fish per pool: 10

(1) The samples must be collected no sooner than 3 weeks after the transfer of the fish from fresh to saltwater.
(2) Samples must be taken from the number of fish that will ensure the detection of VHSV or IHNV with a 95 % confidence if the design prevalence is 10 %.
(3) There shall be minimum one sample for every health inspection.

PART 2

SURVEILLANCE AND CONTROL METHODS FOR KOI HERPESVIRUS DISEASE (KHVD)

I. **Requirements for surveillance and eradication programmes to obtain and maintain disease-free health status with regard to KHVD and to contain infection with koi herpesvirus (KHV)**

I.1. General requirements

When targeted surveillance in wild populations is required in accordance with the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the number and geographical distribution of sampling points shall be determined to obtain a reasonable coverage of the Member State, zone or compartment. The sampling points shall also be representative of the different ecosystems where the wild susceptible populations are located, namely, river systems and lakes.

Targeted surveillance shall rely on the regular monitoring of sites holding susceptible species. Sites shall be monitored when water temperatures have reached levels that are permissive for the development of the disease (> 15 °C) and no sooner than 2 weeks from the date such temperatures have been reached. Any diseased fish or fish showing abnormal behavior that are found on the site shall be sampled and tested.

Whenever possible, fish that have been kept for a prolonged time period at the virus permissive temperature range, namely 2 to 3 weeks at 15 °C to 26 °C, shall be sampled. The following approach may, however, be accepted:

(a) to collect a sub-population at transfer from winter to summer ponds and hold the fish in the same water body as the summer pond until minimum temperature requirements have been obtained, or

(b) to collect samples at harvesting or during other fish handling as part of normal management practices. If possible, samples shall be collected between 24 and 72 hours after such management practices to enhance the chance of KHV detection.

When farms or wild populations have to be subject to health inspections or sampled more than once per year, the intervals between the health inspections or collections of samples shall be as long as possible within the season when the water temperature is likely to reach its highest annual points without exceeding the limit of 28 °C.

All production units, such as ponds and tanks, must be subject to health inspections for the presence of dead, weak or abnormally behaving fish.

Cyprinus carpio and its hybrids, such as *Cyprinus carpio* × *Carassius auratus*, shall be collected when present in the farm.

The fish to be collected as samples shall be selected as follows:

(i) if weak, abnormally behaving or freshly dead but not decomposed fish are present, such fish must be selected;

(ii) if more than one water source is utilised for fish production, fish representing all water sources must be included for sampling;

(iii) the fish selected must include fish collected in such a way that all parts of the farm, as well as all year classes, are proportionally represented in the sample.

I.2. Specific requirements to achieve disease-free health status (Category I) with regard to KHVD

I.2.1. Surveillance programmes

(a) a Member State, zone or a compartment which has Category III health status with regard to KHVD may achieve Category I health status when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within that Member State, zone or compartment comply with the requirements for disease-free status set out in Annex V to that Directive and all those farms, and, when required by the second paragraph of point 2 of Part I of that Annex, sampling points in wild populations selected in accordance with that Part, have been subject to one of the following surveillance programmes:

(i) model A — 2-year surveillance programme:

The farms or sampling points must have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 2.A set out in Section III.

During that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 must have produced negative results for KHV and any suspicion of KHVD must have been ruled out in accordance with the diagnostic methods set out in point III.2;

(ii) model B — 4-year surveillance programme with reduced sample size:

The farms or sampling points must have been subject to health inspections and sampled for a minimum period of four consecutive years as laid down in Table 2.B set out in Section III.

During that 4-year period, the testing of all samples using the diagnostic methods set out in point II.2 must have produced negative results for KHV and any suspicion of KHVD must have been ruled out in accordance with the diagnostic methods set out in point III.2;

(b) if during the implementation of the 4-year surveillance programme set out in point (a), infection with KHV is confirmed in a farm included in that surveillance programme, and therefore its Category II health status has been withdrawn, that farm may immediately regain the Category II health status and continue the implementation of the surveillance programme to obtain disease- free status without implementing an eradication programme as described in point I.2.2 provided that the farm complies with the following conditions:

(i) it is a continental farm whose health status regarding KHVD is independent of the health status of aquatic animal populations in the surrounding natural water as regards that listed disease in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC;

(ii) it has been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period shall be at least 6 weeks;

(iii) it has been restocked with fish sourced from Member States, zones or compartments with a Category I health status with regard to KHVD.

I.2.2. Eradication programmes

I.2.2.1. General requirements

A Member State, zone or a compartment that has Category V health status with regard KHVD, may achieve Category I health status with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within that Member State, zone or compartment have been subject at least to the following eradication programme:

(a) the minimum control measures laid down in Section 4 of Chapter V of Directive 2006/88/EC have effectively been applied, and a containment area as referred to in Article 32(b) of that Directive, comprising a protection zone and surveillance zone, has been established in the vicinity of the farm(s) officially declared infected with KHV.

The containment area must have been defined on a case-by-case basis taking into account factors influencing the risks for the spread of KHVD to farmed and wild fish, such as: the number, rate and distribution the mortalities of fish on the farm infected with KHV; the distance and density of neighbouring farms; the proximity to slaughterhouses; contact farms; the species present at the farms; the farming practices applied in the affected and neighbouring farms; the hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply as regards the geographical demarcation of those zones:

(i) a protection zone shall be established in the immediate vicinity of a farm officially declared infected with KHV and shall correspond to the entire water catchment area of the farm officially declared infected with KHV; the competent authority may limit the extension of the zone to parts of the water catchment area provided that the prevention of the spread of KHVD is not compromised;

(ii) a surveillance zone shall be established outside the protection zone and shall correspond to an extended area surrounding the established protection zone;

(b) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with KHV shall be subject to an official investigation comprising at least the following elements:

(i) the collection of samples for testing of 10 fish, when clinical signs or *post-mortem* signs consistent with KHVD are observed or 30 fish, when clinical or *post-mortem* signs are not observed;

(ii) one health inspection; in those farms, where the tests referred to in point III.2 have produced negative results; health inspections shall continue once per month during the season when the water temperature is likely to reach > 15 °C until the protection zone is withdrawn in accordance with point I.2.2.1(c);

(c) all farms officially declared infected with KHV shall be emptied, cleansed, disinfected and fallowed. The duration of the fallowing period shall be at least 6 weeks. When all farms within the same protection zone officially declared infected have been emptied, at least 3 weeks of synchronised fallowing shall be carried out. This paragraph also applies to new farms officially declared infected during the implementation of the eradication programme.

When fallowing of the officially declared infected farms is carried out, the protection zones shall be converted into surveillance zones.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms within the established protection and surveillance zones. The length of the fallowing period shall be determined by the competent authority following a case-by-case risk evaluation;

(d) All farms officially declared infected with KHV and all other farms fallowed within the established protection and surveillance zones shall be restocked:

(i) with fish sourced from Member States, zones or compartments with a Category I health status with regard to KHVD; or

(ii) for a transitional period until 31 December 2020, with fish from Member States, zones or compartments with an approved KHVD surveillance programme.

Restocking shall only take place when all farms officially declared infected with KHV have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2.1(c);

(e) All farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme and, when surveillance in wild populations is required, sampling points selected in accordance with Point I.1., shall have subsequently been subject at least to the surveillance programme laid down in point I.2.1.

I.2.2.2. Requirements for regaining disease free status for continental compartments comprising one single farm previously declared free of KHVD

A continental compartment comprising one single farm that has Category I health status with regard to KHVD, whose health status with regard to KHVD is independent of the surrounding natural waters in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC, and whose Category I status has been withdrawn in accordance with Article 53(3) of that Directive, may regain Category I health status with regard to KHVD immediately after the competent authority has confirmed that it has complied with the following conditions:

(a) it has been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period must have been be at least 6 weeks;

(b) it has been restocked with fish sourced from Member States, zones or compartments with a Category I health status or compartments with an approved KHVD surveillance programme (Category II health status).

I.3. Specific requirements for the maintenance of Category I status with regard to KHVD

When targeted surveillance is required in order to maintain Category I health status, as provided for in Article 52 of Directive 2006/88/EC, all farms keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment concerned shall be subject to health inspection and sampled in accordance with Table 2.B set out in Section III of this Part, taking into account the risk level of the farm for the contraction of KHV.

The inspection frequency for health inspections of Category I compartments with regard to KHVD placed in continental areas and comprising one or more farms whose health status regarding KHVD is dependent on the health status for that listed disease of surrounding natural waters in accordance with point 2 of Part II of Annex V to Directive 2006/88/EC, shall be in accordance with the number set out for high risk level in that Table 2.C.

In Member States, zones or compartments in which the number of farms is limited and targeted surveillance on these farms does not provide sufficient epidemiological data, the surveillance schemes to maintain disease-free status shall include sampling points selected in accordance with the requirements set out in point I.1.

Those sampling points shall be inspected and sampled by rotation of 50 % of the sampling points each year. The sampling shall be carried out in accordance with Table 2.C set out in Section III. The samples shall be selected, prepared and examined as described in Section II and the laboratory examinations must be negative with regard to the presence of the agent of KHVD.

Disease-free status shall only be maintained as long as all samples tested using the diagnostic methods set out in point II.2 produce negative results for KHVD and any suspicion of KHVD must be ruled out in accordance with the diagnostic methods set out in point III.2.

I.4. Specific requirements for lifting the containment measures provided for in Article 39 of Directive 2006/88/EC to obtain Category III health status with regard to KHVD in Member States, compartments or zones that have Category V health status

A Member State, zone or a compartment that has Category V health status with regard to KHVD may achieve Category III health status with regard to that listed disease provided that:

(a) the requirements set out in points I.2.2.1(a), (b) and(c) have been met. In case fallowing is not technically possible, the farms concerned shall be subject to an alternative measure which provides almost similar guarantee for extermination of KHV from the environment of the farm;

(b) all farms officially declared infected and all other farms fallowed/been subject to alternative measures in accordance with (a) within the established protection and surveillance zones have been restocked with fish sourced from Member States, zones or compartments with a Category I, II or III health status with regard to KHVD;

(c) the restocking has only taken place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed/been subject to alternative measures in accordance with (a).

II. **Diagnostic and sampling methods for surveillance to obtain and maintain disease-free status with regard to KHVD**

II.1. Samples

The tissue material to be examined shall be parts of gill and kidney. Organ pieces from a maximum of two fish may be pooled.

II.2. Diagnostic methods for the surveillance to obtain and maintain disease-free status with regard to KHVD

The diagnostic method to achieve or to maintain disease-free status for KHVD shall be Real-time PCR (qPCR) in accordance with the detailed diagnostic methods and procedures as set out in point II of Part 2 of Annex II.

III. **Diagnostic and sampling methods for official investigations for confirmation or to rule out a suspicion of KHVD**

III.1. Samples

The tissue material to be examined shall be parts of gill and kidney. Organ pieces from a maximum of two fish may be pooled.

III.2. Official investigation and diagnostic methods to rule out and to confirm the presence of infection with KHV

When a suspicion of KHVD is required to be confirmed or ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedure shall be complied with:

(a) the official investigation shall include at least one health inspection and one sampling of 10 fish, when clinical signs or post-mortem signs consistent with infection with KHV are observed or 30 fish when clinical or post-mortem signs are not observed. Samples shall be tested using the diagnostic method set out in point (b) in accordance with the detailed diagnostic methods and procedures set out in point II of Part 2 of Annex II;

(b) the presence of the infection with KHV shall be considered as confirmed if KHV is detected by PCR;

suspicion of KHVD may be ruled out, if this test reveals no further evidence of the presence of KHV.

Table 2.A

Surveillance scheme for zones and compartments for the 2-year control period which precedes the achievement of disease-free status for KHVD as referred to in point I.2.1

		Number of clinical inspections per year (2 years)	Number of laboratory examinations per year (2 years)	Number of fish in the sample
Farms/sampling sites	First 2 years of the surveillance period	2	2	75 [1]
	Maximum number of fish per pool: 2			

[1] Samples must be taken from the number of fish that will ensure the detection of KHV with a 95 % confidence if the design prevalence is 5 %.

Table 2.B

Surveillance scheme for zones and compartments for the 4-year control period which precedes the achievement of disease-free status for KHVD as referred to in point I.2.1

		Number of clinical inspections per year	Number of laboratory examinations per year	Number of fish in the sample
Farms/sampling sites	First 2 years of the surveillance period	1	1	30
Farms/sampling sites	Last 2 years of the surveillance period	2	2	30
	Maximum number of fish per pool: 2			

Table 2.C

Surveillance schemes for zones or compartments to maintain disease-free status for KHVD as referred to in point I.3

Risk level	Number of health inspections	Number of fish in the sample
High	2 every year	30
Medium	1 every year	30
Low	1 every 2 years	30

Maximum number of fish per pool: 2

Table 2.D

Surveillance scheme to maintain KHVD disease-free status in Member States, zones or compartments where the number of farms is limited and targeted surveillance on these farms does not provide sufficient epidemiological data as referred to in point I.3

	Number of clinical inspections per year	Number of laboratory examinations per year	Number of fish in the sample
Sampling points	1 every 2 years	1 every 2 years	30

Maximum number of fish per pool: 2

PART 3

SURVEILLANCE AND CONTROL METHODS FOR INFECTIOUS SALMON ANEMIA (ISA)

I. **Requirements for surveillance and eradication programmes to obtain and to maintain disease-free health statuses with regard to ISA and to contain infection with HPR deleted ISAV**

I.1. General requirements

When health inspections and sampling of farms in accordance with the second paragraph of point 2 of part I of Annex V to Directive 2006/88/EC is required to be carried out more than once per year, the intervals between the health inspections or collection of samples shall be as long as possible.

