

# RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA-INIA)

**SOP/CISA/ASF/IB/1/2008**

**(INMUNOBLOTTING OIE FOR SEROLOGICAL  
DIAGNOSIS OF AFRICAN SWINE FEVER)**

**Rev. 1**

**Date: December 2008**

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## 1. PURPOSE

The main goal of this procedure is to describe the Immunoblotting technique to perform the African swine fever antibody detection.

*Currently this technique is included as OIE confirmatory ASF serological technique in the Chapter 2.6.6 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 Edition.*

## 2. SCOPE

This procedure is applicable to porcine serum sample.

## 3. REFERENCES

### 3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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

- *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees)*. Chapter 2.8.1. OIE, six edition 2008. [[http://www.oie.int/Eng/Normes/Mmanual/2008/pdf/2.08.01\\_ASF.pdf](http://www.oie.int/Eng/Normes/Mmanual/2008/pdf/2.08.01_ASF.pdf)]
- Annual Interlaboratory comparison tests (ILCTs) of Community reference laboratory for ASF
- PG/LCV/001 Procedimiento para la elaboración de documentos, Edición 01.
- Alcaraz C, De Diego M, Pastor MJ, Escribano JM. (1990). Comparison of a radioimmunoprecipitation assay to immunoblotting and ELISA for detection of antibody to African swine fever virus. *J Vet Diagn Invest.* Jul;2(3):191-6.
- 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.
- 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.
- Pastor M. J., Laviada M. D., Sánchez-Vizcaíno J. M. and Escribano J.M. (1989). Detection of African swine fever virus antibodies by Immunoblotting assay. *Can. J. Vet. Res.* 53, 105-107.

#### ASF REVIEWS:

- Arias, M.; Sánchez-Vizcaíno, J.M. (2002). “African Swine Fever (ASF)”. In *Trends in Emerging Viral Infections of Swine*. Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 119-124.
- Arias, M.; Sánchez-Vizcaíno, J.M. (2002). “African Swine Fever Eradication: The Spanish model. In *Trends in Emerging Viral Infections of Swine*”. Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 133-139.
- 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*. [[www.sanidadanimal.info](http://www.sanidadanimal.info)] on line, July, 2002/.

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### 3.2. DOCUMENTS TO USE IN COMPLAIN

- Procedure for the obtaining of ASF cytoplasmatic soluble antigen (SOP/CISA/ASF/AgASFV/1/2008)
- Procedure for the obtaining of nitrocellulose strips with the cytoplasmatic soluble antigen of ASF transferred (SOP/CISA/ASF/SDS-PAGE/1/2008)

## 4. BACKGROUND INFORMATION

### 4.1. ABBREVIATION

Ab: antibody  
 ASF: African swine fever  
 ASFV: African swine fever virus  
 CS-Ag: ASF cytoplasmatic soluble antigen  
 IB: Immunoblotting  
 NC: Reference negative control  
 PC: Reference positive control

### 4.2. BACKGROUND

Immunoblotting (western blotting) 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 immunoblotting technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive. It is recommended in case of sample sera incorrectly handled or bad preserved (inadequate storage or transportation) when simple analysis by ELISA may yield up to a 20% false-negative results.

The viral proteins, electrophoretically separated in PAGE gels, are transferred with a constant current intensity to the nitrocellulose filter. The filter is then cut into strips, which are blocked to saturate 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 were present in the serum sample, the resulting immunocomplexes will be visualized by addition of an A-peroxidase conjugate protein, and 4-chloro-1-naphthol 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 Immunoblotting in all the infected animals. The molecular weights ( $\times 10^{-3}$ ) of these proteins are: IP 23.5, IP 25, IP 25.5, IP 30, IP 31, IP 34 and IP 35. These polypeptides begin to show a positive reaction by Immunoblotting when sera obtained at 7-9 days post ASFV infection are analysed. The positive reaction by Immunoblotting is maintained for several months after an ASF infection in the survived animals.

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## 5. DESCRIPTION

### 5.1. EQUIPMENT AND MATERIALS

#### Materials

- Analytical Balance
- Absorbent paper
- Disposable tips (1-10 µl, 1-200µ, 100-1000 µl).
- Chamber 37°C
- Chronometer
- Distilled water
- Eppendorff (0.5, 1.5 and 2 ml)
- Glass or plastic pipettes for volume of 1-10 ml
- Minincubation trays [Ref. 170-3902. BIORAD].
- Nitrile Gloves
- Ph meter
- Pipetboy acu or equivalent
- Reagent reservoir Polystyrene 50 ml [COSTAR Ref. 4870].
- Shaker plate
- 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 (10ml, 50 ml).
- Table centrifuge
- Vortex

#### Reagents

- **IB Strips:** supplied by ASF reference laboratory. *Storage: room temperature. Expiry date: 12 months.*
- **PC:** positive control supplied at ASF reference laboratory in lyophilized vials of 0,5 ml, 1 ml or 2 ml. *Storage: 4°C lyophilized; Once reconstitute, aliquot and freeze at -20°C. Expiry date: 18 months.*
- **NC:** negative control supplied at ASF reference laboratory in lyophilized vials of 0,5 ml, 1 ml or 2 ml. *Storage: 4°C lyophilized; Once reconstitute, aliquot and freeze at -20°C. Expiry date: 18 months.*
- **Conjugate:** Protein A peroxidase 1mg/ml [REF. 0032400. PIERCE]. *Storage: 4°C lyophilized; once reconstitute, aliquot and freeze at -20°C. Expiry date: indicate in the vial.*
- **PBS-0.05% Tween 20 buffer pH 7.2**

ClNa	[Merck 1.06404]	-----	8,0 g
ClK	[Merck 1.04873]	-----	0,2 g
PO <sub>4</sub> H <sub>2</sub> K	[Merck 1.06586]	-----	0,2 g
PO <sub>4</sub> HN <sub>a</sub> <sub>2</sub>	[Merck 1.04936]	-----	1,15 g
Tween-20	[Merck Ref. 8.22184]	-----	0,5 ml
H <sub>2</sub> O distilled		-----	1000 ml

Check the pH before use. *Store at room temperature.*

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- **Blocking buffer:** PBS/0.05% Tween 20, pH 7,2/milk 2%; *Store at -20°C in aliquots*
- **Substrate solution:** Methanol [MERCK Ref.1.06009]; H<sub>2</sub>O<sub>2</sub> 30% [Panreac Ref. 141077] and 4-chloronaphtol 2.5 gr [SIGMA. Ref. C-8890]. *Storage: room temperature*
- **Washing buffer ; PBS-0.05%Tween 20 / Milk 2% buffer.** Non fat dry milk (NESTLÉ- Sveltesse or Molico); 20 g in 1L of PBS-0.05%Tween 20 buffer pH 7.2, *Store at -20°C in aliquots.*

## 5.2. PREPARATION

### REAGENTS PREPARATION:

- **Controls (PC, and NC):** resuspend controls with distilled water in proportion indicated at the vial. Make aliquots of them and freeze at -20°C. Before add controls to the IB strip, **diluted it 1/40** in PBS-0.05%Tween /2% milk
- **Conjugate:** resuspend in 200 µl of destiled water. Before add it to the IB strip, dilute 1/100 in PBS-0.05%Tween /2% milk
- **Substrate solution;** the substrate solution must be prepared when is going to be used
  - Dissolve 6 mg of 4-chloronaphtol in 2 ml of Methanol.
  - Add slowly 4-chloronaphtol/Methanol solution to 10 ml of PBS buffer pH 7.2, with vigorous agitation (a characteristic precipitate is formed).
  - Then, add 4 µl of H<sub>2</sub>O<sub>2</sub> 30% to the PBS/4-chloronaphtol solution.

### SAMPLE PREPARATION:

**Sample:** blood without anticoagulant or animal serum. Each sample must be identifying with a register number at CISA and with an identification number (sample ID) in case there are more than one sample from the same sender.

1. If sample is blood without anticoagulant must be incubated 1 hour at 37°C and after that, overnight at 4°C for the separation of the coagulum.
2. Discard the coagulum and centrifuge in a table centrifuge [Megafuge 1.0R rotor Heraeus #7570] at 780g (1.500 r.p.m) during 10 minutes.
3. Supernatant put in an eppendorf, which its register entry numbers CISA and the ID of the sample.

## 5.3. METHODS

1. **Blocking the strips:** The nitrocellulose strips are incubated with blocking solution (0.5 ml) for 30 minutes in continuous agitation. This step is for blocking strips and avoid other proteins can fix to them.
2. The **blocking solution** is discard and then it is added 0.5 ml of the test or control serum at a 1/40 dilution in blocking solution. This mixture is incubated for 45 minutes at 37°C, in continuous agitation.
3. **Washing step:** Wash four times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.
4. **Protein A-peroxidase conjugate** (PIERCE) is added (0.5 ml) at 1/1000 dilution -working dilution- in blocking solution. Incubated for 45 minutes at 37°C, in continuous agitation.
5. **Wash** 4 times in blocking buffer; the final wash should be for 5 minutes with continuous agitation.

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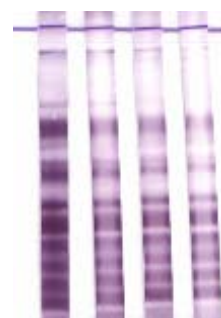
6. The substrate **4-Chloronaphthol** solution (0.5 ml) is added.
7. **The reaction is stopped** after 15 minutes, maximum, with distilled water.

## 5.4. RESULTS

**NOTE:** At the moment of reading results, each strip is analyzed as individual one comparing with control results. Include in each assay PC 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-positive serum that does not clearly react with the proteins mentioned before will be considered negative to ASF antibodies.

**Note:** visualization of specific bands, but not the complete specific pattern showed by the control positive serum.: In endemic areas with a presence of chronic forms and ASF-carrier pigs, a positive reaction of the sera to only one or several (but not all) viral proteins included in the antigen strip could occur. Similarly, at the very beginning of the ASF infection (7-10dpi.) when specific antibodies initiate its appearance, two or three specific bands (but not all) could be visualized. These cases should be only considered under the specific situations mentioned. In these cases, It should be also taken into account that non viral proteins are also included in the antigen strip (coming from the ASF antigen production process), what may lead to a false positive interpretation.



## 5.5. CRITICAL POINTS

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

- **False-negative reactions:** badly conserved sera can origin this type of reaction, because through the time, lost their positivity.
- **False-positive reactions:** sera from animals vaccinated against other viruses can origin this reaction against cellular proteins of the IB strips. In these cases, sera are analyzed by alternative immunoblotting techniques based in recombinant proteins as antigen.
- **Conjugate type (protein A- peroxidase Conjugated):** previous studies performed in CISA-INIA, showed that type of conjugate is important for the sensitivity of the ELISA. The use of protein A-Peroxidase Conjugated of PIERCE, increase the sensibility of the ELISA. We recommend the use of this conjugate for the immunoblotting technique, using strips supplied by CISA-INIA.

## 5.6. 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.
- Always include PC and NC