SECTION 2.3.

DISEASES OF FISH

CHAPTER 2.3.0.

GENERAL INFORMATION

A. SAMPLING

1. Assessing the health status of the epidemiological unit

1.1. Sample material to be used for tests

Sample material and the number of samples to be collected depend on the specific disease or pathogen, the size of the animals and the objective of testing (i.e. diagnosis of overt clinical disease, detection of subclinical infection in apparently healthy animals or sampling for targeted surveillance to demonstrate freedom from infection with a specified pathogen). See the individual disease chapters in the *Aquatic Manual* for specific details of sample requirements.

1.2. Specifications according to fish populations

For details of fish to sample for a specific listed disease, see the relevant disease chapter in the *Aquatic Manual.* The design of a surveillance system for demonstrating disease-free status for a country, zone or compartment should be in accordance with the recommendations of the OIE *Aquatic Code* Chapter 1.4.

Fish to be sampled are selected as follows:

i) Susceptible species should be sampled proportionally or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).

ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include apparently healthy fish collected in such a way that all parts of the farm, as well as all year classes, are proportionally represented in the sample.

1.3. Specifications according to clinical status

For diagnosis of clinical infection for most OIE-listed viruses, appropriate organs to sample include anterior/mid kidney, spleen and either heart or encephalon; for fry whole fish or entire viscera may be used. Disease-specific recommendations are provided in Section 3.2 *Selection of organs or tissues* of the individual chapters. Samples from five to ten clinically diseased fish consistent with the disease of interest should be sufficient for the pathogen test(s) for each epidemiological unit.

For the appropriate organs to sample to detect subclinical infections and for targeted surveillance for case detection or disease freedom, refer to individual disease chapters of the *Aquatic Manual* and chapter 1.4 of the OIE *Aquatic Code*.

1.4. Specifications according to fish size

1.4.1. For the listed viral diseases except infection with koi herpesvirus

Fry and yolk sac fry: Sample the entire fish but remove the yolk sac if present.

Fish 4 to 6 cm: Sample the entire viscera and the kidney. A piece of encephalon can be obtained after severing the head at the level of the rear edge of the operculum and pressing it laterally.

Fish over 6 cm: Sample the kidney, spleen, and heart or encephalon and/or other tissues appropriate for the specific pathogen being tested for (see individual disease chapter in the *Aquatic Manual* for details).

Non-lethal sampling: For non-lethal sampling, appropriate sample types are recommended in Section 3.4 of the specific disease chapter.

2. General processing of samples

2.1. Macroscopic examination

For the listed diseases, macroscopic examination is mostly used for detecting clinical signs of infection with *Aphanomyces invadans* or *Gyrodactylus salaris,* but this is followed by microscopic examination of histological slides for the former or by identification of parasites removed from the skin, fins or gills of fish for the latter.

For viral diseases, clinical signs (including increased mortality rate, surface discolouration, distended abdomen, excess mucous production, exophthalmia, pale gills/anaemia, skin/fin/gill lesions, surface haemorrhages, lethargy, abnormal swimming behaviour and inappetence) and increasing mortality rates are non-specific.

2.2. Preservation of samples for subsequent virological examination

Samples to be submitted are either (i) fresh and chilled on ice or in vials containing cell culture medium for virus isolation, (ii) fixed in a nucleic acid stabilisation solution (e.g. RNA preservative or 80–90% ethanol) for polymerase chain reaction (PCR) detection or (iii) preserved in 10% neutral-buffered formalin fixative for histology and *in-situ* hybridisation. See individual sections below for further details. See the individual disease chapters in the *Aquatic Manual* for specific details of preservation requirements for other types of tests.

2.3. Virological examination

2.3.1. Transportation and antibiotic treatment of samples

Individual or pools of whole fish, organs or secretions ovarian fluids/milt are placed in sterile vials and stored at 4°C or on ice until virus isolation is performed in the laboratory. Virus isolation should optimally be carried out within 24 hours after fish sampling, but is still acceptable for up to 48 hours if the storage temperature is maintained at 0–4°C, or for longer periods for clinical disease samples held frozen at –80°C. Freezing at –20°C for storage should be avoided. For testing of apparently healthy fish, freezing of samples (at any temperature) should be avoided.

Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks' balanced salt solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable antibiotic concentrations are: gentamycin (1000 μg ml^{-1}), or penicillin (800 International Units [IU] ml^{-1}) and streptomycin (800 μg ml^{-1}). Antifungal compounds may also be incorporated into the transport medium at a final concentration of 400 IU ml^{-1}. Serum or albumen (5–10%) may be added to stabilise the virus if the transport time will exceed 12 hours.

2.3.2. Virus isolation

This procedure should be conducted below 15°C (preferably between 0 and 10°C). This can be achieved by using mortars and pestles that have been stored at –20°C or homogenising tissues quickly in a Stomacher or in tubes held in an ice slurry.

1. Decant antibiotic-supplemented medium from the organ sample.
2. Homogenise organ pools (minimum weight of 0.5 g) using a suitable method (e.g. mortar and pestle, glass or electronic homogeniser, Stomacher or validated equivalent) until a paste is obtained and dilute 1/10 (w/v) with transport medium.
3. Centrifuge the homogenate in a refrigerated (2–5°C) centrifuge at 2000–4000 ***g*** for 15 minutes, collect the supernatant and treat for either four hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml^{-1}. If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment makes filtration through membrane filters unnecessary. Alternatively, if gross microbial contamination is suspected, the supernatant can be membrane-filtered (0.45 um) understanding that there may be some loss of virus.
4. Likewise, ovarian fluid/milt samples may be treated with antibiotics to control microbial contamination but should not be diluted more than fivefold in the HBSS and antibiotic medium.
5. Ovarian fluid/milt samples should be centrifuged in the same way as organ homogenates, and their supernatants used directly in subsequent steps.
6. Prepared tissue/ovarian fluids/milt supernatants are used for inoculation of cell cultures for virus isolation and an aliquot may also be used for other tests, for example, PCR.
7. It is recommended to aliquot the homogenised sample material to avoid repeated freeze–thawing of the material. This also ensures reproducibility and comparability of the results.

2.3.3. Treatment to neutralise enzootic viruses

Fish are often subclinically infected with enzootic viruses, such as birnaviruses (e.g. infectious pancreatic necrosis virus [IPNV]), which induce a cytopathic effect in susceptible cell cultures and thus complicate isolation and identification of target pathogens. In such situations, the infectivity of the enzootic viruses should be neutralised, where possible, before testing for the viruses listed in the *Aquatic Code*. However, when it is important to determine whether one of the enzootic viruses is present, samples should be tested with and without the presence of neutralising antibodies (NAbs).

To neutralise aquatic birnaviruses, mix equal volumes (200 µl) of a solution of one or more NAbs against the enzootic birnavirus serotypes with the supernatant to be tested. Allow the mixture to react for 1 hour at 15°C or overnight at 4°C prior to inoculation on to susceptible cell monolayers. The titre of the NAb solution used should be at least 2000 in a 50% plaque reduction test versus the viral serotypes present in the given geographical area.

When samples are from a country, region, fish population or production unit considered to be free from enzootic viral infections, the NAb treatment of the supernatant may be omitted.

This approach can also be used to neutralise other viruses enzootic to the area from where the samples were taken.

2.4. Parasitic examination

See Chapter 2.3.3 Infection with *Gyrodactylus salaris* for specific details.

2.5. Fungal examination

See Chapter 2.3.1 Infection with *Aphanomyces invadans* for specific details.

B. MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF FISH PATHOGENS

1. Fish viruses

1.1. Fish cell lines

The following fish cell lines are used to test for the viral fish pathogens referred to in the *Aquatic Manual:*

Epithelioma papulosum cyprini (EPC)

Bluegill fry (BF-2)

Fathead minnow (FHM)

Rainbow trout gonad (RTG-2)

Chinook salmon embryo (CHSE-214)

Salmon head kidney (SHK-1)

Atlantic salmon kidney (ASK)

Chum salmon heart (CHH-1)

Grunt fin (GF)

Koi fin (KF-1)

Common carp brain (CCB)

Grass carp ovary (GCO)

1.2. Culture media

Traditional Eagle's minimal essential medium (MEM) with Earle's salt supplemented with 10% fetal bovine serum (FBS), antimicrobial agents and 2 mM L-glutamine is the most widely used medium for fish cell culture.

Stoker's medium, however, which is a modified form of the above medium comprising a double-strength concentration of certain amino acids and vitamins, is recommended particularly to enhance cell growth, using the same supplements as above + 10% tryptose phosphate.

These media are buffered with either sodium bicarbonate, 0.16 M tris-hydroxymethyl aminomethane (Tris) HCl, or, preferably, 0.02 M N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES). The use of sodium bicarbonate alone is restricted to those cell cultures made in tightly closed cell culture vessels or cultures incubated in an atmosphere supplemented with CO_2 to maintain the desired pH (7.3–7.6). As an alternative, MEM with Hanks' salts can be used in both closed cell culture flasks and 24-well or 96-well culture plates without the addition of other buffer salts.

Alternatively, Leibovitz medium (L15) supplemented with FBS (5% or 10%), L-glutamine (4 mM) and gentamicin (50 μg ml^{-1}) is recommended for some cell lines, e.g. SHK-1.

For cell growth, the FBS content of the medium is usually 10%, whereas for virus isolation or virus production it may be reduced to 2%.

The composition of the most frequently used antimicrobial agent mixture is penicillin (100 IU ml^{-1}) and dihydrostreptomycin (100 μg ml^{-1}). Add mycostatin (50 IU ml^{-1}) if fungal contamination is likely. Other concentrations or other antimicrobial agents may be used as convenient for the operator depending on the antimicrobial sensitivity of the bacterial or fungal strains encountered.

1.3. Virus positive controls and antigen preparation

1.3.1. Virus production

For the *in-vitro* production of stock cultures of most of these viruses, monolayer cultures of susceptible cells (see relevant sections in the *Aquatic Manual*) in suitable tissue culture vessels (e.g. plastic flasks)

should be inoculated with fairly low multiplicities of infection (m.o.i.), i.e. 10^{-2} to 10^{-3} plaque-forming units (PFU) per cell or equivalent.

The preferred temperatures for virus propagation are included in the table below.

Temperature	Virus
15°C	infectious haematopoietic necrosis virus (IHNV) infectious salmon anaemia virus (ISAV) salmonid alphavirus (SAV) viral haemorrhagic septicaemia virus (VHSV)
20°C	koi herpesvirus (KHV) spring viraemia of carp virus (SVCV)
22°C	epizootic haematopoietic necrosis virus (EHNV)
25°C	red sea bream iridovirus (RSIV)

1.3.2. Preservation and storage of virus stock cultures

1. Centrifuge infected cell cultures at 2–5°C and 2000–4000 ***g*** for 15 minutes then dilute the virus-containing supernatants in order to obtain virus titres averaging 10^6 PFU ml^{-1} or equivalent.
2. Dispense the resulting viral suspensions into sterile vials at volumes of 0.3–0.5 ml each.
3. Freeze and store each series of standard virus stocks at –80°C or in liquid nitrogen vapour phase, and check the titre of each virus stock at regular intervals (6–12 months) if it has not been used during that time period.

Lyophilisation: long-term storage (decades) of standard virus seed strains is achievable by lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% FBS are mixed (v/v) with an equal volume of cryopreservative medium (such as 20% lactalbumin hydrolysate in distilled water) before processing. Seal or plug under vacuum and store at 4°C, in the dark.

At least every 6 months or if decreased cell susceptibility is suspected, titration of reference isolates is performed to verify cell line susceptibility to infection.

2. Techniques

2.1. Direct microscopy

Samples for direct microscopic examination of smears or tissue imprints should be examined as soon as possible after collection. Live specimens should be used whenever possible, or fresh specimens chilled at 4°C, or 10% neutral-buffered formalin-fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection. For *G. salaris,* fresh specimens are examined or fish can be stored in ethanol prior to microscopic examination (see Chapter 2.3.3 Infection with *G. salaris*).

2.2. Histological techniques

2.2.1 Preparation of slides for histological examination

Only live or moribund specimens of fish with clinical lesions should be sampled after humane euthanasia for histology. The removed tissues (<5 mm thick) should be fixed immediately in 10% neutral-buffered formalin. Use at least ten volumes of fixative for each volume of tissue sample and allow to fix for at least 24 hours. After removal from the fixative, tissue samples are then dehydrated in ascending ethanol concentrations, cleared in a wax-miscible agent such as xylene and then embedded in paraffin using standard protocols. Cut sections of approximately 3–5 μm thickness from the block. Mount each section on a glass slide, de-wax in a wax-miscible agent, such as xylene or 'Clearene®', and rehydrate. For most disease examinations, the sections can then be stained with haematoxylin and eosin (H&E) using

standard procedures (Slaoui & Fiette, 2011). For observing granulomas and fungal hyphae as occur in infection with *A. invadans*, a general fungal stain such as Grocott–Gomori may be used instead of H&E.

2.2.2. Preparation of slides for immunohistochemistry

It is important to note that prolonged fixation can mask antigens of interest. Therefore, it is recommended keeping fixation to a minimum whilst still achieving optimal preservation (24–48 hours). This can be reduced further when using small pieces of tissue. Nonetheless, it is recommended to incorporate an antigen retrieval step where possible (Kim *et al.,* 2016). Due to variations that may exist between antibodies and commercially available detection kits, it is probable that individuals will need to optimise the technique for their own purposes. This will include factors such as determination of optimal antibody titre. This is the highest dilution that results in the most intense specific staining whilst achieving the least non-specific "background" staining. In addition, individuals may need to consider amending the duration of reagent incubation.

2.3. Electron microscopy

Electron microscopy (transmission or scanning) is a valuable research tool for the study of aquatic animal diseases (e.g. Hyatt *et al.,* 1991) and for the detection of previously unknown viruses for which there are no specific diagnostic tests. However, these methods are not normally used for the routine diagnosis of the fish diseases listed by OIE so are not described in the *Aquatic Manual*.

2.4. Virus isolation

2.4.1. Introduction

For most viruses, isolation in cell culture followed by identification of the virus using either antibody-based or nucleic acid-based (PCR) methods can be employed in the diagnosis of clinically affected animals or in the surveillance of apparently healthy animals. Isolation of finfish viruses in cultures of a number of established fish cell lines is well-documented (Crane *et al.,* 2005; Devold *et al.,* 2000; Graham *et al.,* 2008; Herath *et al.,* 2009; Lorenzen *et al.,* 1999; Olesen & Vestergård Jørgensen, 1992). However for some viruses, such as KHV, cell culture isolation is not as sensitive as the published PCR-based methods and is not considered to be a reproducible diagnostic method for KHV (Haenen *et al.,* 2004).

The success of pathogen isolation and results of bioassay depend heavily on the quality of samples (level of autolysis of fish samples, time since collection, time and temperature in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternative storage methods should be used only after consultation with the receiving laboratory.

Before transfer to the laboratory, pieces of the organs to be examined for virus isolation should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml transport medium, i.e. cell culture medium with 10% fetal bovine serum (FBS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g). To prepare duplicates (for retesting) it is recommended to aliquot the organ material after homogenisation.

Tubes containing fish tissues in transport medium for cell cultivation should 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 should 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 at least one freeze block 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 should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with current national and international transport regulations, as appropriate.

The virological examination for isolation in cell culture should 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.

See the individual disease chapters in the *Aquatic Manual* for specific details of virus isolation requirements.

2.4.2. Inoculation of cell monolayers

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

Prepared tissue samples (see Section A. *Sampling* above) are inoculated onto cell cultures in at least two dilutions, i.e. the primary dilution and a 1/10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1/100 and 1/1000, respectively (to prevent homologous interference). 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 plate, has to be used. Use of 24-well cell culture plates is recommended, but other units of a similar or larger growth area are also acceptable.

2.4.3. Incubation of cell cultures

Inoculated cell cultures are incubated at the pathogen-specific temperature for 7–14 days. If the colour of the cell culture medium changes from red to yellow indicating medium acidification, pH adjustment with sterile bicarbonate solution, or equivalent substances, has to be performed to ensure cell susceptibility to virus infection.

2.4.4. Microscopy

Using ×40–150 magnification, inoculated cell cultures must be inspected regularly (at least two times a week) for the occurrence of cytopathic effect (CPE). The use of a phase-contrast microscope is recommended. If obvious CPE is observed, virus identification procedures must be initiated immediately.

2.4.5. Sub-cultivation

If no CPE has developed after the primary incubation for 7–14 days, sub-cultivation is performed with fresh cell cultures using a cell area similar to that of the primary culture.

Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to the cell line 7–14 days after inoculation. The pools are then inoculated onto homologous cell cultures undiluted and diluted 1/10 (resulting in final dilutions of 1/10 and 1/100, respectively, of the supernatant) as described above (Section B.2.4.2 *Inoculation of cell monolayers*). For SAV, and other non- or slow CPE-forming viruses that are cell-bound, it is recommended that a freeze–thaw cycle or sonication step be included prior to passage.

Alternatively, aliquots of 10% of the medium constituting the primary culture are inoculated directly into a well with a fresh cell culture (well-to-well sub-cultivation). In the case of salmonid samples, inoculation may be preceded by preincubation of the dilutions with an anti-IPNV antiserum at an appropriate dilution, as described above (see Section A.2.3.3 *Treatment to neutralise enzootic viruses*). The inoculated cultures are then incubated for 7–14 days at the appropriate temperature, with observation, as described above (see Section B.2.4.4 *Microscopy*).

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

If bacterial contamination occurs despite treatment with antibiotics, sub-cultivation must be preceded by centrifugation at 2000–4000 ***g*** for 15–30 minutes at 2–5°C, or filtration of the supernatant through a

0.45 μm filter (low protein-binding membrane). In addition to this, sub-cultivation procedures are the same as for nonspecific cytotoxicity.

If no CPE occurs, the test may be declared negative. For SAV2/SAV3 no apparent CPE is common from field isolates. An IFAT for the detection of SAV antigen is routinely performed.

Where practical difficulties arise (e.g. incubator breakdown, problems with cell cultures, etc.) making it impossible to inoculate cells within 48 hours after tissue sampling, it is possible to store the supernatants at –80°C and carry out virological examination within 14 days. Once supernatants are stored at –80°C, thawing is recommended only once. Another freeze–thaw cycle will substantially reduce virus titres. It is recommended to aliquot the homogenised sample material to avoid repeated freeze–thawing of the material. This also ensures reproducibility and comparability of the results.

2.4.6. Virus identification

Infected cell cultures are used for virus identification by IFAT. Supernatant from cultures demonstrating CPE is used for virus identification by nucleic acid-based techniques. The preferred method for confirmatory identification is by sequence analysis of PCR amplicons (see *Aquatic Manual* chapters on individual pathogens for details).

2.5. Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis

Molecular techniques, including the use of nucleic acid probes for *in-situ* hybridisation, conventional PCR and real-time PCR, have been developed for the identification of many pathogens of aquatic animals. Real-time PCR methods, in general, have high sensitivity and specificity and, following adequate validation, can be used for direct detection of viral nucleic acids in samples prepared from fish tissue. The technique can be used in direct surveillance of apparently healthy populations, if they have a high level of diagnostic sensitivity, as well as in the diagnosis of clinically affected animals (e.g. Garver *et al.,* 2011; Jonstrup *et al.,* 2013). Duplicates of unfixed samples testing positive using real-time PCR can be processed for virus isolation to confirm presence of infectious virus.

When using PCR as a diagnostic method, the design of primers and probe, the use of positive and negative controls, as well as validation of the PCR method chosen are important. Real-time PCR is a powerful technique particularly for analysing relatively high numbers of samples (e.g. for surveillance) via high-throughput testing. Several nucleic acid probe and PCR protocols are included in this version of the *Aquatic Manual* as screening, diagnostic or confirmatory methods for fish and can be undertaken as the standard method. However, following PCR-positive results, where possible, virus isolation should be undertaken to confirm the presence of infectious virus. Conventional PCR with sequencing of PCR products should be used for confirmation of the cultured pathogen identity.

As with all PCR protocols, optimisation may be necessary depending on the reagents, equipment and the plasticware. PCR is prone to false-positive and false-negative results. False-positive results (negative samples giving a positive reaction), may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from previous tests. Therefore, each assay and tissue extraction should include a negative control to rule out contamination. False-negative results (positive samples giving a negative result), may occur due to the presence of a new variant that is not recognised by the PCR primer/probe set, which may lead to unwanted transmission of pathogens and biosecurity failure. Negative molecular results should be investigated further when clinical signs indicate the presence of a specific disease, or other positive test results have indicated that a false negative result may have been obtained.

To minimise the risk of contamination, aerosol-preventing pipette tips should be used for all sample and PCR preparation steps. Additionally, all PCRs should be prepared in a clean area that is separate from the area where the amplifications and gel electrophoresis are performed. Do not share equipment (e.g. laboratory coats and consumables) between areas and, where possible, restrict access between areas. Contaminating PCR products can be carried on equipment, clothes and paper (e.g. workbooks). Also, ensure all work-tops and air-flow hoods used for the extractions and PCR set up are regularly cleaned and decontaminated. To ensure sample integrity, always store the samples (e.g. in a freezer or refrigerator) in a location away from the molecular biology laboratory and reagents.

2.5.1. Sample preparation and types

Samples should be prepared to preserve the nucleic acid of the pathogen and should be handled and packaged with the greatest care to minimise the potential for cross-contamination among the samples or target degradation before the assay can be performed. To prevent contamination, new disposable containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular tests are:

1. *Live iced specimens or chilled specimens:* For specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of ice packs or ice bricks in an insulated box and ship to the laboratory.
2. *Frozen whole specimens:* Select live specimens according to the purpose of sampling, euthanase fish humanely and quick-freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at –20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry ice in an insulated box, and ship to the laboratory. Freezing samples for histological analysis should be avoided.
3. *Alcohol-preserved samples:* In regions where the storage and shipment of fresh (0–4°C) and frozen samples is problematic, 80–90% (v/v) ethanol (analytical grade) or RNA preservative should be used to preserve, store, and transport samples for PCR analysis. Pack for shipment according to the methods described above.
4. *Fixed tissues for* in-situ *hybridisation:* For this purpose, classic methods for preservation of the tissues are adequate. Neutral-buffered formalin is usually a good choice. Fixation for over 24–48 hours should be avoided; samples should be transferred to ethanol following the formalin treatment.

2.5.2. Preservation of RNA and DNA in tissues

Tissue is cut to be less than 0.5 cm in one dimension and submerged in 10 volumes of a suitable nucleic acid preservative (e.g. a 0.5 g sample requires about 5 ml of RNA preservative or 80–90% ethanol). Smaller organs such as kidney, liver and spleen can be stored whole in RNA preservative or 80–90% ethanol. Samples preserved in this way can be stored at 4°C for 1 month, at 25°C for 1 week or indefinitely at –20°C or below.

2.5.3. Nucleic acid extraction

To isolate nucleic acids from tissues preserved in ethanol or an RNA preservative, simply remove the tissue from the fixative or preservative and treat it as though it was just harvested. Most fresh and preserved or fixed tissues can be homogenised (e.g. with a mortar and pestle or in bead-beating tubes) directly in the lysis or extraction buffer provided with commercially available DNA and RNA extraction kits. Commercial kits should be validated or undergo equivalence testing with current validated extraction procedures prior to routine use.

2.5.4. Preparation of slides for *in-situ* hybridisation

For *in-situ* hybridisation, fish tissues should be fixed in neutral-buffered formalin for approximately 24 hours and then embedded in paraffin according to standard histological methods. Sections are cut at a thickness of 5 μm and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 μg ml^{-1}) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For *in-situ* hybridisation tests (see individual chapters for details), it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to non-specific staining/stain dropout, and false negative results due to errors in the staining protocol (Qadiri *et al.,* 2019; Valverde *et al.,* 2017).

3. Additional information to be collected

Sample information should include the collector's name, organisation, date, time, and description of the geographical location. The geographical origin of samples may be described as the name or location of the sampling site or its geographical co-ordinates. There should also be records that provide information to allow trace-backs on the sample movement from the sample site to the storage facility or laboratory and within those facilities.

Information on the preservation method, storage location, and date and time of storage at each storage locker or freezer along with information on the storage temperature (continuously monitored is preferable) should be collected. This information should be tracked with a unique sample code for all samples. For laboratories, the date of receipt, storage location information, date of analysis, analysis notes, and report date should be maintained for all uniquely coded samples. These data will greatly facilitate the tracking of sample problems and provide assurance that the samples were properly handled.

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NB: FIRST ADOPTED IN 1995. MOST RECENT UPDATES ADOPTED IN 2022.