ADIAVET™ VHSV REAL TIME

TEST FOR DETECTION OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

Reference:
ADI581-100 (100 reactions)

VET

English version
NE581-02
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Avec le soutien de
Région Bretagne

ADIAVET™ VHSV 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
2019-09	NE581-01		creation
2021-01	NE581-02	Administrative	Addition of Brittany region logo Modification of ADIAGENE's status as "S.A.R.L." to "S.A.S." Logo ADIAGENE suppressed
2021-01	NE581-02	Technical	II.4. Modification of PBS composition II.4. Addition of the ADIAMAG XL reference V. Applied thermocycler programming: quencher = none VI.2.B. Replacement of terms "positive" by "detected" and "negative" by "undetected".

I. General information

1. Purpose of the test

The ADIAVET™ VHSV REAL TIME kit is used to detect viral haemorrhagic septicaemia virus (VHSV) by enzymatic amplification in real time (PCR), using RNA from organs of fish or supernatant of viral culture.

2. Viral haemorrhagic septicaemia

Viral haemorrhagic septicaemia (VHS) is a major cause of mortality of rainbow trout in breeding. VHS is caused by the viral haemorrhagic septicaemia virus (VSHV), also known as the Egtved virus.
Brown trout, common shade, whitefish and pike are susceptible to this virus, as well as marine species such as turbot and cod. Animals of all ages may be affected, but the disease is more common and more serious in juveniles. The infection is transmitted by water or by direct contact with the secretions (urine) of infected fish. The released virus can travel 10 to 20 km along the current. Fish-eating birds can also act as passive vectors. The acute form of the disease corresponds to the early stages of infection, with clinical signs as follows: rapid increase in mortality (can reach 100%), lethargy, loss of equilibrium with sometimes spiralling swimming, haemorrhages at the base of fins, melanosis, anaemic gills, ascites and dilated abdomen, internal and external petechiae

VHSV is a virus of the Rhabdovirus family and the subfamily of Novirhabdoviruses. It is a packaged virus, single-stranded negative RNA about 12.000 bp.
The reference diagnostic method has long been viral culture followed by identification by immunology or PCR. But this method requires an observation time of 3 weeks to certify the non-detection of viruses in the culture. The use of RT-qPCR allows to know the status of a breeding more quickly.

3. Description and purpose of the test

The ADIAVET ™ VHSV REAL TIME test is based on the reverse transcription (RT) of RNA into complementary DNA. This reaction followed by the gene amplification of the DNA fragments specific for VHSV are in the same tube (One-step RT-qPCR). The ADIAVET ™ VHSV REAL TIME kit can detect simultaneously

- viral haemorrhagic septicaemia virus (FAM-labelled probe)
- An exogenous control "EPC-extraction" which, added at the time of extraction, validates the good progress of this step as well as the PCR reaction (probe labelled with a fluorophore read in the same spectrum as VIC and HEX).

ADIAGENE validated this test with different RNA purification kits (Bio-X Diagnostics, Qiagen and Macherey-Nagel). Other purification kits can be used if they are validated 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
Supernatant of culture	Yes	No

* It depends on the epidemiological case, on the quality of the specimen and specific directives that exist in some countries (follow them).

II. Material and reagents

1. Reagents provided with the kit

REF ADI581-100		
A5	amplification solution	2 x 500 µl tube with green cap (a ready-to-use reagent)
VHSV CTL+	positive control VHSV	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

On receipt, the kit should be stored at <**-15°C**.
It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.
Do not defrost reagents more than 3 times.
Realtime reagents are susceptible to light: store them in the darkness.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of controls

A. « VHSV CTL+ »

« VHSV CTL+ » is an amplification positive control.
Add **200 µl** of **NF-Water** to the **VHSV CTL+** tube. Homogenize tube contents using mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use **5 µl** of **VHSV CTL+** in one of the wells.

B. « EPC-Ext »

EPC-Ext is a no-cible extraction control.
Use of EPC-Ext allows to control both the extraction, purification and amplification steps.
On the first use, aliquot the solution according to the size of the extraction series and store at <-15 ° C.
For each extraction, add **5 µl** of **EPC-Ext** per sample.
This EPC-Ext is the same tube inclus in ADIAVET™ IPNV REAL TIME (ADI641-100) and ADIAVET™ IPNV REAL TIME (ADI571-100) kits.

4. Equipment required but not supplied

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°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)
- Tunsgten or inox beads 3-5 mm.
- Instrument for homogenous mixing of tubes (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
- Scalpel 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 1.7mM, without Ca^{2+}, without Mg^{2+} - another composition could be used after a validation made 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).

- Complementary kits available for adoption of method and PCR (AFNOR NF U47-600):
  - ADIAVET ™ VHSV Extraction Positive Control (Ref. ADI581-8). Supplier reference material for method adoption that can also be used as a sentinel.
  - ADIAVET ™ LDpcr Positive Control - VHSV (Ref. ADI581-LD) Performance Confirmation - LOD_{PCR} of the ADIAVET ™ VHSV REAL TIME Kit.

III. Recommendation before the analysis of samples

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

1. Precautions

Adiagène has elaborated this PCR test with the use of Bio-X Diagnostics, Qiagen and Macherey-Nagel extraction kits. Other extraction kits can be used with a previous validation.

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

Some kits include and/or need the use of toxic reagents. These reagents should be use with gloves and into chemical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes 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 fractions of demineralised and saline water and to autoclave them twice 25 minutes at +120°C or once 60 minutes at +121°C. Take a new fraction for each new manipulation to avoid contamination.

2. Storage of samples and DNA extracts

For the collection, transport and conservation of samples, refer to the recommendations of the Implementing Decision 2015/1554, and to the recommendations of the French national reference laboratory ANSES Ploufragan-Plouzané-Niort. (REF : ANSES/PLOU/MA/4)
After analysis, the samples or residues as well as the extracted nucleic acids are kept at a temperature below -15 ° C for at least 1 month.
Extracted RNAs are sensitive molecules. The extraction is performed at room temperature and must therefore be as fast as possible to avoid damage. The extracted RNAs can be stored at the end of the extraction on ice or at +2/8°C for a few hours, then should be stored at <-65°C.

3. Samples preparation

A. Fish organ pool :

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 4000g.
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. 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 at the moment of the extraction, permited to verify the good progress of this step as well as to verify the amplification for each sample. The "VHSV CTL +" control validate the amplification of the target.
The combination of these different controls allows the validation of all the steps of the analytical process (extraction + amplification), whatever the matrices.
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 must be included per trial (e.g. the normative requirement and recommendation for the development and the validation of veterinary PCR NF U47-600 suggests the use of 1 negative control for 24 samples or 4 negative samples for a 96 wells-plate). This control could be a negative matrix, or a buffer used for dilutions.

B. Positive control of extraction (recommended)

A positive control could be added in each trial. The control is a sample including VHSV. It could come from a positive sample available in the laboratory or from a negative sample spiked with solutions of VHSV. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

ADIAGENE can provide on demand a target positive extraction control consisting of an inactivated and lyophilized viral culture, calibrated between 1 and 100xLD method.
(ADIAVET ™ VHSV Extraction Positive Control, Ref. ADI581-8).
This control, added to a negative template can be used as **sentinel** or after dilution, as a **NED provider to adopt the method.**

IV. Extraction and purification

1. Using Nucleospin® RNA Virus kit

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

	Tissue	Supernatant of viral culture
Lysis	Place **140 µl** of supernatant prepared as previously described in a microtube.	Place **140 µl** of supernatant prepared as previously described 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 to columns and binding to the membrane	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.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column to 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's possible to prepare extemporarily n x (560 µl RAV1+carrier + 5 µl EPC-Ext) then add 565 µl to each sample*

2. 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.

	Tissue	**Supernatant of viral culture**
Lysis	Place **140 µl** of supernatant prepared as previously described in a microtube.	Place **140 µl** of supernatant prepared as previously described in a microtube.
	Add **560 µl** of **AVL 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 to columns and binding to the membrane	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 **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **500 µl** of **AW2 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add **60 µl** of **AVE**. 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's 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 samples analysed including the controls (e.g. positive and negative extraction controls, positive control of amplification (VHSV CTL+) and PCR reagent control (NTC)).

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 darkness.**

e- For each sample, the VHSV CTL+, the extraction negative control (required) and the extraction positive control (recommended) add **5 µl** of denatured purified extract to the **10 µl** of A5 solution.
For the PCR reagent control (NTC), nothing is added to the A5 solution.
Immediately replace purified RNA extracts at +2/8°C or <-15°C. Take care to have no bubbles in the bottom of the wells.

f- Store the plate or the tubes on melting ice or at +2/8°C until the cycler is programmed and start quickly the run after you have placed the plate or the tubes in the cycler.

The VHSV 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 during 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	

* Note 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.

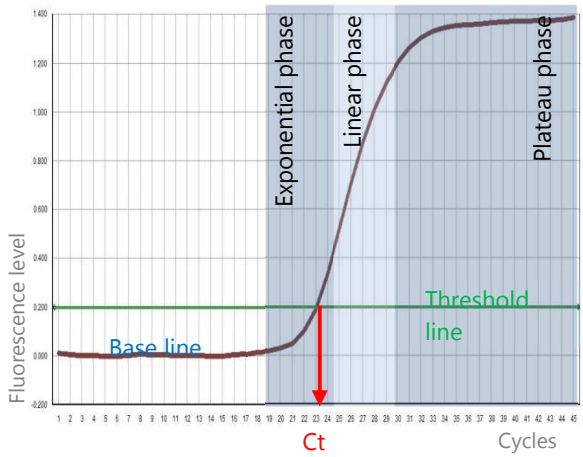
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

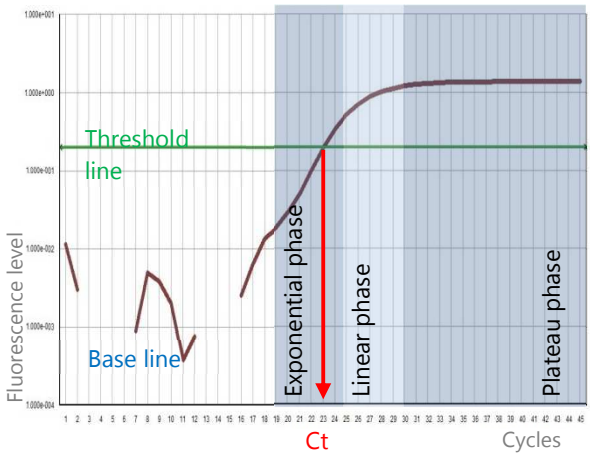
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

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.
Proceed in the same mean for the VIC or HEX curves.

A. Results validation

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

Controls	NTC	Extraction Positive control (VHSV 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 VHSV	No contamination for extraction	Extraction and amplification steps ok

* optionnal

The indicative 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 as valid for each of the samples if at least one characteristic amplification curve is observed in VHSV (FAM) or in 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 as **undetected** if a characteristic amplification curve is observed in VIC or HEX without there being a characteristic amplification curve 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 a deficiency of the extraction of the RNA (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, it is advisable to repeat the test using pure RNA extract and diluted 1 / 10th in Nuclease-free water. In a second step, if the test is still not validated, a new extraction of the total RNA from the sample is desirable.

VII. Bibliographic references

CONCIL 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.
[https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:328:0014:0056:FR:PDF]

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.
[https ://eur-lex.europa.eu/legal-content/fr/TXT/ ?uri=CELEX%3A32015D1554]

Détection du virus de la septicémie hémorragique virale (vSHV) par RT-PCR en temps réel par le Laboratoire de Ploufragan-Plouzané-Niort, laboratoire national de référence pour les maladies règlementées de poissons.
RÉF : ANSES/PLOU/MA/4

OIE. 2016. Chapter 1.3: Manual of Diagnostic Tests for Aquatic Animals (2018)
[http://www.oie.int/fr/normes/manuel-aquatique]

VIII. 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

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