# CENTRO DE INVESTIGACION EN SANIDAD ANIMAL (CISA-INIA)

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# SOP/CISA/ASF/IB/1

## STANDARD OPERATING PROCEDURE FOR THE DETECTION OF ANTIBODIES AGAINST AFRICAN SWINE FEVER BY IMMUNOBLOTTING

## CONTENTS

## 1. PURPOSE

The main goal of this procedure is to describe the Immunoblotting technique to perform the African swine fever antibody detection. It is used as a confirmatory technique for positive and doubtful results obtained by ELISA.

*Currently this technique is included as OIE confirmatory ASF serological test in the Chapter 3.8.1. of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Edition.*

## 2. SCOPE

This procedure is applicable to porcine serum sample.

## 3. REFERENCES

### 3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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

As a basic reference for the elaboration of this procedure the criteria established in the next documents have been taken:

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. Interlaboratory comparison test organized by the EURL for African Swine Fever.

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2. Arias, M.; Escribano, J.M.; Sánchez-Vizcaíno, J.M.(1993). “Persistence of African swine fever antibody reactivity on ELISA and Immunoblotting assays”. Veterinary Record, 133, 189-190.
3. Escribano JM, Pastor MJ, Sánchez-Vizcaíno JM. (1989). “Antibodies to bovine serum albumin in swine sera: implications for false-positive reactions in the serodiagnosis of African swine fever”. Am J Vet Res. Jul; 50(7):1118-22.
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**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. Arias, M.; Sánchez, C.; González, M.A.; Carrasco, L. y Sánchez-Vizcaíno, J.M. (2002). “Peste porcina Africana” In “Curso digital de enfermedades infecciosas porcinas”. On line, July, 2002. [http://www.sanidadanimal.info/cursos/curso/7/7-ppa.htm]
3. Food and Agriculture Organization of the United Nations (FAO). AFRICAN SWINE FEVER: DETECTION AND DIAGNOSIS. A manual for veterinarians. FAO 2017 http://www.fao.org/3/a-i7228e.pdf

### 3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure for the production of Immunoblotting (IB) strips for African swine fever antibody detection **(SOP/CISA/ASF/SDS-PAGE/1).**

- Procedure of samples processing for African swine fever diagnosis **(SOP/CISA/SAMPLE/1).**
- **Procedure to obtain the african swine fever virus (ASFV) cytoplasmic soluble antigen (SOP/CISA/ASF/ASF-Ag/1/**

## 4. BACKGROUND INFORMATION.

### 4.1. ABBREVIATION.

Ag: ASF cytoplasmatic soluble antigen
ASF: African swine fever
ASFV: African swine fever virus
IB: Immunoblotting
PC: Reference positive control
LC: Reference limit control
NC: Reference negative control

### 4.2. BACKGROUND

Immunoblotting (IB) is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization, electrophoretic separation, and transferring of proteins onto membranes (usually nitrocellulose). The membrane is overlaid with a primary antibody for a specific target and then with a secondary antibody labeled. The IB technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive.

For the preparation of the ASF IB strips, the ASFV viral proteins, electrophoretically separated in SDS-PAGE gels, are transferred with a constant current intensity to the nitrocellulose filter. The filter is then cut into strips, which are blocked to sature the remaining protein binding sites. After blocking, the serum is added to allow the antibodies to react with the antigen strip. In the case of specific antibodies against ASF are present in the serum sample, the resulting inmunocomplexes will be visualized by addition of an A-peroxidase conjugate protein, and 4-chloro-1-naphtol as substrate.

The first viral proteins that induce ASF specific antibodies in pigs have been determined by study of the immune response to ASFV infection in *"in vivo"* inoculation experiments. These proteins invariably react by IB in all the infected animals. The molecular weights ($x10^{-3}$) of these proteins are: IP 12.5, IP 23.5, IP 25, IP 25.5, IP 30, IP 31, IP 34 and IP 35. These polypeptides begin to show positive reactions by IB when sera obtained at 7-9 days post ASFV infection are analyzed. The positive reaction by IB is maintained for several months after an ASF infection in the survived animals.

**The ASF-IB it is one of the OIE recommended confirmatory test for samples which have been positive and doubtful by ELISA tests and also in case of sera incorrectly handled or bad preserved (inadequate storage or transportation) when sample analysis by ELISA may yield up to a 20% false-negative results.**

## 5. PROCEDURE DESCRIPTION

**Method validation**:

Since 2003, the EURL organized an annual interlaboratory comparison test (ILCT) to evaluate the available diagnostic techniques at National Reference Laboratories level for ASF.
The panel of samples included in the ILCT must be analized using the routine laboratory techniques. The IB method described in this document has been validated throughout the ILCTs carried out during these years.

## 5.1. EQUIPMENT AND MATERIALS

### MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Aluminium foil.
- Chronometer.
- Distilled water
- Eppendorff tubes (0.5, 1.5 and 2 ml).
- Freezer <-10ºC.
- Freezer ≤-70ºC.
- Fridge 4±3ºC.
- Glass or plastic pipettes for volume of 1-10 ml.
- Latex or nitrile gloves.
- Micropipette disposable tips of 1-20, 20-200 and 200-1000 µl.
- Minincubation trays [Ref. 170-3902. BIORAD or similar characteristics].
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Reagent reservoir Polystyrene 50 ml.
- Shaker incubator 37±2ºC.
- Single channel pipettes 1-10 µl.
- Single channel pipettes 10-100 µl.
- Single channel pipettes 10-200µl.
- Single channel pipettes 200-1000µl.
- Sterile plastic tubes (5ml, 10ml, 50 ml).
- Timer.
- Vortex.

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

- **ASF- IB:** ASF Immunoblotting strips. *Storage: room temperature. Expiry date: 24 months.*
- **ASF-PC**: reference positive control serum supplied by the EURL-ASF in lyophilized vials of 0.5 ml, 1 ml or 2 ml.
  - Prior to reconstitution: stored at 4±3ºC. *Expiry date: 2 years.*
  - Reconstituted: reconstitute with distilled water in the corresponding volume indicated in the vial. Once rehydrated store at <-10ºC. *Expiry date: 18 months.*
- **ASF-LC**: reference limits control serum supplied by the EURL-ASF in lyophilized vials of 0.5 ml, 1 ml or 2 ml.
  - Prior to reconstitution: stored at 4±3ºC. *Expiry date: 2 years.*
  - Reconstituted: reconstitute with distilled water in the corresponding volume indicated in the vial. Once rehydrated store at <-10ºC. *Expiry date: 18 months.*
- **ASF-NC**: reference negative control serum supplied by the EURL-ASF in lyophilized vials of 0.5 ml, 1 ml or 2 ml.
  - Prior to reconstitution: stored at 4 ±3ºC. *Expiry date: 2 years.*
  - Reconstituted: reconstitute with distilled water in the corresponding volume indicated in the vial. Once rehydrated store at <-10ºC. *Expiry date: 18 months.*
- **HRPO-Conjugate:** Protein A peroxidase 1mg/ml [REF. 0032400. PIERCE/THERMO o similar characteristcs). Storage: 4±3ºC lyophilized; once reconstitute aliquot and freeze at <-10ºC. *Expiry date: indicate in the vial.*

### REAGENTS NOT SUPPLIED BY THE EURL-ASF.

- **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.*

- **Phosphate buffered saline (PBS1x)/Tween-20, pH 7.2**$_{(\pm 0.2\ UpH)}$→ Dissolve 0.5 ml of Tween-20 in 1 liter of PBS 1x pH 7.2. $_{(\pm 0.2\ UpH)}$. *Store at room temperature. Check the pH before using. Expiry date: 1 year.*
- **Skim Milk Powder.**
- **Substrate solution :**
  - **Hydrogen peroxidase 30% ($H_2O_2$).**
  - **4-chloronaphtol** [Ref.: C8890 (Sigma) or similar characteristics]
  - **Methanol** [Ref.: 1.06009.1000 (Merck) or similar characteristics]
  - **PBS 1x**
- **Tween-20** [Ref.: 8.22184.1000 (Merck) or similar characteristics]

## 5.2. REAGENT PREPARATION

- **ASF reference sera (PC, LC and NC)**: resuspend the reference sera with distilled water in the proportion indicated in the vial. Once reconstituted, aliquot and freeze at <-10ºC until using to avoid loss of titre. Before adding to the strips, *dilute 1/40 in blocking-washing solution*.
- **Blocking-washing solution:** PBS1x/0.05%Tween 20, pH 7.2$_{(\pm 0.2\ UpH)}$ /milk 2%.
- **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 strips *dilute 1/1000 in blocking-washing solution*.Prepare only the volume necessary for the plate because the volume not used must be discarded.
- **Substrate solution** (must be prepared just before being used). Prepare only the volume required, because the volume not used must be discarded.
  - Dissolve 6 mg$_{(\pm 0.0005mg)}$ of 4-chloronaphtol in 2 ml of Methanol.
  - Add slowly 4-chloronaphtol/Methanol solution to 10 ml of PBS 1x buffer pH 7.2$_{(\pm 0.2\ UpH)}$, with vigorous agitation (a characteristic precipitate is formed).
  - Then, add 4 µl of $H_2O_2$ 30% to the PBS/4-chloronaphtol solution.

