UNIVERSIDAD COMPLUTENSE DE MADRID
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REFERENCE LABORATORY

STANDARD OPERATING PROCEDURE FOR THE DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY REAL-TIME POLYMERASE CHAIN REACTION (PCR)

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1. TISSUE MACERATION

Watch video (Tissue maceration and DNA extraction)

1.1 MATERIALS

- Tray with ice
- Bleach at 50%
- Macerators
- Adhesive tape
- Permanent marker
- Racks for 10 ml tubes
- Racks for microcentrifuge tubes
- Serological pipettes
- Adsorbent paper
- Latex or nitrile gloves
- Pipetboy or similar

1.2 REAGENTS

PBS Buffer pH 7.2:

ClNa [Merck 1.06404]---------- 8 gr
ClK [Merck 1.04873]---------- 0,2 gr
PO_4H_2K [Merck 1.06586] ---------- 0,2 gr
PO_4HNa_2 [Merck 1.04936] ---------- 1,15gr
Distilled water ---------- 1000ml

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

1.3.6 Label the microcentrifuge tubes

1.3.7 Make aliquots of the macerator content in the microcentrifuge tubes

2. DNA EXTRACTION

Watch video (Tissue maceration and DNA extraction)

2.1 MATERIALS

- Tray with ice
- Bleach at 50%
- Permanent marker
- Racks for microcentrifuge tubes
- Latex or nitrile gloves
- Water bath
- Cetrifuge machine
- Isopropanol
- Vortex
- **High Pure PCR Template Preparation Kit** (Roche Diagnostics,ref:11796828001)

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 -20ªc until use.
- **Inhibitor Removal Buffer**: Add 20 ml absolute ethanol to the original vial. Label and date bottle accordingly.
- **Wash buffer:** Add 80 ml absolute ethanol to the orginal vial. Label and date bottle accordingly.

2.2 METHODOLOGY

2.2.1 Label microcetrifuge tubes

2.2.2 Add 200µl of Binding Buffer into microcentrifuge tubes.

2.2.3 Add 40 µl of Proteniase 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 Place the High Pure filter tube in a collection tube. Label the collection tubes and pipette the liquid from the microcentrifuge 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 Inhibitor 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 Inhibitor 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 at -20ºC.

3. REAL-TIME PCR

Watch video (Real-Time PCR)

3.1 MATERIALS AND REAGENTS

- Nuclease-free sterile H_2O, PCR grade.
- QRT-PCR Master Mix 2X Brilliant II (Stratagene).
- 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 mixture described below for the number of samples to be assayed (including positive and negative reaction controls) allowing for at least one extra sample.
- PCR mixture of one sample:

REAGENTS	***VOLUME*** (25 µl reaction)
2X PCR Master Mix	12,5 µl
primer King-s 20 µM	0,5 µl
primer King-a 20µM	0,5 µl
TaqMan probe 10µM	0,63 µl
H_2O	8,87 µl

3.2.2 Add 23 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.