When targeted surveillance in wild populations is required in accordance with the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the number and geographical distribution of sampling points shall be determined to obtain a reasonable coverage of the Member State, zone or compartment. The sampling points shall also be representative of the different ecosystems where the wild susceptible populations are located.

The health inspections shall be carried out in all production units, such as ponds, tanks and net cages, for the presence of dead, weak or abnormally behaving fish. Particular attention shall be paid to the water outlet area where weak fish tend to accumulate because of the water current.

The fish to be collected as samples shall be selected as follows:

(a) only moribund or freshly dead fish, but not decomposed fish shall be selected; in particular fish demonstrating anaemia, bleedings or other clinical signs suggesting circulatory disturbances shall be prioritised for collection;

(b) if Atlantic salmon is among the susceptible species on the site, samples from Atlantic salmon shall be prioritised. If there is no Atlantic salmon in the fish farm, other susceptible species must be sampled;

(c) if more than one water source is utilised for fish production, fish representing all water sources shall be included in the sample;

(d) the fish selected shall include fish collected in such a way that all production units, such as net cages, tanks and ponds, of the farm as well as all year classes are proportionally represented in the sample.

I.2. Specific requirements to achieve Category I health status with regard to ISA

I.2.1. Surveillance programmes

A Member State, zone or compartment which has Category III health status in accordance with part B of Annex III to Directive 2006/88/EC with regard to ISA may achieve Category I health status with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment meet the relevant requirements set out in Annex V to that Directive and all those farms and, when required by the second paragraph of point 2 of Part I of Annex V thereto, sampling points in wild populations selected in accordance with that point, have been subject to the following surveillance programme:

(a) the farms or sampling points have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 3.A set out in Section II;

(b) during that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 must have produced negative results for HPR-deleted ISAV and any suspicion of ISA must have been ruled out in accordance with the diagnostic methods set out in point II.3;

(c) if during the implementation of the surveillance programme, ISA is confirmed in a farm included in that surveillance programme, and therefore its Category II health status has been withdrawn, an eradication programme in accordance with point I.2.2 must have been carried out.

I.2.2. Eradication programmes

I.2.2.1. General requirements

A Member State, zone or a compartment that has Category V health status with regard to ISA may achieve Category I health status with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment have been subject to an eradication programme that complies with the following points (a) to (e).

(a) the minimum control measures laid down in Section 3 of Chapter V of Directive 2006/88/EC have effectively been applied, and in particular a containment area as referred to in Article 32(b) of that Directive, comprising a protection zone and surveillance zone, has been established in the vicinity of the farm(s) officially declared infected with HPR-deleted ISAV or confirmed ISA.

The containment area must have been defined on a case-by-case basis taking into account factors influencing the risks for the spread of ISA to farmed or wild fish, such as: the number, rate and distribution of the mortality of fish on the farm infected with HPR-deleted ISAV or confirmed ISA; the distance and density of neighbouring farms; the proximity to slaughterhouses; contact farms; the species present at the farms; the farming practices applied in the affected and neighbouring farms; the hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply as regards the geographical demarcation of those zones:

(i) a protection zone shall be established in the immediate vicinity of a farm officially declared infected with ISA and shall correspond to:

(1) in coastal areas: an area included in a circle with a radius of at least one tidal excursion or at least 5 km, whichever is larger, centred on the farm officially declared infected with ISA, or an equivalent area determined according to appropriate hydrodynamic or epidemiological data;

(2) in inland areas: the entire water catchment area of the farm officially declared infected with ISA; the competent authority may limit the extension of the zone to parts of the water catchment area provided that the prevention of the spread ISA is not compromised;

(ii) a surveillance zone shall be established outside the protection zone and shall correspond to:

(1) in coastal areas: an area, surrounding the protection zone, of overlapping tidal excursion zones; or an area, surrounding the protection zone, and included in a circle of radius 10 km from the centre of the protection zone; or an equivalent area determined according to appropriate hydrodynamic or epidemiological data; or

(2) in inland areas: an extended area outside the established protection zone;

(b) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with ISA shall be subject to an official investigation comprising at least the following elements:

(i) the collection of samples for testing of minimum 10 moribund fish, when clinical signs or post-mortem signs consistent with ISA are observed, or minimum 30 fish when no clinical or post mortem signs are observed;

(ii) one health inspection; in those farms where the tests referred to in (i) have produced negative results, the health inspections shall continue once per month until the protection zone is withdrawn in accordance with point I.2.2.1(c);

(c) all farms officially declared infected with HPR-deleted ISAV or confirmed ISA shall be emptied, cleansed, disinfected and fallowed for a period of at least 3 months. The protection and surveillance zones may be lifted when all farms within the protection zone are emptied, cleaned, disinfected and followed by a synchronized fallowing period of at least 6 weeks.

When fallowing of the officially declared infected farms is carried out, the protection zones shall be converted into surveillance zones.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms within the established protection and surveillance zones. The length of the fallowing period for those farms shall be determined by the competent authority following a case-by-case risk evaluation;

(d) all farms officially declared infected with HPR-deleted ISAV or confirmed ISA and all other farms fallowed within the established protection and surveillance zones shall be restocked with fish sourced from Member States, zones or compartments with a Category I with regard to ISA.

Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2.1(c);

(e) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the member State, zone or compartment covered by the eradication programme and when surveillance in wild populations is required, sampling points selected in accordance with point I.1, shall subsequently be subject to the surveillance scheme set out in point I.2.1.

I.2.2.2. Requirements concerning regaining disease-free status for continental compartments comprising one single farm that was previously declared as having Category I health status

A continental compartment comprising one single farm that has Category I health status with regard to ISA, whose health status is independent of the surrounding natural waters in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC, and whose Category I health status has been withdrawn in accordance with Article 53(3) of that Directive, may regain it again immediately after the competent authority has confirmed that it complies with the following conditions:

(a) it has been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period shall be at least 6 weeks;

(b) it has been restocked with fish sourced from Member States, zones or compartments with a Category I health status as regards ISA.

I.3. Minimum control measures for the maintenance of Category I status with regard to ISA

When targeted surveillance is required to maintain Category I health status, as provided for in Article 52 of Directive 2006/88/EC, all farms keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment concerned shall be subject to health inspections and sampled in accordance with Table 3.B [1] set out in Section II of this Part, taking into account the risk level of the farm for the contraction of ISA.

When determining the health inspection frequency for category I health status with regard to ISA for compartments which are placed in continental areas and where the health status with regard to ISA is dependent of the health status of surrounding natural waters that are housing Atlantic salmon (*Salmo salar*), the risk for the contraction of ISA shall be regarded as high.

Disease-free status with regard to ISA may only be maintained as long as all samples tested using the diagnostic methods set out in point II.2 have produced negative results for HPR-deleted ISAV and any suspicion of ISA has been ruled out in accordance with the diagnostic methods set out in point II.3.

I.4. Specific requirements to achieve Category III health status with regard to HPR-deleted ISAV in Member States, zones or compartments that previously held Category V health status

A Member State, zone or compartment that has Category V health status with regard to ISA may achieve Category III status provided that:

(a) the requirements set out in point I.2.2.1 (a), (b) and (c) have been met. In case fallowing is not technically possible, the farms shall be subject to an alternative measure which provides almost similar guarantee for extermination of ISAV from the environment of the farm;

(b) all farms officially declared infected and all other farms fallowed or been subject to alternative measures in accordance with (a) within the protection and surveillance zones established, have been restocked with fish sourced from Member States, zones or compartments with a Category I, II or III health status with regard to ISA;

(c) that such restocking has only taken place after all farms officially declared infected have been emptied, cleansed, disinfected and fallowed/been subject to alternative measures in accordance with (a).

(d) no confirmation of HPR-deleted ISAV has occurred during the period of 2 years that follows the completion of the measures referred to in (a), (b) and (c), and suspicions during this period have been ruled out in accordance with the procedures established in point II.3.

[1] Shall not apply to farms only rearing rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) or both and where the water supply is exclusively based on fresh water sources not housing Atlantic salmon (*Salmo salar*).

II. **Diagnostic methods and official investigations**

II.1. Samples

The tissue material to be examined shall be:

(a) Histology: head-kidney, liver, heart, pancreas, intestine, spleen and gill;

(b) Immunohistochemistry: mid-kidney and heart including valves and *bulbus arteriosus*;

(c) RT-qPCR analysis: mid-kidney and heart;

(d) Virus culture: mid-kidney, heart, liver and spleen.

Organ pieces from a maximum of five fish may be pooled.

II.2. Diagnostic methods to obtain or maintain disease-free status with regard to ISA

The diagnostic method to be used to obtain or to maintain disease-free status with regard to ISA in accordance with points I.2 and I.3 shall be RT-qPCR, followed by sequencing of positive samples in accordance with the detailed methods and procedures set out in Part 3 of Annex II.

In the case of a positive result to RT-qPCR, further samples shall be tested before the implementation of the initial control measures provided for in Article 28 of Directive 2006/88/EC.

Those samples shall be tested as follows in accordance with the detailed methods and procedures set out in Part 3 of Annex II:

(a) screening of the samples by RT-qPCR, including sequencing of the HE-gene to verify HPR-deletion;

and

(b) examination in tissue preparations by means of specific antibodies against ISAV (namely IHC on fixed sections or IFAT on tissue imprints); or

(c) isolation and identification of ISAV in cell culture from at least one sample from any fish sampled from the farm.

II.3. Official investigation and diagnostic methods to rule out or to confirm the presence of ISA

When a suspicion of ISA shall be confirmed or ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedure shall be complied with:

(a) the official investigation which shall include at least one health inspection and one sampling of 10 moribund fish, when clinical signs or *post-mortem* signs consistent with ISA are observed. If no clinical signs or *post-mortem* signs consistent with ISA are observed, the health inspection shall be followed by targeted sampling of minimum 30 moribund fish or fresh *post-mortems* with normal constitution in accordance with point I.1. Samples shall be tested in accordance with the diagnostic methods set out in point (b);

(b) in the case of a positive result of RT-qPCR for HPR-deleted ISAV, further samples shall be tested before the implementation of the initial control measures provided in Article 28 of Directive 2006/88/EC. A suspected case of infection with ISA shall be confirmed in accordance with the following criteria using the detailed methods and procedures set out in Part 3 of Annex II:

(i) Detection of ISAV by RT-qPCR, including sequencing of the HE-gene to verify HPR-deletion, and detection of ISAV in tissue preparations by means of specific antibodies against ISAV (namely IHC on fixed sections or IFAT on tissue imprints)

or

(ii) detection of ISAV by RT-qPCR, including sequencing of the HE-gene to verify HPR-deletion, and

isolation and identification of ISAV in cell culture from at least one sample from any fish from the farm;

(c) where the presence of clinical, gross pathological changes or histopathological findings consistent with ISA are observed, the findings must be corroborated by virus detection by two diagnostic methods with independent principles of detection, such as RT-qPCR and IHC, in accordance with Part 3 of Annex II.

The suspicion of ISA may be ruled out, if tests and inspections over a period of 12 months from the date of the suspicion are found to reveal no further evidence of the presence of ISA.

Table 3.A

Surveillance scheme for zones and compartments for the 2-year control period which precedes the achievement of disease-free status for ISA as referred to in point I.2.1

Year of surveillance	Number of health inspections per year (2 years)	Number of laboratory examinations per year (2 years)	Number of fish to be sampled per year
Year 1	6	2 [1]	2 * 75 [2]
Year 2	6	2 [1]	2 * 75 [2]

[1] Samples must be collected and stored and examined during two 1 month test periods per year (namely spring and autumn) or when appropriate in accordance with practical considerations.
[2] Maximum number of fish per pool: 5.

Table 3.B

Surveillance schemes for zones or compartments to maintain disease- free status for ISA as referred to in point I.3 [2]

Risk level	Number of health inspections per year	Number of laboratory examinations per year	Number of fish to be sampled per year
High	2	2 [1]	2 * 30
Medium	1	1 [1]	30
Low	1 every 2 years	1 every 2 years	30 every 2 years

[1] Samples must be collected and examined during two 1 month test periods per year (namely spring and autumn) or when appropriate in accordance with practical considerations.
[2] Shall not apply to farms only rearing rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) or both and where the water supply is exclusively based on fresh water sources not housing Atlantic salmon (*Salmo salar*).

PART 4

SURVEILLANCE AND CONTROL METHODS FOR INFECTION WITH *MARTEILIA REFRINGENS*

I. **Requirements for surveillance and eradication programmes to obtain and to maintain disease-free health statuses with regard to infection with *Marteilia refringens***

I.1. General requirements

Health inspections and, where appropriate, the sampling for laboratory examination shall be carried out in the period of the year when prevalence of the parasite in the Member State, zone or compartment is known to be maximal. When such data is not available, sampling shall be carried out just after the water temperature has exceeded 17 °C.

When molluscs are to be sampled in accordance with the requirements set out in Part 4, the following criteria shall apply:

(a) if *Ostrea* spp. and *Mytilus* spp. are present in the production units or production area, both genera shall be sampled equally in sample size. If only one of those genera is present, that genus shall be sampled. If neither *Ostrea* nor *Mytilus* genera are present, the sample must be representative of all other susceptible species present;

(b) if weak, gaping or freshly dead but not decomposed molluscs are present in the production units, such molluscs shall primarily be selected. If such molluscs are not present, the molluscs selected shall include the oldest healthy molluscs;

(c) when sampling in mollusc farms which utilise more than one water source for mollusc production, mollusc representing all water sources shall be included for sampling in such a way that all parts of the farm are proportionally represented in the sample;

(d) when sampling in mollusc farming areas, mollusc from a sufficient number of sampling points, shall be included in the sample in such a way that all parts of the mollusc farming area are proportionally represented in the sample. The main factors to be considered for the selection of these sampling points are previous sampling points where *Marteilia refringens* was detected, stocking density, water flows, presence of susceptible species, presence of vector species, bathymetry and management practices. Natural beds shall be included in the sampling.

I.2. Specific requirements to achieve Category I health status with regard to *Marteilia refringens*

I.2.1. Surveillance programmes

A Member State, zone or compartment which has Category III health status with regard to infection with *Marteilia refringens* may achieve Category I health status with regard to that listed disease when all farms or mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment have been subject at least to the following surveillance programme comprising health inspections and the collection of samples for testing.

2-year surveillance programme:

(a) the farms or mollusc farming areas have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 4.A set out in Section II;

(b) during that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 have produced negative results for *Marteilia refringens* and any suspicion of *Marteilia refringens* has been ruled out in accordance with the diagnostic methods set out in point II.3;

(c) when *Ostrea edulis*, *Mytilus edulis* or *Mytilus galloprovincialis* sourced from a Member State, zone or compartment of Category I health status are to be included in the sample, they must have been introduced in the farm or mollusc farming area at least in the spring just preceding the period when surveillance programme is carried out.

I.2.2. Eradication programmes

The eradication of *Marteilia refringens* is considered to be impossible in most cases, but where the Member State judges it to be feasible, the following model for an eradication programme shall apply.

A Member State, zone or compartment that has Category V health status as regards infection with *Marteilia refringens* may achieve Category I health status with regard to that listed disease when all farms or mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within that Member State, zone or compartment have been subject at least to the following eradication programme:

(a) the measures laid down in Section 3 of Chapter V of Directive 2006/88/EC have effectively been applied, and in particular a containment area as referred to in Article 32(b) of Directive 2006/88/EC, comprising a protection zone and surveillance zone, has been established in the vicinity of the farm(s) or mollusc farming area(s) officially declared infected with *Marteilia refringens*.

The containment area shall be defined on a case-by-case basis taking into account factors influencing the risks for the spread of *Marteilia refringens*, such as: the number, age, rate and distribution of the mortalities of molluscs on the farm or mollusc farming area infected with *Marteilia refringens* including wild molluscs; the distance and density of neighbouring farms or mollusc farming areas including wild molluscs; the proximity to processing establishments, contact farms or mollusc farming areas; the species, especially susceptible species and vector species, present at the farms or mollusc farming areas; the farming practices applied in the affected and neighbouring farms and mollusc farming areas; the hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply:

(i) a protection zone shall be established in the immediate vicinity of a farm or mollusc farming area officially declared infected with *Marteilia refringens* and shall correspond to an area determined according to appropriate hydrodynamic or epidemiological data;

(ii) a surveillance zone shall be established outside the protection zone and shall correspond to an area, surrounding the protection zone, determined according to appropriate hydrodynamic or epidemiological data;

(b) all farms and mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with *Marteilia refringens* shall be subject to an official investigation comprising at least the collection of samples for the testing of 150 molluscs after the beginning of the transmission period of *Marteilia refringens*. When the transmission period is not known, the sampling shall begin in the period after the temperature of the water exceeds 17 °C;

(c) all farms and mollusc farming areas officially declared infected with *Marteilia refringens* shall be emptied, fallowed and if possible cleansed, and disinfected.

The duration of the fallowing period shall be at least:

(i) 2 months in case of the farms and mollusc farming areas with limited connections with the surrounding waters such as hatcheries and nurseries;

(ii) 2 months in case of the farms and mollusc farming areas with unlimited connections with the surrounding waters provided that the infected molluscs of the susceptible species and those molluscs of the susceptible species with epidemiological links with the infected farm or mollusc farming area have been harvested or removed before the period of the year when the prevalence of *Marteilia refringens* is known to be maximal, or when that period is not known, before the period when water temperature exceeds 17 °C;

(iii) 14 months in case of the farms and mollusc farming areas with unlimited connections with the surrounding waters provided that the infected molluscs of the susceptible species and those molluscs of the susceptible species with epidemiological links with the infected farm or mollusc farming area have not been harvested or removed before the period of the year when the prevalence of *Marteilia refringens* is known to be maximal or when such data is not known, when molluscs of the susceptible species have not been harvested or removed before the period when water temperature exceeds 17 °C.

When all farms and mollusc farming areas officially declared infected are emptied, at least 4 weeks of synchronised fallowing shall be carried out.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms or mollusc farming areas, as appropriate within the established protection and surveillance zones. The length of the fallowing period shall be determined by the competent authority following a case by case risk evaluation;

(d) all farms or mollusc farming areas officially declared infected and all other farms or mollusc farming areas fallowed within the established protection and surveillance zones shall be restocked with molluscs sourced from Member States, zones or compartments with a Category I health status as regards infection with *Marteilia refringens*.

Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2(c);

(e) all farms and mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme shall subsequently be subject to the surveillance scheme set out in point I.2.1 of this Part.

I.3. Specific requirements for the maintenance of disease-free health status (Category I) with regard to infection with *Marteilia refringens*

When targeted surveillance is required to maintain Category I health status, as provided for in Article 52 of Directive 2006/88/EC, all farms or mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment concerned shall be subject to health inspections and sampled in accordance with Table 4.B set out in Section II, taking into account the risk level of the farm or mollusc farming area for the contraction of *Marteilia refringens*.

The disease-free status may only be maintained as long as all samples using the diagnostic methods set out in point II.2 produced negative results for *Marteilia refringens* and any suspicion of *Marteilia refringens* is ruled out in accordance with the diagnostic methods set out in point II.3.

I.4. Requirements for the lifting of the containment measures provided for in Article 39 of Directive 2006/88/EC (change from Category V to Category III health status) with regard to infection with *Marteilia refringens*

A Member State, zone or compartment that has Category V health status with regard to infection with *Marteilia refringens* may achieve Category III health status with regard to that listed disease provided that:

(a) the requirements laid down in points I.2.2(a), (b) and (c) have been met. In case fallowing is not technically possible, the farms shall be subject to an alternative measure which provides almost similar guarantee for extermination of *Marteilia refringens* from the environment of the farm;

(b) all farms or mollusc farming areas officially declared infected and all other farms or mollusc farming areas fallowed/been subject to alternative measures in accordance with (a) within the established protection and surveillance zones have been restocked with molluscs sourced from Member States, zone or compartments with a Category I, II or III health status with regard to infection with *Marteilia refringens*;

(c) restocking has only taken place when all farms or mollusc farming areas officially declared infected have been emptied, cleansed, disinfected and fallowed or been subject to alternative measures in accordance with (a);

(d) no confirmation of infection with *Marteilia refringens* has occurred during the period of 2 years that follows the completion of the measures referred to in (a), (b) and (c), and suspicions during this period have been ruled out according to the procedures established in point II.3.

II. **Diagnostic methods and official investigations**

II.1. Samples

The whole animal shall be submitted to the laboratory for the performance of the diagnostic tests provided for in points II.2 and II.3.

II.2. Diagnostic methods to obtain or maintain disease-free status for infection with *Marteilia refringens*

The diagnostics methods to be used to obtain or maintain disease-free status for infection with *Marteilia refringens* following the detailed diagnostic methods and procedures set out in of Part 4 of Annex II, shall be histopathology, tissue imprints or PCR.

II.3. Official investigation and diagnostic methods to confirm the presence of or to rule out the suspicion of infection with *Marteilia refringens*

When a suspicion of infection with *Marteilia refringens* is required to be confirmed or ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedure shall be complied with:

(a) the official investigation shall include at least one sampling of 30 molluscs of susceptible species if the suspicion is based on a mortality report or if not, 150 molluscs of susceptible species after the beginning of the transmission period of *Marteilia refringens*. When the transmission period is not known, the sampling shall begin in the period after the temperature of the water exceeds 17 °C;

(b) Samples shall be tested using the diagnostic methods set out in point (i) following the detailed diagnostic methods and procedures set out in Section I of Part 4 of Annex II:

(i) the presence of *Marteilia refringens* shall be considered as confirmed when a positive result by histopathology, tissue imprints or *in situ* hybridisation is combined with a positive result PCR completed by sequencing;

(ii) the suspicion of infection with *Marteilia refringens* may be ruled out, if the tests referred to in (i) reveal no further evidence of the presence of *Marteilia refringens*.

Table 4.A

Surveillance scheme for Member States, zones or compartments for the control period which precedes the achievement of disease-free status for *Marteilia refringens* as referred to in point I.2.1

	Number of health inspections per year	Number of laboratory examinations per year	Number of molluscs in the sample
Farms/mollusc farming areas	1	1	150

Table 4.B

Surveillance schemes for Member States, zones or compartments to maintain disease-free status for *Marteilia refringens* as referred to in point I.3

Risk level	Number of health inspections	Number of laboratory examinations	Number of molluscs in the sample
High	1 every year	1 every 2 years	150
Medium	1 every 2 years	1 every 2 years	150
Low	1 every 2 years	1 every 4 years	150

PART 5

SURVEILLANCE AND CONTROL METHODS FOR INFECTION WITH *BONAMIA OSTREAE*

I. **Requirements for surveillance or eradication programmes to obtain and maintain disease-free health statuses with regard to infection with *Bonamia ostreae***

I.1. General requirements

Health inspections and, where appropriate, the sampling of production units shall be carried out in the period of the year when prevalence of *Bonamia ostreae* in the Member State, zone or compartment is known to be maximal. When such data is not available, sampling shall be carried out in winter or at the beginning of spring.

When molluscs are to be sampled in accordance with the requirements set out in Part 5, the following criteria shall apply:

(a) if *Ostrea edulis* are present, only oysters of that species shall be selected for sampling. If *Ostrea edulis* are not present, the sample shall be representative of all other susceptible species present;

(b) if weak, gaping or freshly dead but not decomposed molluscs are present, such molluscs shall primarily be selected. If such molluscs are not present, the molluscs selected shall include the oldest healthy molluscs;

(c) when sampling in farms which utilise more than one water source for mollusc production, mollusc representing all water sources shall be included for sampling in such a way that all parts of the farm are proportionally represented in the sample;

(d) when sampling in mollusc farming areas, molluscs from a sufficient number of sampling points shall be included in the sample. The main factors to be considered for the selection of those sampling points are previous points where *Bonamia ostreae* was detected, stocking density, water flows, the presence of susceptible species, the presence of vector species, bathymetry and management practices. Natural beds within or adjacent to the farming areas shall be included in the sampling.

I.2. Specific requirements to achieve Category I health status as regards *Bonamia ostreae*

I.2.1. Surveillance programmes

A Member State, zone or compartment that has Category III health status with regard to *Bonamia ostreae* may achieve Category I health status again with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment have been subject at least to the following surveillance programme comprising health inspections and the collection of samples for testing.

2-year surveillance programme:

(a) the farms and mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 5.A. set out in this Part;

(b) during that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 produced negative results for *Bonamia ostreae* and any suspicion of *Bonamia ostreae* has been ruled out in accordance with the diagnostic methods set out in point II.3;

(c) when *Ostrea edulis* sourced from a Member State, zone or compartment of Category I health status are to be included in the sample, they must have been introduced into the farm or mollusc farming area at least in the autumn just preceding the period when the surveillance programme is carried out.

I.2.2. Eradication programmes

Eradication of *Bonamia ostreae* is considered to be impossible in most cases, but where the Member State judges it to be feasible, the following model for an eradication programme shall apply.

A Member State, zone or compartment that has Category V health status as regards *Bonamia ostreae* may achieve Category I health status again with regard to that listed disease when all farms or mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment have been subject at least to the following eradication programme:

(a) the minimum control measures laid down in Section 3 of Chapter V of Directive 2006/88/EC have effectively been applied, and in particular a containment area as referred in point (b) of Article 32 of that Directive, comprising a protection zone and surveillance zone, has been established in the vicinity of the farm(s) or mollusc farming area(s) officially declared infected with *Bonamia ostreae*.

The containment area shall be defined on a case-by-case basis taking into account factors influencing the risks for the spread of that listed disease, such as: the number, rate, age and distribution of the mortalities of molluscs on the farm or mollusc farming area infected with *Bonamia ostreae* including wild molluscs; the distance and density of neighbouring farms or mollusc farming areas, including wild molluscs; the proximity to processing establishments, contact farms or mollusc farming areas; the species present at the farms or mollusc farming areas, especially susceptible species and vector species; farming practices applied in the affected and neighbouring farms or mollusc farming areas; hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply:

(i) a protection zone shall be established in the immediate vicinity of a farm or mollusc farming area officially declared infected with *Bonamia ostreae* and shall correspond to an area determined according to appropriate hydrodynamic or epidemiological data;

(ii) a surveillance zone shall be established outside the protection zone and shall correspond to an area surrounding the protection zone, determined according to appropriate hydrodynamic or epidemiological data;

(b) all farms and mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with *Bonamia ostreae* shall be subject to an official investigation comprising at least the collection of samples for testing of 150 molluscs of susceptible species after the beginning of the transmission period of *Bonamia ostreae*. When the transmission period is not known, the sampling shall begin in winter or at the beginning of spring;

(c) all farms and mollusc farming areas officially declared infected with *Bonamia ostreae* shall be emptied, fallowed and if possible cleansed and disinfected. The duration of the fallowing period shall be at least 6 months.

When all farms or mollusc farming areas officially declared infected are emptied, at least 4 weeks of synchronised fallowing shall be carried out.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms or mollusc farming areas, as appropriate within the established protection and surveillance zones. The length of the fallowing period shall be determined by the competent authority following a case by case risk evaluation;

(d) all farms or mollusc farming areas officially declared infected and all other farms or mollusc farming areas fallowed within the established protection and surveillance zones shall be restocked with molluscs sourced from Member States, zones or compartments with a Category I health status with regard to infection with *Bonamia ostreae*. Restocking shall only take place when all farms officially declared infected have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2(c);

(e) all farms and mollusc farming areas keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme must subsequently be subject to the surveillance programme laid down in point I.2.

I.3. Specific requirements for the maintenance of disease-free health status (Category I) with regard to infection with *Bonamia ostreae*

When targeted surveillance is required to maintain Category I health status, as laid down in Article 52 of Directive 2006/88/EC, all farms or mollusc farming areas keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment concerned shall be subject to health inspections and sampled in accordance with Table 5.B set out in Section II of this Part, taking into account the risk level of the farm or mollusc farming area for the contraction of infection with *Bonamia ostreae*.

Disease-free status with regard to infection with *Bonamia ostreae* may only be maintained as long as all samples using the diagnostic methods set out in point II.2 have produced negative results for *Bonamia ostreae* and any suspicion of *Bonamia ostreae* has been ruled out in accordance with the diagnostic methods set out in point II.3.

I.4. Requirements for the lifting of the containment measures provided for in Article 39 of Directive 2006/88/EC (the change from Category V to Category III health status) with regard to infection with *Bonamia ostreae*.

A Member State, zone or compartment that has Category V health status with regard to infection with *Bonamia ostreae* may achieve Category III health status with regard to that disease provided that:

(a) the requirements set out in points I.2.2(a), (b) and (c) have been met. In case fallowing is not technically possible, the farms shall be subject to an alternative measure which provides almost similar guarantee for extermination of *Bonamia ostreae* from the environment of the farm;

(b) all farms or mollusc farming areas officially declared infected and all other farms or mollusc farming areas fallowed/been subject to alternative measures in accordance with (a) within the established protection and surveillance zones have been restocked with molluscs sourced from Member States, zones or compartments with a Category I, II or III health status with regard to infection with *Bonamia ostreae*.

(c) the restocking has only taken place when all farms or mollusc farming areas officially declared infected have been emptied, cleansed, disinfected and fallowed/been subject to alternative measures in accordance with (a);

(d) no confirmation of infection with *Bonamia ostreae* has occurred during the period of 2 years that follows the completion of the measures referred to in (a),(b) and (c), and suspicions during this period have been ruled out according to the procedures established in point II.3.

II. **Diagnostic methods and diagnostic criteria**

II.1. Samples

The whole animal shall be submitted to the laboratory for the performance of the diagnostic tests provided for in points II.2 and II.3.

II.2. Diagnostic methods to obtain or maintain disease-free status for infection with *Bonamia ostreae*

The diagnostics methods to be used to obtain or maintain disease-free status for infection with *Bonamia ostreae*, shall be histopathology, tissue imprints or PCR. When applying these diagnostic methods, the corresponding detailed methods and procedures set out in of Part 5 of Annex II shall be followed.

II.3. Diagnostic criteria to confirm of the presence of or to rule out the suspicion of infection with *Bonamia ostreae*

When a suspicion of infection with *Bonamia ostreae* is required to be confirmed or ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedure shall be complied with:

The official investigation shall include at least one sampling of 30 molluscs of susceptible species if the suspicion is based on a mortality report, or if not 150 molluscs of susceptible species after the beginning of the transmission period of *Bonamia ostreae*. When the transmission period is not known, the sampling shall begin in the winter or at the beginning of spring. The samples shall be tested using the diagnostic methods set out in point (i) following the detailed diagnostic methods and procedures set out in Section I of Part 5 of Annex II.

(i) the presence of *Bonamia ostreae* shall be considered as confirmed when a positive result by histopathology, tissue imprints or *in situ* hybridisation is combined with a positive result by PCR completed by sequencing in accordance with the approved methods and procedures set out in Part 5 of Annex II;

(ii) the suspicion of the presence of infection with *Bonamia ostreae* shall be ruled out, if those tests reveal no further evidence of the presence of *Bonamia ostreae*.

Table 5.A

Surveillance scheme for Member States, zones or compartments for the control period which precedes the achievement of disease-free status for *Bonamia ostreae* as referred to in point I.2.1

	Number of health inspections per year	Number of laboratory examinations per year	Number of molluscs in the sample
Farms/Mollusc farming areas	1	1	150

Table 5.B

Surveillance schemes for Member States, zones or compartments to maintain disease-free status for *Bonamia ostreae* as referred to in point I.3

Risk level	Number of health inspections	Number of laboratory examinations	Number of molluscs in the sample
High	1 every year	1 every 2 years	150
Medium	1 every 2 years	1 every 2 years	150
Low	1 every 2 years	1 every 4 years	150

PART 6

SURVEILLANCE AND CONTROL METHODS FOR WHITE SPOT DISEASE (WSD)

I. **Requirements for surveillance and eradication programmes to obtain and maintain disease-free health statuses with regard to WSD and to contain infection with WSSV**

I.1. General requirements for inspections and sampling

The sampling of crustaceans for laboratory examination shall be carried out whenever the water temperature is likely to reach its highest annual point. That requirement concerning water temperature shall also apply to health inspections where these are feasible and appropriate.

When farmed crustaceans are to be sampled in accordance with the requirements set out in this Part, the following criteria shall apply:

(a) if weak or moribund crustaceans are present in the production units, such crustaceans shall primarily be selected. If such crustaceans are not present, those selected shall include crustaceans of different size cohorts namely juveniles and adults, of the selected susceptible species, proportionally represented in the sample;

(b) if more than one water source is utilised for crustacean production, susceptible crustacean representing all water sources must be included for sampling.

When targeted surveillance in wild populations is required in accordance with the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the number and geographical distribution of the sampling points shall be determined to obtain a reasonable coverage of the Member State, zone or compartment. The sampling points shall also be representative of the different ecosystems where the wild populations of susceptible species are located namely marine, estuary, river and lake systems.

When targeted surveillance in wild populations is required in accordance with the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the crustaceans to be sampled shall be selected as follows:

(i) in marine and estuary systems areas, one or more of the following species shall be selected: *Carcinus maenas, Cancer pagurus, Eriocheir sinensis, Liocarcinus depurator, Liocarcinus puber, Crangon crangon, Homarus gammarus, Palaemon adspersus* or penaeid shrimp species namely *Penaeus japonicus, Penaeus kerathurus, Penaeus semisulcatus*. If those species are not present, the sample must be representative of other susceptible decapod species present. Given the broad susceptible host range, hosts may be selected from genera or families of the Decapoda where susceptibility has been experimentally or naturally demonstrated;

(ii) in river and lake systems, one or more of the following species shall be selected: *Pacifastacus leniusculus, Astacus leptodactylus, Austropotamobius pallipes* or *Orconectes limosus*. If those species are not present, the sample must be representative of other susceptible decapod species present. Given the broad susceptible host range, hosts may be selected from genera or families of the Decapoda where susceptibility has been experimentally or naturally demonstrated;

(iii) if weak or moribund crustaceans are present, such crustaceans shall primarily be selected. If such crustaceans are not present, those selected shall include crustaceans of different size cohorts namely juveniles and adults of the selected susceptible species, proportionally represented in the sample.

I.2. Specific requirements to obtain Category I health status with regard to WSD

I.2.1. Surveillance programmes

(a) a Member State, zone or compartment that has Category III health status with regard to WSD in accordance with part B of Annex III to Directive 2006/88/EC may achieve Category I health status with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment, meet the relevant requirements set out in Annex V to that Directive and all those farms and, when required by the second paragraph of point 2 of Part I of Annex V to Directive 2006/88/EC, the sampling points in wild populations selected in accordance with that point, have been subject to the following 2-year surveillance programme comprising health inspections and collection of samples for testing.

The farms or sampling points have been subject to health inspections and sampled for a minimum period of two consecutive years as laid down in Table 6.A set out in Section II.

During that 2-year period, the testing of all samples using the diagnostic methods set out in point II.2 have produced negative results for infection with WSD and any suspicion of WSD has been ruled out in accordance with the diagnostic methods set out in point II.3.

(b) when during the implementation of the surveillance programme referred to in (a), infection with WSSV is confirmed in a farm included in that surveillance programme, and therefore its Category II health status has been withdrawn, that farm may immediately regain the Category II health status and continue the implementation of the surveillance programme to obtain disease-free status without implementing an eradication programme as set out in point I.2.2. provided that:

(i) it is a continental farm whose health status regarding WSD is independent of the health status regarding that listed disease of the surrounding natural waters in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC;

(ii) it has been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period must be at least 6 weeks;

(iii) it has been restocked with crustaceans sourced from Member States, zones or compartments with a Category I health status with regard to WSD.

I.2.2. Eradication programmes

I.2.2.1. General requirements

A Member State, zone or a compartment that has Category V health status with regard WSD may achieve Category I health status with regard to that listed disease when all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or the compartment have been subject at least to the following eradication programme:

(a) the minimum control measures laid down in Section 4 of Chapter V of Directive 2006/88/EC have effectively been applied, and a containment area referred to in Article 32(b) of that Directive, comprising a protection zone and surveillance zone, has been established in the vicinity of the farm(s) officially declared infected with WSD.

The containment area must have been defined on a case-by-case basis taking into account factors influencing the risks for the spread of WSD to farmed and wild crustaceans, such as: the number, rate and distribution of the mortalities of crustaceans on the farm infected with WSD; the distance and density of neighbouring farms; contact farms; the species present at the farms; the farming practices applied in the affected and neighbouring farms; hydrodynamic conditions and other factors of epidemiological significance identified.

For the establishment of the protection and surveillance zones, the following minimum requirements shall apply:

(i) a protection zone shall be established in the immediate vicinity of a farm officially declared infected with WSD and shall correspond to:

(1) in marine and estuarine areas: the area included in a circle with a radius of at least one tidal excursion or at least 5 km, whichever is larger, centred on the farm officially declared infected with WSD, or an equivalent area determined according to appropriate hydrodynamic or epidemiological data; or

(2) in freshwaters: the entire water catchment area of the farm officially declared infected with WSD; the competent authority may limit the extension of the protection zone to parts of the water catchment area provided that the prevention of the spread of WSD is not compromised;

(ii) a surveillance zone shall be established outside the protection zone and shall correspond:

(1) in marine areas: an area, surrounding the protection zone, of overlapping tidal excursion zones; or an area, surrounding the protection zone, and included in a circle of a radius of 10 km from the centre of the protection zone; or an equivalent area determined according to appropriate hydrodynamic or epidemiological data; or

(2) in freshwaters: as an extended area outside the established protection zone;

(b) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the protection zone not officially declared infected with WSD shall be subject to an official investigation comprising at least the following:

(i) the collection of samples for testing of 10 crustaceans, when clinical signs or post-mortem signs consistent with infection WSD are observed, or 150 crustaceans, when clinical or post-mortem signs are not observed; and

(ii) one health inspection; in those farms where the tests referred to in (i) have produced negative results, health inspections shall continue once per month during the season when the water temperature is likely to reach its highest annual points, until the protection zone has been withdrawn in accordance with point I.2.2.1(c).

(c) all farms officially declared infected with WSD shall be emptied, cleansed disinfected and fallowed. The duration of the fallowing period shall be at least 6 weeks. When all farms officially declared infected are emptied, at least 3 weeks of synchronised fallowing shall be carried out. This paragraph shall also apply to new farms officially declared infected during the implementation of the eradication programme.

When fallowing of the officially declared infected farms is carried out, the protection zones shall be converted into surveillance zones.

The competent authority may decide to require the emptying, cleansing, disinfection and fallowing of other farms within the established protection and surveillance zones. The length of that fallowing period shall be determined by the competent authority following a case by case risk evaluation.

(d) all farms officially declared infected and all other farms fallowed within the established protection and surveillance zones shall be restocked:

(i) with crustaceans sourced from Member States, zones or compartments with a Category I health status as regards WSD; or

(ii) for a transitional period until 31 December 2020, with crustaceans from Member States, zones or compartments with an approved WSD surveillance programme.

Restocking shall only take place when all farms officially declared infected with WSD have been emptied, cleansed, disinfected and fallowed in accordance with point I.2.2.1(c).

(e) all farms keeping susceptible species listed in Part II of Annex IV to Directive 2006/88/EC within the Member State, zone or compartment covered by the eradication programme and, when surveillance in wild populations is required, sampling points selected in accordance with the second paragraph of point 2 of Part I of Annex V to that Directive, must subsequently be subject at least to the programme laid down in point I.2.1.

I.2.2.2. Requirements for regaining disease-free status with regard to WSD for continental compartments comprising one single farm that previously was declared free of WSD

A continental compartment comprising one single farm that has Category I health status with regard to WSD, whose health status with regard to that listed disease is independent of the surrounding natural waters in accordance with point 3 of Part II of Annex V to Directive 2006/88/EC, and whose Category I status has been withdrawn in accordance with Article 53(3) of that Directive, may regain Category I health status immediately after the competent authority has confirmed that the following conditions have been complied with:

(a) the farm with WSD has been emptied, cleansed, disinfected and fallowed; the duration of the fallowing period must have been at least 6 weeks;

(b) the farm with WSD has been restocked with crustaceans sourced from Member States, zones or compartments with a Category I health status with regard to WSD.

I.3. Specific requirements for the maintenance of disease-free health status (Category I) with regard to WSD

When targeted surveillance is required to maintain category I health status, as laid down in Article 52 of Directive 2006/88/EC, all farms keeping susceptible species listed in Part II of Annex IV to that Directive within the Member State, zone or compartment concerned shall undergo a health inspection and sampling in accordance with Table 6 B set out in Section II, taking into account the risk level of the farm for the contraction of WSD.

In Member States, zones or compartments where the number of farms is limited and targeted surveillance on those farms does not provide sufficient epidemiological data, the surveillance programmes to maintain disease-free status shall include sampling points selected in accordance with the requirements laid down in point I.1.

Those sampling points shall be inspected and sampled by rotation of 50 % of the sampling points each year. The sampling shall be carried out in accordance with Table 6 B set out in Section II. The samples shall be selected, prepared and examined in accordance with the diagnostic and sampling methods set out in Section II and the laboratory examinations must have produced negative results as regards to the agent of WSD.

Disease-free status shall only be maintained as long as all samples tested using the diagnostic and sampling methods set out in point II.2 produce negative results for WSD and any suspicion of WSD has been ruled out in accordance with the official investigation and diagnostic methods set out in point II.3.

I.4. Requirements for the lifting of the containment measures provided for in Article 39 of Directive 2006/88/EC (the change from Category V to Category III health status) with regard to WSD

A Member State, zone or a compartment which has Category V health status with regard to WSD may achieve Category III health status with regard to that listed disease provided that:

(a) the requirements set out in points I.2.2.1(a), (b) and (c) have been met. In case fallowing is not technically possible, the farms shall be subject to an alternative measure which provides almost similar guarantee for extermination of WSSV from the environment of the farm;

(b) all farms officially declared infected with WSD and all other farms fallowed/been subject to alternative measures in accordance with (a) within the established protection and surveillance zones have been restocked with crustacean sourced from Member States, zones or compartments with a Category I, II or III health status with regard to WSD.

(c) the restocking has only taken place when all farms officially declared infected with WSD have been emptied, cleansed, disinfected and fallowed/been subject to alternative measures in accordance with (a);

(d) no detection of WSD has occurred during the period of 2 years that follows the completion of the measures set out in paragraphs (a) and (b) and suspicions during this period have been ruled out according to the procedures established in point II.3.

II. **Diagnostic and sampling methods**

II.1. Samples

Samples of integumental epidermis, either dissected or contained within walking legs, pleopods, mouthparts or gills of the test animal shall be fixed in 95 % ethanol prior to the preparation of samples for two-step PCR.

Other samples, fixed for histology and transmission electron microscopy may be collected to support diagnostic data arising from PCR.

II.2. Diagnostic methods to obtain or maintain disease-free status with regard to WSD

The diagnostic method to be used to obtain or to maintain disease-free status with regard to WSD following the detailed methods and procedures set out in Part 6 of Annex II, shall be two-step PCR.

In the case of a positive result of the two-step PCR, the result shall be corroborated by the sequencing of the amplicon before the initial control measures provided for in Article 28 of Directive 2006/88/EC are implemented, if possible under practical conditions by the demonstration of pathognomonic signs of WSD in those susceptible hosts selected, via histology and transmission electron microscopy.

II.3. Official investigation and diagnostic methods to rule out the suspicion of or to confirm the presence of infection with WSD

When the presence of infection with WSD is required to be confirmed or the suspicion of such infection is required be ruled out in accordance with Article 28 of Directive 2006/88/EC, the following inspection, sampling and testing procedure shall be complied with:

(a) the official investigation shall include at least one health inspection and one sampling of 10 crustaceans when clinical or post-mortem signs consistent with infection with WSD are observed or 150 crustaceans when clinical or post-mortem signs are not observed. The samples shall be tested using the diagnostic method set out in point II.2 (two-step PCR);

(b) the presence of WSD shall be considered as confirmed when two-step PCR followed by sequencing, following the detailed methods and procedures set out in this Part 6 of Annex II, is positive for WSSV and, when pathognomonic signs of WSD are present in the selected hosts.

The suspicion of WSD may be ruled out, if those tests reveal no further evidence of the presence of WSD.

Table 6 A

Surveillance scheme for Member States, zones and compartments for the 2-year control period which precedes the achievement of WSD disease-free status as referred to in point I.2.1

	Number of clinical inspections per year	Number of laboratory examinations per year	Number of crustaceans in the sample
Farms/sampling sites	1	1	150

Table 6 B

Surveillance schemes for Member States, zones or compartments to maintain WSD disease-free status as referred to in point I.3

Risk level	Number of health inspections	Number of laboratory examinations	Number of crustaceans in the sample
High	1 every year	1 every 2 years	150
Medium	1 every 2 years	1 every 2 years	150
Low	1 every 2 years	1 every 4 years	150

ANNEX II

DETAILED DIAGNOSTIC METHODS AND PROCEDURES

I. **Introduction**

This Annex sets out the detailed procedures for the diagnostic methods to be used for the laboratory examination in the eradication and surveillance programmes set out in Annex I to this Decision, and in order to confirm or rule out the suspected presence of the following non-exotic diseases listed in Part II to Annex IV to Directive 2006/88/EC ('the listed diseases') in accordance with Article 57 (b) to that Directive:

1.	Viral haemorrhagic septicaemia (VHS)	Part 1
2.	Infectious hematopoietic necrosis (IHN)	Part 1
3.	Koi herpesvirus (KHV) disease	Part 2
4.	Infectious salmon anaemia (ISA)	Part 3
5.	Infection with *Marteilia refringens*	Part 4
6.	Infection with *Bonamia ostreae*	Part 5
7.	White spot disease (WSD)	Part 6

II. **Definitions**

For the purpose of this Annex, 'transport medium' means a cell culture medium with 10 % calf serum and with 200 iu penicillin, 200 µg streptomycin, and 200 µg kanamycin per millilitre or with other antibiotics of proven efficacy.

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.

PART 2

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF KHV DISEASE (KHVD)

I. **Detailed diagnostic methods and procedures for the confirmation of the presence of or to rule out the suspicion of KHVD**

When a laboratory examination is required for the purpose of confirmation of the presence of or to rule out the suspicion of KHVD in accordance with Article 57(b) of Directive 2006/88/EC, using the diagnostic methods set out in Section III of Part 2 of Annex I, the detailed diagnostic methods and procedures set out in points I.1 – I.2 of this Part shall apply.

I.1. Preparation of samples from fish

For diagnostic purposes the fish (sent alive or killed and packed separately in sealed aseptic containers) or alternatively frozen organs or organ pieces preserved in 80 % to absolute ethanol or viral transport medium (to be processed within 48 hours after collection) may be used for testing with conventional PCR or qPCR based methods.

For the detection of KHV, gill and kidney shall be collected; in addition spleen, encephalon and intestine may be included in an additional separated sample. In acute cases, tissue material of up to five fish may be pooled.

Furthermore, non-lethal samples such as blood, gill swabs, gill biopsy, mucus scrape may be used in certain cases (namely very valuable fish may be used in the case of the suspicion of the presence of KHV).

I.1.1. DNA extraction

DNA shall be extracted in accordance with standard procedures.

The commercially available DNA extraction kits that produce high quality DNA suitable for use with the PCR protocols referred to in point I.2 may be used.

I.2. Agent detection and identification by Polymerase chain reaction (PCR) based methods

I.2.1. qPCR for KHV detection

For qPCR detection of KHV, the following qPCR assay shall be used:

Forward primer (KHV-86f): 5′- GACGCCGGAGACCTTGTG -3′;

Reverse primer (KHV-163r): 5′- CGGGTTCTTATTTTTGTCCTTGTT -3′;

and probe (KHV-109p): 5′-FAM- CTTCCTCTGCTCGGCGAGCACG -3′.

Cycling conditions: one cycle of 95 °C at 15 minutes, followed by 40 cycles of 94 °C at 15 seconds and 60 °C for 60 seconds. Negative template controls and positive controls shall be included on each plate run. However, other qPCR versions of proven similar efficacy may be used instead.

I.2.2. Conventional PCR for KHV detection

The assay described in this point targeting the Thymine kinase (TK) gene of KHV shall be used. However, other PCR assays with demonstrated similar sensitivities and specificities to the described assay may be used instead.

Forward primer (KHV-TKf): 5′-GGGTTACCTGTAC GAG-3′;

Reverse primer (KHV-TKr): 5′-CACCCAGTAGATTA TGC-3′.

Cycling conditions: one cycle of 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, 72 °C for one minute and one cycle of 72 °C for 10 minutes. Product size should be 409 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 contamination. Negative template controls and positive controls shall be included on each plate run. However, other PCR versions of proven similar efficacy may be used instead.

The first detection in an area shall be confirmed by sequencing or sent to a to a 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.

II. **Detailed diagnostic methods and procedures for the surveillance of KHVD**

When sampling and laboratory examination for the purpose of obtaining or maintaining certain health statuses with regard to KHVD as set out in Section I of Part 2 of Annex I are carried out using the diagnostic methods set out in Sections II or III of Part 2 of that Annex, the detailed diagnostic methods and procedures set out in the following points II.1 and II.2 of this Part shall apply.

II.1. Preparation of samples from fish

If possible, fish that have been kept for a prolonged time period at the virus permissive temperature range (namely, 2 to 3 weeks at 15 °C to 26 °C) shall be sampled. If possible, samples shall be collected 24 hours but not later than 72 hours after management practices that may reactivate the virus in fish with a carrier status, such as netting or transport, in order to enhance the chance of KHV detection.

For the purpose of the surveillance of KHVD, the fish may be sent alive or killed and packed separately in sealed aseptic containers or alternatively frozen organs or organ pieces preserved in 80 % to 100 % alcohol or viral transport medium (to be processed within 48 hours after collection) may be used for testing PCR based methods. For the surveillance of KHVD, gill and kidney tissue shall be collected.

For the purpose of surveillance of KHVD, pooling shall be avoided where possible. If pooling is necessary, tissue material from a maximum of two fish may be pooled. Larger samples shall be homogenised in mortar and pestle or stomacher, and subsamples retrieved for DNA extraction before clarification. Alternatively, subsamples may be collected from each tissue included in the sample and placed in 'lysis-tubes'.

II.1.1. DNA extraction

DNA shall be extracted in accordance with standard procedures. Commercially available DNA extraction kits that produce high quality DNA suitable for use with the PCR protocols set out in point II.2 may be used.

The acceptable tissue medium ratio shall be 1:9 w/v. 20 to 25 mg tissue material shall be included in the tests.

II.2. Surveillance of KHVD by PCR based methods

For the surveillance of KHV, a qPCR shall be used. If positive samples appear in an area not previously confirmed positive, the test results shall be confirmed either:

(a) by sequencing of a PCR or nested PCR product from the samples.

The obtained clean consensus sequence shall match (by at least 98 %) with these reference sequences.

(b) or alternatively, samples may be sent to a national reference laboratory for confirmation.

II.2.1. qPCR for KHV detection

The qPCR described as follows shall be used:

Forward primer (KHV-86f): 5′- GACGCCGGAGACCTTGTG -3′;

Reverse primer (KHV-163r): 5′- CGGGTTCTTATTTTTGTCCTTGTT -3′;

and probe (KHV-109p): 5′-FAM- CTTCCTCTGCTCGGCGAGCACG -3′.

Cycling conditions: one cycle of 95 °C at 15 minutes, followed by 50 cycles of 94 °C at 15 seconds and 60 °C for 60 seconds.

The results of the qPCR 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. Negative template controls and positive controls shall be included on each plate run. However, other qPCR versions of proven similar efficacy may be used instead.

II.2.2. Conventional PCR for confirmation of KHV detection

For confirmation of the presence of infection with KHV, the generic nested PCR described in the following Table 2.1 shall be used followed by sequencing of the amplified product.

Table 2.1

Primers and conditions for the nested PCR assay targeting all cyprinid herpesviruses (CyHV-1, CyHV-2 and CyHV-3)

Primer name	Sequence	Cycling conditions	Product size
CyHVpol-forward	5′-CCAGCAACATGTGCGACGG-3′	First round PCR *1 cycle:* 95 °C 2 minutes *40 cycles:* 95 °C for 30 seconds 55 °C for 30 seconds 72 °C for 45 seconds *1 cycle:* 72 °C for 10 minutes	362 bp
CyHVpol-reverse	5′-CCGTARTGAGAGTTGGCGCA-3′		

Primer name	Sequence	Cycling conditions	Product size
CyHVpol-internal forward	5′-CGACGGVGGYATCAGCCC-3′	Second round PCR *1 cycle:* 95 °C 2 minutes, *40 cycles:* 95 °C for 30 seconds 55 °C for 30 seconds 72 °C for 45 seconds *1 cycle:* 72 °C for 10 minutes	339 bp
CyHVpol-internal reverse	5′-GAGTTGGCGCAYACYTTCATC-3′		

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. Negative template controls and positive controls shall be included on each plate run. PCR versions of proven similar efficacy may be used instead.

Sequencing may be performed by the laboratory or at external specialised sequencing companies. Sequencing results shall be analysed by aligning the sequences to the known reference sequences of KHV (Gen Bank accession numbers AP008984, DQ657948 and DQ177346). The obtained clean consensus sequence must match at least to 98 % with those reference sequences.

PART 3

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTIOUS SALMON ANEMIA (ISA)

I. **Sampling procedures for the surveillance and control of ISA**

When sampling and laboratory examination are carried out, for the purpose of the surveillance or eradication programmes set out in Part 3 of Annex I or to confirm or rule out the presence of ISA in accordance with Article 57(b) of Directive 2006/88/EC, the detailed methods and procedures set out in points I.1, I.2 and I.3 of this Section shall apply.

I.1. Preparation of samples from fish

For the purpose of laboratory examination for the presence of ISA, fish samples shall not be pooled where possible. However, for the purpose of surveillance for ISA, the pooling of 2 to be 5 fish is accepted.

Samples for Reverse Transcriptase polymerase chain reaction (RT-PCR) analysis shall be taken from all of the fish sampled. A piece of mid-kidney shall be removed from the fish using a sterile instrument and transferred to a microfuge tube containing one ml RNA preservative solution of proven efficacy. Tissue from up to five fish may be collected in one tube of transport solution and shall represent one pooled sample. The weight of tissue in one sample shall be 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 with a normal constitution, exhibiting clinical signs or post-mortem findings consistent with the presence of ISA. Any external or internal lesions shall be sampled and in any case samples of mid-kidney, heart, liver, pancreas, gut, 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 shall be at least 20:1 to ensure satisfactory preservation of the tissues. For immunohistochemistry (IHC), samples from mid-kidney and heart shall be taken.

Tissues for virological examination on cell culture shall be taken from all of the fish sampled. Pieces of the liver, anterior or mid-kidney, heart and spleen shall be removed from the fish using a sterile instrument and transferred to plastic tubes containing 9 ml transport medium. 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.

I.2. Shipment of samples from fish

Whole fish may be transported to the laboratory if the temperature requirements during transportation, as described in paragraph 3 of this point can be fulfilled. Whole fish shall be wrapped in absorbent paper and shipped in a plastic bag, chilled as described in that paragraph.

Live fish may also be shipped, but only under the supervision of the National reference laboratory for fish diseases and taking into account 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, such as thick-walled polystyrene boxes, together with sufficient ice or freeze blocks to ensure chilling of the samples during transportation to the laboratory. Freezing shall be avoided and ice must still be present in the transport box at receipt of the shipment 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 Ribonucleic acid(RNA)later, RNA extraction shall be carried out within the following time frames depending on the temperature the samples are stored at:

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.

If fish tissues are transported in fixative for histological examination, they shall be shipped in leak proof tubes in impact-resistant containers. Freezing of those samples shall be avoided.

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.3. Collection of supplementary diagnostic material

Subject to the approval of the diagnostic laboratory, fish tissues other than those referred to in point I.1 may be collected and prepared for supplementary examination.

II. **Detailed diagnostic methods and procedures for the surveillance and for the confirmation of the presence of or to rule out the suspicion of ISA**

When laboratory examination for the purpose of obtaining or maintaining a certain health status with regard to ISA as set out in Section I of Part 3 of Annex I, or for the purpose of the confirmation of the presence of or to rule out a suspicion of ISA in accordance with Article 57(b) of Directive 2006/88/EC, are carried out, using the diagnostic methods set out in Section II of Part 3 of Annex I, the detailed methods and procedures set out in the following points II.1 to II.5 shall apply.

II.1. Examination of samples by RT-PCR

The diagnostic method to be used for the screening for ISAV shall be RT-qPCR. As the results of the RT-qPCR can vary depending on the conditions under which it is performed, adequate positive and negative controls and amplicons shall be included to avoid any doubts.

II.1.1. Total RNA extraction

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

Total RNA shall be extracted using the phenol-chloroform method or RNA affinity spin columns, according to the manufacturer's instructions.

Purified RNA shall be re-suspended in distilled RNase-free water (namely water treated with 0,1 % diethyl pyrocarbonate).

The concentration and purity of the extracted RNA shall be estimated by measuring the optical density at 260 nm and at 280 nm. An alternative approach may be to include internal controls targeted against the virus genome as referred to in point II.1.3.

II.1.2. RT-PCR for ISAV detection

Several RT-PCR methods may be used for ISAV genome amplification. A two-step RT-PCR may 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, may also be performed. The one-step method shall be used where possible, as the one tube assay minimises the risk of cross-contamination as no transfer of content have to be made and it is regarded to be as sensitive as the two-step method.

The primers and assay described in this point, namely the ILA1 or ILA2 primer pair that target segment 8 and which have been found suitable for detection of ISAV in outbreaks and in carrier fish, shall be used. The ILA2 reverse primer does not match isolates from North America and an alternative primer set shall be used in those cases.

Forward primer (ILA1): 5′-GGCTATCTACCATGAACGAATC-3′;

Reverse primer (ILA2): 5′-GCCAAGTGTAAGTAGCACTCC-3′.

Cycling conditions: one cycle of 50 °C for 30 minutes, 1 cycle of 94 °C for 15 minutes, 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds; one cycle of 72 °C for 5 min. Product size 155 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. Negative template controls and positive controls shall be included on each plate run. However, other RT-PCR versions of proven similar efficacy may be used instead.

II.1.3. RT-qPCR for ISAV detection

The use of RT-qPCR may increase specificity and probably also sensitivity. The method can be performed more rapidly as no gel electrophoresis step is required and it 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 RT-qPCR assay is that it is often not possible to sequence amplified products. However, if there is doubt on the specificity of the amplified product, another ISAV specific assay must be run to verify the result.

The assay described in this point, which is an assay that target segment 8, shall be used. This assay shall cover isolates from the European Union, the European Free Trade Association and North America. The one-step method shall be used where possible, because the one tube assay minimises the risk of cross-contamination.

Forward primer: 5′- CTACACAGCAGGATGCAGATGT -3′;

Reverse primer: 5′- CAGGATGCCGGAAGTCGAT -3′;

and probe: 5′-FAM- CATCGTCGCTGCAGTTC – *MGBNFQ*-3′.

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

II.1.4. Sequencing of amplified PCR products

Forward primer (ILAs6-3F): 5′-ATGAGGGAGGTAGCATTGCA -3′;

Reverse primer (ILAs6-2R): 5′-CATGCTTTCCAACCTGCTAGGA -3′.

Negative template controls and positive controls shall be included on each plate run. Cycling conditions (One-step RT-PCR): one cycle of 50 °C for 30 minutes, one cycle of 94 °C for 15 minutes, 40 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 60 seconds, one cycle of 72 °C for 5 minutes; it shall be adjusted if necessary. Other RT-PCR or RT-qPCR versions of proven similar efficacy may be used instead.

Alternatively, the following method for sequencing HPR in segment 6 may be used:

Forward primer: 5′-GAC-CAG-ACA-AGC-TTA-GGT-AAC-ACA-GA-3′;

Reverse primer: 5′-GAT-GGT-GGA-ATT-CTA-CCT-CTA-GAC-TTG-TA-3′;

Product size: 304 nt if HPR0.

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

The purity of the amplified RT-PCR product shall be checked by gel electrophoresis before sequencing. If only one pure fragment appears, it shall be purified directly from the PCR reaction. If multiple amplified fragments are present, the fragment of interest shall be purified by gel electrophoresis. The purification of PCR fragments from solutions or agarose gels shall be made using PCR fragment affinity spin columns, according to the manufacturer's instructions.

Sequencing shall be performed using amplification primers at external specialised sequencing companies. The results shall be analysed with the search tool BLAST and the sequences shall be compared with other known sequences in the US National Centre for Biotechnical Information (NCBI) nucleotide database.

Sequencing must 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

The tissue may be kept at – 80 °C. The tissue shall only be frozen and thawed once before examination. For surveillance and control purposes, the examination shall be undertaken as fast as possible.

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 1:2 000 in a 50 % plaque neutralisation test. The mixture shall be incubated for one hour at 15 °C. This shall represent the inoculum.

Treatment of all inocula with antiserum to Infectious pancreatic necrosis virus (a virus which in some parts of the Europe occurs in 50 % of fish samples) aims at preventing cytopatic effect (CPE) due to IPN virus from developing in inoculated cell cultures. To 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, such treatment may be carried out. When samples come from production units which are considered free from IPN, the treatment of inocula with antiserum to IPN virus may be omitted.

II.2.2. Inoculation on cell cultures

Atlantic salmon kidney (ASK) cells shall be used for primary ISAV isolation. Other cell lines of proven efficacy 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. The ASK cells seem to support isolation and growth of the hitherto known virus isolates, as long as a low passage level is used. A more distinct cytopathic effect (CPE) may appear in ASK cells than in other susceptible cell lines like SHK-1 (Salmon head kidney-1).

ASK (pass 65 or lower) cells shall be grown in L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine, and 0,08 % (vol:vol) 50 mM 2-mercaptoethanol in multi-well plates. 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, 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 Tissue culture infective dose at the 50 % end point (TCID50 ml^{-1})) shall be inoculated into the first well and mixed. A volume of this material shall be transferred from the first well to the second well to make a 1:10 dilution and mixed. This shall be repeated across the plate to make six 10-fold dilutions. Stock ISAV may be stored at – 80 °C for at least 2 years but once thawed must be used within 3 days. Care shall be taken to prevent cross-contamination of test plates with positive control material. To avoid that risk, positive controls shall be set up and handled separately from test plates. A sensitivity test every 6 months of ASK cells towards ISAV isolates may replace the use of including a positive control at each inoculation.

Samples shall be incubated at 15 ± 2 °C for up to 15 days. Using a microscope, cell cultures shall be examined for CPE twice, between 5 to 7 and 12 to 14 days following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately in accordance with point II.2.4. If no CPE is observed by day 14, an Indirect fluorescent antibody test (IFAT), haemadsorption or RT-PCR shall be performed.

II.2.3. Subcultivation

Subcultivation shall be carried out between days 13 to 15. Culture supernatant shall be added to wells containing fresh actively growing cells in appropriate dilution (1/10) in multi-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 days 14 to 18 following inoculation. If any pool shows CPE, virus identification procedures shall be initiated immediately in accordance with point II.2.4. If no CPE is observed by days 14 to 18, a haemadsorption or an RT-PCR test shall be performed.

If cytotoxicity occurs within the first 7 days of incubation, subcultivation shall be performed at that stage, and the cells shall be incubated for 14 to 18 days and sub cultivated again with a further period of 14 to 18 days incubation. If cytotoxicity occurs after 7 days, subcultivation shall be performed once and the cells shall be incubated to achieve the total period of 28 to 36 days incubation from the primary inoculation.

If bacterial contamination occurs in the primary culture, the test shall be set up again using the tissue homogenate stored at – 80 °C. Prior to inoculation, the tissue homogenate shall be centrifuged at 4 000 × *g* for 15 to 30 minutes at 0 to 6 °C and the supernatant shall be 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.2.4. 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 the identification of ISAV shall be RT-PCR in accordance with point II.1 and Immunofluorescence (IF) in accordance with point II.2.6. If it is considered that other viruses may be present, supplementary virus identification tests shall be carried out. If those tests have not resulted in a definitive identification of the virus within 1 week, the supernatant shall be forwarded for immediate identification to:

(a) the World Organisation for Animal Health (OIE) reference laboratory for ISA, or;

(b) a national reference laboratory or the EU reference laboratory for fish diseases as referred to in Annex VI to Directive 2006/88/EC.

II.2.5. Haemadsorption

As replication of ISAV in cell cultures does not always result in a CPE, every well shall be subject to an RT-PCR test or a haemadsorption test in accordance with this point, or an IF test in accordance with point II.2.6.

Cell culture medium shall be removed from each well, including those of positive and negative controls, and placed in labelled sterile tubes. 500 µl of a 0,2 % (vol:vol) suspension of washed rabbit or horse red blood cells, or a 0,05 % (vol:vol) 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 ASK cells shall be indicative of presumptive infection with an orthomyxovirus. If a haemadsorption test is positive, a virus identification test shall be performed immediately in accordance with point II.2.4.

II.2.6. Immunofluorescence (IF)

ASK (pass 65 or lower) shall be grown in a L-15 medium containing 10 % foetal bovine serum, 2 % (vol:vol) 200 mM L-glutamine, and 0,08 % (vol:vol) 50 mM 2-mercaptoethanol in multi-well plates and used at greater than 50 % confluence. Other cell lines or growth medium of proven efficacy may also be used. 225 µl of putative virus-infected culture supernatant shall be added to each of two wells, mixed and 225 µl transferred to two further wells, namely 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 7 days. When early CPE is observed, or if no CPE is observed within 7 days, the next step shall be fixation. Wells shall be washed with Phosphate buffered saline (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 antibodies (MAb) 3H6F8 and 1OC9F5 against ISAV, or other MAb of proven efficacy 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 Fluorescein isothiocyanate (FITC) conjugate diluted in PBS shall be added to each well and incubated at 37 ± 4 °C for 30 minutes. 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 shall score positive and the negative controls shall score negative.

