

**CENTRO DE INVESTIGACION EN
SANIDAD ANIMAL (CISA-INIA)**

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SOP/CISA/ASF/PCR/1
**STANDARD OPERATING PROCEDURE FOR THE DETECTION
OF AFRICAN SWINE FEVER VIRUS (ASFV) BY
CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)**

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1. PURPOSE

The purpose of this procedure is to rapidly detect the specific presence of African Swine Fever Virus (ASFV) DNA in clinical material by the conventional polymerase chain reaction (PCR) technique.

Currently this technique is included in the Chapter 2.8.1. of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 Edition.

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 tissue homogenates 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 have been inactivated before samples are received in the laboratory.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

1. AFRICAN SWINE FEVER. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). CHAPTER 2.8.1. OIE, 2012
http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.01_ASF.pdf
2. Agüero M, Fernández J, Romero LJ, Zamora MJ, Sánchez C, Belák S, Arias M, Sánchez-Vizcaíno JM. “A highly sensitive and specific gel-based multiplex RT-PCR assay for the simultaneous and differential diagnosis of African swine fever and Classical swine fever in clinical samples”. *Vet Res.* **2004** Sep-Oct;35(5):551-63.
3. M. Agüero, J. Fernández, L. Romero, C. Sánchez, M. Arias, J.M. Sánchez-Vizcaíno.

2003. “Highly Sensitive PCR Assay for Routine Diagnosis of African swine fever virus in Clinical Samples. *J. Clin. Microbiol.*”, vol. 41, no. 9, p. 4431-4434.

ASF REVIEWS:

1. Arias, M., Sánchez-Vizcaíno, J.M. (2012). African swine fever. In: Zimmerman, J., Kariiker, 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. Arias, M.; Sánchez, C.; González, M.A.; Carrasco, L. y Sánchez-Vizcaíno, J.M. (2002). “Peste porcina Africana” In “Curso digital de enfermedades infecciosas porcinas”. On line, July, 2002. <http://www.sanidadanimal.info/cursos/curso/7/7-ppa.htm>
3. Food and Agriculture Organization of the United Nations (FAO). *RECOGNIZING AFRICAN SWINE FEVER. A FIELD MANUAL.* 2000 Edition.
<http://www.fao.org/docrep/004/X8060E/X8060E00.HTM>
4. Oura CA, Edwards L, Batten CA. “Virological diagnosis of African swine fever-Comparative study of available tests”. *Virus Res.* **2012** Nov 3. doi:pii: S0168-1702(12)00411-X. 10.1016/j.virusres.2012.10.022. [Epub ahead of print]

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 reaction positive control

R-: ASFV DNA reaction negative control
PCR: polymerase chain reaction
r.p.m.: revolutions per minute

4.2. BACKGROUND

The polymerase chain reaction (**PCR**) is a molecular genetic technique which allows the specific detection of 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 add complimentary **deoxynucleotides (dNTPs)** to a piece of DNA known as the "template".

Briefly, PCR is a **three-step process** that is carried out in repeated cycles. The initial step is the denaturation, or separation, of the two strands of the DNA molecule. This is accomplished by heating the starting material to temperatures of about 95° C (203° F). Each strand is a template on which a new strand is built. In the second step the temperature is reduced so that the primers can anneal to the template. In the third step the temperature is raised to about 72° C (162° F), and the DNA polymerase begins adding dNTPs to the 3' ends of each primer and generate a section of double-stranded DNA in the region of the gene of interest. At the end of the cycle the temperature is raised and the process begins again. The number of copies doubles after each cycle generating multiple copies of the target DNA. Finally, in the conventional PCR the amplified product will be detected by agarose **gel electrophoresis, ethidium bromide staining** (that is an intercalating dye of double-stranded DNA), and subsequent display by UV light radiation.

The described ASFV conventional PCR method uses a primer set designed in a highly conserved region of the viral genome, VP72, that ensures the detection of a wide range of ASFV isolates **belonging to all the XXII known virus genotypes**. This primer set amplifies a **DNA fragment of 257 base pairs (bp)**, from nucleotide position 88,363 to 88,619 nucleotides of the reference strain BA71V (*GenBank accession no. ASU18466*).

The PCR is a rapid method, that 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.

The ASF conventional PCR described in this procedure has been fully validated by the EURL giving sensitivity and specificity values close to 100%. The PCR is the tool of choice in case of hyperacute, acute or subacute infection with ASF.

5. PROCEDURE DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Conventional thermocycler with heated lid.
- Freezer <-10°C.
- Freezer ≤-70°C.
- Fridge 4±3°C.
- Glass or plastic pipettes for volume of 1-10 ml.
- Latex or nitrile gloves.
- Microcentrifuge for eppendorf tubes.
- Microcentrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- Micropipette disposable tips with aerosol resistant filter of 1-20, 20-200 and 200-1000 µl, sterile.
- Photograph camera and printer.
- Pipetboy acu or equivalent.
- Power supply.
- Single channel pipette 1-10µl.
- Single channel pipette 10-100µl.
- Single channel pipette 10-200µl.

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- Single channel pipette 200-1000µl.
- Tray for horizontal agarose gels, tank, combs, and powder leads.
- Tube racks.
- UV transiluminator.
- Vortex.

REAGENTS.

A) Reagents for DNA amplification step:

- AmpliTaq Gold® DNA polimerase with buffer II and Cl₂Mg [Ref.: N8080243 (Roche) or similar characteristics]. *Store at <-10°C.*
- ASFV primers at a concentration of 20 pmol/µl. *Store <-10°C in aliquots. Expiry date: 1 year.*
 - primer **PPA-1** sequence 5'-AGTTATGGGAAACCCGACCC-3' (forward primer);
 - primer **PPA-2** sequence 5'- CCCTGAATCGGAGCATCCT-3' (reverse primer).

Note: The ASFV primer set (PPA-1/PPA-2) described in this procedure (Agüero et al., 2003) can be combined with a specific primer set for CSFV in a multiplex RT-PCR method that allows the simultaneous and differential detection of both virus genomes in a single reaction (Agüero et al., 2004, Vet.Res. 35: 551-563).

- Deoxyribonucleotide triphosphate (dNTP) mix containing 10 mM of each dNTP [Ref.: 11581295001 (Roche) or similar characteristics]. *Store at <-10°C.*
- Nuclease-free sterile H₂O, 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 <-10°C in aliquots. Expiry date: 6 months.*

- ⇒ **E-→ 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 <-10°C in aliquots. Expiry date: 6 months.*
- ⇒ **R-→ negative DNA target control for the reaction:** distilled water which is included during the PCR process to exclude contaminations.

B) Reagents for amplified DNA detection step:

- Agarose MP 100 [Ref. 1 388 983001 (Roche) or similar characteristics]. *Store at room temperature.*
- Bromophenol blue [Ref.: 1.08122.0025 (Merck) or similar characteristics]. *Store at room temperature.*
- Ethidium bromide 0.625 mg/ml [Ref.: E406 (Amresco) or similar characteristics]. *Store at 4±3°C.*
- Glycerol 87% [Ref. 1.4094.2500 (MERCK) or similar characteristics]. *Store at room temperature.*
- Molecular Weight Marker VI DNA [Ref.: 11062590001 (Roche) or similar characteristics]. *Store at <-10°C.*
- TAE buffer 50x (Tris base, acetic acid and EDTA) [Ref.: A16911000 (AppliChem) or similar characteristics]. *Store at room temperature.*
- Xylene cyanol [Ref.: X4126 (Sigma) or similar characteristics]. *Store at room temperature.*

5.2. PREPARATION

5.2.1. REAGENTS PREPARATION

- **Agarose 2% solution** → Dissolve 2gr (±0.1gr) of agarose MP in 100 ml of TAE 1x and heat in microoven until the agarose appears completely melted.

- **Loading sample buffer 6x** [bromophenol blue 0.25%, xylene cyanol 0.25%, glycerol 30%] → Dissolve 0.1gr (±0.01gr) bromophenol blue + 0.1 gr (± 0.01gr) xylene cyanol in 17.24 ml of glycerol. Adjust with distilled water to a final volume of 50ml. Store at <-10°C in aliquots. Expiry date: 1 year.
- **Electroforesis buffer 1x** → Dilute 40 ml of TAE (50x) in 1,960 ml of distilled water. Store at room temperature. Expiry date: 2 months.
- **Molecular Weight Marker VI DNA** → 200 µl of Marker VI + 200 µl of loading buffer 6x + 400 µl electrophoresis buffer 1x. Store at 4±3°C. Expiry date: 6 month.

5.3. PROCEDURE EXECUTION

5.3.1 DNA amplification procedure

Master mix preparation:

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

Pipetting step	MASTER MIX REAGENTS	1x VOLUME (reaction 25µl)	FINAL CONCENTRATION
1	H ₂ O	17.375µl	
2	PCR Buffer 10X	2.5µl	1X
3	Cl ₂ Mg 25 mM	2µl	2 mM
4	dNTPs 10 mM	0.5µl	0.2 mM
5	Primer PPA-1 20 µM	0.25µl	0.2 µM
6	Primer PPA-2 20 µM	0.25µl	0.2 µM
7	Taq Gold 5 U/µl	0.125µl	0.025 U/µl
Master mix volume		23 µl	

Add 2µl of DNA template to each 0.2 ml PCR tube.
Include R+ control and R- control

After addition of the template, close the reaction tube and spin down the PCR mix. Place all tubes in an automated thermocycler equipped with heated lid. Run the incubation program detailed below.

PCR CYCLE CONDITIONS.

PCR STEP	Temperature	Time	Nº cycles
Activation of TaqGold DNA pol	95°C	10 min	1x
DNA denaturation	95°C	15 sec	
Primer annealing	62°C	30 sec	40 x
Elongation DNA	72°C	30 sec	
Extra elongation step	72°C	7 min	1x
Hold at 4°C.			

Keep the amplified products at 4±3°C until proceed with the electrophoresis (maximun 18 hours).

5.3.2 Agarose gel electrophoresis

1. Make a **2% agarose solution in 1x TAE buffer**. Heat the solution in a microwave oven until the agarose appears completely melted and add the ethidium bromide (BrEt) at a final concentration of 0.5 µg/ml. Shake carefully to homogenate.
2. Prepare the gel tray, sealing the ends and placing the adequate number of combs. Pour the melted agarose into the gel tray. Wait until the gel become solid (aprox 20 minutes).
3. Carefully remove the sealing of the tray and place it in the tank. Add the **electroforesis buffer** until gel is covered. Remove carefully the combs.
4. Add **4 µl of 6x loading buffer** to each tube containing 25 µl of the PCR amplified product.
5. **Load 10 µl of each sample** to one well of the gel.

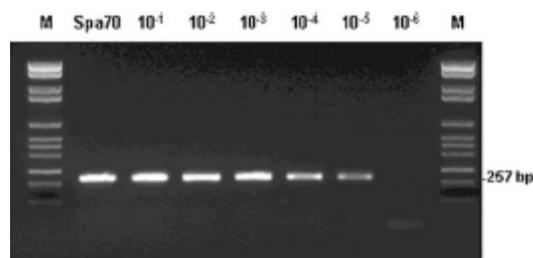
6. Add **6µl of molecular weight marker DNA VI** to one well on each lane of the gel.
7. Connect to power supply (DNA samples will move towards positive electrode). Run the gel at a constant voltage of 150-200 volts for about 30 – 40 minutes.

NOTE: the voltage depends on the percentage and size of the agarose gel. As general rule, it must be considered that for 2% agarose gels set the voltage at 5-10 V/cm²

8. Finally, place the gel on an ultraviolet transilluminator to visualize the bands.

5.4. ANALYSIS AND INTERPRETATION OF RESULTS

When electrophoresis is completed, immediately examine the gel over an UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive controls (**R+**, **E+**). Calculate the size of the PCR products in the test samples and the positive control by reference to the standard molecular weight marker. The PCR product of the positive controls (**R+**, **E+**) has a size of 257 bp (see figure). No bands should be seen in the negative control (**R-**, **E**).



The procedure will be valid if both extraction and reaction positive controls (R+, E+) give a discrete band of the appropriate size corresponding to ASFV DNA amplicon, and both extraction and reaction negative controls do not give a band pattern. Otherwise the procedure must be repeated.

Optional assay: An additional confirmatory assay could be performed by BsmA I restriction endonuclease digestion of the amplified products. For this assay, incubate for 2.5 hours at 55°C a total of 5 µl of amplified DNA product in a final volume of 20 µl digestion mix: 2 µl of 10x buffer, 1 µl of BsmA I (5U/µl) and 12 µl of sterile distilled water. Then, run the samples in a 3% agarose gel as described above. The restriction pattern should include two fragments of 173-177 and 84-80 base pairs in the positive samples.

5.5. 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 and manipulated by agarose gel electrophoresis during the amplicon analysis 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 analysis of PCR products by agarose gel electrophoresis.
- 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 pipette tip each time that a tube containing any sample or DNA is manipulated.

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- Tubes containing amplified product should never be opened and manipulated in other laboratory distinct to that exclusively assigned to their analysis by electrophoresis.

described in this procedure has been fully validated along the ILCTs performed since 2003.

5.6. 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 its 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.
- Ethidium bromide (BrEt) is a known mutagen, mainly when powdered format, and should be handled as a hazardous chemical. It is highly recommended to order as dropper solution to minimize its manipulation. BrEt handling must be performed exclusively in the lab assigned to it following the security measures ordered by the biological security service (gloves, coat, sleeves, protective glasses). In case of any unintended contact, wash immediately with abundant water and call to the biological security service. Also, contact them if any accidental spillage of any reagent containing BrEt occurs.

5.7. 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 conventional PCR

Worksheet CISA/PPA/PCR/1

ID REGISTER:

DATE:

TECHNICIAN:

EXTRACTION KIT BATCH N°:

E+ BATCH N°:

R+ BATCH N°:

AMPLIFICATION PROGRAM:

Pipetting step	MASTER MIX REAGENTS	1x VOLUME (reaction 25µl)	Nx	FINAL CONCENTRATION
1	H ₂ O	17,375µl		
2	Buffer 10X	2,5µl		1X
3	Cl ₂ Mg 25 mM	2µl		2 mM
4	dNTPs 10 mM	0,5µl		0,2 mM
5	Primer PPA-1 20 µM	0,25µl		0,2 µM
6	Primer PPA-2 20 µM	0,25µl		0,2 µM
7	Taq Gold 5 U/µl	0,125µl		0,025 U/µl
	Master mix volume	23 µl		
8	Addition of 2µl of DNA template (test samples and reaction controls)			

ID SAMPLES

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

COMMENTS: