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SOP/CISA/ASF/IPT/1

STANDARD OPERATING PROCEDURE FOR THE DETECTION OF ANTIBODIES AGAINST AFRICAN SWINE FEVER BY INDIRECT IMMUNOPEROXIDASE TECHNIQUE

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1. PURPOSE

The main goal of this procedure is to describe the indirect immunoperoxidase technique for African swine fever virus (ASFV) specific antibody detection on kidney monkey cells infected with adapted ASFV isolates. This technique can be used as **confirmatory technique for positive and doubt ELISA results**.

2. SCOPE

This procedure is applicable to porcine samples, such as: blood, **sera, plasma, fluids exudate and homogenate tissues.**

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. AFRICAN SWINE FEVER. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). Chapter 3.8.1.OIE, 2019 (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.01_ASF.pdf)
2. Gallardo C, Nieto R, Soler A, Pelayo V, Fernández-Pinero J, Markowska-Daniel, Pridotkas G, Nurmoja I, Granta R, Simón A, Pérez C, Martín E, Fernández-Pacheco P, Arias M. Assessment of African Swine Fever Diagnostic Techniques as a Response to the Epidemic Outbreaks in Eastern European Union Countries: How To Improve Surveillance and Control Programs. J Clin Microbiol. 2015 Aug;53(8):2555-65.
3. Gallardo C, Soler A, Nieto R, Carrascosa AL, De Mia GM, Bishop RP, Martins C, Fasina FO, Couacy-Hymman E, Heath L, Pelayo V, Martín E, Simón A, Martín R,Okurut AR, Lekolol I, Okoth E, Arias M. "Comparative evaluation of novel African swine fever virus (ASF) antibody detection techniques derived from specific ASF viral genotypes with the OIE internationally prescribed serological tests". Vet Microbiol. 2013 Feb 22;162(1):32-43. doi: 10.1016/j.vetmic.2012.08.011. Epub 2012 Aug 18.
4. Pan IC, Huang TS, Hess WR. "New method of antibody detection by indirect immunoperoxidase plaque staining for serodiagnosis of African swine fever". J Clin Microbiol. 1982 Oct;16(4):650-5.

ASF REVIEWS:

1. Arias, M., Sánchez-Vizcaíno, J.M. (2012). African swine fever. In: Zimmerman, J., Karriker, L.A., Ramirez, A., Schwartz, K.J, Stevenson, G.W. (Eds), Diseases of swine, 10th Edition. John Wiley and Sons, United States of America, pp. 396-404.
2. Beltrán-Alcrudo, D., Arias, M., Gallardo, C., Kramer, S. &Penrith, M.L. (2017). *African swine fever: detection and diagnosis- A manual for veterinarians.* FAO Animal Production and Health Manual No.19. Rome. Food and Agriculture Production of the United Nations (FAO). 88 page. Available at http://fao.org/3/a-i7228e.pdf.
3. Gallardo C, Fernández-Pinero J, Arias M. *African swine fever (ASF) diagnosis, an essential tool in the epidemiological investigation.* Virus Res.2019 Oct 2;271:197676.

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of samples processing for African swine fever (ASF) diagnosis **(SOP/CISA/SAMPLE/1).**
- Procedure for growing and preparation of established cell lines for ASFV propagation **(SOP/CISA/ASFV CELLs/1).**
- Procedure for ASFV growing and titration in established cell lines. **(SOP/CISA/ASF/TITRATION/1**
- Procedure for the preparation of African swine fever virus-coated 96-well plates for indirect immunoperoxidase test (IPT) **(SOP/CISA/ASF/IPT-PLATES/1).**
- Procedure for the detection of antibodies against African swine fever by indirect ELISA **(SOP/CISA/ASF/ELISA/1).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
C.E.P: cytophatic effect
IPT: indirect immunoperoxidase technique
LC: limit control
m.o.i: multiciplity of infection
NC: negative control
PC: positive control
r.p.m: revolutions per minute
MS: monkey stable cells
VERO: monkey stable cells

4.2. BACKGROUND

The ASFV naturally infects monocytes-macrophages cells. The virus persistence experimentally induced in monkey stable cells (VERO and MS) has been described by the $ClNH_4$ and 5-iodo-2´-desoxiuridin. ASFV replication occurs in cell cytoplasm, although it is needed also the nucleus. Virus enters into the cell by endocytosis.

The **immunoperoxidase technique (IPT)** is an immune-cytochemistry technique on fixed cells to determine the antibody-antigen complex formation through the action of the peroxidase enzyme. In this procedure, VERO or MS cells are infected with ASFV adapted isolates to these cell cultures. The infected cells are fixed and are used as antigens to determine the presence of the specific antibodies against ASF in serum samples.

The IPT has been fully validated at the EURL as alternative confirmatory test for antibody detection to ASF infection either in serum, blood, plasma and exudate and homogenate tissue samples or in a large-scale survey of ASF. The sensitivity and specificity values of 98.20% and 98.95%, respectively are comparable to the Indirect immunofluorescence assays (IFI).

5. DESCRIPTION

Method Validation.

The assay is a validated reference method by CISA and included in the *Manual of Diagnostic Test and Vaccines for Terrestrial Animals (mammals, birds and bees). African swine fever.* OIE (World Organization for Anial Health), 2019, chapter 3.8.1

The Internal verification and quality control (internal and external), will be according the laboratory internal procedures.

5.1. EQUIPMENT AND MATERIALS

MATERIAL

- Analytical Balance (0.1 g).
- Adsorbent paper.
- Aluminium foil.
- Chronometer.
- Distilled water.
- Eppendorff tubes 0.5, 1.5 y 2 ml.
- Freezer <-10 ºC.
- Freezer <-70 ºC.
- Fridge 4 ±3ºC.
- Glass or plastic pipettes for volume of 1-25 ml.
- Latex or nitrile gloves.
- Microcentrifuge for eppendorf tubes.
- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.

- Multichannel pipette 5-50µl.
- Multichannel pipette 50-300 µl.
- Phase Contrast Inverted cell Culture Microscope.
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Reagent reservoir Polystyrene 50 ml.
- Shaker incubator 37±2ºC.
- Sterile glass bottle 250ml and 500 ml.
- Sterile plastic tubes 12 ml and 50 ml.
- Single channel pipettes 1-10 µl.
- Single channel pipettes 10-100 µl.
- Single channel pipettes 10-200µl.
- Single channel pipettes 200-1000µl.
- 96-well microtiter plates (Ref.167008 Thermo Scientific, Ref. 9102 Corning or similar characteristics)
- Vortex.

REAGENTS SUPPLIED BY THE EUROPEAN UNION REFERENCE LABORATORY (EURL-ASF).

- **ASFV-IPT:** ASF-*Coated 96-well plates fixed with ASFV adapted viruses (Ba71V VERO or E70MS) belonging to p72 genotype I. *Store at <-10ºC. Expiry date: 6 months* (plates in strips); 12 months (plates)
- **ASF-PC**: reference positive control serum supplied by the EURL-ASF in vials of 100 microlitres. Store at <-10ºC. *Expiry date: 18 months.*
- **ASF-LC or CV_3**: reference limit control serum supplied by the EURL-ASF in vials of 100 microlitres. Store at <-10ºC. *Expiry date: 18 months.*
- **ASF-NC**: reference negative control serum supplied by the EURL-ASF in vials of 100 microlitres. Store at <-10ºC. *Expiry date: 18 months.*
- **HRPO-Conjugate:** Protein A peroxidase 1mg/ml [REF. 0032400. PIERCE/THERMO or similar characteristics].

INIA-CISA, as EURL, produce ASF-CV_1, ASF-CV_2, ASF-CV_4,verification controls to be use in each assay only for internal use, not to supply externally.

**NOTE; The adapted ASFV Spanish isolates BA71V or E70MS can be supplied to produce the plates at local level according the procedure described in (SOP/CISA/ASF/IPT-PLATES/1).*

REAGENTS NOT SUPPLIED BY THE EURL-ASF

- **Acid acetic glacial** [Ref.: 141008.1611 (PANREAC) o características similares]
- **AEC** (3-aminoethil-carbazol) [Ref. SIGMA A6926 or similar characteristics]
- **Distilled water.**
- **Hydrogen peroxidase 30% (H_2O_2).**
- **N,N-Dimetil formamide** [Ref.: 1.029370500 (Merck) or similar characteristics]
- **Phosphate buffered saline (PBS 1x) pH 7.2**(±0.2 UpH)→ The PBS could be obtained in tablets [Ref.: 524650-1 (CALBIOCHEM) or similar characteristics] or could be prepared as follows:

ClNa [Ref. 1.06404.1000 (MERCK) or similar characteristics]	------	8.0 gr (±0.1)
ClK [Ref. 1.04936.0500 (MERCK) or similar characteristics]	------	0.2 gr (±0.01)
PO_4H_2K [Ref. 1.04873.1000 (MERCK) or similar characteristics]	------	0.2 gr (±0.01)
PO_4HNa_2 [Ref. 1.06586.0500 (MERCK) or similar characteristics]	------	1.15 gr(±0.05)
Distilled water	------	1,000 ml

Store at room temperature. Expiry date: 1 year.

- **Skim Milk Powder (Ref. 06-019-500 Scharlau o similar characteristics).**
- **Serum Fetal Bovine** (SFB) [Ref.: 91S1810-500 Linus or similar characteristics].
- **Sodium acetate tri-hydrated** [Ref.: 1.06267.0500 (Merck) or similar characteristics].
- **Tween-20** [Ref.: 8.22184.1000 (Merck) or similar characteristics].

Reagents preparation not included in this procedure, when necessary, will be described in the internal procedure for reagent preparation.

5.2. REAGENT PREPARATION.

- **Blocking solution**: PBS/0.05%Tween 20, pH 7.2 (±0.2 UpH) /milk 5% (must be prepared just before being used).
- **Conjugate**: resuspend in 200 µl of distilled water. Once reconstituted, aliquot and freeze at <-10ºC until using to avoid loss of titre. Before adding it to the

plate, *dilute at INIA-CISA recommended dilution* (specify for each batch) *in blocking solution (PBS 1x/0.05%* Tween-20, pH7.2/milk 5%). Prepare only the volume necessary for the plate because the volume not used must be discarded.

- **Substrate solution**; the substrate solution must be prepared when is going to be used. Mix 300 µl of stock solution in 5ml of acetate buffer + 5 µl of H_2O_2 (this volume is recommended for one 96 well plate)
  - Stock solution (20 mg/ one tablet AEC (3aminoetilcarbazol) in 2.5 ml dimetilformamide (keep at 4 ±3ºC in dark)
  - Acetate buffer 74 ml solution A+ 176 ml solution B. *Store room temperature. Expiry date 6 months.*
    - **Solution A**; 0.2N acid acetic glacial (1.155 ml acetic in 100ml water). *Store room temperature. Expiry date 6 months.*
    - **Solution B**; 0.2M sodium acetate (2.72 gr (±0.05) AcNa tri-hydrated in 100ml water). *Store room temperature. Expiry date 6 months.*

5.3. SAMPLE PREPARATION

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

5.4. METHODS

1. **Keep the ASFV-IPT plates at room temperature** (18-25ºC) for 30 minutes after defrosting.
2. **Block the plates** by adding 100µl/well of blocking solution. Incubate 1h at 37±2 ºC in continuous agitation**.**
3. During the blocking step (step 2), **pre-incubate the samples and ASF reference sera** (controls)* in a separate 96-well microtiter plates for **1h at 37±2 ºC in continuous agitation**, following this indications:
   a. **For routine confirmatory diagnosis**; prepare a **1/40 dilution** of the samples (serum, blood, tissue exudates and fluids) and ASF reference controls in blocking solution plus 2% of SFB (100 µl/well).
   NOTE: Tissue homogenate can be used at 1/5 dilution in case de sample not exudate.
   b. **For ASF antibody titration**; perform check board titration in two fold dilutions starting from 1/20 of the samples (1/5 in case of testing tissue´s homogenate) in blocking solution plus 2% of SFB (100 µl/well). Controls are included at 1/40 dilution.

**It is recommended to include the ASF reference control sera by duplicate.*

4. After 1h discard the blocking solution **from the ASFV-IPT-plates** and transfer to the plate with the multichannel pipette the **pre-incubated samples and reference sera**.
5. Incubate **45 minutes at 37±2 ºC** in continuous agitation.
6. **Wash three times** by adding 100µl/per well of PBS 1x for 5 minutes at 37±2 ºC in continuous agitation.
7. **Add 100 µl/well of conjugate at INIA-CISA recommended dilution in blocking solution.**
8. Incubate **45 minutes at 37±2 ºC** in continuous agitation.
9. **Wash three times** by adding 100µl/per well of PBS 1x for 5 minutes at 37±2 ºC in continuous agitation.
10. **Add 50 µl/well of substrate solution** and incubate 10 minutes at room temperature (18-25ºC) or until observe that NC begin to take colour.
11. Add adding 100µl/per well of PBS 1x for **stopping** the reaction. It is recommend to perform this step three times to avoid background. Leave the plate in the last PBS wash step.
12. **Read** in the microscopic.

5.5. INTERPRETATION OF THE RESULTS

NOTE: At the moment of reading results, each sample is analyzed as individual one comparing with control results. Include in each assay PC, LC and NC.

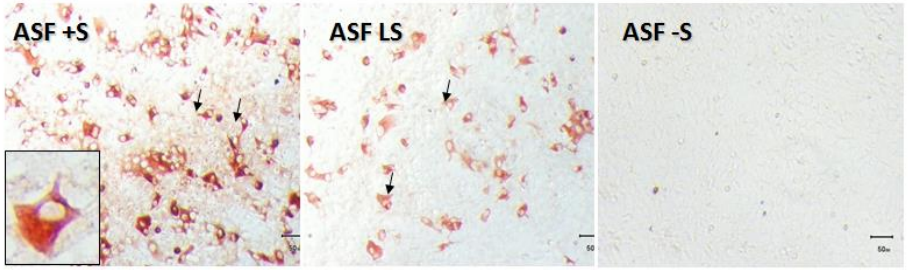
Assay validation:

The test is validated when there are an intensive red cytoplasmic coloration in positive control wells and an absence in case of negative controls. The limit control wells, must have less red coloration than the positive control. (In case of verification controls CV_1 to CV_4 must have a decreasing coloration, detecting, at least, weak red coloration in the CV_4).

Interpretation of the results:

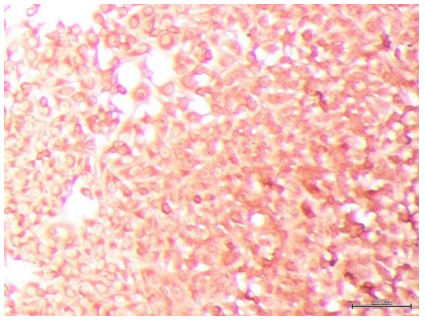
In the wells with positive serum samples against ASF, the Ab will be bind to the ASFV infected forming the Ab-Ag complex that is reveled through the action of the peroxidase with the substrate. An intensive red cytoplasmic coloration will be observed in infected cells.

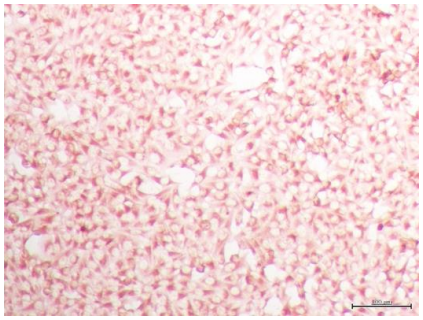
The intensive red cytoplasmic coloration is interpreted as a positive Ab result against ASF and the absence as negative result. A decreasing red coloration will be detected in verification control wells, with a weak positive result in CV_4 well.



5.6. CRITICAL POINTS

During last years, thousands of samples have been analyzed by IPT with an appropriate specificity and sensitivity for ASF serological diagnosis, but in some specific situations related to samples collected from vaccinated animals against other diseases, some slight background can be observed with a nonspecific red coloration in the wells. **In these cases the samples must be analyzed against non-infected cells in parallel with the infected cells.**





5.7. SAFETY CAUTIONS

- Read the protocol previously.
- Storage reagents at the adequate temperature.
- Avoid any reagent contamination.
- Do not use the reagents after the expiry date.
- Do not eat, smoke or drink while the manipulation of reagents.
- Always include PC, LC and NC.
- Do not pipette by mouth
- Use a new tip for each sample.

Worksheet CISA/ASF/IPT/1

ID REGISTER:
DATE:
TECHNICIAN:
ASF-IPT plates BACTH:
ASF-PC BACTH:
ASF-LC BATCH:
ASF-NC BATCH:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

COMENTS: