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SOP/CISA/ASF/ELISA/2

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

CONTENTS

1. PURPOSE

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

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.

In addition as a basic reference for the elaboration of this procedure the criteria established in the next document have been taken:

1. INGEZIM PPA COMPAC (11.PPA.K3): commercial ELISA kit, registration number by Ministry of Agriculture, Fisheries and Food, n° 335 RD July 2002. Ingenasa (Rer. 335RD (INGENASA)).

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- 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
CO: cut off
IB: Immunoblotting
NC: Reference negative control
OD: optical density
PC: Reference positive control

4.2. BACKGROUND

The kit is based on a blocking enzymatic immunoassay (blocking ELISA). A brief description of the technique is showed below:

The antigen is fixed in a solid support (polystyrene plate). When a serum sample contains specific antibodies against the virus, they will bind to the antigen absorbed on plate while if the serum sample does not contain specific antibodies they will not bind the antigen. When a specific monoclonal antibody (Mab) against the viral antigen coated to the plate (conjugated with peroxidase), it will compete with the antibodies of the serum. If the serum samples contains specific antibodies, they will not allow the binding of the labelled Mab to the antigen whereas if it does not contain specific antibodies the Mab will bind to the antigen on the plate. After washing the plate to eliminate all non-fixed material or absence of labelled Mab by adding the specific substrate that in presence of the peroxidase will develop a colorimetric reaction.

The antigen coated to the plate in our kit consists of a purified protein extract from the virus (the VP72), which is the major structural protein from the ASFV and the most antigenic one.

5. PROCEDURE DESCRIPTION

Method Validation.

The assay INGEZIM PPA COMPAC is a validated method by INGENASA, which results are collected in the corresponding "declaration of validated method".

In addition, 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

- Adsorbent paper.
- Aluminium foil.
- Centrifuge
- Chronometer.
- Distilled water
- Disposable tips for micorpipettes.
- Eppendorff tubes (0.5, 1.5 and 2 ml).
- Freezer <-10˚C.
- Freezer ≤-70˚C.
- Fridge 4±3˚C
- 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 96 wells [Ref: 475094 (Nunc)].
- Pipetboy acu or equivalent.
- Reagent reservoir Polystyrene 50 ml.
- Seal plates sealing film.
- Shaker incubator plate 37±2˚C.
- 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 450 nm annexed to a computer program to register and print results.
- Sterile plastic tubes (5ml, 10ml, 50 ml).
- Timer.
- Vortex.

KIT COMPOSITION:

- **96 well microtitration coated plates (divided in 12 strips of 8 wells each)**
- **PC**: inactivated positive control serum.
- **NC**: negative control serum.
- **Conjugate:** peroxidase conjugate 100x concentrated
- **Conjugate diluent (DE01-1) ready to use**
- **Serum diluent (DE31-1) ready to use**
- **Stop solution**
- **Substrate (TMB)**
- **Washing Solution 25x concentrated**

Storage all reagents at 4±3˚C. *Once opened, control sera are stable for one month between +2˚C and +8˚C. In case that they are not going to be used in this period, it is recommended to store in alicuots under -20˚C (Expire date: 12 months).*

5.2. REAGENT PREPARATION

- **Washing solution:** dilute one part of the concentrate washing solution provided in the kit with 24 parts of distilled or deionized water (i.e. 40 ml of concentrate plus 960 ml of dH2O). When ready, this solution remains stable between +2˚C and +8˚C until the expire date described at the label of the concentrated solution.
- **Positive and negative controls**: controls need to be diluted ½ in diluent prior to be used, in the same way than the sera samples. This dilution can be done directly into the plate by adding 50µl of diluent plus 50µl of control sera. Control sera are stable for 1 month at 4±3˚C (If they are not going to be used in this period, it is recommended to store them under -20˚C)
- **Conjugate**: to be prepared before use. Dilute the needed quantity of conjugate provided 1/100 into the supplied conjugate diluent (DE01). Shake very well the solution before the use. Prepare only the quantity needed for each time because the remainder volume has to be refected.

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. Before use, all reagents (except conjugate) must be allowed to come to room temperature.
2. Serum samples addition: if the dilution will be done directly in the plate, add 50µl of supplied serum diluent to each well and after, 50µ of each sample and controls, obtaining ½ dilution of samples. Shake gently to obtain a correct homogenation, avoiding the contamination between Wells. Test controls by duplicate. Seal the plate and incubate for 1 hour at 37 ±2˚C or overnight (18 hours) at 18-25˚C.
3. Washing step: wash 4 times as described in 5.6
4. Conjugate adition:add 100µl of specific conjugate prepared as previous instructions. Seal the plate and incubate for 30 minutes at 37 ±2˚C.
5. Washing step: wash 5 times as described in 5.6
6. Substrate addition: add 100µl of substrate to each well. It is recommended to use a multichannel pipette. Keep for 15 minutes at room temperature (20-25˚C)
7. Add 100µl of stop solution to each well in the same order as the substrate.
8. Read the OD of each well at 450 nm within 5 minutes after the addition of stop solution.

5.5. 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 NC/PC 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 NC is, at least, 4 times greater than the OD of the PC:

OD NC	**=> 4**
OD PC	

Taking into consideration the following criterions:

- **MEAN OD_{NC} ≥ 1.0**
- **MEAN OD_{PC} ≤ 0.250**

Tolerances intervals for the OD values of duplicates must be +/-0.1 in case of PC and NC. The assay will be considered as validated when fix all the criteria. Otherwise, the analysis should be repeated.

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. If samples are being analysed by duplicate, OD of the sample, will be calculated as the arithmetic mean of OD values in both wells.

The CUT OFF is calculated by:

CO POSITIVE= MEAN OD_{NC}-[(MEAN ODNC - MEAN ODPC) x 0.5]
CO NEGATIVE= MEAN OD_{NC}- [(MEAN ODNC - MEAN ODPC) x 0.4]

For calculating the blocking % (X%) of a sample:

$$X\% = \frac{\text{MEAN } OD_{NC} - \text{MEAN } OD_{SAMPLE}}{\text{MEAN } OD_{NC} - \text{MEAN}_{PC}}$$

Interpretation of the results:

- Sera with OD **lower** than the **CO positive** are considered as **postive sera.**
- Sera with OD **greater** than the **CO negative** are considered as **negative sera.**
- Sera with OD **within the range CO negative and CO positive** are considered as **doubtful sera**.

Sera considered as positive/doubtful, must have to be confirmed have to be confirmed by alternative ASF confirmatory antibody detection technique.

5.6. CRITICAL POINTS

1. **For washing steps can be used an automatic plate washer** or with micropipette to dispense 200µl/well. After the incubation times, the washing steps must be done following next instructions:
   i. Throw out the content of the plate by abrupt turnover of the plate to avoid material exchange between wells.
   ii. Dispense 200µl/well of washing solution.
   iii. Shake the plate delicately, avoiding contamination between wells.
   iv. Turn over the plate abruptly to empty the wells.
   v. Repeat the process as much times as is indicated on the instructions on the kit. Prior to empty the content of the las washing step, verify that the next reagent to be added to the plate is ready to use. Do not maintain the plate on dry more time than strictly needed.
   vi. After the last step of washing shake the plate turned over on an absorbent filter paper.
2. Do not use highly haemolysed or contaminated samples. This type of samples can give a false positive result.

5.7. SAFETY CAUTIONS

1. Read the protocol previously.
2. Keep the reagents at indicated temperature before use.
3. Avoid any reagent contamination.
4. Do not use the components after expiration dates.
5. Do not eat, smoke or drink while the manipulation of reagents.
6. Do not pipette by mouth.
7. Use a new tip for each serum sample.

8. Include a positive and negative control per assay.
9. Substrate must be handle with care, it is very sensible to light and contamination. Take the volume needed with a pipette or decantation and never re-use the remaining volume.
10. Stop solution is a strong acid. Handle with care.

Worksheet CISA/ASF/ELISA /2

ID REGISTER:
DATE:
TECHNICIAN:
BACTH:

	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:
POSITIVE *CUT OFF*:
NEGATIVE *CUT OFF:*

COMENTS: