VHSV-IHNV

ELISA kit for antigenic diagnosis of Viral Haemorrhagic Septicaemia and Infectious Haematopoïetic Necrosis Virus
Sandwich test
Diagnostic test for fish

I - INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a disease of farmed rainbow trout, farmed turbot, farmed Japanese flounder as well as several wild freshwater and marine species caused by VHSV rhabdovirus. Disease generally occurs at temperature between 4°C and 14°C. At water temperature between 15°C and 18°C, the disease generally takes a short course with a modest accumulated mortality. Disease rarely occurs at higher temperatures. VHS outbreaks occur during all seasons, but are most common in spring when water temperatures are rising or fluctuating.

The clinical signs of the disease are high mortality (which can reach up to 100% in fry), especially during the young trout's first winter. The subjects exhibit lethargy, melanosis and exophthalmia. The paleness of their gills reflects their anaemic condition. An autopsy will reveal the presence of numerous sites of haemorrhages in the viscera and muscle mass, distended abdomen due to oedema in the peritoneal cavity. VHS can also occur in a nervous form, characterised by severe abnormal swimming behaviour, such as constant flashing and/or spiralling.

IHN is a viral disease caused by a rhabdovirus. It affect most salmonid species, especially the fry and young fish. Susceptible species include: rainbow or steelhead trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*). Pacific salmon including Chinook (*O. tshawytscha*), sockeye (*O. nerka*), chum (*O. keta*), masou (*O. masou*) and coho (*O. kisutch*) and Atlantic salmon (*Salmo salar*).
The clinical disease generally occurs in water at temperature between 8 and 15°C. It is characterized by nervous system and digestive disorders: alternating apathy and spasmodic movements, darkening of the skin, pale gills and distended abdomen. Enteritis is evidenced by long, whitish excrement. Autopsy reveals exophtalmia, ascites and haemorrhages in the muscle mass and viscera. The liver, kidney and spleen are pale. The mortality rates associated with the virus can be high. It is almost impossible to distinguish IHN from VHS on the basis of clinical evidence alone. A differential diagnosis obtained by laboratory investigation thus appears to be indispensable.

The VHS-IHN ELISA test confirms the virus's growth on a susceptible cell line.

II - PRINCIPLE OF THE TEST

The gold standard for detection of IHNV and VHSV is the isolation of the virus in cell culture followed by its immunological or molecular identification. The infected specimens are completely homogenised (either by stomacher, blender or mortar and pestle with sterile sand) and subsequently suspended in an antibiotic-supplemented culture medium. The preparation is centrifuged and a 24-well cell culture plate is inoculated with a serial dilution of the supernatant. After 1 hour's incubation at optimal temperature culture medium is added to each well and the plate is incubated until a cytopathogenic effect is observed. At this point, the plate is frozen. It is ready to be tested by ELISA. The test uses 96-well microtitration plates sensitised by specific antibodies for the VHS and IHN viruses. Rows A and E have been sensitised with anti-VHSV, rows C and G with anti-IHNV and rows B, D, F, H contain non specific antibodies. These control rows allow the differentiation between specific immunological reactions and non specific binding so as to eliminate false positives.
The supernatants are incubated on the microplate for 1 hour at 21°C +/- 3°C.
After this first incubation step, the plate is washed and incubated for 1 hour with the conjugates, peroxidase labelled anti-VHSV and anti-IHNV specific monoclonal antibodies. After this second incubation, the plate is washed again and the chromogen (tetramethylbenzidine) is added. This chromogen has the advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic.
If VHSV or IHNV are present in the cell culture supernatant, the conjugates remain bound to the corresponding microwells and the enzyme catalyses the transformation of the colourless chromogen into a pigmented compound. The intensity of the resulting blue colour is proportionate to the titre of VHSV or IHNV in the supernatant. The enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm recorded using a photometer. The signals recorded for the negative control microwells are subtracted from the corresponding positive microwells. Control antigen is provided with the kit so as to validate the test results.

III - COMPOSITION OF THE KIT

- **Microplates**: Two 96-well microtitration plates. The rows A, C, E, G are sensitised by specific antibodies, the rows B, D, F, H by non specific antibodies.
  Row A: anti-VHSV
  Row B: control
  Row C: anti-IHNV
  Row D: control
  Row E: anti-VHSV
  Row F: control
  Row G: anti-IHNV
  Row H: control
- **Washing solution:** One 100 ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until disappearance of all crystals. Mix the solution well and remove the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- **Conjugates**: Two 12 ml vials of colored conjugates. **These solutions are ready to use.** VHSV (red), IHNV (blue).
- **Positive Controls:** 2 vials of 2 ml colored controls. VHSV (red), IHNV (blue). **These solutions are ready to use.**
- **Single component TMB** One 25-ml bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C protected from light. This solution is ready to use.
- **Stopping solution:** One 15-ml bottle of the 1 *M* phosphoric acid stop solution.

	BIO K 264/2
Microplates	2
Washing solution	1 X 100 ml (20 X)
Conjugates	2 X 12 ml (1 X)
Positive control VHSV (Red)	1 X 2 ml (1 X)
Positive control IHNV (Blue)	1 X 2 ml (1 X)
Single component TMB	1 X 25 ml (1 X)
Stopping solution	1 X 15 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Stomacher or blender or mortar with sterile sand, distilled water, graduated cylinders, beakers, plastic tubes, tube rack, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for "in vitro" diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution may be stored at room temperature. Once diluted, this solution remains stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope's seal airtight. If these precautions are taken, the strips' activity can be conserved up to the kit's shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

For countries belonging to the European Communities, sampling plans and diagnostic methods for the detection and confirmation of viral haemorrhagic septicaemia (VHS) and infectious haematopoietic necrosis (IHN) must be applied (2001/183/EC) – Commission decision of 22 February 2001.

1. Extracting the virus

1.1. Preparing the specimens (see also Manual of Diagnostic Tests for Aquatic Animals 2009 Chapter 2.1.9 4.3.1.2)

Take from moribund fish or fresh corpses approximately 1-gram fragments of spleen, kidney, heart and brain tissue. Mix these fragments with oven-sterilised sand and grind the mixture in a mortar. It is also possible to use stomacher or blender. After complete homogenisation is achieved add 2 ml of culture medium containing 2% foetal calf serum and antibiotics (inoculation medium). For example, one may use a mixture of 200 IU of penicillin, 200 µg of streptomycin and 200 µg of kanamycin per ml of culture medium. This mixture avoids the problems encountered when cell cultures are inoculated with heavily-contaminated specimens. For small fish, the entire corpse may be homogenised in the mortar, ideally after the intestines have been resected.

1.2. Centrifugation of specimens

The homogenised preparation is centrifuged between 2,000 and 4,000 g at 4° C for 15 minutes. The supernatant is collected for the subsequent steps.

1.3. Dilution of specimens

1:10, 1:100 and 1:1 000 dilutions of the supernatant are made using the inoculation medium.

2. Isolating the virus

2.1. Cell line selection

For VHSV, the fish cell lines BF-2 and RTG-2 are recommended. Alternatively, EPC or FHM cells may be used but EPC cells are, in general, less susceptible than BF-2 and RTG-2 to VHSV. The EPC and BF.2 cell lines are susceptible to IHN virus. These cells may be grown in Eagle's modified MEM or with better results in Glasgow's MEM supplemented with 10% foetal calf serum, 10% phosphate tryptose and a mixture of antibiotics at the

standard concentration. If a CO2 incubator is not available, the medium may be buffered at pH 7.4 with 0.16 M Tris-HCl. The optimal temperature for growth is 30° C for the FHM and EPC cells, 25°C for the BF.2 cells and 21° C for the RTG2 cells (de Kinkelin et al., 1986).

2.2. Preparation of the cellular substrate

The cells are kept in a Roux flask at their optimal growth temperature. One to two days before use the cells are treated with trypsin to separate them from their backing, then seeded on a 24-well plate.

As susceptibility to the virus depends on the cells' age, it is advisable to use them 24-48 hours after their transfer to the plate. To guarantee the quality of the diagnosis, the cell layer must be in perfect condition at the time of inoculation with the specimens.

2.3. Inoculation

The culture medium is eliminated by turning the plate upside down over a receptacle. Use a sharp movement so as to avoid adsorption of the culture medium on the outer surface of the well. In carrying out this step, hold the plate at a reasonable distance from the receptacle to avoid all risks of contamination from splashes. After emptying the plate, quickly deposit the different dilutions of specimens, for the cell layer must be kept moist at all times. The specimens must be deposited in the wells very delicately so as not to damage the cell layer. If automatic microtip pipettes or Pasteur pipettes are used, place the tip of the pipette against the side wall of the well and release the sample material slowly. 200 µl aliquots of the different dilutions are placed in each well. Incubate the plate at 15° C for 1 hour.

2.4. Addition of inoculation medium

At the end of the viral adsorption period add gently to each well 1 ml of the 2% foetal calf serum culture medium (inoculation medium).

2.5. Incubating the plate

The plate is kept in an incubator (under 5% CO2) at the optimum temperature for viral growth (15°C). It is inspected daily until a cytopathogenic effect is observed. This consists of the development of dense, spherical cells, the destruction of which results in the formation of plaques.

3. Identifying the virus

3.1- Bring all the reagents to 21°C +/- 3°C before use. Thaw the culture plate that was prepared from the samples.

3.2-Dilute the concentrated washing solution 20 fold in distilled water. Be sure that all crystals have disappeared before dilution.

3.3-Add 100-µl aliquots of the supernatants to the wells as follows: sample 1 in wells from E1 to H1, sample 2 in wells from A2 to D2 etc.

For the positive reference example: Positive reference VHSV in wells A1-B1, positive reference IHNV in wells C1-D1

3.4-Cover with a lid and incubate the plate at 21°± 3°C for one hour.

3.5-Rinse the plate with the washing solution prepared as instructed in the section “Composition of the Kit”. To do this, dispose of the microplate’s contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed to the next step.

3.6-Add 100 µl of conjugate solutions per well anti-VHSV in rows A, B, E and F and anti-IHNV in rows C, D, G and H. Cover with a lid and incubate the plate at 21°± 3°C for one hour.

3.7-Wash the plate as described in step 3.5 above.

3.8-Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated. Incubate at 21°C +/- 3°C and away from light for 10 minutes. Do not cover. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.

3.9-Add 50 µl of stop solution to each well. The blue colour will change into a yellow colour.

3.10-Read the optical densities by means of a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect measurements

VII – INTERPRETING THE RESULTS

Calculate the net optical density for each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control.
Proceed in the same way for the positive controls antigens.
The test is validated only if the positive controls antigens yield a difference in optical density at 10 minutes that is greater than the values given on the QC data sheet.

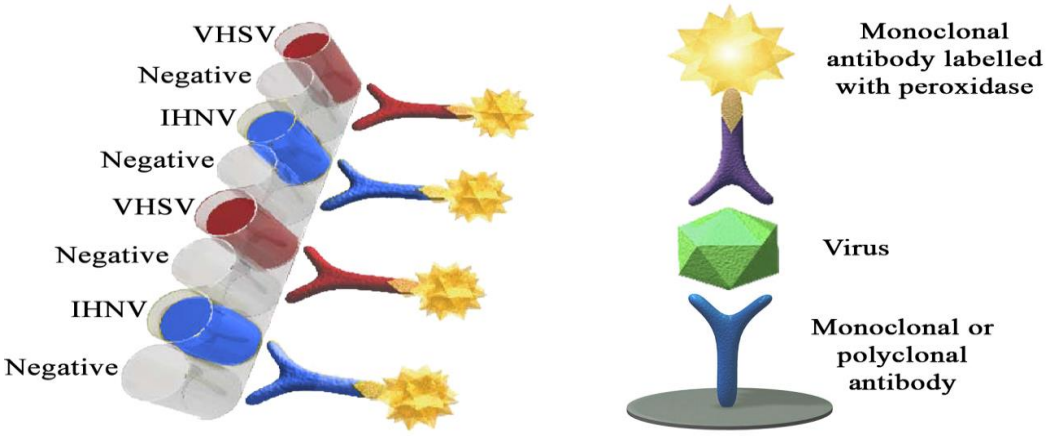
Divide each resulting value by the corresponding value obtained for the corresponding positive control and multiply this result by 100 to express it as a percentage.

$$Val = \frac{\text{Delta OD spl} * 100}{\text{Delta OD pos}}$$

Using the first table in the quality control procedure, determine each sample's status (positive, negative).

VIII – ORDERING INFORMATION

Multiscreen AgELISA VHSV-IHNV	2 X 24 samples	BIO K 264/2



IX – REFERENCES

Manual of Diagnostics Tests for Aquatic Animals 2009 Chapter 2.1.4 Infectious Haematopoietic Necrosis 1-17

Manual of Diagnostics Tests for Aquatic Animals 2009 Chapter 2.1.9 Viral Haemorrhagic Septicaemia 1-23

COMMISSION DECISION of 22 February 2001 laying down the sampling plans and diagnostic methods for the detection and confirmation of certain fish diseases and repealing
Decision 92/532/EEC