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SOP/CISA/ASF/DNA EXTRACTION/1

STANDARD OPERATING PROCEDURE FOR THE EXTRACTION OF AFRICAN SWINE FEVER VIRUS (ASFV) DNA

CONTENTS

1. PURPOSE

The purpose of this procedure is to describe the **nucleic acid extraction method** of the African swine fever virus (ASFV) DNA in clinical material using the commercial nucleic acid extraction kit ***"High Pure PCR Template Preparation Kit "*** [Ref. 11796828001 (ROCHE)] for further amplication by polymerase chain reaction (PCR) techniques.

Currently this technique is included in the World Organisation for Animal Health (OIE), 2019. African swine fever. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Vol 2 Chapter 3.8.1

2. SCOPE

This procedure can be applied in any kind of porcine clinical sample such as EDTA-blood, serum and organs and in cell culture supernatants, even if the latter have been kept in warm conditions and undergone a degree of putrefaction. It is recommended, in the case of homogenates, to analyze them in duplicate undiluted and at a 1/10 dilution.

Also it is applicable to homogenated soft ticks (*Ornithodorous genus).*

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

For the elaboration of this procedure it has been used all the internal procedure included in the INIA-CISA quality system accredited under the UN-EN ISO/IEC17025:2017

In addition as a basic reference for the preparation of this procedure it has been used the following documents:

1. World Organisation for Animal Health (OIE), 2019a. African swine fever. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Vol 2 Chapter 3.8.1
2. Protocol ***High Pure PCR Template Preparation Kit***: Commercial nucleic acid ROCHE [http://www.roche-applied-science.com/proddata/gpip/3_6_8_48_1_1.html]

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7. M. Agüero, J. Fernández, L. Romero, C. Sánchez, M. Arias, J.M. Sánchez-Vizcaíno. **2003.** "Highly Sensitive PCR Assay for Routine Diagnosis of African Swine Fever Virus in Clinical Samples". J. Clin. Microbiol., vol. 41, no. 9, p. 4431-4434.

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of samples processing for African swine fever diagnosis **(SOP/CISA/SAMPLE/1).**

- Procedure for the detection of African swine fever virus by conventional polymerase chain reaction (PCR) **(SOP/CISA/ASF/PCR/1).**
- Procedure for the detection of African swine fever virus by real time polymerase chain reaction (PCR) **(SOP/CISA/ASF/PCR/2).**
- Procedure for the detection of African swine fever virus by real time polymerase chain reaction (PCR) **(SOP/CISA/ASF/PCR/3).**
- Procedure for ASFV genotyping **(SOP/CISA/ASF/GENOTYPING/1)**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION.

ASF: African swine fever
ASFV: African swine fever virus
DNA: Deoxyribonucleic acid
E+: ASFV positive extraction control
E-: ASFV negative extraction control
PCR: polymerase chain reaction.
r.p.m.: revolutions per minute

4.2. BACKGROUND.

This procedure described the ASFV DNA extraction using the commercial **High Pure PCR Template Preparation Kit *(Roche Diagnostics)*.** This procedure has the advantage that can be used for the extraction of both ASFV DNA and CSFV RNA, which enables the simultaneous detection of both viruses in a single reaction if a multiplex PCR assay is used (Agüero *et* al., 2004). During the DNA extraction, firstly, cells are lysed during a short incubation with proteinase K in the presence of a chaotropic salt (guanidine-HCl), which immediately inactivates all nucleases. Cellular nucleic acids bind selectively to special glass fibers pre-packed in the High Pure Purification Filter Tube. Bound nucleic acids are purified in a series of rapid "wash-and-spin" steps to remove contaminating cellular components. A special Inhibitor Removal Buffer has been included which allows even the application of heparinized sample material with - 100 U/ml of Heparin. Finally, sterile water elution releases the nucleic acids from the glass fiber. The nucleic acid obtained is used as template for further PCRs.

5. PROCEDURE DESCRIPTION

Method Validation.

Since 2003 the EURL for ASF (INIA-CISA) organize the Annual interlaboratory comparison test (ILCT) to evaluate the ASF diagnostic techniques available at the EU National reference laboratories (NRLs). The ILCT comprises a panel of blind samples to be tested using all the methods routinely employed at the NRLs. This procedure has been fully validated over the last ILCT editions.

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Chronometer
- Freezer <-10ºC.
- Freezer ≤-70ºC.
- Fridge 4±3ºC.
- Heating block or water bath (72±2ºC).
- Single channel pipette 1-10µl.
- Single channel pipette 2-20µl.
- Single channel pipette 20-200µl.
- Single channel pipette 100-1000µl.
- Microcentrifuge for eppendorf tubes.
- Tube racks.

- Vortex.

Disposable material:

- Micropipette tips of 1-200 and 200-1000 µl, sterile.
- Micropipette tips with aerosol resistant filter of 1-10, 2-20, 20-200 and 100-1000 µl, sterile.
- Microcentrifuge tubes of volumes 0.2, 0.5, 1.5, and 2 ml, sterile.
- Latex or nitrile gloves.

REAGENTS INCLUDED IN THE KIT *(store at room temperature).*

- **Binding Buffer (20 ml)** [6 M guaninidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 (v/v), pH 4.4].
- **Proteinase K, recombinant PCR grade 20 mg/mL** (lyophilized).
- ***Inhibitor Removal Buffer* (33 ml)** [5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6].
- **Wash Buffer (20 ml)** [20 mM NaCl, 2 mM Tris-HCl, pH 7.5].
- **High Pure Filter Tubes:** two bags with 50 polypropylene tubes with two layers of glass fiber fleece, for use of up to 700 µl sample volume.
- **Collection Tubes:** eight bags with 50 polypropylene tubes (2 ml).

REAGENTS NOT INCLUDED IN THE KIT.

- **Absolute isopropanol** (≥ 99%) [Ref.: I9516 (Sigma) or similar characteristics].
- **Absolute ethanol** [Ref.: 1.00983.1000 (Merck) or similar characteristics].
- Distilled H_2O, sterile, PCR grade.
- ***Positive and negative controls:*** at least one positive and one negative control should be included in each nucleic acid extraction run.
  - ⇒ **E+→ ASFV positive sample target control for the extraction:** ASFV positive sample (serum, EDTA-blood, 1/10 tissue homogenates or culture supernatants) diluted in negative sample. It's highly recommended that the positive control is about the detection limit of the technique to track the yield of the DNA extraction procedure. *Store <-10ºC in aliquot.Expiry date: 6 months.*
  - ⇒ **E-→ negative sample control for the extraction:** distilled water which is included during the extraction process to exclude contaminations.

5.2. PREPARATION

5.2.1. SAMPLE PREPARATION

Sample preparation is performed according is described in the sample processing procedure for ASF diagnosis **(SOP/CISA/ASF/SAMPLE/1).**

5.2.1. REAGENTS PREPARATION

- Lyophilized proteinase K: dissolve proteinase K in 4.5 ml of sterile distilled water, and aliquot the solution in 500 µl vials. *Store at <-10ºC until use.*
- Inhibitor Removal Buffer: Add 20 ml absolute ethanol to the original vial. Label and date bottle accordingly. *Store at room temperature.*
- Washing buffer: Add 80 ml absolute ethanol to the original vial. Label and date bottle accordingly. *Store at room temperature.*

5.3. METHODS.

Note: Before starting the purification, warm the Elution Buffer (distilled water)to 72±2ºC.

1. Into a 1.5ml microcentrifuge tube add:
   - **200 µl** of **binding buffer**
   - **40 µl of proteinase K 20 mg/mL**.
   - **200 µl of the sample**. Include in each extraction procedure the E+ y E- (200 µl H_2O).
2. Mix inverting the tubes and incubate for **10 minutes at 72±2ºC**. Briefly centrifuge (spin) the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
3. Add **100 µl of isopropanol** to the sample tube.

4. Mix in vortex and and spin the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
5. Insert one **High Pure Filter Tube into one Collection Tube and pipette the sample into the upper buffer reservoir** of the Filter Tube.
6. Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge and **centrifuge for 1 min. at 8,000 g.** With blood samples, repeat the centrifugation step if sample remains in the filter tube.
7. Discard the collection tube and place the filter tube into a clean collection tube.
8. Add **500 µl of Inhibitor Removal Buffer** to the upper reservoir and **centrifuge for 1 min. at 8,000 g**
9. Discard the collection tube and place the filter tube into a clean collection tube.
10. Add **450 µl of the wash buffer** to the upper reservoir and **centrifuge for 1 min. at 8,000 g.**
11. Discard the collection tube and place the filter tube into a clean collection tube.
12. Repeat the washing step.
13. Discard the collection tube and place the filter tube into a clean collection tube. **Centrifuge for 10 seconds at full speed (~ 13,000 g)** to remove residual wash buffer.
14. Discard the collection tube and place the filter tube in a clean 1.5 ml microcentrifuge tube.
15. For the elution of nucleic acids, add **50 µl of prewarmed (72±2ºC.) sterile distilled water** to the upper reservoir (be careful not to use the Elution Buffer included in the kit for CSFV RNA). **Centrifuge for 1 min. at 8,000 g.**
16. **The microcentrifuge tube contains the eluted, purified DNA**, which can be used directly or store at <-10ºC (*expiry date: 12 months*) for future use or at 4±3ºC if is going to be used in the PCR procedures within 1-2 hours.

5.4. CRITICAL POINTS

Because of PCR is a highly sensitive technique, the most critical point along all the stpes including the extraction procedure is the considerable risk of carry-over contamination, and the false positive results that could be obtained in this situation. The contamination could be due to the ASFV itself present in the positive analysed samples or in the positive controls included in the DNA extraction procedure. **It is mandatory that personnel working on PCR follow and carry out some strict work rules in order to minimize the contamination risk associated to PCR technique**:

- All steps of sample analysis by PCR should be performed in separate locations, using equipment and material specific for each one: sample preparation, DNA extraction, PCR mix preparation, and analysis of PCR products by agarose gel electrophoresis.
- Personnel must work always with clean nitrile or latex gloves in the PCR laboratory.
- Whenever personnel goes into a different PCR area, should be remove the gloves and take clean ones.
- The material will be of exclusive use for the PCR procedure step in which is located/labeled.
- Use a new pippete tip each time that a tube containing any sample or DNA is manipulated.
- Tubes containing amplified product should never be opened and manipulated in other laboratory distinct to that exclusively assigned to their analysis by electrophoresis.

5.5. SECURITY MEASURES

- Read and follow carefully the complete procedure.
- Keep reagents to the appropriate temperature before and after use.
- Do not pool reagents or instructions from different kits.
- Avoid any contamination of reagents.
- Do not use any reagent after it expiration date has passed.

- Do not eat, drink or smoke in the laboratory.
- Do not pipette by mouth.
- Wear always protective disposable nitrile or latex gloves.
- Binding Buffer, included in DNA extraction kit, contains guanidinium hydrochloride and Triton X-100, which are irritants. Inhibitor Removal Buffer and Wash Buffer contain guanidinium hydrochloride which is an irritant. Do not let the Binding Buffer, Inhibitor Removal Buffer, or Wash Buffer touch your skin, eyes, or mucous membranes. If contact does occur, wash the affected area immediately with large amounts of water. If you spill the reagent, dilute the spill with water before wiping it up.