ADIAVET™ PRV REAL TIME

TEST FOR THE DETECTION OF PSEUDORABIES VIRUS (Aujeszky's disease) BY REALTIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI072-100 (100 reactions)

VET

NOTE
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English version
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ADIAVET™ PRV REAL TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
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
2012/05	NE072-05	N/A	First publication
2014/12	NE072-06	Technical change	Addition of "Index of symbols" section, in page 11.
2014/12	NE072-06	Technical change	Removal of reference ADI072-50 (50 reactions)
2016/07	NE072-07	Administrative	Changing logos
2016/07	NE072-07	Administrative	Biosearch legal mention
2016/07	NE072-07	Administrative	Addition of table "Analysis options according to the specimen"

II. General information

1. Purpose of the test

ADIAVET™ PRV REAL TIME kit is intended to detect the Pseudorabies Virus (PRV), in other words Aujeszky's Disease Virus, using real-time Polymerase Chain Reaction (PCR) technology from nasal swab, tissue and brain specimens of dog, pig and wild boar.

2. Pathogen

Pseudorabies virus is the causing agent of Aujeszky's disease. The principal host of this double strand DNA virus (family: *herpesviridae;* subfamily: *alphaherpesvirinae)* is swine.
More rarely, it may also infect other mammals like cattle, little ruminants, carnivores and rodents. However, it isn't pathogenic for humans.
PRV virus affects the central nervous system and organs of the respiratory system. In swine, Aujeszky's disease shows itself under three forms depending on animal's age: a nervous form, a respiratory form and a genital form. In other mammals, it causes infection of the central nervous system leading very quickly to the death of the animal.
The diagnosis of Aujeszky's disease can be established by the detection of the virus (by cell culture or by PCR) from tonsils, lymphatic nodes, lungs, brain, spinal cord or nasal swabs. It is also possible to detect the presence of antibodies using a serological test.

3. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ PRV REAL TIME kit enables the simultaneous detection of:
- the PRV virus (probe labelled in FAM),
- an exogen control EPC-Ext added during the extraction that allows validating extraction and amplification steps (probe labelled with a fluorochrome read in the same spectra as VIC or HEX).

ADIAGENE validated the test using DNA purification kits (MACHEREY-NAGEL, QIAGEN). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis
Nasal swab	☑
Tissue (tonsil, ganglion, lung …)	☑
Brain	☑

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagent	ADI072-100
A5	Amplification solution	2 x 1000 µl green tubes
PRV CTL+	Positive control Pseudorabies Virus	1 purple tube
EPC-Ext	External control of extraction	2 x 300 µl yellow tubes

2. Validity and storage

On receipt, the kit should be stored at **<-15°C**.
It is recommended to make fractions of Amplification solution A5 if it should be defreezed more than 3 times. **Do not defreeze 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. Use of PRV CTL+

Add **200 µl** of Nuclease-free water to the PRV CTL+ tube included in the kit. Homogenize tube contents using a 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 PRV CTL+ in a well.

B. Use of EPC-Ext

The EPC-ext will follow all the extraction process.
Make fractions and store the solution at <-15°C (EPC-Ext may demean itself up to 3 defrosting: make fractions of 50 µl).
For each extraction, it is recommended to add 5 µl of EPC-Ext solution in each sample.

4. Equipment required but not supplied

Caution: material used should be Nuclease-free or autoclaved twice 25 minutes at +120°C or 60 minutes once at +121°C.
- Thermal cycler with PCR consumables: 0.2 ml PCR tubes or closed 96-well PCR plates with optical quality
- A centrifuge for microtubes
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Water bath or heating block
- Ethanol 96-100%
- Sterile saline water (NaCl 8.5 g/l)
- Powder-free latex gloves
- Instrument for homogenous mixing of tubes
- 1 - 10 µl, 20 - 200 µl and 200 - 1000 µl pipettes
- Nuclease-free filter tips
- Metal beads 3 mm (Qiagen ref. 69997)
- Universal laboratory mixer mill
- Scalpel blades
- **DNA extraction kit (individual columns)**
    - QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)

or

    - NucleoSpin® Tissue (Macherey-Nagel, 50 tests: ref.740952.50 or 250 tests: ref. 740952.250)

IV. Recommandation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiagène has elaborated this PCR test with the use of 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 water and to autoclave them 25 minutes at +120°C. Take a new fraction for each new manipulation to avoid contamination.

We recommend to include at least a negative extraction control (= extraction without sample) by run of extraction.
A sample positive in PRV virus (culture or field sample) can be included and extracted in each run, it will be considered as positive extraction control.

2. Storage of samples and DNA extracts

Tissues and nasal swabs are stored a couple of days at +2/8°C. Up to 2 days, it is recommended to store them at <-15°C.
Extracted DNAs are quite sensitive molecules. Crude extracts should be stored at the end of extraction at +2/8°C for 24 hours, then at <-15°C.

