

# RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA-INIA)

## SOP/CISA/ASFV/GENOTYPING/1/2008

### (STANDAR PROCEDURE OPERATION FOR AFRICAN SWINE FEVER VIRUS GENOTYPING)

**Rev. 1**

**Date: December 2008**

REV.	DATE	EPIGRAPH	CAUSE OF CHANGE
<u>Performed</u>  <b>Signature:</b> Carmina Gallardo Frontaura  Researcher, Laboratory Coordinator EU reference laboratory for ASF  <b>Date:</b>		<u>Reviewed</u>  <b>Signature.:</b> Miguel Ángel Jiménez Clavero  Heath of Service for P3 coordination  <b>Date:</b>	<u>Approved</u>  <b>Signature:</b> Marisa Arias Neira  CISA Technical Director  <b>Date:</b>

RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA – INIA)	AFRICAN SWINE FEVER GENOTYPING PROCEDURE	SOP/CISA/ASF/GENOTYPING/1/2008
		Rev. 1
		Page 2 of 4

<b>INDEX</b>	
<b>1.</b>	<b>OBJECT</b>
<b>2.</b>	<b>SCOPE</b>
<b>3.</b>	<b>REFERENCES</b>
<b>4.</b>	<b>BACKGROUND INFORMATION</b>
<b>5.</b>	<b>PROCEDURE DESCRIPTION</b>
<b>5.1.</b>	<b>EQUIPMENT AND MATERIALS</b>
<b>5.2.</b>	<b>PREPARACIÓN</b>
<b>5.3.</b>	<b>METHODS</b>
<b>5.4.</b>	<b>RESULTS AND INTERPRETATION OF RESULTS</b>
<b>5.5.</b>	<b>CRITICAL POINTS</b>
<b>5.6.</b>	<b>SAFETY CAUTIONS</b>
<b>5.7.</b>	<b>QUALITY CONTROL</b>
<b>6.</b>	<b>APPENDIX</b>

RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA – INIA)	AFRICAN SWINE FEVER GENOTYPING PROCEDURE	SOP/CISA/ASF/GENOTYPING/1/2008
		Rev. 1
		Page 3 of 4

## 1. OBJECT

This procedure has the objective to describe the genotyping procedure to perform the molecular characterization of African swine fever isolates.

## 2. SCOPE

This procedure is applicable to African swine fever isolates.

## 3. REFERENCES

### DOCUMENTS USED IN THE PROCEDURE REDACTION

1. Almazan F, Murguia JR, Rodriguez JM, Delavega I, Vinuela E (1995) A set of African swine fever virus tandem repeats shares similarities with Sar-like sequences. *J Gen Virol* 76: 729–740
2. Bastos ADS, Penrith ML, Cruciere C, Edrich JL, Hutchings G, Roger F, Couacy-Hymann E, Thomson GR (2003) Genotyping field strains of African swine fever virus by partial p72 gene characterisation. *Arch Virol* 148: 693–706
3. Boshoff CI, Bastos AD, Gerber LJ, Vosloo W. (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999). *Vet Microbiol.* Mar 31;121(1-2):45-55.
4. Gallardo C, Mwaengo DM, Macharia JM, Arias M, Taracha EA, Soler A, Okoth E, Martín E, Kasiti J, Bishop RP (2009) “Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes” *Virus Genes*, Feb;38(1):85-95.
5. Nix RJ, Gallardo C, Hutchings G, Blanco E, Dixon LK. (2006). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. *Arch Virol.* Dec;151(12):2475-94.

### DOCUMENTS TO BE USED TOGETHER WITH THIS PROCEDURE

- Procedure for antigen detection by conventional polymerase chain reaction (PCR) (SOP/CISA/ASF/PCR/1/2008)
- Procedure for Cloning of PCR amplicons in competent cells of *E. coli* using pGEMT easy vector (SOP/CISA/ASFV/pGMT cloning/1/2008).

## 4. BACKGROUND INFORMATION

African Swine Fever (ASF) was first reported in 1921 in Kenya as a disease of domestic pigs, and was confined to Africa until 1957 at which time an outbreak of ASF occurred in Portugal (Lisbon). Since then, many outbreaks were reported in other parts of Europe, the Caribbean and South America. In June 2007, the disease was first confirmed in the Caucasus region in Georgia. Two months later (August 2007) ASF was reported from Armenia and in December 2007 from Russia. Currently, ASF is endemic in the Caucasus region, Sardinia (Italy), and many African countries.

The ASF genome consists of a linear double-stranded DNA molecule of 170 to 190 kilobase pairs with terminal inverted repetitions and terminal hairpin loops. A high degree of variability in genome size and restriction fragment patterns is observed when different ASFV isolates are compared. Restriction enzyme site mapping and sequence analysis of virus genomes have established that the central region of the ASFV genome is relatively conserved but large length variations occur at the termini, particularly within 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome. Many of the length variations are associated with the loss or gain of copies within multigene families. In addition, smaller length

RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA – INIA)	AFRICAN SWINE FEVER GENOTYPING PROCEDURE	SOP/CISA/ASF/GENOTYPING/1/2008
		Rev. 1
		Page 4 of 4

variations are associated with the number of tandem repeats located at loci both within coding regions and in intergenic regions between genes. ASFV molecular polymorphism is investigated by partial sequencing of the gene *B646L* that encodes the major capsid protein p72 and 22 distinct genotypes have so far been defined. Recent studies confirm complete gene encoding the p54 protein as a valuable additional genotyping method for molecular epidemiological studies of p72 genotype I viruses, particularly in West Africa where this genotype predominates. The higher level of resolution of the viral discrimination possible using p54 - gene sequencing was confirmed by the separation of viruses within the homogeneous p72 genotype I, comprising viruses from West Africa, Europe, South America and the Caribbean, were separated into four clearly distinct p54 genotypes allowing discrimination between West African isolates from Europe, South America and the Caribbean. Although the p72 and p54 are useful for classification of major genotypes, PCR amplification and sequencing of more variable genome regions located in the common conserved central area of the ASFV genome have been used to distinguish between closely related isolates and identify virus subgroups within several of the 22 p72 genotypes. The molecular basis for this variability are the presence of the tandemly repeated sequences (TRS), such as intergenic repeat arrays similar to chromosomal minisatellite sequences and repeated sequences within the several ORFs of ASFV showing the TRS identified in the *B602L* gene (CVR) as the most variable locus.

**The genotyping strategy on ASFV isolates employed at ASF Community Reference Laboratory (CRL) involved sequencing of the gene encoding the VP72 and VP54 to place isolates into major subgroups, following by sub-typing through analyzing the TRS located in CVR region on the ASFV genome.**

## 5. DESCRIPTION

### 5.1. EQUIPMENT AND MATERIALS

#### ▪ **Materials.**

- Automated 3730 DNA sequence analyzer” (Applied Biosystems).
- Bath, termoblock or heater.
- Microcentrifuge for eppendorf tubes.
- Tubes racks.
- Thermal cycler.
- Tray for horizontal agarose gels and accessories.
- Source of electricity.
- Tubes shaker or vortex.
- Photograph camera and printer.
- Single channel pipettes of volumes 1-20, 20-200 and 200-1000 µl
- UV light source.

#### ▪ **Disposable material:**

- Micropipette tips of 1-200 and 200-1000 µl.
- Micropipette tips with aerosol resistant filter of 1-20, 20-200 and 200-1000 µl
- Eppendorf tubes of volumes 0.2, 0.5, 1.5, and 2 ml.
- Latex or nitrile gloves.

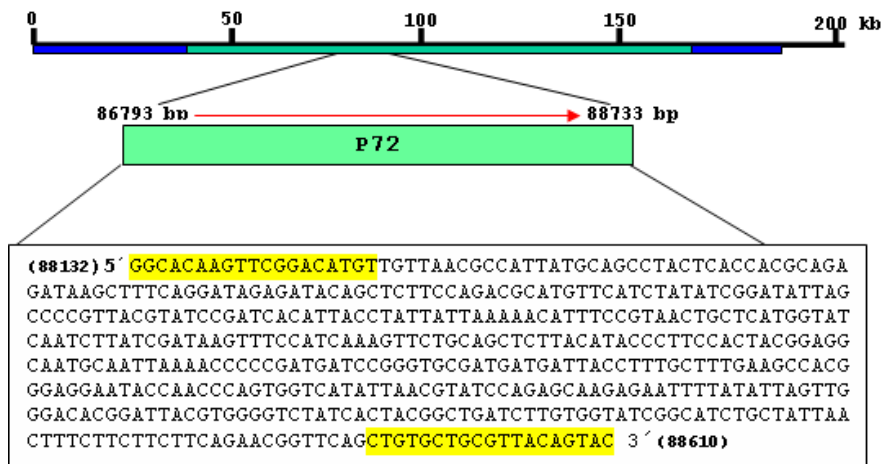
#### ▪ **Reagents.**

- Taq Gold DNA Polymerase, 10x PCR Buffer II and Cl2Mg. (ROCHE Ref. N808-0245)
- PCR nucleotide mix (10mM of each dNTP). (ROCHE Ref. 11581295001)
- Agarose MP 100 gr. (ROCHE Ref. 1 388 983).
- 10x Loading buffer (0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol).
- TAE buffer (50x). (Appllichem Ref. A1691,1000)

- o Ethidium bromide. (AMRESCO Ref. E406-5ML)
- o Molecular Weight Marker DNA (VI). (ROCHE Ref. 11062590001)
- o Sterile distilled water

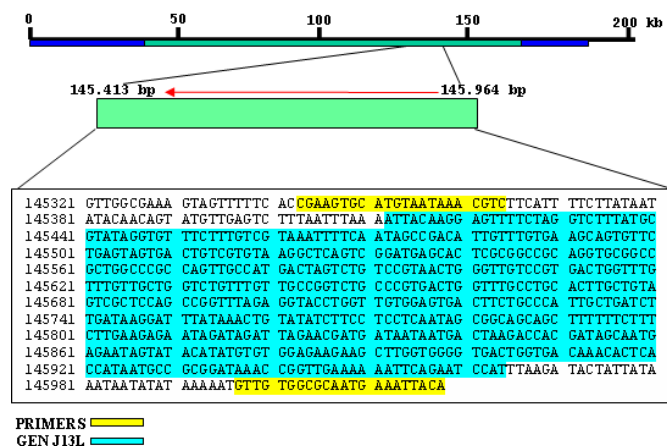
**Genotyping primers:** Three different set of primers derived from several genes are used to PCR amplify specific regions of the different ASF isolates.

1. The **C-terminal region of p72 protein is amplified using primers p72-U** [5'-GGCACAAGTTCGGACATGT - 3'] and **p72-D** [5'-GTACTGTAACGCAGCACAG- 3']. These primers amplify **478 bp from the protein p72 of ASFV (Ba71V isolate)** that is shown in the Figure 1. This region has been previously described by A.D.S.Bastos 2003.



**Fig. 1:** Sequence obtained using p72U/D primers set (marked in yellow) inside P72 protein.

2. The **complete gene (E183L) that encodes the ASFV protein p54 is amplified using primers ASF89** [5'-TGTAATTTTCATTGCGCCACAAC - 3'] and **ASF722** [5'-CGAAGTGCATGTAATAAACGTC - 3']. These primers that amplify **676 bp flanks the complete VP54 sequence of ASFV (Ba71V isolate)** that is shown in the Figure 2.



**Fig. 2:** Sequence obtained using ASF89/722 primers set (marked in yellow) flanking the P54 protein.

3. The primer pairs **ORF9L-F** [5'- CCGATTTAACAGATCCTGAGCGCATT- 3'] and **ORF9L-R** [5'-TCTTCATGCTCAAAGTGCATACCT - 3'] are used to amplify the central variable region (CVR) on ASFV genome. These primers amplify **393 bp (Ba71V isolate)** included in the **B602L gen** (Fig 4). In this region are located repeated amino acid tetramers that vary in number and type among different

ASFV isolates. Analysis of the number and composition of these tandem tetramers within the CVR may prove useful for identifying and/or grouping ASFV isolates.

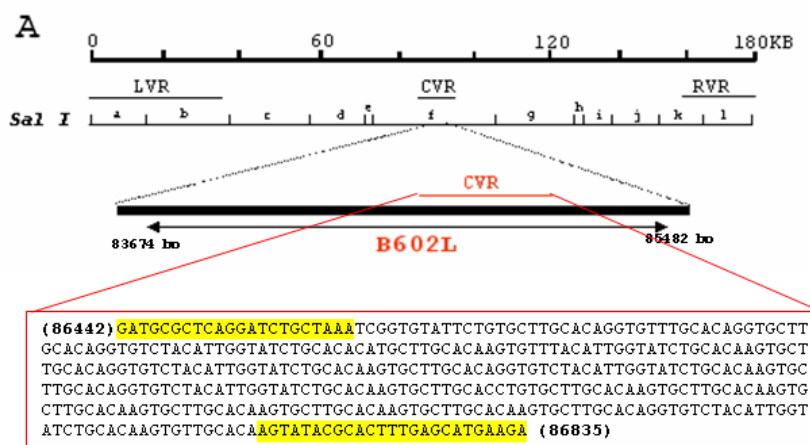


Fig. 3: Sequence obtained after PCR amplification of primers set ORF9L/9F (marked in yellow) inside B602L gene.

### 5.3. METHODS

#### a) PCR amplification

**Condition for the PCRs assay are as follows:** 10-50 ng of sample DNA, 1x PCR buffer II (50 mM KCl, 10 mM Tris-HCl), 2.5 mM MgCl<sub>2</sub>, 0.2 mM concentrations of the four deoxynucleoside triphosphates dNTPs (Roche Molecular Biochemicals), 0.4 μM concentrations of the primers and 0.625 U of Taq Gold polymerase (Applied Biosystems), in a total volume of 50 μl (Table 1).

REAGENTS	FINAL CONCENTRATION	VOLUME
Buffer 10 x	1x	5 μl
<b>Mg Cl<sub>2</sub> 25 mM</b>	<b>2.5 mM</b>	5 μl
DNTPs 10 mM	0.2mM	1 μl
Taq gold DNA pol.	0.625 U	0.25 μl
<b>Primer 20 M</b>	<b>0.4 μM</b>	1 μl
H <sub>2</sub> O		36.75. μl
<b>FINAL VOLUME OF REACTION =50 μL (46μl mix +4μl DNA)</b>		

The PCR reactions performed in a thermal cycler is (i) Denaturation for 5 min at 95°C; (ii) forty cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C; (iii) Incubation for 10 min at 72°C.

b) **Nucleotide sequencing:** Amplicons of the expected size are excised and purified by Quiaex gel extraction (QUIAGEN) according the manufactures instructions. After that the sequencing of the specific PCR products is performed as follows:

- The C-terminal region of p72 protein and the complete sequence of VP54 protein are directly sequencing using the same primers as were used in amplification in an automated sequencer 3730 DNA analyzer” (Applied Biosystems).
- The amplicons obtained from the CVR on ASFV genome are firstly cloning into a pGMT easy vector according the manufactures instructions and sequenced using primers specific for the pGMT vector (SP6/T7) in an automated sequencer 3730 DNA analyzer” (Applied Biosystems).

<b>RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA – INIA)</b>	<b>AFRICAN SWINE FEVER GENOTYPING PROCEDURE</b>	SOP/CISA/ASF/GENOTYPING/1/2008
		Rev. 1
		Page 7 of 4

*c) Nucleotide analysis:* Sequence alignment is performed with the CLUSTAL W package and phylogenetic and molecular evolutionary analyses is conducted using MEGA version 4.0 and Philip.