ADIAVET™ IPNV REAL TIME

TEST FOR DETECTION OF INFECTIOUS PANCREATIC NECROSIS VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

Reference:
ADI641-100 (100 reactions)

VET

English version
NE641-01
2021-01

Avec le soutien de
Région Bretagne

ADIAVET™ IPNV REAL TIME

Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change product	Addition, revision and/or removal of information related to the
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2021-01	NE641-01	N/A	creation

I. General information

1. Purpose of the test

The ADIAVET™ IPNV REAL TIME kit is used to detect Infectious Pancreatic Necrosis Virus (IPNV) by enzymatic amplification in real time (PCR), using RNA from organs, eggs, semen, coelomic liquid of fish or supernatant of viral culture.

2. Infectious pancreatic necrosis

Infectious pancreatic necrosis (IPN) is a highly infectious viral disease affecting fish farms. It appears mainly in young salmonids as well as among pike fry, but almost all freshwater and saltwater fish are susceptible to disease, as are molluscs. Infectious pancreatic necrosis is present in parts of Europe, America and Asia.
Sensitivity to NPI varies according to age since young fish are more likely to catch the disease, compared to older ones. Asymptomatic carrier parents as well as contaminated eggs are the main reservoirs of IPN. The infectious agent is enriched inside the eggs and semen and is transmitted to fry. Fish can carry and transmit the infectious agent for several generations and years without showing any symptom
Affected fish swim in spirals or corkscrews, or are found on the flank at the bottom of the basin. They have a dark coloration, exophthalmos, a swollen belly and strings of whitish excrement.
IPN is caused by the infectious pancreatic necrosis virus (IPNV) which belongs to the Birnaviridae family. It is a packaged virus, made up of 2 double RNA segments strands. Segment A codes for a viral capsid protein (VP2), an internal protein (VP3), a viral protease (VP4) and a non-structural protein VP5. Segment B codes for an RNA polymerase (VP1) (Dobos, 1995).
Aquabirnaviruses are divided into 2 serogroups based on cross-neutralization tests: serogroup A (A1-A9) includes most of the isolates associated with the disease, serogroup B includes exclusively B1 serotypes (Hill & Way 1995). A more recent phylogenic classification, based on the VP2 gene, makes it possible to reduce the 9 serotypes of serogroup A in 6 genogroups (Blake and all 2001). In Europe, genogroups 2 and 5 are the most represented.
The standard diagnostic method has long been viral culture followed by immunological, sero-neutralization or RT-PCR identification. This method requires 2 weeks to certify virus detection failure in the culture. The use of RT-qPCR allows faster and more precise identification of the breeding status compared to viral cultivation.

3. Description and purpose of the test

The first step of this test is the reverse transcription (RT) of RNA into complementary DNA. The cDNA obtained is then amplified by PCR using a DNA polymerase and specific primers. Both enzymatic reactions happen in a single tube (One-step RT-qPCR).
The amplified products are detected in real time with specific labeling of hydrolysis probes (5'-exonuclease technology).

ADIAVET™ IPNV REAL TIME can detect simultaneously:

- Infectious pancreatic necrosis virus (FAM-labelled probe)
- An exogenous control (EPC-extraction) added at the time of extraction. It allows the validation of the extraction process and of the PCR reaction (probe labelled with a fluorophore read in the same spectrum as VIC and HEX).

ADIAGENE has validated this test with different RNA purification kits (Bio-X Diagnostics, Qiagen and Macherey-Nagel). Other purification kits can be used after validation by the user.

Analysis options according to the specimen:

Specimen	Individual analysis	Pool of sample is possible*
Organ pool: spleen, kidney, heart or brain	Yes	Yes, up to 10 fishes
Eggs, semens, coelomic liquid	Yes	No
Supernatant of culture	Yes	No

* Varies according to the epidemiological situation, the quality of the specimen and specific directives existing in some countries (follow them).

II. Material and reagents

1. Reagents provided with the kit

REF ADI641-100		
A5	amplification solution	2 x 500 µL tube with green cap (a ready-to-use reagent)
IPNV CTL+	positive control IPNV	1 tube with purple cap (to reconstitute)
EPC-Ext	Exogeneous extraction control	2 x 300 µL tube with yellow cap (a ready-to-use reagent)
NF-Water	Nuclease free Water	1 x 1000 µL tube with white cap (a ready-to-use reagent)

2. Validity and storage

After reception, the kit should be stored at <**-15°C**.
It is recommended to make aliquots of the A5 solution to prevent it from being defrosted more than 3 times. **Do not defrost reagents more than 3 times.**
Real-time reagents are sensitive to light: they should be stored in the dark.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents from two different batches.

3. Use of controls

A. « IPNV CTL+ »

« IPNV CTL+ » is an amplification positive control.
Add **200 µL** of **NF-Water** to the **IPNV CTL+** tube. Homogenize with a vortex for 20 seconds. Divide this solution by 6 or 12 µL aliquots and store at <-15°C.
For each analysis, we recommend using **5 µL** of **IPNV CTL+** in one of the wells.

B. « EPC-Ext »

EPC-Ext is a no-target extraction control.
Use of EPC-Ext allows the validation of the extraction, purification and amplification steps.
At first use, aliquot the solution according to the size of the extraction series and store at <-15 ° C.
Add **5 µL** of **EPC-Ext** per sample extracted.
This EPC-Ext is the same tube included in ADIAVET™ IHNV REAL TIME (ADI641-100) and ADIAVET™ VHSV REAL TIME (ADI581-100) kits.

4. Equipment required but not supplied in the kit

Material should be Nuclease-Free (e.g. autoclaved 25 minutes twice at +121°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 mL PCR tubes or closed 96-wells PCR plates with optical quality
- Class II Microbiological Safety Cabinet
- A centrifuge for microtubes, 50mL tubes or 96-wells plates
- Beads crusher (Mixer Mill, Fasr Prep or Rybolyser)
- Vortex
- 1 - 10 µL pipette, 20 - 200 µL pipette and 200 - 1000 µL pipette
- Nuclease-Free filter tips
- Nuclease-Free microtubes: 1.5 mL and 2 mL
- Powder-free latex gloves
- Tunsgten or inox beads 3 mm.
- Razor blades
- 96-100% ethanol solution
- Nuclease-Free water

PBS 1X buffer pH = 7.4 (recommended composition, NaCl 150 mM, Na_2HPO_4 5 mM, KH_2PO_4 1.7 mM, without Ca^{2+}, without Mg^{2+} - another composition can be used after validation by the user)

- Extraction kits for RNA/DNA:
  - **Material needed for individual column extraction**
    - Nucleospin® RNA Virus (Macherey-Nagel, 50 extractions: ref. 740956.50; 250 extractions: ref. 740956.250)
    - QIAamp® Viral RNA kit (Qiagen, 50 extractions: ref. 52904; 250 extractions: ref. 52906)
  - **Automated DNA/RNA extraction kit (magnetic beads)**
    - ADIAMAG (Bio-X Diagnostics; 200 extractions: ref. NADI003; 800 extractions: ref. NADI003-XL).

III. Recommendation before the analysis of samples

Before starting the test, read the entire protocol and follow it carefully.

1. Precautions

Adiagène has validated this PCR test with Bio-X Diagnostics, Qiagen and Macherey-Nagel extraction kits. Other extraction kits can be used after validation by the user.

Follow the supplier's instructions for the storage, preparation and use of the extraction reagents.

Some kits include and/or need toxic reagents. These reagents should be manipulated with gloves and in achemical hood.

Only appropriately trained personnel should perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends on rigorous respect of good laboratory practices.

The PCR generates large amount of DNA. A few molecules of amplified products are sufficient to generate a positive result. Hence, it is important to reserve 2 rooms, one for the manipulation of the samples to be tested, and the other one for amplified products analysis. PCR tubes should not be opened after amplification.
Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using aliquots of demineralised and saline water and autoclaving 25 minutes at +121°C twice or 60 minutes at +121°C. To avoid contamination, use a new aliquot for any new experiment.

2. Storage of samples and RNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at <-15°C.
Extracted RNAs are sensitive molecules. The extraction is performed at room temperature and must therefore be as fast as possible to avoid damage. After extraction, RNAs can be stored at +2/8°C for a few hours, but should be placed at <-65°C for long-term storage.

3. Samples preparation

A. Fish organ pool, eggs and semens:

Homogenize, by stomacher, mixer or mortar and pestle with sterile sand, re-suspend in the original transport medium with a ratio of 10% w / v, centrifuge for 15 minutes at 4000 g.
or
Grind [1 or 2] 0.1 g of sample with 1 mL of 1X PBS buffer.
[1] For example: with a Mixer Mill type ball mill: add a tungsten bead (3 mm), grind for 2 minutes at 30 Hz then centrifuge 6,000 g / 2 minutes.
[2] For example: with a Fast Prep type vibratory grinder: in a Lysing Matrix D tube, grind 2 times 20 seconds at 6m / sec with a 5 minutes pause on ice between the 2 grindings, then centrifuge 5,000 g / 3 minutes.

See § IV for RNA extraction and purification.

B. Coelomic liquid :

Use 100 or 140 µL of coelomic liquid according to the chosen extraction method.
See § IV for the extraction and purification of RNAs.

C. Supernatant of viral culture

Viral cultures are extracted after centrifugation for 15 minutes between 2000 and 4000 g.
See § IV for RNA extraction and purification.

4. Controls to include

Several controls must be included during each extraction run.
The non-target EPC-Ext, added during extraction, allows the validation of the extraction process and of the amplification for each sample. The "IPNV CTL +" allows validation of the target amplification.
The combination of these different controls allows the validation of all the steps of the analytical process (extraction + amplification), irrespective of the matrix.
Other controls must or may be added:

A. Negative control of extraction (required)

To verify the absence of cross-contamination, at least one negative control has to be included per assay (the norm NF U47-600 recommends 1 negative control for 24 samples or 4 negative samples for a 96 wells-plate). This control can be a negative matrix or a buffer used for dilutions.

B. Positive control of extraction (recommended)

A positive control (a sample including IPNV) can be added to each assay. It can be a positive sample available in the laboratory or a negative sample spiked with IPNV solutions. This positive control will be detected close to the limit of detection of the method and allow comparison of the results obtained in different assays.

IV. Extraction and purification

1. Using QIAamp Viral RNA kit

All the centrifugations are performed at room temperature.
Before the beginning of extraction, pre-warm the AVL buffer + RNA carrier at +56°C.

<table>
<tr><th></th><th>Tissue, eggs, semens, Supernatant of viral culture</th><th>Coelomic liquid</th></tr>
<tr><td rowspan="2">Lysis</td><td>Place 140 µL of surpernatant prepared as previously descibed, in a microtube.</td><td>Place 140 µL of coelomic liquid in a microtube.</td></tr>
<tr><td colspan="2">Add 560 µL of AVL buffer + RNA Carrier + 5 µL EPC-Ext*.
Homogenize ~15 seconds and incubate 10 minutes at room temperature.</td></tr>
<tr><td>Binding preparation</td><td colspan="2">Add 560 µL of ethanol 100%.
Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).</td></tr>
<tr><td>Transfer on columns and membrane binding</td><td colspan="2">Identify columns, apply 630 µL of the obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g.
Put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>1st wash</td><td colspan="2">Change the collection tube and add 500 µL of AW1 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="2">Change the collection tube and add 500 µL of AW2 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Dry column</td><td colspan="2">Change the collection tube.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="2">Transfer the column on a microtube. Add 60 µL of AVE.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="2">Close the tubes, identify and store on ice if using immediately or at <-65°C.</td></tr>
</table>

** It is possible to prepare extemporarily n x (560 µl AVL+carrier + 5 µl EPC-Ext) then add 565 µl to each sample.*

2. Nucleospin® RNA Virus kit

All the centrifugations are performed at room temperature.
Before extraction, pre-warm the RAV1 buffer + RNA carrier at +56°C.

	Tissue, eggs, semens, Supernatant of viral culture	**Coelomic liquid**
Lysis	Place **140 µL** of surpernatant prepared as previously descibed, in a microtube.	Place **140 µL** of coelomic liquid in a microtube.
	Add **560 µL** of **RAV1 buffer** + **RNA Carrier + 5 µL EPC-Ext*** Homogenize ~15 seconds and incubate 10 minutes at room temperature.	
Binding preparation	Add **560 µL** of **ethanol 100%**. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer on columns and membrane binding	Identify columns, apply **630 µL** of the obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g. Put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.	
1st wash	Change the collection tube and add **500 µL** of **RAW buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **500 µL** of **RAV3 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Dry column	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column on a microtube. Add **60 µL** of **Nuclease-free water**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-65°C.	

* *It is possible to prepare extemporarily n x (560 µl RAV1+carrier + 5 µl EPC-Ext) then add 565 µl to each sample.*

3. Using ADIAMAG kits - DNA/RNA magnetic beads kit

See the NEKF user manual available on the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

V. Amplification

a - Determine the number of samples to analyse (including the controls).

b- **Denaturation of viral RNAs** (double stranded):
The denaturation step is mandatory and must be carried out **extemporaneously** before amplification.
For each sample and control, place an RNA aliquot in a 0.2 mL tube or in a PCR plate. Briefly spin the microtubes or PCR plate.
Heat the microtubes or the PCR plate for **3 minutes at + 95 ° C**, then place immediately on ice.

c- Defrost the A5 solution reagent at room temperature. Homogenize. Dispense **10 µL** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-Free tip.

d- **Immediately replace the A5 solution tube at <-15°C and in the dark.**

e- Add **5 µL** of denatured purified extract to each sample and controls (except for the NTC in which NF-water should be added).
Immediately replace purified RNA extracts at <-15°C (at -65°C for a long storage)
Make sure there are no bubbles at the bottom of the wells. If bubbles are present, briefly spin the tubes or plates.

f- Store the tubes or plates on ice until the cycler is programmed and start quickly the run after you have placed the tubes or plates in the cycler.

The IPNV target is read in FAM, the Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read at the end of the elongation step.

The following programs are defined for **ABI Prism** thermocyclers (like 7500, StepOne…) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **MX3005P** and **ARIAMX** of **Agilent** and for **CFX96** of **BioRad**.

Standard program	
10 min. 45°C	
10 min. 95°C	
15 sec 95°C*	45 cycles
1 min. 60°C	

* 30 secondes for the MX3005P
For ABI7500 et QS5 applied's thermocyler, select the quenchers = « none ».
Contact us if you wish to use other thermalcyclers.

VI. Interpretation of results

1. Definitions

The **« base line »** corresponds to the background of fluorescence and qualifies the non-characteristic part of the curve observed during the first cycles.

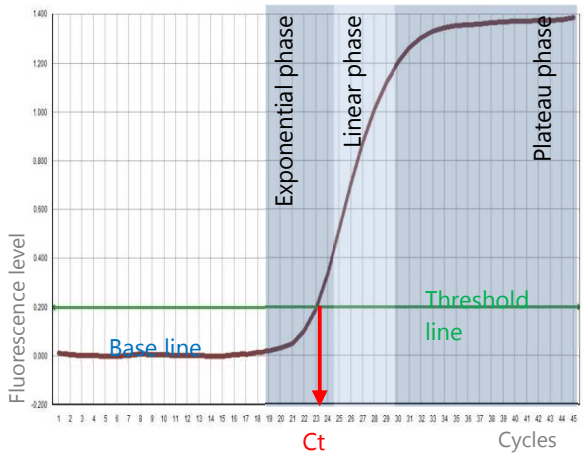
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The **« threshold line »** has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

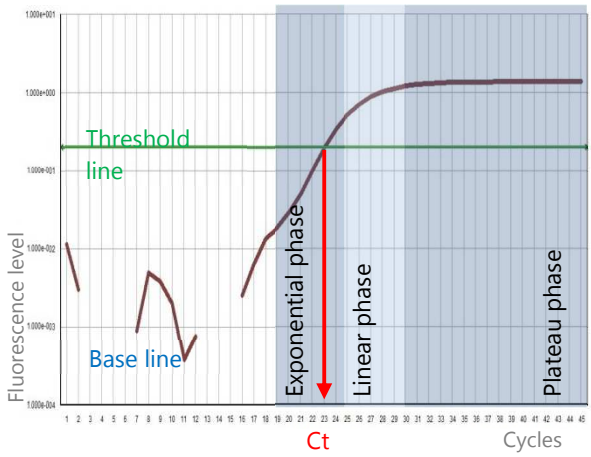
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, to the crossing point of the threshold line and fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequence initially present.

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above.
Repeat for the VIC or HEX curves.

A. Results validation

Amplification is considered valid if the following results are obtained for the controls:

Controls	NTC	Extraction Positive control (IPNV CTL+)	Negative control of extraction	Extraction Positive Control*
FAM amplification	no	yes	no	yes
HEX amplification	no	Yes/no	yes	yes
Validation of	No contamination for amplification	Amplification of IPNV target	No contamination for extraction	Extraction and amplification steps

* optionnal

The values of Ct expected in FAM and VIC/HEX for the positive control ("CTL +") are indicated on the certificate of analysis of the kit.

B. Results interpretation

RNA extraction and amplification are considered valid for each sample if at least one characteristic amplification curve is observed for IPNV (FAM) or internal control (VIC/HEX).

Exemple	A	B	C
FAM amplification	No	Yes	No
HEX amplification	Yes	Yes / No	No
Résults	Undetected	Detected	Undetermined

The sample is considered **undetected** if a characteristic amplification curve is observed in VIC or HEX but not in FAM (example A).

The sample is considered **detected** if a characteristic amplification curve is observed in FAM (example B). Internal Control can be co-amplified.

The total absence of a characteristic amplification curve for a sample (example C) indicates an issue withRNA extraction (loss or destruction of the RNA) or a defective real-time RT-PCR (presence of inhibitors in the sample, program error or lack of sample). In this case, we recommend repeating the test using pure RNA extract and a ten-fold dilution in Nuclease-Free water. If the test is still not validated, a new RNA extraction is recommended.

VII. Symbols index

Symbole	Signification
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

ADIAGENE S.A.S.
9, rue Gabriel Calloët-Kerbrat
22440 Ploufragan - France

RCS 417 876 299
Tel. +33 (0)2 96 68 40 20
www.biox.com