CENTRO DE
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OIE
Reference Laboratory

SEROLOGICAL DIAGNOSIS OF AFRICAN SWINE FEVER BY OIE ELISA INDIRECT TEST

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OIE ELISA INDIRECT TEST for SEROLOGICAL DIAGNOSIS of AFRICAN SWINE FEVER

*Equipment required

- Chamber 37ºC
- ELISA reader with 620 nm wavelength filter.
- Analytical Balance
- Ph meter
- Table centrifuge
- Tubes shaker or vortex mixer

*General laboratory material

- Latex or nitrile gloves
- Permanent marker
- Racks for microcentrifuge tubes

1. MATERIALS AND REAGENTS

- Single channel pipettes 10, 200 and 1000 µl
- Multichannel pipettes
- Aluminium foil
- NUNC-Polysorp microtiter plate (ref: 469957.NUNC)
- Distilled water
- Reagent reservoir Polystyrene 50ml (ref: 4870.COSTAR)
- Steril plastic tubes (10ml, 50ml)

- **Ag**: Antigen supplied by ASF reference laboratory in lyophilized vials. Store once reconstituted at recommended dilution and freeze at -20ºC. Expiry date: 18 months.
- **PC**: reference positive control serum supplied by ASF reference laboratory in lyophilized vials. Store once reconstituted aliquots at recommended dilution and freeze them at -20ºC. Expiry date: 18 months.
- **LC**: reference limit control control serum supplied by ASF reference laboratory in lyophilized vials. Store once reconstituted aliquots at recommended dilution and freeze them at -20ºC. Expiry date: 18 months.

- **NC**: reference negative control serum supplied by ASF reference laboratory in lyophilized vials. Store once reconstituted aliquots at recommended dilution and freeze at -20ºC. Expiry date: 18 months.

- **CONJUGATE**: Protein-A peroxidase 1mg/ml (Pierce ref: 0032400 or SIGMA ALDRICH ref: P-8651)

- **CARBONATE/BICARBONATE BUFFER** 0,05M (pH 9,6):

Na2CO3 [Merck 1.06392]---------- 1.59 gr
NaHCO3 [Merck 10 6329]---------- 2.93 gr
Distilled water ---------- 1 L

Store at room temperature. Check the pH(9.6) before use:

- To increase pH: Sodium bicarbonate
- To decrease pH: Sodium carbonate

- **HYDROGEN PEROXIDASE** (H_2O_2): Use at 30 % diluted in distilled water.

- **WASHING SOLUTION**: PBS BUFFER pH 7.2/ TWEEN20 (0.05%):

PBS tablets (ref 524650 CALBIOCHEM) or house made:
ClNa [Merck 1.06404]---------- 8 gr
ClK [Merck 1.04873]---------- 0.2 gr
PO4H2K [Merck 1.06586] ---------- 0.2 gr
PO4HNa2 [Merck 1.04936] ---------- 1.15gr
H_2O distilled ----------until 1000 ml

+ Tween-20 [Merck 8.22184] ---------- 0.5 ml

Store at room temperature. Check the pH before use.

- **SUBSTRATE SOLUTION:**

Is made by DMAB and MBTH solutions (explained below). For the preparation of these two solutions, is previously needed to prepare the Phosphate buffer. For preparing 1l of DMAB and 1l of MBTH, 2 l of Phosphate buffer are required:

- Phosphate buffer 0.1 M pH 7:
  PO4H2K ---------- 5.3 g
  PO4HNa2 ---------- 8.65 g
  H_2O distilled ---------- 1 l

- DMAB -3- Dimethylaminobenzoic acid:

Add 13.315 gr DMAB in 900 ml of phosphate buffer 0.1 M pH 7
Mix during 1 hour at room temperature, adjust pH to 7 with NaOH 5M. Adjust the final volume to 1 L. Filter and prepare aliquots of 10ml (required volume for one plate) and 5ml (volume required for half plate). Store them at -20ºC in darkness.

- MBTH -3- Methyl-2-benthiazolinone hydrazone hydrochloride monohidrate:

Add 0.3646 gr MBTH in 900 ml of phosphate buffer 0.1 M pH 7.
Mix during 1 hour at room temperature, adjust pH to 6.25 with concentrate HCl. After that, adjust the final volume to 1 litre. Filter and prepare aliquots of 10ml (required volume for one plate) and 5ml (volume required for half plate). Store them at -20ºC in darkness.

- **STOP SOLUTION** (Sulphuric acid 3N):
  Sulphuric Acid ---------- 16.1 ml (in 200 ml distilled water).

  Store at room temperature.

2. METHODOLOGY:

❖ NOTE: Before the ELISA assay, plates must be sensitised with the ASF-antigen. In this video plates were already sensitised.

Sensitisation of microtiter plates with ASF antigen:

- Dilute the soluble antigen in carbonate/bicarbonate buffer pH 9.6 at the concentration recommended by the laboratory supplier.

e.g: (Ag 1/1600): 6.25µl of Ag + 9.99 ml carbonate/bicarbonate buffer pH 9.6

- Add 100 µl of diluted antigen per well of a NUNC-Polysorp microtiter plate.
- Incubate at 4 ºC for 18h (overnight)

The sensitised and dry plates can be stored at 4ºC for one day or at -20ºC for several months.

2.1 Wash the plates four times with washing buffer. Blot them onto paper towels.

2.2 Dilute test and control sera (1/30) in PBS/Tween-20 solution

- Label the **NON sensitised plate**, where we are going to dilute test and control sera.
- Add 96.6 µl of PBS-Tween-20 to the **NON sensitised** plates

- Add 3.4 µl of test and control sera.

2.3 Transfer 100 µl of each diluted serum to the sensitised plate wells (the one coated with the antigen). A recommended plate design includes duplicate control sera.

2.4 Cover the plate and incubate for 1 h at 37 ºC in agitation.

2.5 Wash the plates four times with washing buffer. Then blot them onto paper towels.

2.6 Conjugate preparation (1/5000):

Add 2µl Protein A to 10 ml (9998 µl) of PBS-tween20
(Volume for one plate)

2.7 Add 100 µl of conjugate per well.

2.8 Cover the plate and incubate for 1 h at 37 ºC in agitation.

2.9 Wash the plates four times with washing buffer. Then blot them onto paper towels.

2.10 Substrate solution preparation:

10ml of DMAB + 10 ml of MBTH + 5 µl H_2O_2 (30%)
(Volume for one plate)
Or

5ml of DMAB + 5 ml of MBTH + 2.5 µl H_2O_2 (30%)
(Volume for half plate)

2.11 Add 200 µl of substrate solution per well.

2.12 Cover the plate with aluminium foil. Incubate for 10-15 minutes in darkness at room temperature.

2.13 Stop the reaction by addition of 50 µl stop solution per well.

2.14 Reading plates. The results can be obtained using a spectrophotometer UV/VIS to read microtiter plates at 620 nm wavelengths.

3. INTERPRETATION OF THE RESULTS

○ VALIDATION OF THE TEST:

The test could be considered valid when the OD of the PC (Positive Control) is, at least, 4 times higher than the OD of the NC (Negative Control).

DO PC ≥4 DO NC

- Value of OD PC must be ≥ 1.0
- Value of OD NC must be ≤ 0.250
- Value of OD LC must be in range of Cut Off, with a value of OD up to 0,7.

If the test is not valid, the following steps are not necessary because no conclusions could be obtained from the OD results.

Once the test is valid, the cut off values must be calculated based on the following formulas. Finally, the OD values of each sample must be compared with both cut offs, being classified on greater, lower or between both cut offs, and consequently on positive, negative or doubtful sera.

○ CUT OFF CALCULATION:

CUT OFF= (OD negative serum) + (OD Positive serum x 0,2)

- Negative sera: OD below the CUT OFF -0,1.
- Positive sera: OD higher than CUT OFF + 0,100.
- Ambiguous sera: OD between CUT OFF +/- 0,100. They have to be confirmed by IB technique.

**Recommendation*: positive and doubtful results should be confirmed by confirmatory techniques: immunoblotting, indirect immunofluorescence or immnuoperoxidase test.

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