RESEARCH CENTER FOR ANIMAL HEALTH AND SAFETY (CISA-INIA)

SOP/CISA/ASF/SDS-PAGE/1/2008

(STANDAR PROCEDURE OPERATION FOR PROTEIN SEPARATION BY DESNATURALIZATION ELECTROPHORESIS FOR AFRICAN SWINE FEVER)

Rev. 1

Date: December 2008

REV.	DATE	EPIGRAPH	CAUSE OF CHANGE

Performed	Reviewed	Approved
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1. PURPOSE

The main goal of this procedure is to describe the method to separate ASF protein by electrophoresis SDS-PAGE and its immunotransference to nitrocellulose to obtain IB strips.

2. SCOPE

This procedure is applicable to ASF soluble cytoplasmatic Ag.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

As a basic reference for the elaboration of this procedure it has been taken the criteria established in the next documents:

- PG/LCV/001 Procedure for the preparation of documents, Edición 01
- Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). Capítulo 2.8.1. OIE, sexta edición, 2008. [http://www.oie.int/esp/normes/mmanual/A_00035.htm]
- Interlaboratory Comparison Test performed by the Community Reference Laboratory.
- Towbin H. Staehelin T. Gordon J. (1979). Electrophoretic transfer of proteins from gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76, 4350-4354.
- Neal Burnette W. (1981). Western Blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein. A. Anal. Biochem. 112, 195-203.
- Gershoni J. M. and Palade G. E. (1983). Protein blotting: principles and applications Anal. Biochem. 131, 1-15.
- Jhonson T. K., Yuen L., Denell R. E. and Consigli R. (1983). Efficient transfer of proteins from acetic-Urea and isoelectric-focusing gels to nitrocellulose membrane filters with retention of protein antigenicity. Biochemistry, 133, 126-131.
- Pastor M. J., Laviada M. D., Sánchez-Vizcaíno J. M. and Escribano J.M. (1989). Detection of African swine fever virus antibodies by Immunoblotting assay. Can. J. Vet. Res. 53, 105-107

PPA reviews:

- Arias, M.; Sánchez-Vizcaíno, J.M. (2002). "African Swine Fever (ASF)". In Trends in Emerging Viral Infections of Swine. Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 119-124.
- Arias, M.; Sánchez-Vizcaíno, J.M. (2002). "African Swine Fever Eradication: The Spanish model. In Trends in Emerging Viral Infections of Swine". Iowa State University press, ISBN: 0813803837. Eds. A. Morilla, K-J Yoon, J. Zimmerman. Pp 133-139.
- 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". [www.sanidadanimal.info] on line, July, 2002/.

3.2. DOCUMENTS TO BE USED TOGETHER WITH THIS PROCEDURE

- Procedure for the obtainig of ASF soluble citoplasmatic Ag (PNT/CISA/ASF/AgASFV/1/2008).
- Procedure for serologic diagnosis of ASF: Immunoblotting (PNT/CISA/ASF/IB/1/2008).

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
PAGE: 'polyacrilamide gel electrophoresis'
Ag: ASF cytoplasmatic soluble antigen
SDS: sodium-dodecyl-sulfate
IB: Immunoblotting
PC: positive control
NC: negative control
LC: limit control
H_20 d: distilled water

4.2. BACKGROUND

Gel electrophoresis is a useful method to separate and/or identify proteins and nucleic acids. In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated largely on the basis of polypeptide length, and so their molecular weight can also be estimated. SDS does however denature the protein, so activity stains cannot be used to identify particular enzymes. Described below is the protocol for preparing and using Laemmli discontinuous gels. In this system, two sequential gels are actually used; the top gel, called the stacking gel, is slightly acidic (pH 6.8) and has a low (5.5%) acrylamide concentration to make a porous gel. Under these conditions proteins separate poorly but form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (pH 8.8), and has a higher polyacrylamide content (in our case, 12%), which causes the gel to have narrower channels or pores. As a protein, concentrated into sharp bands by the stacking gel, travels through the separating gel, the narrower pores have a sieving effect, allowing smaller proteins to travel more easily and hence rapidly, than larger proteins.

This method is used to separate the ASFV viral proteins that are transferred with a constant current intensity to the nitrocellulose filter to produce the IB strips that are going to be used as confirmatory technique in the ASF serological diagnosis.

5. DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS

- **Vertical Electrophoresis System**: PROTEAN II xi CELL 20 cm, 1.0 mm spacers (4), 15 well combs (2) [REF 165-1813 Bio-rad]
- **Accessories**:

  - Absorbent paper
  - Buffer tank [REF. 165-1807 Bio-rad]
  - Casting stand with gaskets [REF 165-1911 Bio-rad]
  - Central cooling core with gaskets [REF 165-1806 Bio-rad]
  - Combs 1 mm: Blank REF 165-1892 Bio-rad or 2D reference well [REF 165-1897 Bio-rad]
  - Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)
  - Filter Millipore 0.45 µm
  - Filter paper Whatman
  - Glass bottles (100 ml, 250 ml, 500 ml)
  - Inner Plates 20 cm cell, 20 x 20 (2) [REF 165-1823 Bio-rad]
  - Latch Assembly kit Black [REF 100-5430 Bio-rad]
  - Lid with power cables [REF. 165-1808 Bio-rad]
  - Notched inner plate 20 cm cell [REF 165-1833 Bio-rad]
  - Nitrile Gloves
  - Outer Plates 20 cm cell, 22.23 x 20 cm (2) [REF 165-1824 Bio-rad]
  - Pipetboy acu or equivalent
  - Pipettes (5 ml, 10 ml, 25 ml)
  - Power Pac HC High-current power supply [BIORAD Ref 164-5052]
  - Precision balance
  - Reagent reservoir Polystyrene 50 ml [COSTAR Ref. 4870].
  - Replacement gaskets for central cooling core (2) [REF 165-1913 Bio-rad]
  - Replacement gaskets, for casting stand (2) [REF 165-1912 Bio-rad]
  - Sandwich clamps 20 cm set (2) [REF 165-1902 Bio-rad]
  - Spacers 20 cm 1mm set 4 [REF 165-1848 Bio-rad]
  - Single channel pipette 1-10 µl
  - Single channel pipette 10-200µl
  - Single channel pipette 200-1000 µl

- Sterile plastic tubes (10ml, 50 ml)
- Table centrifuge
- Tips
- Trans-Blot SD Semidry transfer Cell [BIORAD Ref 170-3940]
- Thermoblock
- Vortex

]

REAGENTS

- **Ag:** ASFV soluble cytoplasmatic antigen.
- Distilled water**.**
- Acetic acid (glacial)[MERCK Ref. 1.00063.1000]
- Acetone [PANREAC 21.1007]
- Acrilamide [SERVA Ref 10675]
- Ammonium Persulfate (PA) [BIORAD Ref 162-0115]
- β-mercaptoethanol [MERCK Ref. 805740]
- Bromphenol Blue [MERCK Ref. 1.08122.0005]
- Ethanol [PANREAC 12.1086.1214]
- Glicine [MERCK Ref 1.04201.1000]
- Glicerol [PANREAC Ref 131339.1212]
- Hidroximetil amino metano (TRIS) [MERCK Ref. 1.08387.2500]
- Hydrocloric acid fuming (HCl) [MERCK Ref. 1.00317.1000]
- Methanol [MERCK Ref. 1.06009.1000]
- N, N´-diallitartar diamina (DATD) [BIORAD Ref 161-0620]
- N,N,N,N'-tetrametilnediamina (TEMED) [BIORAD Ref 161-0800]
- Nitrocelulose [BIORAD Ref 162-0115]
- Red Ponceau [SIGMA Ref. P3504]
- Sodium dodecyl sulfate (Electrophoresis Purity reagent) (SDS) [BIORAD Ref. 161-0301]

- **ACRILAMIDE 30%**

	V_F *1000 ml*	V_F *500 ml*
Acrilamide	280 gr ……………	140 gr
N, N´-diallitartar diamina (DATD) …….	7,35gr ……………	3,675 gr
H_2O (MilliQ) (c.s.p) ……………………...	600 ml ……………	300 ml

**Heat at 37º C and add H_2O up 1000 ml or 500 ml.*
**Filter with millipore 0.45µm.*
**Store at 4ºC in dark conditions.*
CAUTION!! Weigh acrylamide and DATD with gloves and face pack

- **AMMONIUM PERSULFATE (PA) 10 %**

PA ………………….. 100 mg
H_2O (MilliQ) …………. 1ml
Store at -20ºC in aliquots

- **ELECTROPHORESIS BUFFER TRIS-GLICIN 10X**

Tris …………... 30 g
Glicine …………… 144 g

SDS 20% …………… 50 ml
H2O (c.s.p) …............... 500 ml
Once dissolved, complete with water till the foam disappear.
Store at room temperature

- **PBS buffer pH 7.2** .

