CENTRO DE INVESTIGACION EN SANIDAD ANIMAL (CISA-INIA)

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SOP/CISA/ASF/ELISA/1

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

CONTENTS

1. PURPOSE

The main goal of this procedure is to describe the indirect ELISA technique to perform the African swine fever (ASF) antibody detection.

Currently this technique is included as the OIE prescribed ASF serological technique for international trade in the Chapter 2.8.1. of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 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 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 2.8.1. OIE, 2012 [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.01_ASF.pdf]
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3. Escribano, J.M.; Pastor, M.J.; Sánchez-Vizcaíno, J.M. (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. 50 (7), 1118-1122.
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8. Sánchez-Vizcaíno, J.M.; Tabarés, E.; Salvador, E.; Sánchez Botija, A. (1982). "Semipurified structural viral protein for the detection of African swine fever antibodies by the indirect ELISA technique". Current Topics in Veterinary Medicine and Animal Science, 22, 214-222.

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). RECOGNIZING AFRICAN SWINE FEVER. A FIELD MANUAL. 2000 Edition. [http://www.fao.org/docrep/004/X8060E/X8060E00.HTM]

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

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

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
Ag: ASF cytoplasmatic soluble antigen
CO: cut off
PC: Reference positive control
LC: Reference limit control
NC: Reference negative control
OD: optical density
IB: Immunoblotting
r.p.m.: revolutions per minute

4.2. BACKGROUND

ASF-specific antibody detection is recommended for **subacute and chronic forms** as well as for large-scale testing and ASF eradication programmes, for several reasons:

- antibodies are **rapidly produced in the infected pig**. In these pigs antibodies are usually detectable in serum samples from seven to ten days after infection;
- **no vaccines** are available against ASF. This means that ASF-specific antibodies are only induced by ASF virus infection;
- the **long-lasting antibodies response**. In pigs that have recovered from the disease, specific antibodies can be detected at high levels for many months or even for the lifetime of some of these pigs.

Specific ASF antibodies of maternal origin can be detected in piglets during the first weeks of life. The half-life of maternal antibodies in piglets is about three weeks. If antibodies are found in piglets older than three months, ASF antibodies are very unlikely to be of maternal origin.

The technique more used for specific antibody detection of ASF is the ELISA, which is based in the use of antibodies or antigens labelled with an enzyme, so the resulting conjugates have enzymatic and immunologic activity. Being one of the components (antigen or antibody) labelled with an enzyme and insolubilized, the antibody-antigen reaction will be immobilized and it is easy develop by addition of specific substrate that could be read in a spectrophotometer.

There are a widely types of ELISA. In case of ASF, it is used **indirect ELISA.** The antigen is fixed in the plate. Samples with antibodies against ASF will recognise antigen so an antigen-antibody complex will be formed. After that, the conjugate is added and fix with the antigen-antibody complex. With several washing steps, all material not fixed is removed. Adding substrate we can obtain de result of the technique: develop of colour in wells, indicates ASF antibody presence.

The indirect ELISA it is the OIE recommended confirmatory test for international trade which has been fully validated by the EURL giving sensitivity and specificity values of 95.8% and 97.3% respectively. Large number of samples can be tested in a short time with this method.

5. DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Aluminium foil.

- Chronometer.
- 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.
- Multichannel pipette 5-50µl.
- Multichannel pipette 50-300 µl.
- NUNC-Polysorp microtiter plate [Ref: 475094 (Nunc)].
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Reagent reservoir Polystyrene 50 ml.
- Seal plates sealing film.
- Shaker incubator plate 37±2ºC.
- Single channel pipette 1-10µl.
- Single channel pipette 10-100µl.
- Single channel pipette 10-200µl.
- Single channel pipette 200-1000µl.
- Spectrophotometer UV/VIS with filter 620 nm annexed to a computer program to register and print results.
- Sterile plastic tubes (5ml, 10ml, 50 ml).
- Vortex.

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

- **ASFV-Ag:** ASFV semipurified soluble cytoplasmatic antigen supplied by the EURL-ASF in lyophilized vials of 0.5 ml or 1 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 aliquot and store at ≤-70ºC. *Expiry date: 18 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 limit 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]. *Storage:* 4±3ºC *lyophilized; once reconstitute aliquot and freeze at* <-10ºC*. Expiry date: indicate in the vial.*

REAGENTS NOT SUPPLIED BY THE EURL-ASF

- **Carbonate/bicarbonate buffer 0.05M pH 9.6;** Mix the carbonate/bicarbonate sodium buffers until pH9.6 (±0.2 UpH) with:
  - ⇑ **pH: Sodium bicarbonate ($NaHCO_3$) pH 8.3**(±0.2 UpH) **buffer →** Dissolve 2.93 gr (±0.05) of $NaHCO_3$ (Ref. 1.06329.1000 (MERCK) or similar characteristics) in 500ml of distilled water. *Store at room temperature. Expiry date: 1 year.*

- ⇓ **pH: Sodium carbonate (Na_2CO_3) pH 11**$_{(\pm 0.2\ UpH)}$ **buffer** → Dissolve 1.59gr $_{(\pm 0.05)}$ of Na_2CO_3 [Ref. 1.06392.1000 (MERCK) or similar characteristics] in 500ml of distilled water. *Store at room temperature. Expiry date: 1 year.*

- **Phosphate buffered saline (PBS 1x) pH 7.2**$_{(\pm 0.2\ UpH)}$ → 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 $_{(\pm 0.1)}$
ClK [Ref. 1.04936.0500 (MERCK) or similar characteristics]	------	0.2 gr $_{(\pm 0.01)}$
$P0_4H_2K$ [Ref. 1.04873.1000 (MERCK) or similar characteristics]	------	0.2 gr $_{(\pm 0.01)}$
$P0_4HNa_2$ [Ref. 1.06586.0500 (MERCK) or similar characteristics]	------	1.15 gr$_{(\pm 0.05)}$
Distilled water	------	1,000 ml

Store at room temperature. Expiry date: 1 year.

- **Substrate solution (DMAB-MBTH-H_2O_2)**
  - **Hydrogen peroxidase 30% (H_2O_2).**
  - **DMAB** (3-Dimethylaminobenzoic acid) [Ref. D-0787 (SIGMA) or similar characteristics]. Dissolve 13.3 gr $_{(\pm 0.1gr)}$ of DMAB in 900 ml of phosphate buffer 0.1M pH 7 $_{(\pm 0.2\ UpH)}$ [5.3 gr P04H2K + 8.65 gr P04HNa2 in 1L distilled water]. Mix during 1 hour at room temperature, adjust pH to 7 $_{(\pm 0.2\ UpH)}$ with NaOH 5M. Adjust the final volume to 1 litre. Filter and prepare aliquots of 10ml, 5ml and 3ml. *Store at <-10ºC in darkness. Expiry date: 1 year.*
  - **MBTH** (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate) [REF: 12973-9 (SIGMA) or similar characteristics]. Dissolve 0.36gr $_{(\pm 0.01gr)}$ of MBTH in 900 ml of phosphate buffer 0.1M pH 7 $_{(\pm 0.2\ UpH)}$ [5.3 gr $P0_4H_2K$ + 8.65 gr $P0_4HNa_2$ in 1L distilled water]. Mix during 1 hour at room temperature, adjust pH to 6.25 $_{(\pm 0.2\ UpH)}$ with concentrate HCl. Adjust the final volume to 1 litre. Filter and prepare aliquots of 10ml, 5ml and 3ml. *Store at <-10ºC in darkness. Expiry date: 1 year.*

- **Sulphuric Acid 95-97%** [Ref. 1.00731.1000 (MERCK)) or similar characteristics]

- **Tween-20** [Ref.: 8.22184.1000 (Merck) or similar characteristics]

5.2. PREPARATION.

5.2.1. SAMPLE PREPARATION.

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

5.2.2. REAGENTS PREPARATION

- **ASFV-Ag:** resuspend the Ag in distilled water in the proportion indicated in the vial. Once reconstituted, aliquot and freeze at ≤-70ºC until using to avoid loss of titre. Before adding it to the plate, dilute in carbonate/bicarbonate buffer 0.05M pH 9.6 $_{(\pm 0.2\ UpH)}$. The working dilution is provided by the EURL in the vial.

- **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 plate, *dilute 1/30 in washing solution*.

- **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 1/5000 in washing solution* Prepare only the volume necessary for the plate because the volume not used must be discarded.

- **Stop Solution (sulphuric acid 3N):** 16.1 ml of sulphuric Acid 95-97% in 183.9 ml distilled water. *Store at room temperature. Expiry date: 1 year.*

- **Substrate solution** (must be prepared just before being used). For one plate mix 10 ml of DMAB + 10 ml MBTH + 5µl of H_2O_2 30%. Prepare only the volume required, because the volume not used must be discarded.

- **Washing solution: Phosphate buffered saline (PBS1x)/Tween-20, pH 7.2** (±0.2 UpH) → Dissolve 0.5 ml of Tween-20 in 1 liter of PBS 1x pH 7.2 (±0.2 UpH). *Store at room temperature. Check the pH before using. Expiry date: 1 year.*

5.3. METHODS

1. **Sensitisation of microtitre plates with antigen**: Dilute the soluble antigen in carbonate/bicarbonate buffer pH 9.6 (±0.2 UpH) at the EURL-recommended working dilution. Add 100 µl per well of a NUNC-Polysorp microtiter plate. **Incubate at 4±3ºC for 16-18 h (overnight).**
2. **Washing plates:** Wash the plates four times with washing buffer. Plates are flicked free of antigen, then blotted onto paper towels**.** The sensitised and dry plates can be used directly or stored at <-10ºC for 18 months.
3. **Addition of sera:** add 100 µl/per well of test and reference sera (PC, NC and LC) diluted 1/30 in washing solution. Includes blank control well (100µl of washing solution). Test the reference sera and blank control well by duplicated. Incubate 1 hour at 37±2 ºC in agitation.
4. **Wash the plates:** Wash the plates four times with washing buffer. Then blot them onto paper towels.
5. **Addition of HRPO-conjugate:** add 100 µl/per well of protein A peroxidase conjugate, diluted at 1/5000 in washing solution. Incubate 1 hour at 37±2 ºC in agitation.
6. **Wash the plates:** Wash the plates four times with washing buffer. Then blot them onto paper towels.
7. **Addition of substrate solution:** add 200 µl/per well of substrate solution **[10 ml DMAB + 10 ml MBTH + 5µl of H2O2 30%]**. Incubate between 5-10 minutes at room temperature (18-25ºC) or until NC wells begin to take colour.
8. **Stop** the reaction by addition of 50 µl stopping solution per well.
9. **Reading plates**: The results can be obtained using a spectrophotometer UV/VIS to read microtitre plates at 620 nm wavelengths.

5.4. ANALYSIS AND INTERPRETATION OF THE RESULTS.

NOTE: At the moment of reading results, each plate is analyzed as individual: the reference controls are used to calculate the CO and the relation among the PC/NC value. In this way, sera will be analyzed respect to the controls of its plate.

VALIDATION OF THE TEST:

The test is validated when the optical density (OD) of the PC is, at least, 4 times greater than the OD of the NC:

OD PC / **OD NC**	**=> 4**

Taking into consideration the following criterions:

- **MEAN OD_{PC} ≥ 1.0** *(the tolerance interval for the OD values of duplicate PC must be +/-0.3)*
- **MEAN OD_{NC} ≤ 0.250** *(the tolerance interval for the OD values of duplicate NC must be +/-0.1)*
- **MEAN OD_{LC} ≥ CO – 0.1**
- **MEAN OD_{BLANK} ≤ 0.1**

Cut Off Calculation: To achieve a correct interpretation of the results it is necessary to calculate the CUT OFF which will define negative, ambiguous and positive sera.

The CUT OFF is calculated by a simple equation:

CUT OFF= MEAN OD_{NC} + [MEAN OD_{PC} x 0.2]

Interpretation of the results:

- Sera with OD **lower** than the **CUT OFF - 0.1** are considered as **negative sera.**
- Sera with OD **greater** than the **CUT OFF + 0.1** are considered as **positive sera.**
- Sera with OD **within the range CO – 0.1 ≤ OD sample ≤ CO + 0.1** are considered as **ambiguous sera**, and they have to be confirmed by alternative ASF confirmatory antibody detection technique.

5.4. CRITICAL POINTS

Although the OIE-indirect ELISA has been proved to be sensible and specific enough for ASF antibody detection there are some critical points:

- **Badly conserved sera could origin false-positive reaction** because the antibodies of these sera stick to the wells of the plate in nonspecific form, producing a positive result even without antigen fixed in the well. These sera are negative when are analysed by alternative ASF antibody confirmatory tests such as Immunoblotting or Indirect immunoperoxidase test. Even the incidence of these sera in the ASF diagnosis is very low, it is very important to extreme cautions when the samples are collecting and to store in appropriate conditions. In the case of sera collected from countries where is difficult to maintain the cold chain this critical point must be to take into consideration in the analysis of the ELISA results.

- **Type of immunoplates used for coating the ASF-Ag**: the type of plastic material is critical for the sensitivity and specificity of this technique. Studies performed at the EURL have demonstrated than greater sensitivity is obtained when POLYSORP 96 wells of NUNC plates are used.

- **Type of conjugate**: previous studies performed at the EURL, showed that type of conjugate is important for the sensitivity of the ELISA. The use of protein A-Peroxidase Conjugated of PIERCE, increase the sensitivity of the ELISA. We recommend the use of this kind of conjugate for the indirect ELISA described in this procedure.

5.5. SAFETY CAUTIONS

- Read the protocol previously.
- Avoid any reagent contamination.
- Do not use the components after expiration dates.
- 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.
- Substrate must be handle with care, it is very sensible to light and contamination.
- Stop solution is a strong acid. Handle with care.

Worksheet CISA/ASF/ELISA /1

ID REGISTER:
DATE:
TECHNICIAN:
ASF-Ag 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												

RESULTS:

MEAN OD POSITIVE CONTROL:
MEAN OD NEGATIVE CONTROL:
MEAN OD LIMIT CONTROL:
***CUT OFF*:**

COMENTS: