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SOP/CISA/ASF/GENOTYPING/ASFVGII/1

MULTI GENE-APPROACH METHOD FOR AFRICAN SWINE FEVER VIRUS (ASFV) GENOTYPE II ISOLATES GENOTYPING

CONTENTS

1. PURPOSE

The purpose of this procedure is to describe of molecular characterization **of African swine fever virus (ASFV) isolates belonging to genotype II using a multigene approach.**

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.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

1. Gallardo, C., Fernández-Pinero, J., Pelayo, V., Gazaev, I., Markowska-Daniel, I., Pridotkas, G., Nieto, R., Fernández-Pacheco, P., Bokhan, S., Nevolko, O., Drozhzhe, Z., Pérez, C., Soler, A., Kolvasov, D., & Arias, M. (2014). Genetic variation among African swine fever genotype II viruses, eastern and central Europe. Emerging infectious diseases, 20(9), 1544–1547. https://doi.org/10.3201/eid2009.140554
2. Giammarioli M, Torresi C, Biccheri R, et al. Genetic Characterization of African Swine Fever Italian Clusters in the 2022-2023 Epidemic Wave by a Multi-Gene Approach. Viruses. 2024;16(8):1185. Published 2024 Jul 24. doi:10.3390/v16081185
3. Giammarioli M, Torresi C, Biccheri R, et al. Genetic Characterization of African Swine Fever Italian Clusters in the 2022-2023 Epidemic Wave by a Multi-Gene Approach. Viruses. 2024;16(8):1185. Published 2024 Jul 24. doi:10.3390/v16081185
4. Mazur-Panasiuk N, Walczak M, Juszkiewicz M, Woźniakowski G. The Spillover of African Swine Fever in Western Poland Revealed Its Estimated Origin on the Basis of O174L, K145R, MGF 505-5R and IGR I73R/I329L Genomic Sequences. Viruses. 2020 Sep 27;12(10):1094. doi: 10.3390/v12101094. PMID: 32992547; PMCID: PMC7601147.
5. Nix, R. J., Gallardo, C., Hutchings, G., Blanco, E., & Dixon, L. K. (2006). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. Archives of virology, 151(12), 2475–2494. https://doi.org/10.1007/s00705-006-0794-z

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. ABREVIATION

ASF: African swine fever
ASFV: African swine fever virus
Bp: base pairs
CVR: central variable region
DNA: Deoxyribonucleic acid
E+: ASFV positive extraction control
E-: ASFV negative extraction control
Kbp: kilobase pairs
PCR: polymerase chain reaction.
R+: ASFV DNA reaction positive control
R-: ASFV DNA reaction negative control
r.p.m.: revolutions per minute

4.2. BACKGROUND

Molecular epidemiology has proved useful in investigating epidemiological patterns of ASF as well as the likely origin of the disease when introduced into new regions. The ASFV genome consists in a double-stranded DNA molecule of 170 to 193 kilobase pairs encoding among 151 to 167 genes depending on ASFV strain. Restriction enzyme site mapping and sequence analysis of virus genomes have established that the central region is conserved but large length variations occur at

the terminal ends, particularly within 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome.

While whole genome sequencing is the gold standard for identifying new genetic markers, **targeted sequencing of specific loci with significant variations can serve as a rapid and cost-effective alternative for tracking outbreaks and studying disease evolution in endemic area**s. The most common method for genotyping African swine fever virus (ASFV) during outbreaks involves analyzing the C-terminal end of the B646L/p72 gene, which helps classify ASFV into its major genotypes. Additionally, sequencing the E183L gene, which encodes the p54 protein, and the CVR region within the B602L gene, aids in distinguishing between geographically and temporally linked p72 genotypes, particularly within genotype II.

However, finer discrimination is necessary for viruses belonging to p72 genotype II, which is responsible for the ongoing Eurasian pandemic of ASF. These protocol describes a **multi-gene approach sequencing method** that can be used in routine genotyping to determine the origin of new introductions in ASF-free areas and track infection dynamics in endemic areas where genotype II is circulating. The method is based on the combination of the sequencing results obtained after the analysis of **six variable ASFV regions** as described below.

