qPCR kit for the detection of African Swine Fever (ASFV) in Biological samples
100 Reactions
Cat No. LT-AFSV-RT100

1. INTRODUCTION

This is a real-time PCR kit suited for the detection of DNA of African Swine Fever Virus (ASFV) in biological samples in a simple way, with high levels of sensitivity and specificity.

The kit contains all necessary reagents and enzymes in two mixes. When mixed together, they are at the required concentrations designed to detect (and optionally quantify) ASFV, simply by adding the DNA of the problem sample. This kit makes use of a UPL® probe labelled with FAM. On the other hand, each Master Mix provides a positive internal control (PIC) with primers and a Taqman probe marked with VIC, allowing detection of false negatives due to an inhibition of the PCR. In addition, the master mix contains a passive reference fluorochrome, ROX, allowing the normalisation of the signal avoiding possible quantification errors deriving from pipetting differences. When using Applied Biosystems' ABI PRISM PCR models in real time, this ROX is necessary to prevent interferences between the data collected from various emission channels.

The kit includes an ASFV positive control for detection. The kit doesn´t include a standard positive control with a known number of ASFV copies (ask our commercial department for standard control). This control allows generating a standard curve which links the number of pathogen copies with the cycle threshold (Ct) value.

The kit has been tested for every system from Applied Biosystems (StepOne, HT7300, HT7500) and LC480 from Roche. It is possible to work with other thermocyclers from other commercial houses, if they have at least two fluorescence channels, but Ct values may experiment changes.

2. KIT COMPOSITION:

COMPONENTS	Nº VIALS	VOLUMEN/VIAL
Mixture A (ASFV specific primers)	2	600 µl
Mixture B (enzyme mixture)	2	600µl
Positive Control A1 – ASFV Amplification control	1	60 µl

3. GUIDELINES FOR THE CORRECT CONSERVATION OF THE KIT COMPONENTS

On receiving the kit, keep it at -20ºC until use. The components of the kit are stable for 1 year from the manufacturing date (see expiry date on packaging).

4. MATERIALS REQUIRED BUT NOT SUPPLIED

- Dust-free disposable gloves (without talcum powder).
- Microcentrifuge.
- Tube shaker.
- Real-time thermocycler.
- Micropipettes (0.5-1000 µl).
- Sterile pipette tips (with filter).
- Sterile DNAses / RNAses free water.

5. PRECAUTIONS TO AVOID CONTAMINATION

The following points should be read with care:

- Disposable items must be DNAse and RNAse-free.
- Use DNAse and RNAse-free distilled, autoclaved water (25 min., 120ºC).
- Use sterile filtered tips.
- Ensure good homogenisation of the kit components once defrosted.
- Maintain the A and B mixes stored in ice at all times. Exposing them to temperatures above 4ºC reduces the efficacy of the PCR.
- Repeated cycles of freezing and defrosting may reduce the sensitivity of the reagents. Protect them from exposure to light until use.

To avoid contaminations giving rise to false positives it is important to:

- Physically separate the positive PCR control from the remaining reagents of the kit.
- To carry out any handling of samples to be tested in a different location/room from the one where the amplified products are being analyzed.
- Add the positive PCR control in a different location/room from the one where the mix is added and where the samples to be tested are being handled.

6. SAMPLES COLLECTION AND TRANSPORT

The assay could be performed using any biological sample considered of interest by the clinician. Our technique has been standardised and contrasted with a considerable number of blood, sera, spleen and liver.

The following are some recommendations and/or limitations relating to the method of obtaining and sending the samples to the laboratory:

- The samples must be cooled from the moment they are obtained until they are processed. Processing needs to occur within 24 hours after obtaining the samples. In case a longer time is envisaged, we recommend freezing the samples.

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7. DNA EXTRACTION

Any extraction procedure should be used which yields good-quality of the extracted material.

In case you have no optimized extraction procedures, we could provide extraction protocol and buffer under request.

We have tested the following methods for the DNA extraction:

- Method of Chomczynski and Sacchi (Phenol extraction).
- Automatic magnetic methods (Mag Max TM96 total NA isolation Ref AM1840).
- Colum purification methods: QIAamp® Viral RNA Mini Kit (QIAGEN), High pure PCR template Preparation kit (ROCHE).

There are several commercial houses that provide high quality extraction kits that could be used following the manufacturer instructions, like: Life-technologies, ROCHE, Quiagen, Marchery Nalgene, others.

CAUTION: If amplification is not going to be done immediately, preserve the DNA at -20 ° C .

8. AMPLIFICATION OF THE GENETIC MATERIAL

REQUIRED MATERIALS

- Crushed ice.
- DNA extracted from the samples.
- Mixture A – **KEEP IN CRUSHED ICE AT ALL TIMES.**
- Mixture B - **KEEP IN CRUSHED ICE AT ALL TIMES.**
- Positive amplification control A1 (ASFV).
- DNAase-RNAase-free water.

PROCEDURE

1. Prepare and identify as many tubes for the amplification as samples to be processed, adding an additional tube for the positive amplification control, and another one for the negative control.
2. Take mixtures A and B out from the cooler keeping them in crushed ice. Make sure that they are correctly homogenised before taking out the required volume for the assay.
3. Prepare an appropriate amount of amplification mixture for the number of samples to be processed. The volume of each reagent to be mixed for each of the samples is:

	Mixture A	Mixture B	Final Master Mix Volume
Per sample	10 µl	10 µl	20 µl
For 10 samples	100 µl	100 µl	200 µl

The tube used for mixing should be kept in crushed ice at all times. Likewise, it is recommendable to prepare an excess amount of mixture (calculate an extra 10% for all reagents) in order to compensate for possible losses of volume during pipetting.

4. Once mixture is prepared, homogenise correctly. Place the tubes previously labelled in crushed ice and add 20 µl of the mixture prepared in this way to each tube.

Add 2 µl of previously extracted DNA samples to each tube, 2 µl of positive control A1 (amplification control for ASFV), to the corresponding tube and 2 µl of water to the tube labelled as negative control. Carefully mix the contents of each tube and ensure that all the liquid has been deposited at the bottom of the tube. If not, tubes may be centrifuged lightly until this occurs.

6. Set the thermocycler to the following conditions:

	Temperature (ºC)	Time	Cycles
Desnaturalization	95	5 min	1
Amplification	95	10 sg	45
	60 *	30 sg	

7. Reading the fluorescence takes place during the elongation step (marked in the table)*, and the channels through which the fluorescence data are collected are detailed in table :

	Reporter	Quencher
ASFV	FAM	None
I.C.	VIC	None
Passive reference	ROX	

9. ANALYSIS AND INTERPRETATION OF RESULTS

Analysis of results

Each problem sample analyzed yields data regarding the fluorescence originated from channel FAM. Automatic threshold analysis (threshold and baseline) is recommended.

If this option is not viable, the analysis can be carried out manually following the thermocycler instructions.

The assay will be considered as valid when the C+ have a Ct value within the range 32 ± 4 and the C- Ct ≥ 45 in the FAM channel

Possible results are summarised in the table:

FAM channel: pathogen detection

A positive result during amplification implies a typical fluorescence curve with a Ct value <45.

Samples with Ct´s comprised between 40 and 45 should be regarded as doubtful. In that case we recommend:

- Repeating the analysis.
- Sequencing the amplified product.

VIC channel: Internal Control detection

A positive result during amplification implies a typical fluorescence curve with a Ct value <45. Normally, values are comprised within a range of 30 <Ct < 45

Table: Possible results.

Cases	Channel FAM	Channel VIC	Results
A	+	+	Positive
B	+	-	Positive
C	-	+	Negative
D*	-	-	Null/inhibited

*Repeat the analysis diluting DNA 1/40 to avoid inhibitors, before considering the sample as negative.

Table: Results validation

Case	Problem sample	Negative extraction control	Positive PCR control	Negative PCR control	Result of problem sample
1	+	-	+	-	+
2	-	-	+	-	-
3		+			Nulo
4		-		+	Nulo

Case 1. Both the problem sample and the positive PCR control exhibit a positive result, whereas the negative extraction and PCR controls yield a negative result.

Positive result: the sample contains ASFV.

Case 2. The positive PCR control exhibits a positive result, whereas the problem sample and the negative extraction and PCR controls yield a negative result.

Negative result: the sample does not contain any ASFV.

Case 3. The negative extraction control yields a positive result.

Result invalidated or null. Go back to extraction step.

Case 4. The negative extraction control exhibits a negative result, whereas the negative PCR control yields a positive result.

Result invalidated or null. Go back to amplification step.

10. TROUBLESHOOTING

Detection of fluorescence signal on FAM channel in the negative extraction control.

Possible cause	Solution
Contamination during extraction process	• Repeat the DNA extraction process and PCR using new reagents. • Ensure that the workplace, equipment and all instruments used for performing the PCR are decontaminated, i.e. free from nucleic acids.

Detection of fluorescence signal on FAM channel (pathogen) in the negative PCR control.

Possible cause	Solution
Contamination during PCR preparation.	• Repeat PCR reaction with new reagents and, if possible, various replicates of each sample. • If tubes are being used, close each one immediately after adding the sample. • As a strict rule, the positive control must be added last and in a separate physical location from where the problem samples are added. • Ensure that the workplace, equipment and all instruments used for performing the PCR are decontaminated, i.e. free of nucleic acids.

Absence of fluorescence signal on FAM channel in the positive PCR controls.

Possible cause	Solution
Wrong fluorochrome channel selected	• Check that you select the FAM channel for all samples and controls to be analyzed.

11. QUALITY CONTROL:

Each production batch of this kit, has been checked under our ISO-certified Quality Management System.