INgene q PPA

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Kit qPCR para la detección del Virus de la Peste Porcina Africana (VPPA) en muestras biológicas. / qPCR kit for the detection of African Swine Fever Virus (ASFV) in Biological samples.

Real Time PCR (TaqMan technology). 100 Reacciones

Producto fabricado en Eurofins Ingenasa

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1. INTRODUCTION

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

African swine fever infection causes a lethal haemorrhagic disease in domestic and wild pigs, with important productive losses. African swine fever virus (ASFV) is a large, enveloped, icosahedral double-stranded DNA virus that belongs to the *Asfaviridae* family, genus *Asfivirus*.

In the past, outbreaks were detected in Africa, parts of Europe, South America and the Caribbean. More recently, since 2007, the disease has been reported in several African countries, Asia and Europe, in both domestic and wild pigs.

2. INTENDED USE

INgene q PPA is a real-time PCR kit suited for the detection of DNA of African Swine Fever Virus (ASFV) DNA in biological samples of clinical interest, such as blood, serum, spleen and liver.

The kit has been used since 2014 to monitor domestic and wild pigs in Eastern Europe and China.

For veterinary use only

3. PRINCIPLE OF THE ASSAY

INgene q PPA is an assay for amplification and detection of the ASFV genetic material based on the polymerase chain reaction (PCR). The assay consists on a duplex real-time PCR in a one-well format. The kit contains all necessary reagents and enzymes to amplify and simultaneously detect ASFV genetic material in extracted DNA samples. The reagents are included in two mixes, that once combined in the indicated proportions, are in optimal concentrations to detect (and optionally quantify) the genetic material of ASFV, simply by adding the extracted DNA of the problem sample. This kit contains specific primers and a TaqMan probe labelled with FAM fluorochrome for the specific detection of ASFV. In addition, the reaction mix includes an internal control (I.C.) that is amplified with specific primers and detected with a TaqMan probe labelled with HEX, allowing identification of false negatives due to PCR inhibition. Furthermore, the master mix contains a passive reference fluorochrome, ROX, to allow signal normalisation in the real-time PCR platforms where this is required. The amount of ROX in the master mix is adjusted for the thermocyclers that are indicated to use LOW ROX (such as Applied Biosystems Quantstudio5). In the case that higher ROX levels are required it should be supplemented by the end user.

The kit includes an ASFV positive control for detection. If a standard positive control with a known number of ASFV copies is required, it can be available upon request. This control could be used to generate a standard curve in order to correlate the number of pathogen copies with the cycle threshold (Ct) value.

The kit has been validated in the following PCR platforms from Applied Biosystems™ : Quantstudio™ 5 ; StepOnePlus™ and LightCycler® 480 II from Roche. It is compatible with other thermocyclers, if they have the appropriate fluorescence channels, but Ct values may vary among them.

4. KIT COMPOSITION:

COMPONENTS	Nº VIALS	VOLUMEN/VIAL
Mixture A (ASFV and I.C. specific primers and probes)	2	300 µl
Mixture B (enzyme mix)	2	600µl
Positive Control A1 – ASFV Amplification control	1	120 µl

5. CONSERVATION OF THE KIT COMPONENTS

All components have to be stored at -20ºC immediately after arrival and until the moment of use, and protected from light. The components of the kit are stable for 1 year from the manufacturing date (see expiry date on packaging).

6. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Powder-free disposable gloves (
- Microcentrifuge
- Tube shaker
- Real-time thermocycler
- Micropipettes (0.5-1000 µl)
- Sterile pipette tips (with filter)
- DNAse/RNase-free water, molecular grade

7. WORKING GUIDELINES AND PRECAUTIONS TO AVOID CONTAMINATION

The following working guidelines should be carefully observed:

- Use DNAse/RNase-free disposable consumables.
- Use DNAse/RNase-free water, molecular grade
- Use sterile filtered tips.
- Ensure good homogenisation of the kit components once defrosted.
- During use, keep all the kit components on ice/cooling block.
- Avoid repeated cycles of freezing and defrosting of the kit components, as it can reduce kit performance. Protect kit reagents from light exposure until their immediate use.

Special precautions to avoid ambient or cross-contaminations that could cause false positive results:

- The positive PCR control should be stored separately from the rest of kit reagents.
  Store the kit components in dedicated areas separated from samples or amplification products.

Use separate working areas with dedicated material and unidirectional workflow, for every step of the process: (1) Sample manipulation and extraction; (2) qPCR setup; (3) qPCR amplification and (4) just in the case needed, analysis of amplified products.

- If the above recommendation is not feasible, separate at least two separated areas, pre-PCR (steps 1 and 2) and post-PCR (steps 3 and 4).
- During the qPCR setup, the positive control should be manipulated and dispensed in the last place, avoiding the contact with the other kit components.

8. SAMPLES COLLECTION AND TRANSPORT

INgene q PPA is a real-time PCR kit suited for the detection of DNA of African Swine Fever Virus (ASFV) DNA in biological samples of clinical interest. Our assay has been optimized and specifically tested for blood, sera, spleen and liver samples, as those specimens are of clinical importance.

Samples should be collected and handled carefully to avoid contamination and should arrive in the testing laboratory as soon as possible, following national regulations for their transport. The samples must be cooled from the moment they are obtained and during the transport until they are processed. Processing needs to occur within 24 hours after obtaining the samples. In case a longer time is envisaged, freezing of samples is recommended.

9. DNA EXTRACTION

Any extraction procedure which yields good-quality of the extracted material could be used. Parallel to the extraction of the genetic material of the samples, it is advised to perform a mock-extraction, in which the biological sample is replaced by nuclease-free water, to check possible contamination during the extraction procedure.

Ingenasa has tested the assay with the following methods for the DNA extraction:

- Method of Chomczynski and Sacchi (Phenol extraction)*.
- Automatic magnetic methods (MagMAX™ Total NA Isolation Kit, Applied Biosystems™)
- Colum-based purification methods The QIAamp® Viral RNA Mini Kit (QIAGEN). High pure PCR template Preparation kit (ROCHE)
- Several commercial suppliers could provide high quality nucleic-acid extraction, following the manufacturer instructions, such as: Applied Biosystems™, ROCHE, QIAGEN, MACHEREY-NAGEL, others.

  *In the case of using phenolic extractions, it might be needed to reduce/dilute the DNA input in the qPCR reaction to avoid potential inhibition.

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

10. AMPLIFICATION OF THE GENETIC MATERIAL

REQUIRED MATERIALS

- Ice bucket or cooling block
- DNA extracted from the samples.
- Mixture A and B from the kit– KEEP ON ICE AND PROTECTED FROM LIGHT meanwhile PCR setup, store at -20C afterwards.
- Positive amplification control A1 (ASFV).
- DNAase-RNAase-free water (molecular grade)
- Tubes/plates with optical caps/sealing compatible with thermocycler.
- DNAase-RNAase-free tubes (1,5ml).
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PROCEDURE

1. Prepare as many tubes or PCR plate wells for the amplification as samples to be processed, adding additional tubes for the positive and the negative amplification controls. Identify positions avoiding direct label on the tubes/plates at it could interfere with optical reading.
2. Thaw and keep mixes A and B on ice/cooling block. Make sure that they are correctly homogenised before pipetting. Prepare a fresh amplification mix by adding in one tube the amount needed for the total number of samples to be processed, including the necessary controls. Keep the mixes A and B (and the amplification mix) always on ice/cooling block and protected from light.
3. Prepare the amplification mix on ice as follows. It is advisable to prepare an excess amount of mix (calculate an extra 10% for all reagents) in order to compensate for possible losses of volume during pipetting:

	Mixture A	Mixture B	Master Mix	Final Master mix volume (+10%)
Per sample	5 µl	10 µl	15 µl	16,5 µl
For 10 samples	50 µl	100 µl	150 µl	165 µl

4. Once mixture is prepared, homogenise it correctly. Place the PCR plate or strips on ice/cooling block and add 15 µl of the final reaction mix to each well.
5. Add 5ul of each extracted sample to the corresponding tube or reaction well of the PCR plate, or alternatively, of each of the reaction controls indicated

Type of sample/control	**component**	**Volume ,** µl
sample	extracted DNA from biological samples	5 µl
extraction control	extraction of a water control, in parallel to the samples	5 µl
positive control (C+)	positive amplification control A1 (ASFV.)	5 µl
negative control	nuclease free water	5 µl

6. Close/seal the tubes/plate and mix carefully the content, make sure the liquid is at the bottom of the tubes/wells. Otherwise, spin down briefly in a centrifuge.

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8. Insert the plate/tubes in the real-time instrument and follow the specific instructions to programme the reaction according to the following conditions:

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

9. Reading of fluorescence takes place during the hybridisation/elongation step (asterisk in the table)*, using the indicated fluorescence channels:

	Reporter	Quencher
ASFV	FAM™	None
I.C.	HEX/JOE/VIC	None
Passive reference	ROX	

11. ANALYSIS AND INTERPRETATION OF RESULTS

At the end of the run, results should be analysed with the software of the thermocycler, following the manufacturer instructions

From each sample, fluorescence data originated from channels FAM™ and HEX™/JOE™/VIC® is obtained. It is recommended in first place to select the automatic analysis mode for threshold and baseline determination. Alternatively, in the case that the automatic threshold is set incorrectly, adjust it manually following the thermocycler instructions. The threshold should be placed above the background and in the initial phase of the exponential curve. The baseline start and end cycles, should be set before any significant fluorescence is detected.

Interpretation of fluorescence data:

FAM channel: pathogen detection: A positive result should show a typical amplification curve with a Ct value <45.

HEX 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

In both channels: a negative result implies absence of amplification (undetected) or a Ct >=45

.Criteria for the kit validation:

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

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Before interpreting the samples result, check the validation criteria of the controls. :

control	FAM™ channel Pathogen detection	HEX/JOE/VIC channel Internal Control detection	Result
Negative control	undetected (ND), **Ct ≥45**	Not relevant	Valid PCR
	Ct<45	Not relevant	Invalid PCR (see section 12.1)
Positive control (C+)	Ct 32+/- 4	Not relevant	Valid PCR
	Ct<28 o Ct>36	Not relevant	Invalid PCR (see section 12.2)
Extraction control	undetected (ND), **Ct ≥45**	Not relevant	Valid extraction control
	Ct<45	Not relevant	Invalid extraction (see section 12.3)

In the case that the validation criteria of the controls are met, the interpretation of the sample results should be as follows:

Table: Interpretation of sample results

case	sample	Detection channels		Result	Interpretation
		FAM	HEX		
1	Problem sample	Positive (Ct<45)	Positive (Ct<45) or Negative (Ct>=45)	Positive	sample contains DNA of ASFV
2	Problem sample	Positive (Ct>=40 y CT<45)	Positive (Ct<45) or Negative (Ct>=45)	Doubtful	the detected levels of ASFV genetic material are very low and it is recommended to repeat the test to verify this result
3	Problem sample	Negative (Ct>=45)	Positive (Ct<45)	Negative	sample does NOT contain DNA of ASFV
4	Problem sample	Negative (Ct>=45)	Negative (Ct>=45)	Invalid	Possible inhibition (see section 12.4)

12. TROUBLESHOOTING

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

Possible cause	Solution
Contamination during PCR setup	• Repeat PCR reaction with new reagents • If tubes are being used, close each one immediately after adding the sample. • As a strict rule, the positive control must be manipulated and added in last place and avoiding any contact with the rest of reaction components • Ensure that the workplace, equipment and all instruments used for performing the PCR are decontaminated, i.e. free of nucleic acids.

12.2 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.

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

Possible cause	Solution
Wrong fluorochrome channel selected	• Check that FAM channel is selected for all samples and controls to be analysed.
Wrong analysis settings (automatic or manually selected)	• Verify the adequate positioning of the threshold and baseline, and the corrected selection of passive fluorescence dye (ROX) in the case needed, according to the instrument requirements.

12.4 Absence of fluorescence signal in both fluorescence channels FAM y HEX in the case of negative samples

Possible cause	Solution
PCR inhibition	• Dilute 1/40 the extracted DNA material and repeat the PCR reaction. • In the case that inhibition persists with the diluted sample, repeat the DNA extraction from the biological sample

13. QUALITY CONTROL:

Each production batch of this kit has been validated under our ISO-9001 and 14001certified Quality Management System.

14. TECHNICAL ASSISTANCE: AND ORDERS

For further information concerning the assay and its performance, please contact:

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