II.3. Examination of other tissues

The technique referred to in point II.2.6 may 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 shall remain the same for each tissue, although for some tissues it may be preferable to omit the propidium iodide staining and rely on the 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 in clinically diseased Atlantic salmon are variable, but may include the following:

(a) numerous erythrocytes in the central venous sinus and lamellar capillaries of the gills, where erythrocyte thrombi also may be formed;

(b) multifocal to confluent petechiae or hepatocyte necrosis or both at some distance from larger vessels in the liver; multifocal accumulation of erythrocytes in dilated hepatic sinusoids;

(c) accumulation of erythrocytes in blood vessels of the intestinal lamina propria and eventually haemorrhage into the lamina propria;

(d) spleen stroma distended by erythrocyte accumulation;

(e) slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney;

(f) erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

II.5. Immunohistochemistry (IHC)

Polyclonal antibody against ISAV nucleoprotein shall be used on paraffin sections from formalin-fixed tissue. The organs to be examined shall be mid-kidney and heart (transitional area including all three chambers and valves). Suspected cases due to pathological signs shall be verified with a positive IHC. Histological sections shall be prepared in accordance with standard methods.

(1) Preparation of tissue sections

The tissues shall be fixed in neutral phosphate-buffered 10 % formalin for at least 1 day, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 5 µm thick sections (for IHC placed on poly-L-lysine-coated slides) shall be heated at 56 °C to 58 °C (maximum 60 °C) for 20 minutes, dewaxed in xylene, rehydrated through a graded ethanol series, and stained with haematoxylin and eosin for pathomorphology and IHC in accordance with point (2).

(2) Staining procedure for IHC

All incubations shall be carried out at room temperature on a rocking platform, unless otherwise provided for in this decision:

(a) antigen retrieval shall be done by boiling sections in 0,1 M citrate buffer pH 6,0 for 2 × 6 minutes followed by blocking with 5 % non-fat dry milk and 2 % goat serum in 50 mM TBS (TBS; Tris/HCl 50 mM, NaCl 150 mM, pH 7,6) for 20 minutes;

(b) sections shall then be incubated overnight with primary antibody (monospecific rabbit antibody against ISAV nucleoprotein) diluted in TBS with 1 % non-fat dry milk, followed by three washes in TBS with 0,1 % Tween 20;

(c) for detection of bound antibodies, sections shall be incubated with Alkaline phosphatase-conjugated antibodies to rabbit IgG for 60 minutes. Following a final wash, Fast Red (1 mg ml^{-1}) and Naphthol AS-MX phosphate (0,2 mg ml^{-1}) with 1 mM Levamisole in 0,1 M TBS (pH 8,2) shall be added to develop for 20 minutes. Sections shall then be washed in tap water before counterstaining with Harris haematoxylin and mounted in aqueous mounting medium. ISAV positive and ISAV negative tissue sections shall be included as controls in every setup.

(3) Interpretation of the result of IHC

The interpretation of the result of the IHC test shall be as set out in points (a) and (b):

(a) control sections shall be considered as positive, when it is observed that the control sections have clearly visible red-coloured (reddish) cytoplasmic and intranuclear staining of endothelia cells in blood vessels of endocardium. A test sample section shall only be regarded as positive if such clear, intranuclear red staining of endothelial cells is found;

(b) control sections shall be considered as negative if they don't have any significant colour reaction.

Since the intranuclear localisation is particular to the orthomyxovirus nucleoprotein during a stage of virus replication but concurrent cytoplasmic staining is often dominant, cytoplasmic and other staining patterns without intranuclear localisation shall be considered as nonspecific or inconclusive.

The strongest positive staining reactions are usually obtained in endothelial cells of heart and kidney. Endothelial staining reactions within very extensive haemorrhagic lesions may be slight or absent, possibly because of lysis of infected endothelial cells.

PART 4

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH *MARTEILIA REFRINGENS*

I. **Detailed diagnostic methods and procedures for the diagnosis of infection with *Marteilia refringens***

When sampling and laboratory examination are carried out for the purpose of obtaining or maintaining a health status with regard to infection with *Marteilia refringens* as set out in Section I of Part 4 of Annex I or to confirm or rule out the presence of that listed disease in accordance with Article 57(b) of Directive 2006/88/EC, using the diagnostic methods set out in Section II of Part 4 of Annex I, the detailed diagnostic methods and procedures set out in points I.1, I.2 and I.3 of this Part shall apply.

I.1. Sampling procedure

Gaping or freshly dead individual molluscs shall be sampled by priority, to increase the chances of finding infected animals.

Once sampled, oysters or mussels shall be maintained at 4 °C or on refrigerated ice for no more than 24 hours if samples include gaping molluscs and no more than 72 hours if not, in a plastic bag including a label with details related to the nature and the origin of the oysters or mussels. Gaping or freshly dead molluscs shall be maintained separately from other molluscs.

A 3 to 5 mm thick section of tissues including gills and heart tissue shall be used for the diagnostic of *Marteilia refringens* by histology. A piece of digestive gland shall be used for some tests, including imprints and polymerase chain reaction (PCR).

I.2. Microscopic techniques

I.2.1. Cytology (imprint cytology)

After drying digestive gland tissues on absorbent paper, several imprints shall be made on a glass slide. Slides shall be air-dried, fixed in methanol or in absolute ethanol and stained using a commercially available blood-staining kit such as Diff-Quik®/Hemacolor®, in accordance with the manufacturer's instructions. After rinsing in tap water and drying, the slides shall be mounted with a cover-slip using an appropriate synthetic resin. The slides shall be observed first at × 200 magnification and then under oil immersion at × 1 000 magnification.

A positive result shall be the observation of cells ranging in size up to between 30 to 40 µm. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. Pale halos around large, strongly stained (refringent) granules and, in larger cells, cell within cell arrangements are observed.

The technique is not parasite species specific.

I.2.2. Histology

Sections of tissue that include gills, digestive gland, mantle, and gonad shall be fixed for at least 24 hours in Davidson's fixative followed by normal processing for paraffin histology and staining, for example with haematoxylin and eosin. Observations shall be made at increasing magnifications up to × 1 000.

A positive result shall be the observation of cells ranging in size from 4 up to 40 µm. Early stages consist of multinucleate, spheric to elongated cells. These are mainly found in the epithelium of oesophagus and stomach and sometimes of labial palps. Sporulation involves divisions of cells within cells and takes place in the digestive gland tubules and ducts. Refringent granules appear in the course of sporulation, but are not observed in early stages. In late phases of infection, sporangia are observed free in the lumen of the digestive tract. The cytoplasm stains basophilic, whereas the nucleus stains eosinophilic. The granules can range from deep orange to deep red.

The technique is not parasite species specific.

I.3. Molecular techniques

I.3.1. DNA extraction

DNA shall be extracted in accordance with standard procedures.

DNA extraction kits, which are available commercially and usually produce high quality DNA suitable for use with the PCR protocols as described in point I.3.2, may be used.

I.3.2. Polymerase chain reaction (PCR)

Several PCR protocols have been developed and published.

PCR primers that target the internal transcribed spacer (ITS1) region shall be used as they are able to amplify only *M. refringens*.

PCR shall be carried out in a 50 µl volume. PCR mixtures shall contain buffer (500 mM KCl, 100 mM Tris/HCl [pH 9,0 at 25 °C] and 1 % Triton® X-100), 2,5 mM MgCl2, 0,2 mM dNTP mix, 1 µM forward and reverse primers, 0,02 units μl^{-1} Taq DNA polymerase, and 10 to100 ng of extracted DNA. After denaturation of DNA at 94 °C for five minutes, 30 cycles shall be run as follows: denaturation at 94 °C for one minute, annealing at 55 °C for one minute, and elongation at 72 °C for one minute per kilo-base pair. A final elongation step of 10 minutes at 72 °C shall be performed. For the detection of *M. refringens*, PCR shall be performed with primers that target the ITS1 region (5′-CCG-CAC-ACG-TTC-TTC-ACT-CC-3′ and 5′-CTC-GCG-AGT-TTC-GAC-AGA-CG-3′).

Positive controls shall consist of genomic DNA from a highly infected host or plasmidic DNA including the target region.

Negative controls shall consist of genomic DNA from non-infected hosts and PCR reactives without target DNA.

A positive result shall be positive PCR amplification at the expected size (412 bp), with all negative controls being negative and all positive controls being positive.

I.3.3. In-situ hybridisation (ISH)

Several ISH protocols have been developed and published.

Probe that targets the SSU of the rRNA gene complex shall be used because it has been validated against histology.

Sections of tissue that include gills, digestive gland, shall be fixed for at least 24 hours in Davidson's fixative followed by normal processing for paraffin histology. Sections of 5 µm shall be cut and placed on aminoalkyl-silane-coated slides, which shall then be baked overnight in an oven at 40 °C. The sections shall be dewaxed by immersing in xylene for 10 minutes. This step shall be repeated once and then the solvent shall be eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections shall then be dehydrated by immersion in a graded ethanol series. The sections shall be treated with proteinase K (100 µg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37 °C for 30 minutes. Slides shall be dehydrated by immersion in a graded ethanol series and then air dried. Sections shall be incubated with 100 µl of hybridisation buffer (4 × SSC [standard saline citrate], 50 % formamide, 1 × Denhardt's solution, 250 µg ml^{-1} yeast tRNA, 10 % dextran sulphate) containing 10 ng (1 µl of the PCR reactants prepared as described in point I.3.2 using primers CCG-GTG-CCA-GGT-ATA-TCT-CG and TTC-GGG-TGG-TCT-TGA-AAG-GC) of the digoxigenin-labelled probe. Sections shall be covered with *in-situ* plastic cover-slips and placed on a heating block at 95 °C for five minutes. Slides shall then be cooled on ice for one minute before overnight hybridisation at 42 °C in a humid chamber. Sections shall be washed twice for five minutes in 2 × SSC at room temperature, and once for 10 minutes in 0,4 × SSC at 42 °C. The detection steps shall be performed according to the manufacturer's instructions. The slides shall then be rinsed in sterile distilled water (dH2O). The sections shall be counter-stained with Bismarck Brown Yellow, rinsed in dH2O, and cover-slips shall be applied using an aqueous mounting medium.

Positive and negative controls shall be sections from known infected and non-infected hosts respectively.

A positive result shall be demonstrated by the purple-black labelling of *M. refringens* cells within known target tissues, with all negative controls being negative and all positive controls being positive.

I.3.4. Sequencing

Sequencing shall be carried out as one of the final steps for confirmatory diagnostic. Targeted regions are the SSU rDNA and ITS1.

II. **Detailed diagnostic methods and procedures for surveillance and confirmation of infection with *Marteilia refringens***

For the purpose of surveillance programmes and to confirm the presence of an infection with *Marteilia refringens* or to rule out a suspicion of that listed disease, in accordance with the requirements laid down in Section II of Part 4 to Annex I, the diagnostic methods and corresponding procedures to be used shall be in accordance with the guidelines laid down in Table 4.1 as follows:

Table 4.1

Guidelines for the use of diagnostic methods for the surveillance programmes and to confirm or rule out infection with *Marteilia refringens*

Method	Targeted surveillance	Presumptive diagnosis	Confirmatory diagnosis
Digestive gland imprints	X	X	X, or
Histopathology	X		X, or
In situ hybridisation			X, and
PCR	X	X	X, and
Sequencing			X

PART 5

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH *BONAMIA OSTREAE*

I. **Procedures for diagnosis of Infection with *Bonamia ostreae***

When sampling and laboratory examination are carried out for the purpose of obtaining or maintaining a certain health status with regard to *Bonamia ostreae* as set out in Section I of Part 5 of Annex I or to confirm or rule out the presence of that listed disease in accordance with Article 57(b) of Directive 2006/88/EC, using the diagnostic methods set out in Section II of Part 5 of Annex I, the detailed diagnostic methods and procedures set out in following points I.1, I.2 and I.3 shall apply.

I.1. Sample process

Gaping or freshly dead individual molluscs shall be sampled by priority, to increase the chances of finding infected animals.

Once sampled, oysters shall be maintained at 4 °C or on refrigerated ice for no more than 24 hours if samples include gaping molluscs and 72 hours if not, in a plastic bag including a label with details related to the nature and the origin of the oysters. Gaping or freshly dead molluscs shall be maintained separately from other molluscs.

A 3 to 5 mm thick section of tissues including gills and heart tissue shall be used for the diagnostic of *Bonamia ostreae* by histology. A piece of digestive gland shall be used for some tests, including imprints and polymerase chain reaction (PCR).

I.2. Microscopic techniques

I.2.1. Cytology (imprint cytology)

After drying gills or heart tissues on absorbent paper, several imprints shall be made on a glass slide. Slides shall be air-dried, fixed in methanol or in absolute ethanol and stained using a commercially available blood-staining kit (namely such as Diff- Quik®/Hemacolor®.), in accordance with the manufacturer's instructions. After rinsing in tap water and drying, the slides shall be mounted with a cover-slip using an appropriate synthetic resin. Slides shall be observed first at × 200 magnification and then under oil immersion at × 1 000 magnification.

A positive result shall be the presence of small spherical or ovoid organisms (2 to 5 µm wide) within haemocytes. However, the parasite might also occur extracellularly. These organisms show a basophilic cytoplasm and an eosinophilic nucleus (colours may vary with the stain used) and, because they spread on the slide, they can appear wider on imprints than on histological examination. Multinucleated cells may be observed. The technique is not parasite species specific.

I.2.2. Histology

Sections of tissue that include gills and digestive gland, shall be fixed for a period of at least 24 hours in Davidson's fixative followed by normal processing for paraffin histology and staining, for example with haematoxylin and eosin. Observations shall be made at increasing magnifications up to × 1 000.

A positive result shall be the presence of parasites as very small cells of 2 to 5 µm wide within the haemocytes or free in the connective tissue or sinuses of gill, gut and mantle epithelium, often associated with an intense inflammatory reaction. To avoid any doubt, the parasite shall be observed inside the haemocyte for a positive diagnosis. The technique is not parasite species specific.

I.3. Molecular techniques

I.3.1. DNA extraction

DNA shall be extracted in accordance with standard procedures.

DNA extraction kits, which are available commercially and usually produce high quality DNA suitable for use with the PCR protocols as detailed below, may be used.

I.3.2. Polymerase chain reaction (PCR)

Several PCR protocols have been developed and published.

Two PCR protocols targeting the small subunit (SSU) rDNA may be used:

(a) the first one is a conventional PCR that amplify several members of the *Haplosporidia* including *Bonamia spp*. Primers designated Bo and Boas are 5′-CAT-TTA-ATT-GGT-CGG-GCC-GC-3′ and 5′-CTG-ATC-GTC-TTC-GAT-CCC-CC-3′ respectively, and amplify a 300 bp product. PCR mixtures contain buffer (500 mM KCl, 100 mM Tris/HCl [pH 9,0 at 25 °C] and 1 % Triton® X-100), 2,5 mM MgCl2, 0,2 mM dNTP mix, 1 µM forward and reverse primers, 0,02 units μl^{-1} Taq DNA polymerase, and 0,2 ng μl^{-1} of the DNA template in a total volume of 50 µl. Samples shall be denatured in a thermocycler for five minutes at 94 °C before being submitted to 30 cycles (94 °C for one minute, 55 °C for one minute, 72 °C for one minute) followed by a final extension of 10 minutes at 72 °C.

Positive controls shall consist of genomic DNA from a highly infected host or plasmidic DNA including the target region.

Negative controls shall consist of genomic DNA from non-infected hosts and PCR reactives without target DNA.

A positive result shall be positive PCR amplification at the expected size (namely, 300 bp), with all negative controls being negative and all positive controls being positive;

(b) the second PCR protocol is a SYBR® Green Real time PCR assay. It allows the specific detection of *B. ostreae* (described below) and may be combined with a SYBR® Green Real time PCR assay allowing the specific detection of *B. exitiosa* (Ramilo et al. 2013).

Primers BOSTRE-F (5′- TTACGTCCCTGCCCTTTGTA-3′) and BOSTRE-R (5′- TCGCGGTTGAATTTTATCGT--3′) amplify a 208 bp product. PCR mixtures contain SYBR® Green Master Mix (1X), 0,3 µM forward and reverse primers, and 200 ng of extracted DNA. Samples shall be denatured in a Real Time Detection System for 10 minutes at 95 °C before being submitted to 35 cycles (95 °C for 30 seconds, 55 °C for 45 seconds and 72 °C for one minute). Melting temperature curve analysis shall be carried out with temperature increments of 0,5 °C/s starting at 55 °C and ending at 95 °C and recording fluorescence at each change of temperature.

Positive controls shall consist in genomic DNA from a highly infected host or plasmidic DNA including the target region.

Negative controls shall consist in genomic DNA from non-infected hosts and PCR reactives without target DNA.

A positive result shall be positive PCR amplification with a unique melting temperature peak (78,25 ± 0,25 °C in the conditions published by Ramilo et al. 2013), with all negative controls being negative and all positive controls being positive.

I.3.3. *In-situ* hybridisation (ISH)

Several ISH protocols have been developed and published.

A probe that targets the SSU of the rDNA gene complex shall be used although it was shown to cross react with some other members of the family *Haplosporidia*.

Sections of tissue that include gills and digestive gland shall be fixed for a period of at least 24 hours in Davidson's fixative followed by normal processing for paraffin histology. Sections of 5 µm shall be cut and placed on aminoalkylsilane-coated slides, which shall then be baked overnight in an oven at 40 °C. The sections shall be dewaxed by immersing in xylene for 10 minutes. This step shall be repeated once and then the solvent shall be eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections shall then be dehydrated by immersion in a graded ethanol series. The sections shall be treated with proteinase K (100 µg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37 °C for 30 minutes. Slides shall be dehydrated by immersion in a graded ethanol series and then air dried. Sections shall be incubated with 100 µl of hybridisation buffer (4 × SSC [standard saline citrate], 50 % formamide, 1 × Denhardt's solution, 250 µg ml^{-1} yeast tRNA, 10 % dextran sulphate) containing 20 ng (2 µl of the PCR reaction prepared as described in point I.3.2 using primers Bo and Boas) of the digoxigenin-labelled probe. Sections shall be covered with *in-situ* plastic cover-slips and placed on a heating block at 95 °C for five minutes. Slides shall then be cooled on ice for one minute before overnight hybridisation at 42 °C in a humid chamber. Sections shall be washed twice for five minutes in 2 × SSC at room temperature, and once for 10 minutes in 0,4 × SSC at 42 °C. The detection steps shall be performed according to the manufacturer's instructions. The slides shall then be rinsed in sterile distilled water (dH2O). The sections shall be counter-stained with Bismarck Brown Yellow, rinsed in dH2O, and cover-slips shall be applied using an aqueous mounting medium.

Positive and negative controls shall be sections from known infected and non-infected hosts respectively.

A positive result shall correspond to labelled parasites inside the haemocytes, with all negative controls negative and all positive controls positive.

I.3.4. Sequencing

Sequencing shall be carried out as one of the final steps for confirmatory diagnostic. Targeted regions shall be the SSU rDNA and ITS1.

II. **Procedures for surveillance and confirmation of infection with *Bonamia ostreae***

For the purpose of surveillance and confirmation of the presence of or to rule out a suspicion of infection with *Bonamia ostreae* in accordance with the requirements laid down in Section II of Part 5 to Annex I, the diagnostic methods and corresponding procedures to be used shall be in accordance with the guidelines laid down in the following Table 5.1.

Table 5.1

Guidelines for the use of diagnostic methods for the surveillance programmes and to rule out or confirm infection with *Bonamia ostreae*

Method	Targeted surveillance	Presumptive diagnosis	Confirmatory diagnosis
Heart or gill imprints	X	X	X, or
Histopathology	X		X, or
In situ hybridisation			X, and
PCR	X	X	X, and
Sequencing			X

PART 6

DETAILED DIAGNOSTIC METHODS AND PROCEDURES FOR SURVEILLANCE AND CONFIRMATION OF WHITE SPOT DISEASE (WSD)

1. **Diagnostic procedures for detection of WSSV**

When sampling and laboratory examination for the purpose of the surveillance and eradication programmes set out in Section I of Part 6 of Annex I and for the confirmation of the presence of or to rule out a suspicion of infection with WSSV are carried out in accordance with Article 57(b) of Directive 2006/88/EC, using the diagnostic methods set out in Section II of Part 6 of Annex I, the detailed diagnostic methods and procedures set out in points 2 to 7 of this Part shall apply.

The methods and procedures described in this Part of Annex II are adapted from the ISO 17025 accredited test applied at the European Union Reference Laboratory for Crustacean Diseases. Alternative approaches, using equivalent conditions or kits produced by different manufacturers, but which offer equivalent sensitivity and specificity to those described in this Part may be applied. In all instances, PCR amplified product shall be sequenced to confirm identity as White spot syndrome virus (WSSV).

2. **Sample process**

Tissue (pleopods and gills) containing WSSV from crustaceans may be stored in ethanol, RNAlater or snap frozen at – 80 °C. The stages required for the identification of WSSV from tissue samples shall be as follows: Homogenisation of the tissue, extraction of the DNA, specific amplification of WSSV DNA using PCR, visualisation of the amplified product on a gel, purification of the DNA and sequencing to confirm the identity of the pathogen.

3. **Tissue homogenisation**

The disruption of the tissues and the preparation of a homogenate in an appropriate buffer shall be carried out using the Fast Prep tissue disruptor and Lysing matrix A tubes (MP Biomedicals). The tissue shall be weighed, placed in Lysing Matrix A tubes, diluted 1 in 10 w/v or according to manufacturer's instructions, in an appropriate buffer (G2 and 10 µl Proteinase K for use with DNA Tissue kit (Qiagen)) and homogenised using the Fast Prep 24 homogeniser for two minutes. Homogenised samples shall be incubated at 56 °C for a minimum period of four hours or overnight. Samples shall be vortexed, centrifuged at 9 000 rpm for two minutes and 50 µl of supernatant or a volume equivalent to 5 mg of tissue (weight of tissue optimal for extraction kit) shall be added to a sample tube for DNA extraction and volume made up to 200 µl using G2 buffer.

4. **DNA extraction**

Total DNA shall be extracted using a DNA tissue extraction kit and the EZ1 Advanced XL Biorobot (Qiagen) following the manufacturers' instructions. An extraction control (Calf Thymus DNA) and a negative control (G2 buffer) shall run with each batch of samples. DNA shall be eluted into 50 µl volume. To ensure that the extraction has completed successfully, the DNA concentration for all samples and controls shall be determined using a Nano Drop machine. Extracted DNA shall be frozen at – 20 °C if not required immediately.

5. **WSSV Polymerase Chain Reaction (PCR)**

The method to be used for the detection of WSSV shall be the protocol for the detection of WSSV by nested PCR set out in the following paragraphs which amplifies a 1 447 bp and an 848 bp amplicon of the 18s rRNA gene in the first and second round PCRs respectively.

The 1st round PCR reaction shall be set up in a 50 µl volume which contains final concentrations of 1 × GoTaq Buffer (Promega), 5 mM $MgCl_2$, 1 pmol/µl WSSV 146 F1 primer, 1 pmol/µl WSSV 146 R1 primer (Table 1), 0,25 mM dNTPs, 1,25U Taq polymerase and 2,5 µl of DNA. Each sample shall be run in duplicate alongside a negative extraction control, a negative PCR control, (2,5 µl H_2O added instead of DNA) and a positive control. The positive control shall be diluted WSSV plasmid made and validated for use in-house (available from the EURL).

The 2nd round WSSV PCR reaction shall be set up in the same way as the first round but using the WSSV 146 F2/R2 primer set and using a second positive control to check that this stage of the PCR has worked.

Primer	Sequence
WSSV 146 F1	ACTACTAACTTCAGCCTATCTAG
WSSV 146 R1	TAATGCGGGTGTAATGTTCTTACGA
WSSV 146 F2	GTAACTGCCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

Both 1st and 2nd round PCRs shall be run using the following cycling conditions on a DNA Engine Tetrad 2 Peltier Thermal Cycler (or equivalent). An initial denaturation step of 94 °C for two minutes followed by 94 °C for 30 seconds, 62 °C for 30 seconds, 72 °C for 30 seconds repeated for 30 cycles, a 72 °C extension step for two minutes and held at 4 °C.

6. **Gel electrophoresis**

Amplified PCR products from both the 1st and 2nd round PCRs shall be visualised on 2 % agarose gels made using TAE buffer. 15 µl of each sample shall be run at 120 Volts for approximately 20 minutes and viewed under UV light. Positive samples will produce a band of 1 447 bp in the 1st round PCR and 848bp in the second round PCR. Samples of that size shall be cut out and placed into a 1,5 ml microfuge tube. The DNA contained within the gel slices shall be purified using Promega Wizard® SV Gel and PCR clean-Up System according to manufacturers' instructions. The concentration of the DNA shall be estimated using a Nano Drop machine. The purified DNA shall be frozen at – 20 °C if not being used immediately.

7. **Sequencing of PCR products**

DNA shall be sequenced using the Big Dye Terminator Kit v3,1 (Applied Biosystems). The total volume in each reaction is 20 µl, the final concentrations being 1 × Big Dye terminator, 1 × sequencing buffer, 10 pmol/µl forward or reverse primer and 10 µl of purified DNA (diluted to approximately 10 ng/µl) run using the following cycling conditions on a DNA Engine Tetrad 2 Peltier Thermal Cycler (or equivalent): 94 °C for 30 seconds followed by 96 °C for 10 seconds, 50 °C for 10 seconds and 60 °C for four minutes, cycling the last three steps 30 times.

The PCR products shall be precipitated using a sodium acetate method where 20 µl DNA is added to 10 µl NaAc, 70 µl H_2O and 250 µl ethanol, vortexed and centrifuged at 13 000 rpm for 20 minutes, the supernatant shall be removed and the pellet shall be washed with 200 µl absolute ethanol, centrifuging at 13 000 rpm for five minutes. The pellet shall be dried for five minutes at 37 °C. 25 µl of Hi-Di formamide shall be added to the pellets, heated to 95 °C for two minutes and vortexed thoroughly. Samples shall be sequenced using the ABI3130xl Avant Genetic analyser according to manufacturers' instructions. Sequencing results shall be analysed using Sequencher software and sequences matched to sequences on the NCBI database using the BLAST function.