CENTRO DE
VIGILANCIA SANITARIA VETERINARIA
Universidad Complutense

OIE
Reference Laboratory

DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)

jmvizcaino@visavet.ucm.es
Av/ Puerta de Hierro s/n.
28040 Madrid.

Tel: (34) 913944082
Fax: (34) 913943908

DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL POLYMERASE CHAIN REACTION (PCR)

*Equipment required

- Heating block or water bath
- Tubes shaker or vortex mixer
- Microcentrifugue for eppendorf tubes
- Convencional thermocycler with heated lid
- Tray for horizontal agarose gels, tank, combs, and powder leads
- UV transiluminator
- Microwave
- 4ºC fridge
- -20ºC or lower freezer

*General laboratory material

- Latex or nitrile gloves
- Permanent marker
- Tray with ice
- Bleach at 50%
- Racks for 10 ml tubes
- Racks for microcentrifuge tubes

1. TISSUE MACERATION

1.1 MATERIALS

- Macerators
- Serological pipettes
- Pipetboy or similar

1.2 REAGENTS

PBS pH 7.2: tablets (ref 524650 CALBIOCHEM) or house made:

ClNa [Merck 1.06404]---------- 8 gr

ClK [Merck 1.04873]---------- 0.2 gr

PO4H2K [Merck 1.06586] ---------- 0.2 gr

PO4HNa2 [Merck 1.04936] ---------- 1.15gr

H_2O distilled ----------until 1000 ml

Check the pH before use. Store at 4ºC

1.3 METHODOLOGY

1.3.1 Number the tissue trays

1.3.2 Cut approximately 1 gr of tissue

1.3.3 Label the macerators

1.3.4 Introduce the tissue samples into the macerators

1.3.5 Add 10 ml of PBS 1X, and macerate the tissues (dilution 1/10)

1.3.6 Label the microcentrifuge tubes

1.3.7 Make aliquots of the macerate in microcentrifuge tubes and keep them frozen

2. DNA EXTRACTION

2.1 MATERIALS

- Micropipettes of volumes 1-20, 20-200, and 200-1000 µl
- Micropipette tips with aerosol resistant filter of 1-20, 20-200, and 200-1000 µl
- Microcentrifuge tubes of volumes 0,2, 0,5, 1,5, and 2 ml, sterile
- **High Pure PCR Template Preparation Kit** (Roche Diagnostics,ref:11796828001)
- Isopropanol
- Absolute Ethanol
- Nuclease-free sterile H_2O, PCR grade.

Preparation of working solutions:

- **Lyophilized proteinase K**: dissolve proteinase K in 4.5 ml sterile distilled water. Aliquot the solution in 500 µl vials. Store at -20ºC until their use. It is stable at -20ºC for one year.
- **Inhibitor Removal Buffer**: Add 20 ml absolute ethanol to the original vial. Label and date bottle accordingly. Store it at room temperature.
- **Wash buffer:** Add 80 ml absolute ethanol to the original vial. Label and date bottle accordingly. Store it at room temperature.

2.2 METHODOLOGY

2.2.1 Label micro centrifuge tubes

2.2.2 Add 200µl of Binding Buffer into micro centrifuge tubes.

2.2.3 Add 40 µl of Proteinase K.

2.2.4 Add 200 µl of sample.

2.2.5 Mix immediately by inversion.

2.2.6 Incubate for 10 minutes at 72ºC

2.2.7 Briefly centrifuge the tubes.

2.2.8 Add 100 µl of isopropanol to the sample tube. Place the High Pure filter tube in a collection tube. Label the collection tubes and pipette the liquid from the micro centrifuge tubes in the upper reservoir.

2.2.9 Centrifuge for 1 minute at 8000 rpm.

2.2.10 Discard the collection tube and place the filter tube into a clean collection tube

2.2.11 Add 500 µl of Inhibitor Removal Buffer

2.2.12 Centrifuge for 1 minute at 8000 rpm.

2.2.13 Discard the collection tube and place the filter tube into a clean collection tube

2.2.14 Add 450 µl of Wash Buffer

2.2.15 Centrifuge for 1 minute at 8000 rpm.

2.2.16 Discard the collection tube and place the filter tube into a clean collection tube

2.2.17 Add 450 µl of Wash Buffer again

2.2.18 Centrifuge for 1 minute at 8000 rpm.

2.2.19 Discard the collection tube and place the filter tube into a clean collection tube

2.2.20 Centrifuge for 10 seconds at 13000 rpm.

2.2.21 Prewarmed the sterile distilled water (70ºC)

2.2.22 Label new microcentrifuge tubes to store the DNA.

2.2.23 Place the filter tube in the labeled clean microcentrifuge tubes (1.5ml)

2.2.24 Add 50 µl of sterile distilled water, prewarmed at 70ºC

2.2.25 Centrifuge 1 min. at 8000rpm.

2.2.26 Discard the filter and store the DNA frozen at -20ºC.

3. CONVENTIONAL PCR

3.1 MATERIALS AND REAGENTS

- Micropipettes of volumes 1-20, 20-200, and 200-1000 µl
- Micropipette tips with aerosol resistant filter of 1-20, 20-200, and 200-1000 µl
- Microcentrifuge tubes of volumes 0.2 and 1.5 ml sterile
- Nuclease-free sterile H_2O, PCR grade.
- Taq Glod DNA polymerase, 10X PCR Buffer II, and Cl_2Mg (Applied Biosystems ref. nº N808-0241)
- PCR nucleotide mix containing 10 mM of each dNTP (Roche Diagnostics ref. nº 11581295001)
- Primers at a concentration of 20 pmol/ µl:
    - primer **PPA-1** sequence 5'-AGTTATGGGAAACCCGACCC-3' (forward)
    - primer **PPA-2** sequence 5'-CCCTGAATCGGAGCATCCT-3' (reverse)
- Positive ASFV DNA controls

For gel electrophoresis:

- Agarose (e.g. Biotools ref: 20.012)
- TAE 1XBuffer (e.g.Premixed TAE Buffer, 10X, Roche Diagnostics ref: 11 666 690 001)
- SYBR Safe (Invitrogen ref: S33102-400 µl) or ethidium bromide
- 10X loading buffer. For example:
    - Xinencianol (MERCK, ref:110590.0005)----------0.02gr
    - Azul de Bromofenol (PANREAC, ref: 251165.1604)-------0.02gr
    - Glicerol (PANREAC, ref: 131339.1211)----------3ml
    - Distilled water----------7ml

- DNA weight marker for amplicon size 257 bp (e.g. Biotools, ref:31.006 or Roche Diagnostics, ref: 0.019-1.11)

3.2 METHODOLOGY

A) DNA AMPLIFICATION

A.1 *MASTER MIX PREPARATION:*

- In a sterile 1.5 ml microcentrifuge tube, prepare the PCR reaction mix described below for the number of samples to be assayed (including positive and negative reaction controls). Make the calculations including for at least one extra sample due to the pipetting errors.
- PCR mix for one sample:

REAGENTS	***VOLUME*** (25 µl reaction)
Buffer 10X II	2.5 µl
Cl_2Mg (25mM)	2 µl
DNTP's (10 mM)	0.5 µl
primer PPA-1 20 µM	0.25 µl
primer PPA-2 20 µM	0.25 µl
Amplitaq Taq Gold (5 U/ µl)	0.125 µl
H_2O	17.37 µl

A.2 Add 23 µl of the PCR reaction mix to the required number of 0.2 ml PCR tubes.

A.3 Add 2 µl of DNA template to each PCR tube. Include a positive reaction control (2 µl of DNA) and a negative control (2 µl of distilled water) for each PCR run.

A.4 Close the reaction tubes and mix the content.

A.5 Place all tubes in an automated thermocycler equipped with heated lid. Run the **incubation program** detailed below:

Activation of polymerase	95ºC---10min	
DNA denaturation	95ºC---15sec	
Primer annealing	62ºC---30sec	x 40 cycles
DNA elongation	72ºC---30 sec	
Extra elongation step	72ºC---7min.	
Hold at 4ºC		

B) AGAROSE GEL ELECTROPHORESIS:

B.1 Weight 2 gr of agarose to prepare a 2% agarose gel

B.2 Add 2 gr of agarose in 100 ml of TAE 1x Buffer

B.3 Heat the solution in the microwave until agarose is completely melted.

B.4 Add 10 µl of SYBR safe for each 100 µl of agarose. Shake carefully to disperse.

B.5 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).

B.6 Carefully remove the sealing of tray and place it into the tank. Remove carefully the combs. Add 1X TAE buffer to the tank until the gel is completely covered by TAE solution.

B.7 Add 2.5 µl of 10X loading buffer to each tube

B.8 Load 10 µl of each PCR sample to each well of the gel.

B.9 Add 10 µl of molecular DNA weight marker to one well one each lane of the gel.

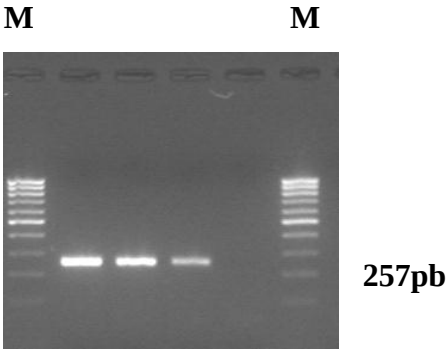
B.10 Connect the power supply. Run the gel at a constant voltage of 120-130 volts for about 30 minutes. (DNA samples have to move towards positive electrode)

B.11 For the lecture of the gel place it on an ultraviolet transilluminator.

3.3 RESULTS INTERPRETATION

A positive sample will present a discrete band that should have co-migrated with the PCR product of the Positive control. The molecular weight marker must be used as a reference to calculate the molecular weight of the product. This assay amplifies a DNA fragment of **257bp** of the ASFV VP72 genome region.

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 more information, please contact us:
jmvizcaino@visavet.ucm.es
linamur@vet.ucm.es

VISAVET Center and Department of Animal Health
Facultad de Veterinaria. Universidad Complutense de Madrid
Av/ Puerta de Hierro s/n.
28040 Madrid, Spain
Tel: (34) 913944082
Fax: (34) 913943908