ADIAVET™ BVDV REAL TIME

TEST FOR THE DETECTION OF THE BOVINE VIRAL DIARRHOEA VIRUS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-PCR TEST)

References:
ADI105-100 (100 reactions)
ADI105-500 (500 reactions)

VET

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English version
NE105-13
2020/01

ADIAVET™ BVDV 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
2014/07	NE105-07	Technical change	Addition of technical details related to use of ADIAPURE™ TLB kit, in page 8, § V-1.
2014/07	NE105-07	Administrative	Revision of paragraphs order, page 8 to 18, § V.
2014/12	NE105-08	Technical change	Addition of "Index of symbols" section, in page 23.
2014/12	NE105-08	Technical change	Removal of reference ADI105-50 (50 reactions)
2015/04	NE105-09	Technical change	Addition of safety instructions, in page 8, §V.1.
2016/07	NE105-10	Administrative	Changing logos
2016/07	NE105-10	Administrative	Biosearch legal mention
2016/07	NE105-10	Administrative	Addition of table "Analysis options according to the specimen"
2017/01	NE105-11	Technical change	Addition of reference ADI105-500 (500 reaction), §III.1 Addition of reference ADIAPURE™ TLB 400 reactions, §III.4 Addition pool of 25 with ADIAPURE™ TLB, § V.1 Modification of results interpretation §VII.2.B
2019/05	NE105-12	Technical change	Modification of milk protocol with QIAamp® viral RNA (§ V-3) Removal of NucleoSpin RNA, QIAamp 96 DNA Blood Addition of Fast program of PCR (§VI)
2019/06	NE105-12	Correction	Update the size of milk pool Update reference of magnetic beads RNA/DNA kit (Bio-X Diagnostics) Correction of the name "positive" by "Detected" and the name "negative" by "No detected"
2020/01	NE105-13	Technical change	Addition of a NF-Water tube in the kit

II. General information

1. Purpose of the test

ADIAVET™ BVDV REAL TIME kit is intended to detect the Bovine Viral Diarrhoea Virus (BVDV) and the Border Disease Virus (BDV), using real-time Polymerase Chain Reaction (PCR) technology, from whole blood, serum, and tissue specimens of bovine, ovine, caprine and wild cervid, from ear notch of bovine, as well as from milk specimen of bovine, ovine and caprine.

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). BVDV, which induces mucosal disease in bovine, causes economic losses in cattle.

Many countries have started eradication programs of this disease, which involves a perfect management of infected animals. Indeed, those must be detected earlier with a high reliability. However, the prenatal infection of a calf between the 60th and the 120th day of gestation leads to the birth of a persistently infected (PI) animal. These contagious animals are seronegative all their live and positive by virology. The detection of the virus by antigenemy is only possible several weeks after their birth because of the persistence of colostral antibodies. The earlier detection of these persistently infected animals is still necessary in eradication programs.

Since the discovery of DNA in vitro amplification in 1985 (PCR), many scientists have developed virus screening tests using genomic amplification of the RNA also called RT-PCR. Most of these tests allow the detection of minute quantities of BVDV in blood or organs of infected animals, even with less than three months old animals.

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™ BVDV REAL TIME kit enables the simultaneous detection of:

- BVDV, BDV and CSFV (probe labelled in FAM),
- RNaseP, 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 or HEX).

ADIAGENE recommends using this test with RNA purification kits (Adiagene, Qiagen or 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 sample is possible*, up to
Whole blood	☑	50
Serum	☑	50
Tissue (placenta, spleen, fœtal tissues…)	☑	☒
Ear notch**	☑	25
Milk	☑	Bulk

* It depends on the epidemiological case and on the quality of the specimen.
** Ear notch: ear notches' RNA can be extracted using ADIAPURE™ TLB kit (Bio-X Diagnostics, ref. ADIADP10E1-100 or ADIADP10E1-400).

III. Material and reagents

1. Reagents provided with the kit

REF ADI105-100		
A5	Amplification Solution	2 x 1000 µl tubes green caps (a ready-to-use reagent)
BVDV CTL+	Positive control BVDV	1 tube purple caps (to reconstitute)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)
REF ADI105-500		
A5	Amplification Solution	10 x 1000 µl tubes green caps (a ready-to-use reagent)
BVDV CTL+	Positive control BVDV	2 tubes purple caps (to reconstitute)
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 BVDV CTL+

Add **200 µl** of **NF-Water** to the **BVDV 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 **BVDV CTL+** in one of the wells.

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, tubes of 10 or 15 ml, 96-wells plate
- Universal laboratory mixer mill
- Etuve, heating baths or block heaters
- 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
- Sterile tube of 5, 10 or 15 ml
- Latex or nitrile powder-free gloves
- Metal (tungsten or stainless) beads 3 mm
- Scalpel blades
- 96-100% ethanol solution
- ß-mercaptoethanol 14.5 M

- Equipment required according to the extraction protocol

Extraction kit references		Additional references	Whole blood 50	Serum 50	Milk bulk	Tissue (placenta, spleen, fœtal tissues…) 1	Ear notch 25
ADIAMAG Magnetic beads	Bio-X Diagnostics: Ref. NADI003	- Lysis Buffer LB3 (for pool of ear notches): Bio-X Diagnostics: ref. NADI004	+	+	+	+	+
ADIAPURE™ TLB	Bio-X Diagnostics, ref. ADIADP10E1-100 ref. ADIADP10E1-400						+
QIAamp® Viral RNA	Qiagen, 50 extractions: ref. 52904 or 250 extractions: ref. 52906		+	+	+	+	+
NucleoSpin® RNA Virus	Macherey-Nagel, 50 extractions: ref. 740956.50 or 250 extractions: ref. 740956.250	- Buffer RL1 (optionnal,for ear notches) : Macherey-Nagel, 50 ml: ref.740385.50 Or Bio-X Diagnostics ref.740385	+	+	+		+
Nucleospin® 96 Virus	Macherey-Nagel, 2x96 extractions: ref. 740691.2 or 4x96 extractions: ref. 740691.4	- MN Square-well Block: Macherey-Nagel, 4 plates: ref. 740476	+	+			
RNeasy® Mini Kit	Qiagen, 50 extractions: ref. 74104 or 250 extractions: ref. 74106	- Buffer EL: Qiagen: ref. 79217 (for blood on anticoagulant)	+	+	+	+	+

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 and Bio-X Diagnotics. 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 a 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. Samples of blood with anticoagulant reagent must not be frozen.
Extracted RNAs are quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradations. 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 (RNaseP) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The BVDV 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 BVDV. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of BVDV. 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 ADIAMAG kit

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

2. Using ADIAPURE™ TLB kit (RNA extraction from ears notches without purification)

Extract the ear tissue sample from the ear tag, e.g. in the collector tube.
Add

- **280 µl of L1 lysis buffet ADIAPURE™ TLB**
- **20 µl of L2 lysis buffer ADIAPURE™ TLB** *.

**A pre-mix of the both reagents could be prepared then 300 µl are added to each sample. The pre-mix is stable up to 5 days stored at +2/8°C.*

Homogenize.

Incubate

- **20 minutes** at **65°C**
- **15 minutes** at **95°C**
- **To ensure the accuracy of subsequent pipetting, allow the samples to cool** (e.g., 15-30 minutes at room temperature or 5-20 minutes at +2/4°C, according to the number of samples per assay)

NB1: Analysis on pooled samples is possible; mix in equal volume until 25 samples (e.g. 50 µl) and homogeneise.

NB2: In case of a new analysis, each individual supernatant can be store at +2/8°C for 24 hours, then store them at <-15°C.
Directly continue with the amplification (see §VI.).

* Contains a reagent at a concentration considered as dangerous: Proteinase K ,1,00%
Signal word: **DANGER**

H334
P261 / P280 / P342 + P311
Hazard statement(s):
H334 : May cause allergy or asthma symptoms or breathing difficulties if inhaled.
Precautionary statement(s):
P261 : Avoid breathing dust/fume/gas/mist/vapours/spray.
P280 : Wear protective gloves/protective clothing/eye protection/face protection.
P342+P311 : If experiencing respiratory symptoms: Call a POISON CENTER/doctor.
Note:

A black diamond shape may appear on outer container labels. When located next to a danger symbol, read the danger symbol and the reference H and P number to hazard and precautionary statements. Otherwise, do not consider it as a symbol.

3. Using QIAamp® Viral RNA kit

All the centrifugations are performed at room temperature.

<table>
<tr><th></th><th>EDTA blood</th><th>Sera</th><th>Tissue</th><th>Milk</th><th>Ear notche</th></tr>
<tr><td>Sample prépared</td><td>100 µl</td><td>140 µl</td><td>0.1 g</td><td>100 µl</td><td>1 ear notche tissue sample</td></tr>
<tr><td rowspan="2">Lysis</td><td colspan="4">Add 560 µl of AVL buffer + Carrier RNA.</td><td>Add 350 µl of AVL buffer + Carrier RNA.</td></tr>
<tr><td>Homogenize 15 seconds and incubate 10 minutes at room temperature.</td><td>Grind. [1]
Centrifuge 2 minutes at 6 000 g.
Transfer the supernatant in a microtube.</td><td>Homogenize 15 seconds until the suspension of the pellet.</td><td>Homogenize 15 seconds and incubate 10 minutes at room temperature.[2]
Transfer 100 µl of individual or pooled [3] surpenatant in a microtube.</td></tr>
<tr><td>Binding preparation</td><td colspan="5">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 to columns and binding to the membrane</td><td colspan="5">Identify columns, apply 630 µl of the obtained solution to the corresponding column.
Centrifuge 1 minute at 10 000 g.
Change the collection tube and 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="5">Change the collection tube and add 500 µl of buffer AW1.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="5">Change the collection tube and add 500 µl of buffer AW2.
Centrifuge 1 minutes at 10 000 g.</td></tr>
<tr><td>Column dry step</td><td colspan="5">Change the collection tube.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="5">Transfer the column to a microtube. Add 60 µl of buffer AVE.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="5">Close the tubes, identify and store on ice if using immediately or at <-15°C.</td></tr>
</table>

[1] For example, using a Mixer Mill: add 1 metal bead (3 mm), grind 2 minutes at 30 Hz.

[2] In case of a new analysis, each individual supernatant must be **quickly** store at <-15°C. For the new analysis, defreeze the supernatant, take 100 µl and store quickly at <-15°C.

[3] Analysis pooled is possible; mix in equal volume until 25 samples (e.g. 50 µl) and homogeneise.

4. Using Nucleospin® RNA Virus kit

A. Sample preparation

It is possible to extract pools of samples. See table page 6 to know the higher number of samples in pools according to the matrix.

a) From EDTA blood

Transfer **100 µl** of blood or pool of in a microtube.
Continue following the table below.

b) From sera

Transfer **140 µl** of serum or pool of in a microtube.
Continue following the table below.

c) From milk

Transfer **6 ml** of milk or bulk milk in a 15 ml tube.
Centrifuge 15 minutes at 3 500 g, at +2/8°C.
Get rid of the cream with a spatula and discard the whey.
NB: the pellet can be store at <-15°C until the extraction.
Continue following the table below.

d) From ear notche

One or other of the following methods can be used to extract RNA from the ear tissue sample.

1st method
Extract the ear tissue sample from the ear tag, e.g. in the collector tube.
Continue following the table below.

2nd method
Extract the ear tissue sample from the ear tag, e.g. in the collector tube.
Add **350 µl** of **RL1 buffer**. Homogenize and incubate 15 minutes at room temperature.
NB1: Analysis pooled is possible; homogenize in equal volume until 25 samples (e.g. 50 µl) and homogenize.
NB2: With this method, each individual supernatant can be store at room temperature or at +2/8°C for 24h in case of a new analysis, then store them at <-15°C.
Transfer **100 µl** of **supernatant** in a microtube.
Continue following the table below.

B. Extraction

All the centrifugations are performed at room temperature.

	Ear notche 1st method	Ear notche 2nd method	EDTA blood	Sera	Milk
Sample prépared	1 ear sample tissue	100 µl of supernatant	100 µl	140 µl	Pellet
Lysis	Add **350 µl** of **buffer RAV1** + **RNA Carrier**.	Add **560 µl** of **buffer RAV1** + **RNA Carrier**.			
	Homogenize and incubate **10 minutes** at room temperature.[1] Transfer **100 µl** of **individual** or **pooled** [2] **supernatant** in a microtube.	Homogenize and incubate **10 minutes** at room temperature.			Homogenize until the suspension of the pellet.
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. Centrifuge 1 minute at 10 000 g. Change the collection tube and 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**. Centrifuge 1 minute at 10 000 g.				
2nd wash	Change the collection tube and add **500 µl** of **RAV3 buffer**. 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 minutes 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.				

[1] In case of a new analysis, each individual supernatant must be **quickly** store at <-15°C. For the new analysis, defreeze the supernatant, take 100 µl and store quickly at <-15°C.

[2] Analysis pooled is possible; mix in equal volume until 25 samples (e.g. 50 µl) and homogeneise.

5. Using Nucleospin® 96 Virus

It is possible to extract pools of samples. See table page 6 to know the higher number of samples in pools according to the matrix.
Three MN Square well block plates are included in each kit. They are used as 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.
The 96 well plate (ELISA like) isn't include in the kit.
All centrifugations are achieved at 5900 tr/min (5600 to 5800 g) and at room temperature.
Before the beginning of extraction, pre-warm:

- the RAV1 buffer + RNA carrier at +56°C.
- the Nuclease-free water at +70°C.

	EDTA blood sample	**Sera**
Lysis	Place **100 µl** of **blood** or pool of bloods in each well of a Round-well Block plate.	Place **140 µl** of **serum** or pool of serums in each well of a Round-well Block plate.
	Add **560 µl** of **buffer RAV1 + RNA carrier** pre-warmed at +56°C + **20 µl of proteinase K**. Close the plate with an adhesive seal Self-adhering PE Foil. Homogenize ~15 seconds with a plate agitator. Incubate 10 minutes at +70°C. Centrifuge briefly to discard condensation.	
Binding preparation	Place **560 µl** of **ethanol 100 %** in an MN Square well Block plate. Carefully remove the adhesive seal of the Round-well Block plate containing the samples and transfer the **whole content** of in the MN Square well Block plate containing ethanol. Homogenize the mix 5-times (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. Transfert 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 **buffer RAW** 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 **buffer RAV3** 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 (ELISA-like). 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 **Nuclease-free water** pre-warmed at +70°C in each well of the Nucleospin® Virus Binding plate. Do not use the **buffer RE.** Centrifuge 2 minutes.	
Storage	Remove 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.	

6. Using RNeasy® kit

A. Sample preparation

It is possible to extract pools of samples. See table page 6 to know the higher number of samples in pools according to the matrix.

a) From blood with anticoagulant

Transfer **0.5 ml** of blood or **0.5 ml** of a pooled sample in a 15 ml tube.
Add **2.5 ml** of **EL buffer** and homogenize.
Place the tubes on melting ice during 15 minutes (homogenize twice during this step).
Centrifuge 10 minutes at 1 000 g, at +2/8°C.
Discard the supernatant.
Add **2 ml** of **EL buffer** to the pellet and homogenize.
Centrifuge 10 minutes at 1 000 g, at +2/8°C. Discard the supernatant.
NB: the pellet can be store at <-15°C until the extraction.
Continue following the table below.

b) From non-centrifuged and non-frozen sera

Transfer **1 ml** of sera or pool of in a microtube.
Centrifuge 10 minutes at 3 000 g, at +2/8°C.
Discard the supernatant.
Continue following the table below.

c) From milk

Transfer **6 ml** of milk or bulk milk in a 15 ml tube.
Centrifuge 15 minutes at 3 500 g, at +2/8°C.
Get rid of the cream with a spatula and discard the whey.
NB: the pellet can be store at <-15°C until the extraction.
Continue following the table below.

d) From tissue

Transfer **0.1 g** of minced tissue in a microtube.
Continue following the table below.

e) From ear notche

Extract the ear tissue sample from the ear tag, e.g. in the collector tube
Continue following the table below.

B. Extraction

All the centrifugations are performed at room temperature.

	Blood on anti-coagulant **Sera** **Milk**	**Tissue (speen, ganglion, buccal mucosa…)**	**Ear notche**
Prepared sample	Pellet	**0.1 g** of minced tissue	1 ear notche
Lysis step	Add **350 µl** of **buffer RLT + ß-Mercaptoethanol (10 µl/ml).** Homogenize by pipetting at least 10 times or by using a mixer such as vortex (15 seconds).	Add **350 µl** of **buffer RLT + ß-Mercaptoethanol (10 µl/ml).** Incubate 15 minutes at room temperature. Homogenize now and then.	Add **350 µl** of **buffer RLT**. Homogenize and incubate 15 minutes at room temperature. [1] Transfer **100 µl** of **individual** or **pooled** [2] **surpenatant** in a microtube.
Binding preparation	Add **350 µl** of **ethanol 70%.** Homogenize by pipetting at least 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 1 minute at 10 000 g.		
1st wash	Change the collection tube and add **700 µl** of **buffer RW1** to the column. Centrifuge 1 minute at 10 000 g.		
2nd wash	Change the collection tube and add **500 µl** of **buffer RPE** to the column. Centrifuge 1 minute at 10 000 g.		
Column dry step	Change the collection tube. Centrifuge 1 minute at 10 000 g.		
Elution	Transfer the column to a microtube. Add **60 µl** of **Nuclease-free water**. Incubate ~2 minutes 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.		

