CENTRO DE INVESTIGACION EN SANIDAD ANIMAL (CISA-INIA)

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SOP/CISA/ASF/PCR/3/

STANDARD OPERATION PROCEDURE FOR THE DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY REAL-TIME POLYMERASE CHAIN REACTION (PCR) USING UNIVERSAL PROBE LIBRARY (UPL)

CONTENTS

1. PURPOSE

The purpose of this procedure is to rapidly detect the specific presence of African Swine Fever Virus (ASFV) DNA in porcine clinical material by a real-time polymerase chain reaction (PCR) technique using a Universal Probe Library (UPL) probe **(UPL-PCR)**.

This method has been validated at the EURL and is described in Fernández-Pinero, J.; Gallardo, C.; Elizalde, M.; Robles, A.; Gómez, C.; Bishop, R.; Heath, L.; Couacy-Hymann, E.; Fasina, F.O.; Pelayo, V.; Soler, A.; Arias, M. (2013). Molecular diagnosis of African swine fever (ASF) by a new real-time PCR using Universal Probe Library (UPL). Transbound Emerg. Dis., 60: 48-58.

2. SCOPE

This procedure is applied to the ASFV DNA extracted following the procedure described in the **SOP/CISA/ASF/DNA EXTRACTION/1** *("Standard operating procedure for the extraction of African Swine Fever Virus (ASFV) DNA"*) in any kind of porcine clinical sample such as EDTA-blood, serum and organs and in cell culture supernatants. It is particularly useful for identifying ASFV DNA in porcine tissues that are unsuitable for virus isolation or antigen detection, because they have undergone putrefaction, or when there is good reason to believe that virus may has been inactivated before samples are received in the laboratory. This PCR technique is highly sensitive, and its detection limit is below 20 DNA copies. It can be also applied for the detection of the ASFV in tick´s homogenate.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

1. Gallardo C, Nieto R, Soler A, Pelayo V, Fernández-Pinero J, Markowska-Daniel, Pridotkas G, Nurmoja I, Granta R, Simón A, Pérez C, Martín E, Fernández-Pacheco P, Arias M. Assessment of African Swine Fever Diagnostic Techniques as a Response to the Epidemic Outbreaks in Eastern European Union Countries: How To Improve Surveillance and Control Programs. J Clin Microbiol. 2015 Aug;53(8):2555-65.
2. Fernández-Pinero, J.; Gallardo, C.; Elizalde, M.; Robles, A.; Gómez, C.; Bishop, R.; Heath, L.; Couacy-Hymann, E.; Fasina, F.O.; Pelayo, V.; Soler, A.; Arias, M. *(2013). Molecular diagnosis of African swine fever (ASF) by a new real-time PCR using Universal Probe Library (UPL).* Transbound Emerg. Dis., 60: 48-58.

ASF REVIEWS:

1. Arias, M., Sánchez-Vizcaíno, J.M. (2012). African swine fever. In: Zimmerman, J., Karriker, L.A., Ramirez, A., Schwartz, K.J, Stevenson, G.W. (Eds), Diseases of swine, 10th Edition. John Wiley and Sons, United States of America, pp. 396-404.
2. Food and Agriculture Organization of the United Nations (FAO). AFRICAN SWINE FEVER: DETECTION AND DIAGNOSIS. A manual for veterinarians. FAO 2017 http://www.fao.org/3/a-i7228e.pdf

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of samples processing for African swine fever (ASF) diagnosis **(SOP/CISA/SAMPLE/1).**
- Procedure for the extraction of African Swine Fever Virus (ASFV) DNA (**SOP/CISA/ASF/DNA EXTRACTION/1).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
DNA: Deoxyribonucleic acid

E+: ASFV positive extraction control
E-: ASFV negative extraction control
R+: ASFV DNA positive reaction control
R-: ASFV DNA negative reaction control
PCR: polymerase chain reaction
r.p.m.: revolutions per minute
UPL: Universal probe library
UPL-PCR: real-time PCR using a UPL probe

4.2. BACKGROUND

Polymerase chain reaction **(PCR)** is a molecular genetic technique which allows the specific detection of ASFV DNA by enzyme-based amplification of a short viral genome fragment defined by a specific primer set. Under controlled conditions, multiple copies of DNA are generated by the action of the **DNA polymerase enzyme**, that adds complimentary **deoxynucleotides (dNTPs)** to a piece of DNA known as the "template".
Real-time PCR is an advanced amplification method, which allows the automated detection of the amplified product, reducing the risk of carry-over contamination with increased specificity and in most cases, even sensitivity.

PCR method requires a first step of viral DNA extraction from the original material to be analysed, which will be the template for the PCR. In real-time PCR, the appearance of amplified product is monitored continuously, in special equipments, with the incorporation in the reaction mix of a fluorescent dye that will give a fluorescence signal in a proportional way to the amplicon accumulation. By determination of fluorescence signal intensity in each amplification cycle, a sigmoid-shaped curve, that represents the amplicon appearance along the PCR, will be obtained.

Universal Probe Library (UPL, *Roche Applied Science)* comprises a collection of 165 pre-synthesized fluorogenic hydrolysis locked nucleic acid (LNA) probes for use in real-time PCR assays, originally designed for gene expression analysis and offered as a universal detection system. In this case, the PCR specificity is attained by the strict combination of specific forward and reverse primers and the appropriate UPL probe. UPL probes are 8-9 LNA residues in length, labeled with FAM dye at the 5' and a dark quencher dye at the 3'.
The described **UPL-PCR method** uses an ASFV specific primer set combined with an appropriate UPL probe (UPL#162, Roche Applied Science) directed to a highly conserved region of the viral genome, VP72, which ensure the detection of a wide range of ASFV isolates **belonging to the different p72 viral genotypes** (19 of the 22 known genotypes tested so far). The primers amplify a DNA fragment of **68 bp, from nucleotide position 893 to 960 of the complete VP72 gene sequence** of the reference strain ASFV Spain70 (*GenBank accession no. S89966*). The UPL probe employed for amplified product detection **(UPL#162) is commercially available** from *Roche Applied Science*, and is labelled with a reporter at 5' end [6-carboxy-fluorescein (FAM)] and a dark quencher molecule at 3' end.

PCR is a rapid method, which can be performed in less than four hours, and highly sensitive, allowing the viral detection even before the appearance of clinical symptoms in the infected animals. PCR is the tool of choice in case of **hyperacute, acute or subacute infection with ASFV.**

5. PROCEDURE DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Freezer <-10ºC.
- Freezer ≤-70ºC.

- Fridge 4±3ºC.
- Latex or nitrile gloves.
- Microcentrifuge for eppendorf tubes.
- Microcentrifuge tubes of volumes 0.2 (optical quality), 0.5, 1.5, and 2 ml, sterile.
- Microcentrifuge tubes of volume 0.5 ml, amber colour.
- PC connected to thermocycler.
- Real-time thermocycler [MX3005P, Stratagene or similar characteristics].
- Single channel pipette 1-10µl.
- Single channel pipette 10-100µl.
- Single channel pipette 10-200µl.
- Single channel pipette 200-1000µl
- Tube racks.
- Vortex

REAGENTS.

- **LightCycler 480 Probes Master kit,** commercially available from *Roche Applied Science (Ref. 04 707 494 001, 500 reactions*). *Store at <-10ºC.*
- **UPL-162 probe,** commercially available from *Roche Applied Science (Ref. 04694490001)* at a concentration of 10 pmol/µl: 5'-6FAM-GGCCAGGA-dark quencher-3'. *Store <-10ºC in aliquot, kept always away from light. Expiry date: 1 year*
- **Primers** at a concentration of 20 pmol/µl. *Store <-10ºC in aliquots. Expiry date: 1 year:*
  - primer **ASF-162/I2** sequence 5'-CCCAGGRGATAAAATGACTG-3' (forward primer);
  - primer **ASF-162/V2** sequence 5'-CACTRGTTCCCTCCACCGATA-3' (reverse primer).
- Nuclease-free sterile H_2O, PCR grade.
- ***Positive and negative controls:*** the following controls must be included in each PCR run:
  - ⇒ **E+→ ASFV positive sample target control for the extraction:** ASFV positive sample (serum, EDTA-blood, 1/10 tissue homogenates or culture supernatants) diluted in negative sample. It's highly recommended that the positive control is about the detection limit of the technique to track the yield of the DNA extraction procedure. *Store <-10ºC in aliquots. Expiry date: 6 months.*
  - ⇒ **E-→ ASFV negative sample control for the extraction:** distilled water which is included during the extraction process to exclude contaminations.
  - ⇒ **R+→ASFV positive DNA target control for the reaction:** ASFV positive DNA**.** It's highly recommended that the positive control is about the detection limit of the technique to track the yield of the PCR procedure *Store <-10ºC in aliquots. Expiry date: 6 months.*
  - ⇒ **R-→ ASFV negative DNA target control for the reaction:** distilled water which is included during the PCR process to exclude contaminations.

5.2. METHODS

General aspects:

- The assay amplifies a DNA fragment of 68 bp of the ASFV VP72 genome region.
- The PCR is carried out in a volume of 20 µl.
- UPL probe must be kept away from light (it should be placed in amber tubes)
- 2 µl of DNA sample is added to each reaction tube after master mix preparation.
- At least, one positive reaction control (2 µl of ASFV DNA, in the range of the detection limit of the technique) and one negative reaction control (2 µl of distilled water) should be included in each PCR run.

Master mix preparation:

In a sterile 1.5 ml microcentrifuge tube, prepare the PCR reaction mixture described below for the number of samples to be assayed (including R+ and R- controls) allowing for at least two extra samples.

Pippeting step	MASTER MIX REAGENTS	1x VOLUME (reaction 20µl)	FINAL CONCENTRATION
1	H_2O	7 µl	
2	Master mix 2X	10 µl	1X
3	Primer **ASF-162/I2** 20 µM	0.4µl	0.4 µM
4	Primer **ASF-162/V2** 20 µM	0.4µl	0.4 µM
5	UPL-162 probe 10 µM	0.2 µl	0.1 µM
Master mix volume		**18 µl**	

- Add 18 µl of the PCR reaction mix to the required number of 0.2 ml optical PCR tubes.
- Add 2µl of DNA template to each PCR tube. Include **R+ control** (2 µl of ASFV DNA) and **R- control** (2 µl of distilled water).
- After addition of the template, close the reaction tube and spin down the PCR mix. Place all tubes in an automated real-time thermocycler. Run the incubation program detailed below.

PCR CYCLE CONDITIONS.

PCR STEP	Temperature	Time	Nº cycles
Activation of DNA polymerase	95ºC	5 min	1x
DNA denaturation **Primer annealing/elongation**	**95ºC** **60ºC**	**10 sec** **30 sec**	**45 x**
Program the fluorescence collection in FAM channel at the end of each cycle.			

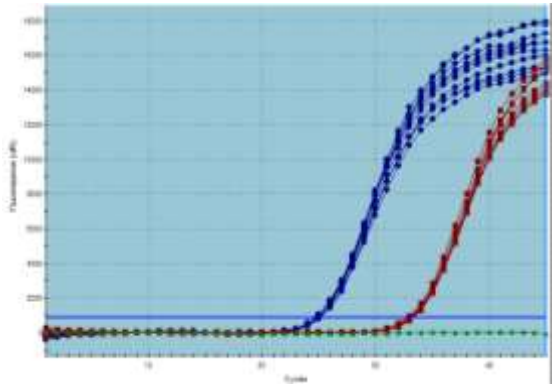
5.3. ANALYSIS AND INTERPRETATION OF RESULTS

The point where the fluorescence measurement is above the background signal and goes into detectable level is called the cycle threshold (Ct). This will be the starting fluorescence point to consider a sample as positive. The Ct value is inversely proportional to the starting amount of DNA template present in the reaction mixture that means in the analysed sample. The Ct value is automatically determined by the software of the thermocycler.

The assay will be considered as validated when the E+ and R+ have a Ct value within the range of 32±4 and the E– and R– Ct ≥40. Otherwise, the analysis should be repeated.

Interpretation of the results:

- In a **positive sample**, a sigmoid-shaped amplification curve will be obtained, indicating the cycle number versus read fluorescence level, where the Ct value will be under 35 **(Ct value <35).**
- A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value (**Ct value ≥40)**
- Samples giving a **Ct value ≥35** are considered as **weak samples** if a sigmoidal plot is observed. In this case and to confirm the results, the extracted DNA from the weak sample must be tested by duplicated in a second PCR run. Sample will be considered as positive in case of the Ct value <40 in, at least, one duplicate.



5.4. CRITICAL POINTS

Because of PCR is a highly sensitive technique, the most critical point along all the analysis procedure is the considerable risk of carry-over contamination, and the false positive results that could be obtained in this situation. The contamination could be due to the ASFV itself present in the positive analysed samples or in the positive controls included in the DNA extraction procedure; also, it could be due to ASFV DNA obtained after amplification of a previous PCR. **It is mandatory that personnel working on PCR follow and carry out some strict work rules in order to minimize the contamination risk associated to PCR technique**:

- All steps of sample analysis by PCR should be performed in separate locations, using equipment and material specific for each one: sample preparation, DNA extraction, PCR mix preparation, and removal of PCR products.
- Personnel must work always with clean nitrile or latex gloves in the PCR laboratory.
- Whenever personnel goes into a different PCR area, should be remove the gloves and take clean ones.
- The material will be of exclusive use for the PCR procedure step in which is located/labelled.
- Use a new pippete tip each time that a tube containing any sample or DNA is manipulated.
- Tubes containing amplified product should never be opened and manipulated in other laboratory distinct to that exclusively assigned to their analysis by electrophoresis, where they will be discarded.

5.5. SECURITY MEASURES

- Read and follow carefully the complete procedure.
- Keep reagents to the appropriate temperature before and after use.
- Do not pool reagents or instructions from different kits.
- Avoid any contamination of reagents.
- Do not use any reagent after it expiration date has passed.
- Do not eat, drink or smoke in the laboratory.
- Do not pipette by mouth.
- Wear always protective disposable nitrile or latex gloves.
- Probes used for amplified product detection are highly sensitive to light, so they should be manipulated for the minimum time, and must be kept always out of light (it is recommended to use amber colour tubes to keep probes stock and to prepare reaction mix).

5.6. QUALITY CONTROL

Annually, the European Union Reference Laboratory for ASF, CISA-INIA, Valdeolmos, Madrid, Spain, organizes an Interlaboratory Comparison Test (ILCT) to evaluate the diagnostic techniques for ASF available in all National Reference Laboratories of the EU.

The ILCT consists of a number of blind clinical material to be tested by the different diagnostic techniques in use in the laboratory. The real time-PCR described in this procedure has been fully validated at the EURL and along the last ILCTs editions performed by the EURL.

Worksheet CISA/ASF/PCR/3

ID REGISTER:
DATE:
TECHNICIAN:
EXTRACTION KIT BATCH Nº:
E+ BATCH Nº:
R+ BATCH Nº:
AMPLIFICATION PROGRAM:

Pippeting step	MASTER MIX REAGENTS	1x VOLUME (reaction 20µl)	Nx	FINAL CONCENTRATION
1	H_2O	7µl		
2	Master mix 2X	10 µl		1X
3	Primer **ASF-162/I2** 20 µM	0,4µl		0,4 µM
4	Primer **ASF-162/V2** 20 µM	0,4µl		0,4 µM
5	UPL-162 probe 10 µM	0,2µl		0,1 µM
	Master mix volume	**18 µl**		
8	**Addition of 2µl of DNA template** (test samples and reaction controls)			

ID SAMPLES											