ADIAVET™ CSFV REAL TIME

TEST FOR THE DETECTION OF THE CLASSICAL SWINE FEVER VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

References:
ADI223-100 (100 reactions)

VET

English version
NE223-01
2017/01

ADIAVET™ CSFV 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
2017/01	NE223-01	Administrative	First publication

II. General information

1. Purpose of the test

ADIAVET™ CSFV REAL TIME kit is intended to detect the Classical Swine Fever Virus (CSFV) using real-time Polymerase Chain Reaction (PCR) technology from tissue, whole blood and serum specimens of pig and wild boar, as well as from viral culture.

2. Pathogen

Pestiviruses consist in a single strand of positive sense RNA. Bovine Viral Diarrhoea Virus (BVDV), Classical Swine Fever (CSFV) and border disease virus (BDV) in sheep are also members of the pestivirus genus which belongs to the Flaviviridae family (like hepatitis C). CSFV is listed in the A OIE list; it causes heavy epizooties by pigs, and so presents a risk for environment. Generally, all precaution measures have to be taken to avoid viral particles to spread out of laboratory. Refer to the reference centre of your country to know the requirements for biosecurity (experiments in a certified laboratory).

3. Description and purpose of the test

This test is based first on the reverse transcription (RT) of RNA into complementary DNA. Then, cDNA is amplified (PCR) by a DNA polymerase using specific primers. Both enzymatic reactions occur in the same tube (One-step RT-PCR).
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ CSFV REAL TIME kit enables the simultaneous detection of:

- CSFV (probe labelled in FAM),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous RNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE validated the test using RNA purification kits (Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis	Pool of samples is possible*, up to
Tissue (spleen, tonsil, ganglion…)	☑	10
Whole blood	☑	20
Serum	☑	20
Viral culture	☑	☒

***** It depends on the epidemiological case and on the quality of the specimen.

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagent	ADI223-100
A5	Amplification solution	2 x 1000 µl green tubes
CSFV CTL+	Positive control Classical Swine Fever	1 purple tube
CSFV CTL-	Negative control Classical Swine Fever	1 purple tube

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. CSFV CTL+

Add **200 µl** of Nuclease-free water to the CSFV CTL+ tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Use this solution like a sample RNA extract. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use 5 µl of CSFV CTL+ in a well.

B. CSFV CTL-

Add **200 µl** of Nuclease-free water to the CSFV CTL- tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Use this solution like a sample RNA extract. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use 5 µl of CSFV CTL- in a well.

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
- Centrifuge for microtubes, 96-wells plate
- Universal laboratory mixer mill
- Instrument for homogenous mixing of tubes
- 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
- Metal beads 3 or 5 mm
- 96-100% ethanol solution
- Nuclease-free water

- RNA extraction kit (individual and plate columns)
- RNeasy® Mini Kit (Qiagen, 50 extractions: ref. 74104 or 250 extractions: ref. 74106)
- NucleoSpin® RNA (Macherey-Nagel, 50 extractions: ref. 740955.50 or 250 extractions: ref. 740955.250)
- QIAamp® Viral RNA (Qiagen, 50 extractions: ref. 52904 ou 250 extractions : ref. 52906)
- Nucleospin® 96 Virus (Macherey-Nagel, 2x96 extractions: ref. 740691.2 ou 4x96 extractions: ref. 740691.4) with MN Square-well Block (Macherey-Nagel, 4 plates ref. 740476), optional.

-Automated extraction kit using magnetic beads (Bio-X Diagnostics, 2x96 tests: ref. OC-MNPACKKF96)

IV. Recommendation before the analysis of samples

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

1. Precautions

Adiagene has elaborated this PCR test with the use of extraction kits from Qiagen, Macherey-Nagel. 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 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 quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradations. We then recommend to read the entire protocol before starting the test and to rigorously respect it. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for few hours, then at <-15°C.

3. Samples preparation

See § IV for the extraction and purification of RNA.

4. Controls to include

The use of controls allows verifying 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.

All the steps of the analysis procedure (extraction+amplification), for all the types of samples, are validated with the association of the controls included in the kit.

- The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The CSFV CTL+ allows validating the amplification of the target.

Other controls must or could be added:

- **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.

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

A positive control could be added in each trial. The control is a sample including CSFV. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of CSFV. 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 RNeasy® kit

All the centrifugations are performed at room temperature.
Avoid putting fragments on the column and increase the centrifuge times if the mix seems too dense or heterogenic.

	Liquids biological samples (bloods, viral cultures, sera...)	**Tissues (spleen, ganglion, tonsils...)**
Lysis	**100 µl** of **sample** (individual or pool) + **400 µl** of **RLT buffer** in a microtube. Homogenize ~15 seconds. Incubate 10 minutes at room temperature.	**20-30 mg** of **sample** (individual or pool) + **500 µl** of **RLT buffer** **+ 1 metal bead** in a microtube. Disrupt 2 minutes at 30 Hz. Centrifuge 2 minutes at 6000 g. Transfer **300 µl** of **supernatant** in a microtube.
Binding preparation	Add **500 µl** of **ethanol 70%**. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Add **300 µl** of **ethanol 70%**. 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 **700 µl** of the obtained solution to the corresponding column. Centrifuge 2 minutes at 10 000 g.	
1st wash	Change the collection tube and add **500 µl** of **RW1 buffer**. Centrifuge 2 minutes at 10 000 g.	
2nd wash	Change the collection tube and add **500 µl** of **RPE buffer**. Centrifuge 2 minutes at 10 000 g.	
3rd wash	Change the collection tube and add **500 µl** of **RPE buffer**. Centrifuge 5 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add **50 µl** of **Nuclease-free water**. Incubate ~1 minutes at room temperature and centrifuge 2 minutes at 10 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

2. Using Nucleospin® RNA kit

All the centrifugations are performed at room temperature.
Avoid putting fragments on the column and increase the centrifuge times if the mix seems too dense or heterogenic.

	Liquids biological samples (bloods, viral cultures, sera...)	**Tissues (spleen, ganglion, tonsils...)**
Lysis	**100 µl** of **sample** (individual or pool) + **350 µl** of **RA1 buffer** in a microtube. Homogenize ~15 seconds. Incubate 10 minutes at room temperature.	**20-30 mg** of **sample** (individual or pool) + **350 µl** of **RA1 buffer** + **1 metal bead** in a microtube. Disrupt 2 minutes at 30 Hz. Centrifuge 1 minute at 1 000 g. Transfer **300 µl** of **supernatant** in a microtube.
Centrifugation (optional)	If the obtained mix is viscous, hard to pipette and likely to plug the column, place the whole mix on a « Nucleospin® Filter L » with a microtube as collecting tube. Centrifuge at 1 minute 11 000 g. Store the filtrate to follow the purification protocol.	
Binding preparation	Add **450 µl** of **ethanol 70%**. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Add **300 µl** of **ethanol 70%**. 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 **700 µl** of the **obtained solution** to the corresponding column. Centrifuge 2 minutes at 10 000 g.	Identify columns, apply the **whole obtained solution** to the corresponding column. Centrifuge 2 minutes at 10 000 g.
1st wash	Change the collection tube and add **200 µl** of **RAW2 buffer**. Centrifuge 2 minutes at 10 000 g.	
2nd wash	Change the collection tube and add **600 µl** of **RA3 buffer**. Centrifuge 2 minutes at 10 000 g.	
3rd wash	Change the collection tube and add **250 µl** of **RA3 buffer**. Centrifuge 5 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add **60 µl** of **Nuclease-free water**. Incubate ~1 minutes at room temperature and centrifuge 2 minutes at 10 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

3. Using QIAamp® Viral RNA kit

All the centrifugations are performed at room temperature.
Avoid putting fragments on the column and increase the centrifuge times if the mix seems too dense or heterogenic.

	Liquids biological samples (bloods, viral cultures, sera...)	**Tissues (spleen, ganglion, tonsils...)**
Lysis	**100 µl** of **sample** (individual or pool) + **560 µl** of **AVL buffer+Carrier** in a microtube. Homogenize ~15 seconds. Incubate 10 minutes at room temperature.	**20-30 mg** of **sample** (individual or pool) + **560 µl** of **AVL buffer+Carrier** **+ 1 metal bead** in a microtube. Disrupt 2 minutes at 30 Hz. Centrifuge 2 minutes at 6000 g. Transfer **the totality of supernatant** in a microtube.
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 buffer**. 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 <-15°C.	

4. Using Nucleospin® 96 Virus

Three MN Square well block plates are included in each kit. These plates are either mix plates or recovery plates. Once they have been used, they can be emptied, decontaminated with HCl 0.4 M during 1 minute, washed with distilled water and autoclaved.

All centrifugations are achieved at 5900 tr/min (5600 to 5800 g) and at room temperature.

Before starting the extraction, place the RAV1+RNA carrier buffer and the Nuclease-free water at +70°C in a water bath or a heating block.

	Blood
Lysis	Place **100 µl** of **sample** (individual or pool) and in each well of a Round-well Block plate. For extraction negative controls, use **100 µl** of **PBS 1X**.
	Add **400 µl** of pre-warmed **RAV1 buffer + RNA carrier** + **20 µl of proteinase K** Close the plate with an adhesive seal Self-adhering PE Foil. Mix gently during 15 seconds with a plate agitator. Incubate 10 minutes at +70°C.
Binding preparation	Add **400 µl** of **ethanol 100 %** in an MN Square well Block plate. Carefully remove the adhesive seal of the Round-well Block plate and transfer the **whole content** of each well in the MN Square well Block plate containing ethanol. Homogenize the mix 5-fold (very important) with a multichannel pipette P1000.
Transfer to columns and binding to the membrane	Place a Nucleospin® Virus Binding plate (blue) on a new MN Square well Block plate. Place the **whole mix** with a multi pipette P1000 on the Nucleospin® Virus Binding plate. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 2 minutes. If the whole mix has not filtered, centrifuge one more time 3 minutes.
1st wash	Place the Nucleospin® Virus Binding plate on a new MN Square well Block plate. Remove the adhesive seal from the Nucleospin® Virus Binding plate. Add **500 µl** of **RAW buffer** in each well. Place a new adhesive seal Self adhering PE foil on the plate. Centrifuge 2 minutes.
2nd wash	Remove the adhesive seal of the Nucleospin® Virus Binding Plate. Add **900 µl** of **RAV3 buffer** in each well. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 5 minutes.
Column dry step	Place the Nucleospin® Virus Binding Plate on an empty and dry 96 well plate. Centrifuge 10 minutes.
Elution	Place the Nucleospin® Virus Binding Plate on the Rack plate with MN tube strips. Remove the adhesive seal from the plate. Add **100 µl** of pre-warmed **Nuclease-free water** in each well of the Nucleospin® Virus Binding plate. Do not use the **RE buffer.** Centrifuge 2 minutes.
Storage	Discard the Nucleospin® Virus Binding plate. Close the Rack plate with MN tube strips with Caps for strips. Store it on melting ice if analysis is immediately achieved, then at <-15°C.

5. Using RNA/DNA 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.

VI. Amplification

a- Determine the number samples analysed including the controls (e.g. positive and negative extraction controls, positive and negative control of amplification (CSFV CTL+ and CSFV CTL-) and PCR reagent control (NTC)).

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

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

d- For each sample, the extraction negative control (obligatory) and the extraction positive control (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.
For the positive control, add **5 µl** of the solution obtained in § II-3.A to the 20 µl of A5 solution.
For the negative control, add **5 µl** of the solution obtained in § II-3.B to the 20 µl of A5 solution.
For the PCR reagent control (NTC), nothing is added to the A5 solution.
Immediately replace purified RNA extracts on melting ice or at **<-15°C**. Take care to have no bubbles in the bottom of the wells.

e- 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 CSFV 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 (at 60°C).

The following programs, according to the thermal cyclers, are defined:

<table>
<tr><th colspan="2">Standard program</th><th colspan="2">Short program</th></tr>
<tr><td colspan="2">ABI7500* -Thermofisher
AriaMx - MX3005P - Agilent Agilent
LightCycler 480 - Roche Diagnostic</td><td colspan="2">ABI7500* - Thermofisher
AriaMx - MX3005P - Agilent
CFX96 Touch - Biorad</td></tr>
<tr><td colspan="2">10 min. 45°C</td><td colspan="2">10 min. 45°C</td></tr>
<tr><td colspan="2">10 min. 95°C</td><td colspan="2">10 min. 95°C</td></tr>
<tr><td>15 sec 95°C***</td><td rowspan="2">45 cycles</td><td>5 sec 95°C</td><td rowspan="2">45 cycles</td></tr>
<tr><td>1 min. 60°C</td><td>30 sec 60°C **</td></tr>
</table>

* Check « emulation 9600 » option if it's exist.
** Note 32 secondes for the ABI7500 thermofisher
*** Note 30 secondes for the MX3005P

Contact us if you wish to use other thermalcyclers.

VII. 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.

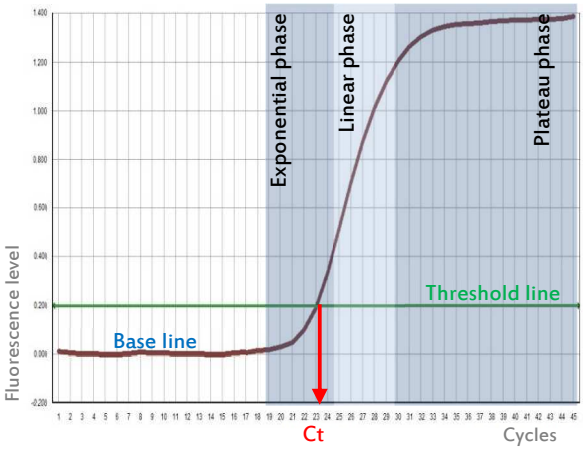
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

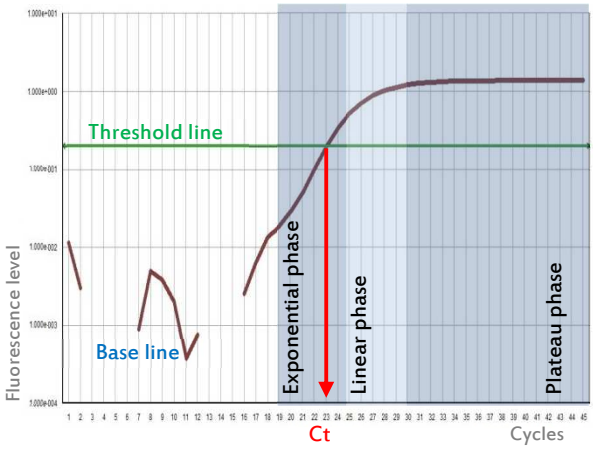
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

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

Controls	Reagent control (NTC)	CSFV CTL+	CSFV CTL-	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	no	yes
VIC/HEX amplification	no	no/yes	yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the PPC target	Amplification of the IPC target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the CSFV CTL+ and CSFV CTL- were indicated in the certificate of analysis of the kit.

B. Result interpretation

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

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or 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 (C example).

A total absence of characteristic amplification curve for a sample (example D) shows a defective RNA extraction (lost or destruction of RNA) or a deficient real-time 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 RNA in 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

Bio-X Diagnostics, the logos, ADIAGENE and ADIAVET™ are used, pending and/or registered trademarks belonging to ADIAGENE and/or Bio-X Diagnostics, or one of its subsidiaries, or one of its companies. Any other name or trademark is the property of its respective owner.

ADIAGENE
38 rue de Paris
22000 Saint-Brieuc - France

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