CENTRO DE
VIGILANCIA SANITARIA VETERINARIA
Universidad Complutense

OIE
Reference Laboratory

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

*Equipment required

- Heating block or water bath
- Tubes shaker or vortex mixer
- Microcentrifugue for eppendorf tubes
- Real time thermocycler connected with a computer with the appropriate software
- 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. REAL-TIME PCR

3.1 MATERIALS AND REAGENTS

- Nuclease-free sterile H_2O, PCR grade.
- KAPA PROBE FAST Universal qPCR Kit, (Cu KK4702)
- Taq Man probe at a concentration of 10 pmol/µl:

5'-FAM-CCACGGGAGGAATACCAACCCAGTG-TAMRA -3´

- Primers at a concentration of 20 pmol/µl:
  - primer **King-s** sequence 5'-CTGCTCATGGTATCAATCTTATCGA -3' (forward)
  - primer **King-a** sequence 5'-GATACCACAAGATCRGCCGT -3' (reverse)

3.2 METHODOLOGY:

3.2.1 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) allowing for at least one extra sample.
- PCR mix of one sample:

REAGENTS	***VOLUME*** (10 µl reaction)
Master Mix KAPA	5 µl
primer King-s 10 µM	0,4µl
primer King-a 10µM	0,4µl
TaqMan probe 10µM	0,4µl
H_2O	1,8µl

3.2.2 Add 8 µl of the PCR reaction mix to the required number of 0,2 ml PCR tubes.

3.2.3 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.

3.2.4 Close the reaction tubes and mix them.

3.2.5 Place all tubes in an automated Real-Time thermocycler. Run the **incubation program** detailed below:

Activation of DNA pol.	95ºC---3min.	
DNA denaturation	95ºC---10sec	45 cycles
Primer annealing/elongation	58ºC---30sec	

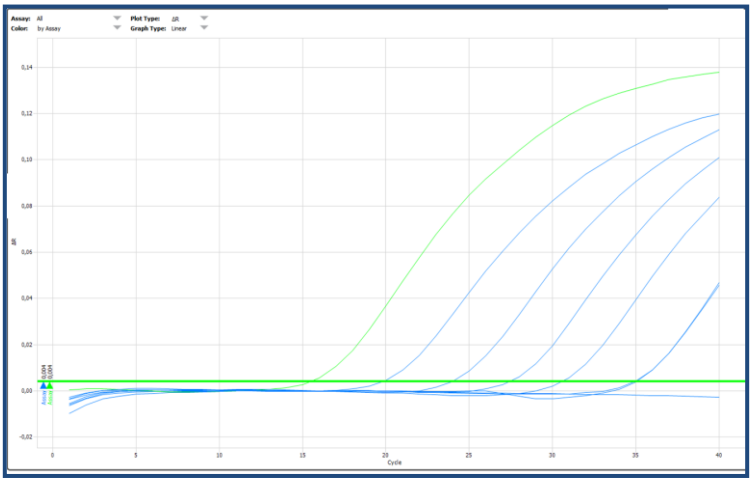
Program the fluorescence collection in FAM channel at the end of each cycle.

3.3 INTERPRETATION OF RESULTS

The procedure will be valid if both extraction and reaction positive controls give a Ct value of 32±2, and both extraction and reaction negative controls do not show any Ct value.

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 40. A negative sample will maintain the fluorescence profile under background fluorescence level and the equipment will not report any Ct value.

Ct value >38 samples should be considered as doubtful result if a sigmoidal plot is observed and the analysis should be repeated for confirmation. Ct value >38 samples should be considered as negative if the amplification plot has a linear shape.



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