[1] In case of a new analysis, each individual supernatant can be store at room temperature or at +2/8°C for 24h, then store them at <-15°C.

[2] Analysis pooled is possible; mix in equal volume until 25 samples (e.g. 50 µl) and homogeneise.

VI. Amplification

a- Determine the number samples analysed including the controls (e.g. positive and negative extraction controls, positive control of amplification (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 CTL+, add **5 µl** of the solution obtained in § II-3 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 BVDV 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 (1 minute at 60°C).

The BVDV 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** thermalcyclers (like 7500, StepOne…) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **MX3005P** and **ARIAMX** of **Agilent, LightCycleur** of **Roche Diagnotics** and for **CFX96** of **BioRad**.

Standard program		Fast program	
10 min. 45°C		10 min. 45°C	
10 min. 95°C		10 min. 95°C	
15 sec 95°C**	45 cycles	5 sec 95°C	45 cycles
1 min. 60°C		30 sec 60°C *	

* 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.

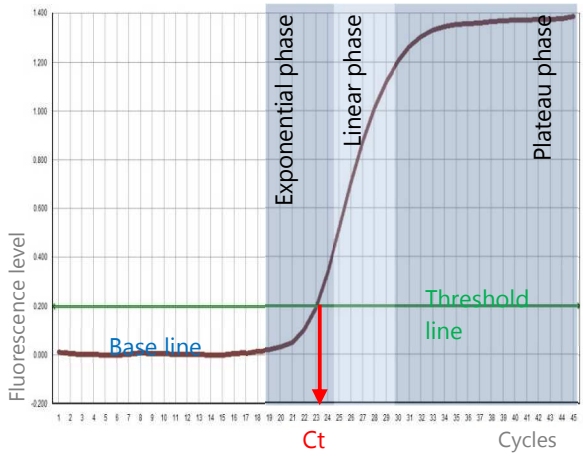
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

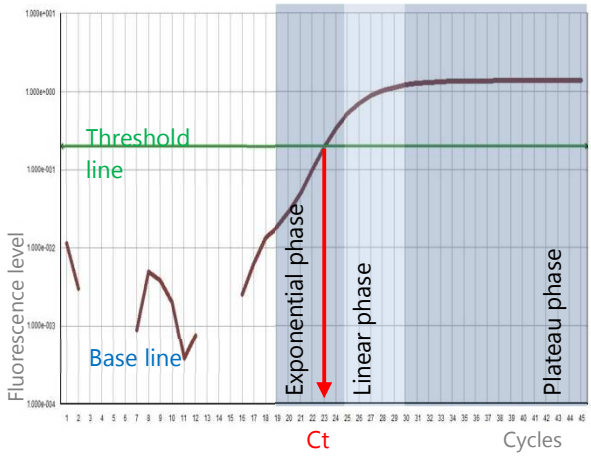
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present 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)	BVDV CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the 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 BVDV (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	No detected	Detected	Detected	Undetermined

The sample is considered as **No detected** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (example A).

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

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.

Special case of ears notches in direct lysis with the ADIAPURE ™ TLB buffer:

Ear ears notches are usually analyzed as a mixture.
This mixture may contain partially or completely inhibitory samples of the PCR reaction and mask positive result.

Thus, for direct lysis analysis with the ADIAPURE ™ TLB buffer, we propose the following interpretation and decision-making scheme.

Exemple	A	B	C	D
Amplification FAM	No	Yes	No	No
Amplification VIC/HEX	Ct < 27*	Non/Oui	Non	Ct > 27*
Results	No detected	Detected	Inhibited	Potentially inhibited
Recommendations			**Mixture analysis:** Test samples individually **Individual analysis** Test the pure and diluted sample (1/10)	**Mixture analysis:** Test samples individually **Individual analysis** Test the diluted sample (1/10)

** The value of Ct of the internal control mentioned below may vary depending on the thermal cycler.*

EXAMPLE A The sample is considered as **No detected** if a characteristic amplification curve with a Ct of less than 27 is observed in VIC or HEX without any characteristic amplification curve in FAM.

Example B: The sample is considered as **Detected** if a characteristic amplification curve is observed in FAM. Internal Control can be co-amplified.

Example C: The complete absence of a characteristic amplification curve for a sample indicates a deficiency in the extraction of the RNA (loss or destruction of the RNA) or a defective real-time RT-PCR (inhibitors in the sample, program error or lack of sample).

Example D: Samples with an IPC Ct greater than 27 are either partially inhibited or poor in host cells.

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

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