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SOP/CISA/ASF/ASF-Ag/1

STANDAR OPERATING PROCEDURE TO OBTAIN THE AFRICAN SWINE FEVER VIRUS (ASFV) CYTOPLASMIC SOLUBLE ANTIGEN

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1. PURPOSE

This procedure describes the semi purification of the African Swine Fever Virus (ASFV) to obtain the soluble cytoplasmatic semi purified antigen used as antigen in the indirect ELISA and Immunoblotting ASF antibody detection techniques.

2. SCOPE

This procedure is applicable to Monkey Stable cells infected with the African Swine Fever Virus E70 Spanish isolate adapted to Monkey Stable cells after 48 passages.

Currently this technique is included in the Chapter 2.8.1. of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012 Edition.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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ASF REVIEWS:

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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 for the detection of antibodies against African swine fever by indirect ELISA **(SOP/CISA/ASF/ELISA/1).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus

ASF-Ag: ASF cytoplasmatic soluble antigen
MS: monkey stable cells
m.o.i: mulciplity of infection
r.p.m.: revolutions per minute
IB: Immunoblotting
V_f: Final volumen

4.2. BACKGROUND

African swine fever virus (ASFV) replicates in the cytoplasm of *monkey stable cell line* (MS). The soluble cytoplasmic fraction is obtained after the infection of cell cultures with ASFV, in presence of porcine serum, and semipurified to be used as antigen in antibody detection techniques. The Spanish strain of ASFV isolated in 1970 (*E70*) and adapted to grow in a *monkey stable cell* line (MS) is the virus used for antigen production.

In the ASFV semipurified antigen are represented those proteins that induces antibodies at early at late times post-infection and includes the most antigenic proteins p72, p54 and p30. Briefly, the proteins p243, p172, p73, p34, p30, and p12 induced antibodies soon after infection, whereas proteins such as p23.5 and p15 induced antibodies up to 12 days after infection. The protein p12, the major ASFV-induced protein in infected cells, seems to be the first protein to induce antibodies in infected pigs.

5. PROCEDURE DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- Analytical Balance.
- Adsorbent paper.
- Aluminium foil.
- Chronometer.
- Centrifuge [SORVALL RC6/ rotor SLA-1500 SUPER-LITE or similar characteristics].
- Centrifuge tubes.
- Cell cultures flasks, T25, T75 and T175 cm^2 flasks [Falcon].
- CO_2 (±0.5%) incubator/ 37±3ºC.
- Eppendorff tubes 0.5, 1.5 y 2 ml.
- Freezer <-10 ºC.
- Freezer <-70 ºC.
- Fridge 4 ±3ºC.
- Glass or plastic pipettes for volume of 1-25 ml.
- Laminar flow cabin class II.
- Latex or nitrile gloves.
- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.
- MiniSart filtre 0.45 µm (Ref. Sartorius 16555).
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Shaker incubator 37±2ºC.
- Sterile glass bottle 100, 250ml and 500 ml.
- Sterile plastic tubes 12 ml and 50 ml.
- Single channel pipettes 1-10 µl.
- Single channel pipettes 10-100 µl.
- Single channel pipettes 10-200µl.
- Single channel pipettes 200-1000µl.
- Table centrifuge [Megafuge1.0R (rotor Heraeus #7570) or similar characteristics].
- Ultracentrífuge [SORVALL WX ULTRA / 100 rotor SW-41-1 or similar characteristics].
- Vortex.

REAGENTS.

- **EMEM cell culture medium** [Ref BF-12-136F (BioWhittaker) or similar characteristics], Eagle Medium supplemented with 10% of porcine sera filtered with MiniSart filtre (0.45 µm) and sterile [Sigma P-9783 or similar characteristics], 1% non-essential amino acid solution 100x [Ref.: BE13-114E (BioWhittaker) or similar characteristics], 1%

glutamine [4mM] [Ref.: BE17-605E (BioWhittaker)] or similar characteristics], and gentamicin sulfate 50mg/ml [Ref.: 17-518Z (BioWhittaker) or similar characteristics].

- **Ethylenediaminetetra-acetic acid (EDTA)** [Sigma E-5391 or similar characteristics].
- **Hidrocloride acid fuming 37% GR for analysis (HCl)** [Merck 1.00317 or similar characteristics].
- **2-Mercapto-ethanol** [Merck Ref. 805740 or similar characteristics].
- **Monkey stable cells (MS)** [ATCC/ECACC 91070510].
- **Nonidet P40 (NP)** [Merck 492016 or similar characteristics].
- **Sucrose for biochemistry** [Merck 1.07687 or similar characteristics].
- **Sucrose for density gradient ultracentrifuge** [Merck 1.07654 or similar characteristics].
- **Sodium Chloride** [Merck 1.06404 or similar characteristics].
- **Thimerosal (NaCl)** [SigmaT 4687 or similar characteristics].
- **Tris (hydroxymethyl)-aminomethano** [Merck 1.08387 or similar characteristics].

5.2. PREPARATION

5.2.1. REAGENTS PREPARATION

- **EDTA 0.2M solution** → 0.90 gr $_{(\pm 0.01)}$ of EDTA in 10 ml of distilled water (V_f 10 ml). *Store at 4±3ºC. Expiry date 1 month.*
- **NaCl 5M solution** → 7.30 gr $_{(\pm 0.05)}$ of NaCl in 25 ml of distilled water (V_f 25 ml). *Store at room temperature. Expiry date 1 month.*
- **NP40 10% solution** →20 ml of NP40 in 180 ml of distilled water (V_f 200 ml). *Store at room temperature. Expiry date 3 months.*
- **Sucrose 0.34 M in Tris HCl 5mM pH8** $_{(\pm 0.2UpH)}$ →34.9 gr $_{(\pm 0.5)}$ sucrose for biochemistry in 1.5 ml of Tris HCl 1M until 300 ml of distilled water (V_f 300 ml). *Store at* <-10 ºC*. Expiry date 1 month.*
- **Sucrose 0.067 M en Tris HCl 5mM pH8** $_{(\pm 0.2UpH)}$ →6.88 gr $_{(\pm 0.05)}$ sucrose for biochemistry in 1.5 ml of Tris HCl 1M until 300 ml of distilled water (V_f 300 ml). *Store at* <-10 ºC*. Expiry date 1 month.*
- **Sucrose 64% (w/v) solution in Tris HCl 0.4M pH8** $_{(\pm 0.2UpH)}$ → Add 128 gr $_{(\pm 1)}$ of sucrose density gradient in 80 ml of Tris HCl 1M and complete with distilled water until 200 ml (V_f 200 ml). *Store at* <-10 ºC*. Expiry date 1 month.*
- **Sucrose 20% (w/w) solution in Tris HCl 50mM pH8** $_{(\pm 0.2UpH)}$ → Add 60 gr $_{(\pm 0.5)}$ of sucrose density gradient in 20 ml of Tris HCl 1M and complete with distilled water until the total mass equals 300 gr (w/w). *Store at* <-10 ºC*. Expiry date 1 month.*
- **TEN solution** → 175 µl Tris HCl 1M + 700 µl EDTA 0.2M + 250 µl β-mercaptoetanol+ 2,37 ml of distilled water. *It must be prepared when is going to be used.*
- **Tris HCl 1M pH8** $_{(\pm 0.2UpH)}$ (V_f 500 ml) → 60.57 g $_{(\pm 0.5)}$ Tris in 300 ml of distilled water (V_f 300 ml). Adjust pH to 8$_{(\pm 0.2UpH)}$ with HCl and complete volume with water. *Store at room temperature. Expiry date 3 months.*

5.3. METHODS

1. **Infect 80% confluent MS cells grown in T175 cm^2 flasks** with the adapted ASFV Spanish isolate E70MS48 at 10 m.o.i. in medium without serum.
2. **Incubate for two hours at 37±2 ºC** in continuous agitation (adsorption of the virus). After two hours complete the corresponding volume of the 175 cm^2 flasks with medium containing 2% pig serum.
3. **Incubate** for 36-48 hours at 37±3 ºC.
4. **Harvest the cells** at 36-48 hours post-infection, when the CPE is extensive, and centrifuge at 650 g for 5 minutes (1,500 r.p.m./ 20 minutes centrifuge SORVAL rotor SLA1500).
5. **Remove the** supernatant and w**ash the cell debris in 0.34 M sucrose solution (~ 20ml)** and centrifuge at 1,000 g for 5 minutes (1,500 r.p.m/10 minutes Megafuge1.0R (rotor Heraeus #7570) to pellet cells.
6. **Remove the** supernatant and resuspend the cell pellet in **0.067 M sucrose solution** (1.8 ml per 175 cm^2 flask), and leave for **10 minutes on ice** with agitation after 5 minutes.

7. **Add NP40 10%** solution to a final concentration of 1% (w/v), [**1/9** of the total volume] and leave for **10 minutes on ice** (with agitation after 5 minutes) to lyses the cells.
8. **Add 1/7** of the total volume of **sucrose 64% (w/v)** and centrifuge at **1,200 g for 10 minutes** [2,500 r.p.m./10 minutes table centrifuge Megafuge1.0R (rotor Heraeus #7570] to pellet nuclei.
9. Collect the supernatant and add **1/19** of the total volume of **TEN buffer** and **1/10** of the total volume of **NaCl 5M**.
10. Incubate for **15 minutes on ice**. In this step the viral particles are disaggregated and the NaCl maintain the isotonicity.
11. The total volume is distributed in the ultracentrifuge tubes over a layer of **20% (w/w) sucrose solution**.
12. **Centrifuge at 100,000 g** for 1 hour at 4 ±1 ºC [25,000 r.p.m./1h 5min SORVALL WX ULTRA / 100 rotor SW-41-1]
13. **Remove** the band immediately above the sucrose layer **(ASF-Ag)** and add **Thimerosal** for a final concentration of 0.1%. *Store at <-70 ºC in aliquots of 100µl. Expiry date 18 months.*

5.4. ANALYSIS AND INTERPRETATION OF RESULTS

The band immediately above the sucrose layer is the ASF semi purified antigen (ASF-Ag).

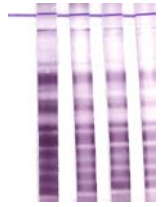
The **suitability of each batch of ASF-Ag** for the **OIE-indirect ELISA** (SOP/CISA/ASF/ELISA/1) must be demonstrated by *checkerboard titration* against the ASF reference positive, limit and negative sera (PC, LC and NC) to determine the optimal antigen dilution to use in coating ELISA plates. A new batch of ASF-Ag is consider as **optimal** when at **1:1600 dilution (0.5-0.9µg/per well)** and **using a single dilution of sera at 1/30,** the absorbance corresponding to positive reference sera (PC) is **4 times greater** than that corresponding to the negative reference serum (NC) and the limit control is within the cut off (CO) range estimated as is described in the SOP/CISA/ASF/ELISA/1.

Ag titration ↓

	1/30 dilution PC	LC	NC	*OPTICAL DENSITY (O.D 620nm)* 4	5	6
A	1/200	1/200	1/200	0.149	0.086	0.061
B	1/400	1/400	1/400	0.163	0.093	0.076
C	1/800	1/800	1/800	0.572	0.195	0.100
D	1/1600	1/1600	1/1600	1.460	0.429	0.157
E	1/3200	1/3200	1/3200	0.991	0.279	0.145
F	1/6400	1/6400	1/6400	0.771	0.238	0.135
G	1/12800	1/12800	1/12800	0.431	0.167	0.114
H	1/25600	1/25600	Blank	0.226	0.127	0.078

OD_{PC} = 1.460 ≥ 1.0	PC/NC = 9.29 > 4
OD_{NC} = 0.157 ≤ 0.250	
OD_{LC} = 0.457 ≥ CO (0.449) – 0.1	

The suitability of each batch of ASF-Ag also must be demonstrated in the Immunoblotting assay according is described in the procedure for the production of Immunoblotting (IB) strips for ASF antibody detection (SOP/CISA/ASF/IB-STRIPS/1). A new batch of ASF-Ag is consider as **optimal for using in the IB strips production** when the ASF-PC reacts against the ASFV viral proteins with molecular weights



($x10^{-3}$) ranged from IP 12.5 IP 23.5, IP 25, IP 25.5, IP 30, IP 31, IP 34 and IP 35, showing the specific pattern.

5.5. SECURITY MEASURES

- Do not use any reagent after it expiration date has passed.
- Do not eat, drink or smoke in the laboratory.
- Avoid any contamination of the cell culture.
- Do not pipette by mouth.
- Wear always protective disposable gloves Read and follow carefully the complete procedure.
- Keep reagents to the appropriate temperature before and after using.