UNIVERSIDAD COMPLUTENSE DE MADRID
Facultad de Veterinaria
Departamento de Sanidad Animal

REFERENCE LABORATORY

STANDARD PROCEDURE OPERATION ELISA FOR SEROLOGICAL DIAGNOSIS FOR AFRICAN SWINE FEVER

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1. MATERIALS AND REAGENTS

Watch video (Indirect ELISA-OIE)

- Single channel pipettes 1-10 µl
- Single channel pipettes 10-100 µl
- Single channel pipettes 10-200 µl
- Single channel pipettes 200-1000 µl
- Adsorbent paper
- Aluminium foil
- Multichannel pipette 5-50µl
- Multichannel pipette 50-300µl
- NUNC-Polysorp microtiter plate (ref: 469957.NUNC)
- Chamber 37ºC
- Analytical Balance
- Distilled water
- Minincubationn trays (ref:170-3902.BIORAD)
- Ph meter
- Tubes shaker or vortex mixer
- Reagent reservoir Polystyrene 50ml (ref: 4870.COSTAR)
- Steril plastic tubes (10ml,50ml)
- Spectrophotometre UV/VIS with filter 620 nm annexed to a computer program to register result.
- Table centrifuge
- Latex or nitrile gloves

• **Ag**: Antigen supplied by ASF reference laboratory in lyophilized vials of 0,5 ml, 1ml or 2ml. Store once reconstituted aliquot and freze at -20ºC. Expiry date: 18 months.
• **PC**: reference positive control serum supplied by ASF reference laboratory in lyophilized vials of 0,5 ml, 1ml or 2ml. Store once reconstituted aliquot and freeze at -20ºC. Expiry date: 18 months.
• **LC:** reference limit control control serum supplied by ASF reference laboratory in lyophilized vials of 0,5 ml, 1ml or 2ml. Store once reconstituted aliquot and freeze at -20ºC. Expiry date: 18 months.
• **NC:** reference negative control serum supplied by ASF reference laboratory in lyophilized vials of 0,5 ml, 1ml or 2ml. Store once reconstituted aliquot and freeze at -20ºC. Expiry date: 18 months.
• **CONJUGATE:** Proteina A peroxidase 1mg/ml

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

1,59 gr ---------- Na_2CO_3 [Merck 1.06392]
2,93 gr ---------- $NaHCO_3$ [Merck 10 6329]
1 L. ---------- Distilled water

Store at room temperature. Check the pH(9,6) before use:

⇧ pH: Sodium bicarbonate

⇩ pH: Sodium carbonate

- **HYDROGEN PEROXIDASE (H2O2)**

To use at 30 %.

- **WASHING SOLUTION; PBS-TWEEN BUFFER Ph 7,2:**

ClNa [Merck 1.06404]---------- 8 gr
ClK [Merck 1.04873]---------- 0,2 gr
PO_4H_2K [Merck 1.06586] ---------- 0,2 gr
PO_4HNa_2 [Merck 1.04936] ---------- 1,15gr
Tween-20 [Merck 8.22184] ---------- 0,5 ml
H_2O destilled ---------- 1000 ml

Store at room temperature. Check the pH before use.

- **SUBSTRATE SOLUTION:**

❖ **DMAB** -3- Dimethylaminobenzoic acid:
Dissolve 13,315 gr DMAB in 900 ml of ***phosphate buffer 0,1 M pH 7***

-Phosphate buffer 0,1 M pH 7:
5,3 g ---------- PO_4H_2K
8,65 g ---------- PO_4HNa_2
1 l. ---------- H_2O destilled

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 and 5ml. *Storage at -20ºC in darkness*.

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

  Dissolve 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 and 5ml. *Storage at -20ºC in darkness*.

- **STOP SOLUTION (Sulphuric acid 3N):**

  Sulphuric Acid ---------- 16,1 ml (in 200 ml distilled water).
  Storage at room temperature.

2. METHODOLOGY:

Watch video (Indirect ELISA-OIE)

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

Sensitisation of microtiter plates with antigen:

- Dilute the soluble antigen in carbonate/bicarbonate buffer pH 9,6. at the recommended concentration.

e.g.: (Ag 1/1600): 6,25µl of Ag + 9,99 ml carbonate/bicarbonate buffer pH 9,6

- Add 100 µl 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

- Add 96,6 µl of PBS 0,5 tween-20 to the **NON sensitised** plates

- Label the NON sensitised plate, where we are going to dilute test and control sera.
- Add 3,4 µl of test and control sera.

2.3 Add 100 µl of each diluted serum in duplicate to plate wells. 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 9998 µl of PBS 0,5 Tween 20
(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)

2.11 Add 200 µl of substrate solution per well.

2.12 Cover the plate with aluminium foil. Incubate for approximately 5-10 minutes in darkness at room temperature or until observe that Negative Control begin to take colour.

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. 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.

- 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.