UNIVERSIDAD COMPLUTENSE DE MADRID
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REFERENCE LABORATORY

STANDARD OPERATING PROCEDURE FOR THE DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY CONVENTIONAL 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

1.3METHODOLOGY

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. CONVENTIONAL PCR

Watch video (Conventional PCR)

3.1 MATERIALS AND REAGENTS

- Micropipettes of volumes 1-20, 20-200, and 200-1000 µl
- Tubes shaker or vortex mixer
- Microcentrifugue for eppendorf tubes
- Heating block or water bath
- Tube Racks
- Convencional thermocycler with heated lid
- Tray for horizontal agarose gels, tank, combs, and powder leads
- Power supply
- UV transiluminator
- 4 ºC fridge
- -20ºC or lower freezer
- -70ºC or lower freezer
- 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
- Latex or nitrile gloves
- Nuclease-free sterile H_2O, PCR grade.

- Taq Glod DNA polymerase, 10X PCR Buffer II, and Cl_2Mg (Applied Biosystems ref. nº N808-0245)
- 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)

3.2 METHODOLOGY

A) DNA AMPLIFICATION

A.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 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 20µM	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 them.

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

Activation of Taq Gold DNA pol.	95ºC---10min.	
DNA denaturation	95ºC---15sec	40 cycles
Primer annealing	62ºC---30sec	
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 prerpare 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 3 µl of SYBR safe (Invitrogen ref. nº. S33102-400 µl) 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 in the tank. Remove carefully the combs. Add 1X TAE buffer until gel is covered.

B.7 Add 2,5 µl of 10X loading buffer to each tube

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

B.9 Add 10 µl of molecular weight marker DNA VI 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 a ultraviolet transilluminator.

3.4 RESULTS INTERPETATION

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 **257pb** 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.