## 5.3. SAMPLE PREPARATION

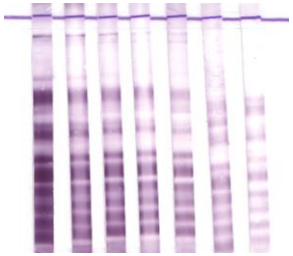
Sample preparation is performed according is described in the sample s processing procedure for ASF diagnosis **(SOP/CISA/ASF/SAMPLE/1).**

## .4. METHODS

1. **Blocking the strips**: The nitrocellulose strips are incubated with blocking solution (0.4 ml/ per strip) for **30 minutes at 37±2 ºC** in continuous agitation. This step is for blocking strips and avoids nonspecific unions of other proteins.
2. **Addition of sera:** Discard the blocking solution and add 0.4 ml/per strip of test and reference sera (PC, NC and LC) diluted **1/40 in blocking solution**. Incubate for **45 minutes at 37±2 ºC** in continuous agitation.
3. **Washing step**: Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation 37±2 ºC.
4. **Addition of HRPO-conjugate:** Add 0.4 ml/per strip of protein A-peroxidase conjugate **diluted at 1/1000 in blocking solution**. Incubate for **45 minutes at 37±2 ºC** in continuous agitation.
5. **Washing step**: Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation 37±2 ºC.
6. **Add 0.4 ml /per strip of substrate solution** [4-chloronaphtol/Methanol/$H_2O_2$] **(prepared as indicated in section 5.2) and** incubate for approximately 5-10 minutes at room temperature (18-25ºC) or until NC begin to take colour
7. **The reaction is stopped** after 5-10 minutes, with distilled water.

## 5.5 ANALYSIS AND INTERPRETATION OF THE RESULTS.

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



Sera showing a specific pattern of reaction similar as the antigen strips stained with positive control serum will be considered as positive to the ASF antibodies. Any ELISA- ambiguous or positive serum that does not clearly react with the proteins mentioned before will be considered negative to ASF antibodies.

In the next figure is showed the characteristic pattern obtained using the reference positive control serum diluted from 1/2 to 1/128 in negative sera. The dilutions 1/32 and 1/64 corresponds with de LCs supplied by the EURL-ASF

**Note:** *visualization of specific bands, but not the complete specific pattern showed by the control positive serum.* In ASF endemic areas with a presence of chronic forms and ASF-carrier pigs, a positive reaction of the sera against individual (but not all) viral proteins included in the antigen strip could occur. Similarly could occur at early stages of the ASF infection (7-10 days post infection) with the visualization of antibodies against ASFV early proteins such as Vp12 or Vp30. These cases should be only considered under the specific situations in endemic areas and must not be considered in ASF-free areas.

***If in the assay appears un-specific spots in the IB strips the assay is considered not validated and must be repeated.***

## 5.6. CRITICAL POINTS

In the last years a large number of sera have been tested with this technique with good results of specificity and sensitivity for the diagnosis of ASF. But there are some critical points which must be considered:

- **False-negative reactions**: badly conserved sera and/or undergone putrefaction can origin false negative reaction in IB technique because the samples lost positivity, although it is maintain when is used the ELISA test.
- **False-positive reactions**: sera from animals vaccinated against other viruses can origin false positive reaction against cellular proteins represented in the IB strips produced with ASF-semipurified virus. In these cases, sera can be analyzed by alternative confirmatory tests such as Indirect Immunoperoxidase test (IPT) or Indirect Inmunofluorescence test (IFI).
- **Conjugate type (protein A- peroxidase conjugated):** previous studies performed at the EURL, showed that type of conjugate is important for the sensitivity of the assay. The EURL recommends the use of this type of conjugate for the IB technique using the strips supplied by the EURL.

## 5.7. SAFETY CAUTIONS

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

***Worksheet CISA/ASF/IB/1***

**ID REGISTER:**
**DATE:**
**TECHNICIAN:**
**ASF-IB BACTH:**
**ASF-PC BACTH:**
**ASF-LC BATCH:**
**ASF-NC BATCH:**

**ID SAMPLES**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | |
| **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** |
| | | | | | | | | | | | | | | | |

## RESULTS

**COMMENTS:**