ID region	Variants amongst genotype II-ASFVs	Sequencing	Reference
CVR	Four variants /Number of TRS and SNPs	Partial *B602L* gene	Nix et al., 2006; Gallardo et al., 2023
IGR *I78R-I329L*	Four variants /Number of TRS	Intergenic region I78R-I329L genes	Gallardo et al., 2014
MGF	Nine variants/Number of TRS	Intergenic region MGF505 9R-10R genes	Giammaroli et al., 2024
K145R	Two variants/SNPs	Partial *K145R* gene	Mazur-Panasiuk et al., 2020
O174L	Three variants/Number of TRS and SNP	Complete *O174L* gene	Giammaroli et al., 2024
ECO2 *I329L-I215L*	Four variants /SNPs	Intergenic region and partial *I215L* gene	Gallardo et al., 2023

Tandem repeat sequence (TRS); Single nucleotide polymorphism (SNP)

5. PROCEDURE DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Conventional thermocycler with heated lid.
- Freezer <-10ºC.
- Freezer ≤-70ºC.
- Fridge 4±3ºC
- Glass or plastic pipettes for volume of 1-10 ml.
- Heating block or water bath (72±2ºC).
- 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
- Single channel pipette 1-10µl
- Single channel pipette 10-100µl.
- Single channel pipette 10-200µl.
- Single channel pipette 200-1000µl
- Pipetboy acu or equivalent.
- Power supply.
- 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 Cl_2Mg [Ref.: N8080243 (Roche) or similar characteristics]). *Store at <-10ºC.*
- ***ASFV genotpying primers*** *Store <-10ºC in aliquots (expiry date: 1 year).* Three different set of primers are using to amply six independent regions of the ASFV genome comprising:

ID	Name	Sequence 5´→3´	Position Referring to Georgia 2007/1 (FR682468.2)	Amplicon Length (nt)
CVR	CVR1	ACTTTGAAACAGGAAACWAATGATG	102,943-102,968	491
	CVR2	ATATTTTGTAATATGTGGGCTGCTG	102,520-102,524	
IGR	Eco1A	CTATTTATCCCCCRCTTTGG	173,272-173292	356
	Eco1B	TCGTCATCCTGAGACAGCAG	173,607-173,627	
O174L	O174L-F	TGGCTCAGACGATATTTCAACTC	128,160–128,182	673
	O174L-R	GCCTCCACCACTTGAACCAT	128,832–128,813	
K145R	K145R-F	TTTCAGGCTGAAAACTTTTTAT	65,030–65,051	282
	K145R-R	AAAGTTTTCAATGGTTGTTAGC	65,312–65,291	
MGF	505U	AGAAACCGCAGATGAATGTA	45,069-45,089	551
	505L	TACAGCCCTAGTTGTTGAAG	45-567-45,587	
ECO2	Eco2A	TCCTACCTGTTAAGCCACTTCC	174,452–174,472	604
	Eco2B	GCAAATGTGGATGCAGCTAA	175,035–175,055	

- Deoxyribonucleotide triphosphate (dNTP) mix containing 10 mM of each dNTP [Ref.: 11581295001 (Roche) or similar characteristics]. *Store at <-10ºC.*
- 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:** positive ASFV isolate. *Store <-10ºC 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**.** *Store <-10ºC 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.*
- FlashGel Loading Dye 5X [Ref.:50462 (Lonza) or similar characteristics]. *Store at room temperature.*
- GelRed Nucleic Acid Gel Stain [ref 4003, Biotium or similar characteristics]. *Store at room temperature in alicuots of 100µl*
- Molecular Weight Marker VI DNA [Ref.: 11062590001 (Roche) or similar characteristics]. *Store at <-10ºC.*
- TAE buffer 50x (Tris base, acetic acid and EDTA) [Ref.: A16911000 (AppliChem) or similar characteristics]. *Store at room temperature.*

5.2. PREPARATION

5.2.1. REAGENTS PREPARATION

- **Agarose 2% solution** → Disolve 2gr (±0.1gr) of agarose MP in 100 ml of TAE 1x and heat in microowen until the agarose appears completely melted.
- **FlashGel Loading Dye 5X →** Dilute 1 ml of Flash Gel 5x in 4 ml of distilled water to a final volume of 5ml. *Store at < 4ºC (expiry date: 1 year).*
- **Electroforesis buffer 1x**→ Dilute 40 ml of TAE (50x) in 1,960 liter of distilled water. *Store at room temperature (expiry date: 2 months).*
- **Molecular Weight Marker VI DNA** → 200 µl of Marker VI + 100 µl of loading buffer 5x + 700 µl destilated water grade PCR . *Store at 4ºC (±3ºC) (expiry date: 6 months).*

5.3. METHODS

5.3.1 DNA amplification procedures.

1. 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.

Pippeting step	MASTER MIX REAGENTS	1x VOLUME (reaction 25µl)	FINAL CONCENTRATION
1	H_2O	16.37µl	
2	PCR Buffer 10X	2.5µl	1X
3	Cl_2Mg 25 mM	2.5µl	2.5 mM
4	dNTPs 10 mM	0.5µl	0.2 mM
5	Primers **forward** 20 µM	0.5µl	0.4 µM
6	Primer **reverse** 20 µM	0.5µl	0.4 µM
7	Taq Gold 5 U/µl	0.13µl	0.025 U/µl
	Master mix volume	**23 µl**	
Add **2µl of DNA template** to each PCR tube. Include **R+ control** and **R- control**			

2. After addition of the DNA template, close the reaction tubes 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	Nª cycles
Activation of TaqGold DNA pol	95ºC	10 min	1x
DNA denaturation	**95ºC**	**30 sec**	
Primer annealing	**55ºC**	**1 min**	**40 x**
Elongation DNA	**72ºC**	**1 min**	
Extra elongation step	72ºC	10 min	1x
Hold at 4°C.			

Keep the amplified products at 4±3ºC 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 nucleic acid staining (Gel red) in 1/10,000 dilution. (Ex add 10 microlitres of Gel Red in 100ml of agarose). 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 buffe**r until gel is covered. Remove carefully the combs.
4. Add **5 µl of 1x loading buffer** to 5 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 50 – 60 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.

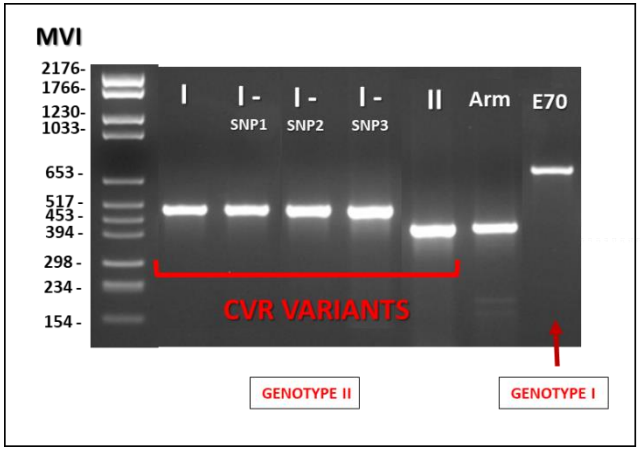
5.4. ANALYSIS AND INTERPRETATION OF RESULTS

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 control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard marker. No bands should be seen in the negative control. The procedure will be valid if both extraction and reaction positive controls 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.

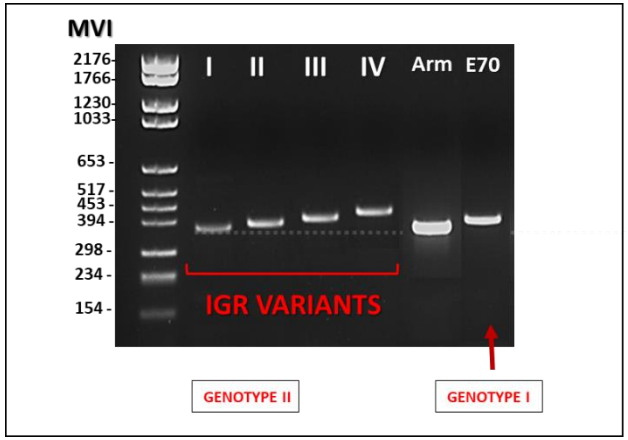
For optimal sequencing results, the target submitted for Sanger sequencing should exhibit a single product, or band, as confirmed by the gel electrophoresis procedure. If one "band" is present, indicating a homogeneous product, the amplicon can be purified and used for sequencing.

For PCR reactions in which more than one product is formed, the unique band of interest should first be isolated. Several gel purification methods are available to isolate the single band of interest. Examples include physical methods such as electrophoresis into a preformed trough, enzymatic methods such as agarose digestion, or purification methods using columns or magnetic beads. Although in theory it is possible, given the specificity of the sequencing primers, to generate NA sequence information from a single amplicon included in a multiplex PCR reaction, this is an approach that is prone to primer interference, lower product yields, and increased analysis complexity, and therefore is not recommended for diagnostic applications. In most cases, repeating the PCR reaction using one specific pair of primers will resolve the problem changing the cycle conditions. When the original template concentration is very low, re-amplifying the single target from diluted multiplex PCR amplicons can often yield the desired concentration for sequencing.

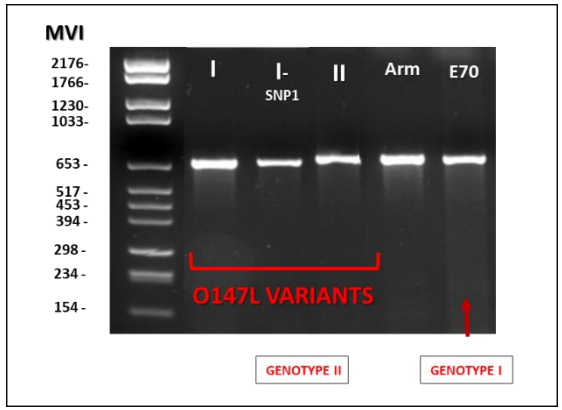
- **CVR REGION: product size Georgia strain 491bp**



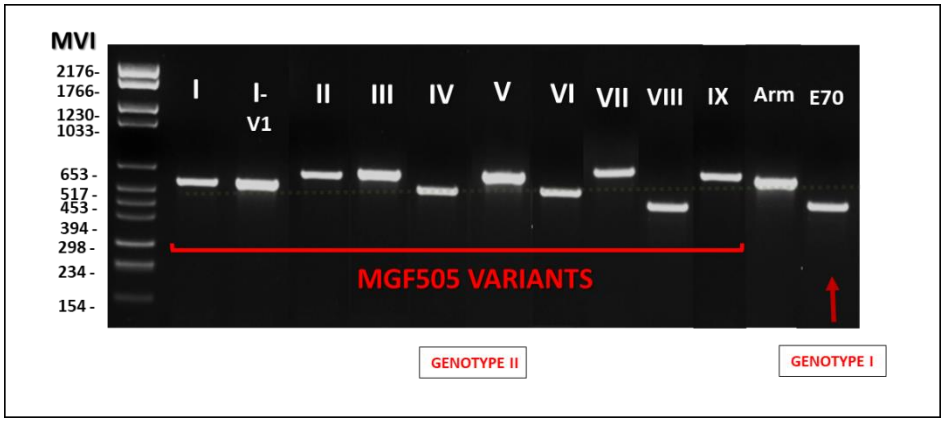
- **IGR REGION: product size Georgia strain 356bp**



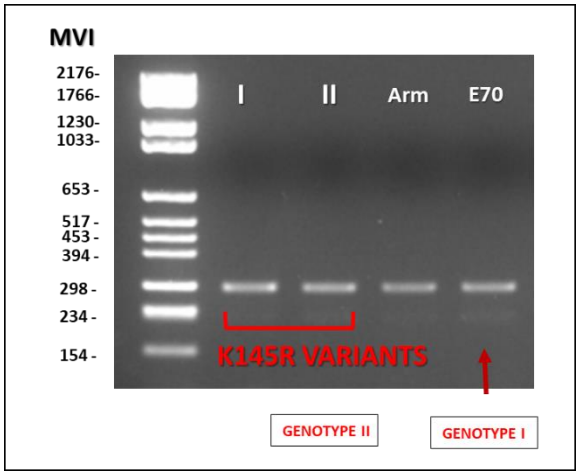
- **O174L REGION: product size Georgia strain 673bp**



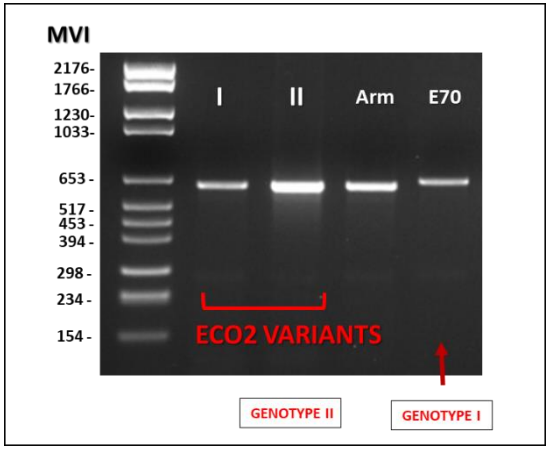
- **MGF505 REGION: product size Georgia strain 551bp**



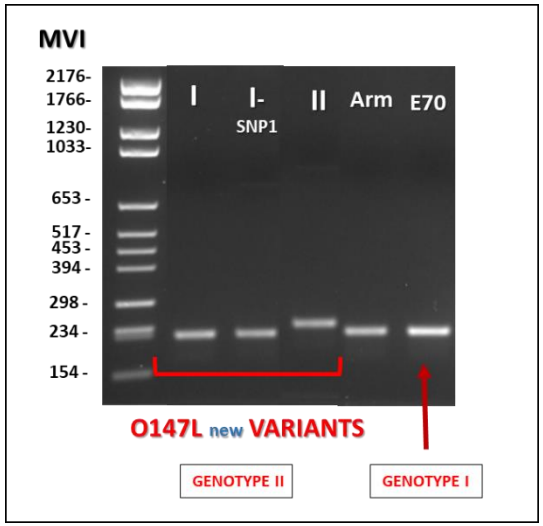
- **K145R REGION: product size Georgia strain 282bp**



- **ECO2 REGION: product size Georgia strain 604bp**



- **O174L new REGION: product size Georgia strain 209bp**



ID	Name	Sequence 5´→3´	Amplicon Length (nt)
CVR	CVR1	ACTTTGAAACAGGAAACWAATGATG	491
	CVR2	ATATTTTGTAATATGTGGGCTGCTG	
IGR	Eco1A	CTATTTATCCCCCRCTTTGG	356
	Eco1B	TCGTCATCCTGAGACAGCAG	
O174L	O174L-F	TGGCTCAGACGATATTTCAACTC	673
	O174L-R	GCCTCCACCACTTGAACCAT	
K145R	K145R-F	TTTCAGGCTGAAAACTTTTTAT	282
	K145R-R	AAAGTTTTCAATGGTTGTTAGC	

ID	Name	Sequence 5´→3´	Amplicon Length (nt)
MGF	505U	AGAAACCGCAGATGAATGTA	551
	505L	TACAGCCCTAGTTGTTGAAG	
ECO2	Eco2A	TCCTACCTGTTAAGCCACTTCC	604
	Eco2B	GCAAATGTGGATGCAGCTAA	
O174L new	O174L-FN	ACGTTTCTTAGGTATGCGATACGT	209
	O174L-RN	CCTTAGCCGAGGAAAAGCCA	