3. Controls to include

The use of controls allows to verify the reliability of the results.
The controls are included per trial of analysis. A trial is defined as all the samples treated in the same conditions.
The use of the controls follows the recommendation of the normative requirements and recommendations for the development and the validation of veterinary PCR (NF U47-600).
All the steps of the analysis procedure (extraction+amplification), for the type of sample, are validated with the association of the controls included in the kit.

- The exogen control EPC-Ext added during the extraction allows to verify the extraction and amplification steps of each sample.
- The PRV CTL+ allows to validate the amplification of the target.

Other controls must or could be added.

- **Negative control of extraction (obligatory)**

To verify the absence of cross-contamination, at least one negative control must be included per trial. The control is a negative sample, for example a buffer used for dilution.

- **Positive control of extraction (recommended)**

A positif control could be added in each trial. The control is a sample incuding Pseudorabies Virus. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of Pseudorabies Virus. 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.

V. Extraction and purification

1. Using the QIAamp® DNA mini kit

All the centrifugations are performed at room temperature.
Before the beginning of extraction, turn on one or two heating systems at temperatures mentioned below.

<table>
<tr><th></th><th colspan="2">Tissues/brain</th><th rowspan="2">Nasal Swabs</th></tr>
<tr><th></th><th>Without grinding</th><th>With grinding</th></tr>
<tr><td>Samples preparation</td><td>Place 20-30 mg of minced tissue in a microtube.</td><td>Place 0.1 g of minced tissue in a 2 ml-microtube.</td><td>Add 2 ml of sterile saline water or MEM medium to each tube of swab.</td></tr>
<tr><td rowspan="2">Lysis</td><td>Add 180 µl of buffer ATL, 20 µl of proteinase K and 5 µl of EPC-Ext.
Homogenize.
Incubate 30 minutes at +70°C (or a night at +56°C).</td><td>Add a metal bead.
Add 1 ml of sterile saline water or MEM medium.
Mix by grinding twice 3 minutes at 30 Hz with a pause (e.g. 1 minute) between both.
Centrifuge 2 minutes at 6 000 g.</td><td>Knead the swab through the transport tube and/or homogenize.
Transfert the supernatant in a 2 ml-microtube.
Squeeze each swab to collect the maximum of liquid. (*)</td></tr>
<tr><td>Add 200 µl of buffer AL.
Homogenize.
Incubate 10 minutes at +70°C.</td><td colspan="2">Transfer 200 µl of supernatant.
Add 180 µl of buffer AL, 20 µl of proteinase K and 5 µl of EPC-Ext.
Homogenize.
Incubate 10 minutes at +70°C.
Centrifuge 1 minute at 10 000 g (facultative for nasal swabs).
Transfer the supernatant in a new microtube.</td></tr>
<tr><td>Binding preparation</td><td colspan="3">Add 210 µl of ethanol 100%.
Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="3">Identify columns, apply the the whole obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>AW1 washing</td><td colspan="3">Change the collection tube and add 500 µl of buffer AW1 to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>AW2 washing</td><td colspan="3">Change the collection tube and add 500 µl of buffer AW2 to the column.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Column dry</td><td colspan="3">Change the collection tube.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="3">Transfer the column to a microtube.
Add 200 µl of buffer AE.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="3">Close the tubes, identify and store them at +2/8°C if using immediately, or at <-15°C.</td></tr>
</table>

(*) At the end of the extraction, store at -70°C +/-10°C for a new analysis or for a viral culture.

2. Using the NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.
Before the beginning of extraction, turn on one or two heating systems at temperatures mentioned below.

<table>
<tr><th></th><th colspan="2">Tissues/brain</th><th rowspan="2">Nasal Swabs</th></tr>
<tr><th></th><th>Without grinding</th><th>With grinding</th></tr>
<tr><td>Samples preparation</td><td>Place 20-30 mg of minced tissue in a microtube.</td><td>Place 0.1 g of minced tissue in a 2 ml-microtube.</td><td>Add 2 ml of sterile saline water or MEM medium to each tube of swab.</td></tr>
<tr><td rowspan="2">Lyse</td><td>Add 180 µl of buffer T1, 25 µl of proteinase K and 5 µl of EPC-Ext.
Homogenize.
Incubate 30 minutes at +70°C (or a night at +56°C).</td><td>Add a metal bead.
Add 1 ml of sterile saline water or MEM medium.
Mix by grinding twice 3 minutes at 30 Hz with a pause (e.g. 1 minute) between both.
Centrifuge 2 minutes at 6 000 g.</td><td>Knead the swab through the transport tube and/or homogenize.
Transfert the supernatant in a 2 ml-microtube.
Squeeze each swab to collect the maximum of liquid. (*)</td></tr>
<tr><td>Add 200 µl of buffer B3.
Homogenize.
Incubate 10 minutes at +70°C.</td><td colspan="2">Transfer 200 µl of supernatant.
Add 180 µl of buffer B3, 25 µl of proteinase K and 5 µl of EPC-Ext.
Homogenize.
Incubate 10 minutes at +70°C.
Centrifuge 1 minute at 10 000 g (facultative for nasal swabs).
Transfer the supernatant in a new microtube.</td></tr>
<tr><td>Binding preparation</td><td colspan="3">Add 210 µl of ethanol 100%.
Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="3">Identify columns, apply the the whole obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>AW1 washing</td><td colspan="3">Change the collection tube and add 500 µl of buffer BW to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>AW2 washing</td><td colspan="3">Change the collection tube and add 500 µl of buffer B5 to the column.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Column dry</td><td colspan="3">Change the collection tube.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="3">Transfer the column to a microtube.
Add 200 µl of buffer BE.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="3">Close the tubes, identify and store them at +2/8°C if using immediately, or at <-15°C.</td></tr>
</table>

(*) At the end of the extraction, store at -70°C +/-10°C for a new analysis or for a viral culture.

VI. Amplification

a- Determine the number of PCR tubes required. We recommend including in addition to the negative extraction control, a positive control and a reagent control (no template control).

b- Defreeze the A5 solution at room temperature. Homogenize. Dispense **20 µl** in each PCR tubes or PCR plate wells with a micropipette with an RNase-free tip.

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

d- For each sample, add **5 µl** of DNA extract into the **20 µl** of A5 solution. For the positive control, add **5 µl** of the solution (§II.3.) into the 20 µl of A5 solution. **Immediately replace the DNA extracts at +2/8°C or -20°C +/-5°C.** Take care to have no bubbles in the bottom of the wells.

e- Once all the tubes have been prepared, perform the realtime PCR amplification.

Start the run as soon as possible after the loading of the plate or the tubes in the thermalcycler.
The Pseudorabies virus target is read in FAM and the EPC-Ext 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 (1 minute at 60°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:
2 minutes 50°C
10 minutes 95°C
15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:
2 minutes 50°C
10 minutes 95°C
30 seconds at 95°C and 1 minute at 60°C during 45 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*
**** NOTE:*** *The use of LightCycler thermalcyclers requires a calibration manipulation. Adiagene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Results interpretation

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.

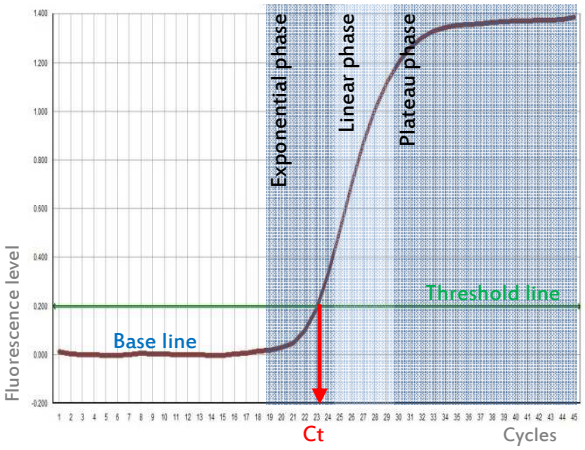
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

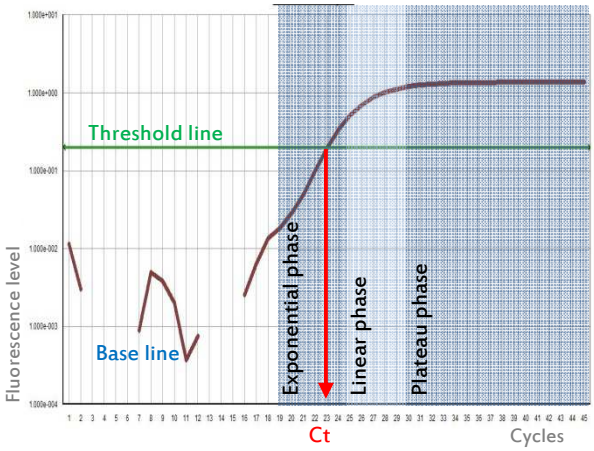
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Reading and validation 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.

DNA extraction and amplification are considered to be **valid** if the following results are obtained for the controls:

Témoins	PRV CTL+	Reagent control	Negative extraction control *	Positive extraction control *
VIC/HEX Amplification	Yes	No	Yes	Yes
FAM Amplification	Yes	No	No	Yes
Validation of	Amplification of the target PRV and EPC	Absence of contamination for amplification	Absence of contamination for extraction	Extraction and amplification steps

* Optionnal

DNA extraction and amplification are considered to be valid for each sample if at least a characteristic amplification curve is observed for PRV target (FAM) or for the internal control (VIC/HEX).

Example	A	B	C
VIC/HEX amplification	Yes	Yes/No	No
FAM amplification	No	Yes	No
Result	Negative	Positive	Undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC/HEX without any amplification in FAM (A example).

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

A total absence of characteristic amplification curve for a sample (example C) shows a defective DNA extraction (lost or destruction of DNA) or a deficient Realtime PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted DNA in sterile Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	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

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

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