IPNV3

ELISA kit for antigenic diagnosis of Infectious Pancreatic Necrosis Virus
Sandwich test
Diagnostic test for fish
Double wells

I - INTRODUCTION

The viral aetiology of infectious pancreatic necrosis (IPN) was proven by Wolf et al. (1960). Three major groups of IPN virus are recognised: ATCC VR 299 (Wolf et al., 1968), Ab and Sp (Vestergard-Jorgensen and Kehlet, 1971). These three groups differ in their seroneutralisation reactions, their ability to grow on various fish cell lines and their pathogenicity. These IPN viruses are much more resistant to various environmental physico-chemical conditions than other Salmonidae viruses, such as VHS. Infection causes sometimes highly variable mortality in the fry from their first feed to the age of about 1,800 degree-days (Dorson and Torchy, 1981). Clinically, the fry exhibit peculiar swimming behavior characterised by rotation around a horizontal axis, abdominal distension that is especially visible in the gastric region, and a mucus-filled digestive tube that is empty of food (Wood et al., 1955). Although IPN virus is primarily pathogenic for certain Salmonidae fry, Dorson et al. (1987) have proven the susceptibility of young pike fry (Esox lucius) to the virus, which has also been isolated from a host of other fish species that show no external clinical signs of infection (many cyprinids, eels, etc.). These fish may be reservoirs and physical vectors of the IPN virus (Dorson, 1982).

II - PRINCIPLE OF THE TEST

The infected specimens are ground up in a mortar with the help of sand, then put in solution in an antibiotic-supplemented culture medium. It is also possible to use stomacher or blender. 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 monoclonal antibodies for the IPN virus. Rows A, C, E, G have been sensitised with these antibodies 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 conjugate, a peroxidase labelled anti-IPNV specific polyclonal antibody. 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 IPNV is present in the cell culture supernatant, the conjugate remains 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 IPNV 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. A positive control antigen is provided with the kit so as to validate the test results.

III - COMPOSITION OF THE KIT

- **Microplates**: 96-well microtitration plates. Rows A, C, E, G are sensitised by anti-IPNV specific antibodies, while rows B, D, F, H are sensitised by the non specific antibodies.
- **Washing solution**: Bottle 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. Store the diluted solution between +2°C and +8°C.
- **Conjugate**: Vial of coloured conjugate. **This solution is ready to use.**
- **Positive Control:** The reagent is ready to use.
- **Single component TMB:** Bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C protected from light. **This solution is ready to use.**
- **Stopping solution**: Bottle of the 1 *M* phosphoric acid stop solution.

	BIO K 282/1	BIO K 282/2
Microplates	1	2
Washing solution	1 X 100 ml (20 X)	1 X 100 ml (20 X)
Conjugate	1 X 12 ml (1 X)	1 X 25 ml (1 X)
Positive control	1 X 2 ml (1 X)	1 X 4 ml (1 X)
Single component TMB	1 X 12 ml (1 X)	1 X 25 ml (1 X)
Stopping solution	1 X 6 ml (1 X)	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

1. Extracting the virus

1.1. Preparing the specimens

Take from moribund fish or fresh corpses approximately 1-gram fragments of pyloric caecum, spleen, kidney 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 at between 2,000g and 4,000g 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

The FHM, EPC, BF.2, and RTG2 cell lines are susceptible to serotypes Sp and ATCC VR299 of the virus causing infectious pancreatic necrosis. Serotype Ab will grow only on the BF.2 and RTG2 cell lines. 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 Klinkelin et al., 1986).

2.2. Preparing 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 vulnerability 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.

.3. Inoculation with the specimens

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 spindle-shaped cells, the destruction of which results in the formation of plaques.

2.6. Freezing the plate
The plate is frozen in order to release the virus from cells

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.
Keep this solution between +2°C and +8°C when not used.

3.3-Add 100-µl aliquots of the supernatants to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1, etc. Proceed in the same manner for the positive reference (ex.: G1 and H1).

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 go on to the next step.

3.6-Distribute the conjugate solution at the rate of 100 µl per well. 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.

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

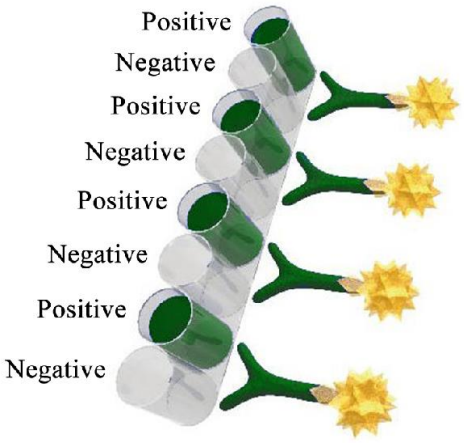
Calculate the net optical density of each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control.
Proceed in the same way for the positive control antigen.
The test is validated only if the positive control antigen yields a difference in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

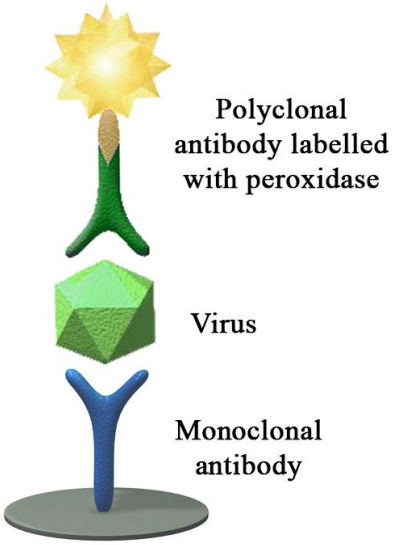
Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage. Using the first table in the quality control procedure, determine each sample's status (positive, negative).

$$\text{Val(ue)} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

VIII – ORDERING INFORMATION

Monoscreen AgELISA IPNV3	1 X 48 tests	BIO K 282/1
	2 X 48 tests	BIO K 282/2





IX – REFERENCES

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