NaCl [Merck 1.06404]…….. ……………… 8.0 g
KCl (Merck 1.04936) ………………………. 0.2 g
KH_2PO_4 [Merck 1.04873]……………………… 0.2 g
Na $_2HPO_4$ [Merck 1.04936] ...…………………. 1.15 g
Distilled water to ……………………………… 1000 ml
Store at room temperature

- **PONCEAU RED**

25 ml acetic acid + 475 ml H2O miliQ (55 v/v)

0.5 gr ponceau red (Final concentration 0.1%) +500 ml acetic acid 5%
Store at room temperature

- **LOADING SAMPLE BUFFER 4X (SR4X)**

Concentration 1X	Concentration 4X
SDS 2%	SDS 8%
Glicerol 10%	Glicerol 40%
Tris-HCl pH 7 80 mM	Trs HCl pH 7 320 mM
Bromophenol blue 0.01%	Bromophenol Blue 0.04%
β-mercaptoethanol 5%	β-mercaptoethanol 20%

100 ml de TR-4X ⇒ H_2O (MilliQ) ……………. 15 ml
Tris HCl pH 7 ……………. 32 ml
SDS …………………….. 8 gr
Glicerol 100% ……………. 40 ml
Azul de bromophenol 0,04 %. 0.4 ml
β-mercaptoethanol ……. 5 ml
*Aliquot and store at –20ºC.

- **SDS 10% (Sodium dodecyl-sulfate)**

SDS ………………….. 10 g
H_2O (MilliQ) …………. 80 ml
When dissolved, complete with H_2O (MilliQ) till foam disappear.
Store at room temperature

- **TRANSFER BUFFER (10X) (TT 10X)**

Tris ……… …. 30.3 gr
Glicine ………….. 114 gr
H_2O c.s.p ………….. 1 litro

Store at room temperature

- **TRANSFER BUFFER (1X)**

TT 10 X	……… ….	50 ml
Methanol	………..	100 ml
H_2O c.s.p	…………..	350 ml

Store at room temperatura

- **TRIS (hidroximetil amino methane) - HCl 1.5 M (pH 8,8)**

Tris	……… …	181,71 g	………..………	54,5 g
H_2O (c.s.p)	…………….	1 litro	……………..	300 ml

Check pH to 8.5 with ClH
Store at room Temperature

- **TRIS (hidroximetil-amino-methane) -HCl 1 M (pH 6.8)**

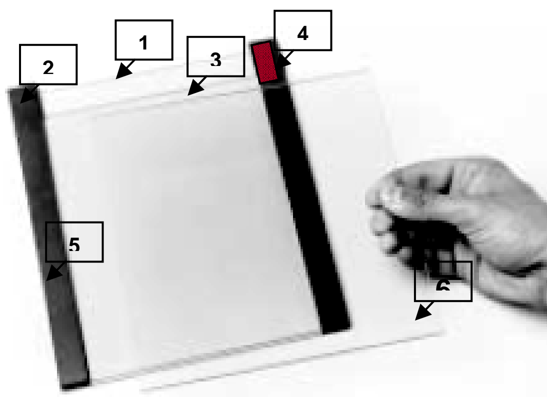
Tris	……………..	12,1 g	……………..	36, 3 g
H_2O (c.s.p)	…………….	100 ml	……………..	300 ml

Check pH to 6.8 with ClH
Store at room Temperature

5.2. PREPARATION

Before beginning, put thermoblock at 100ºC.

- **Clean** glasses well with ethanol and acetone. Glasses may be placed as follow:



1. Outer plate
2. Spacers
3. Inner plate
4. Replacement glaskets*
5. Spacers*
6. Inner plate*

Fig. 1

- **Put glasses in the "sandwich clamps"** (fig. 2) and put them in the casting stand for the assembly of the gels (fig. 3). Check the position of gels adding water.

Fig. 2

Fig. 3

5.2. SAMPLE PREPARATION

SAMPLE: soluble cytoplasmatic Ag ASFV obtained as described SOP/CISA/ASF/AgASFV

The amounts indicated bellow is per gel

- 500 µl sample (Ag ASF) + 125 µl TR4X
- Mix
- Heat at 100ºC during 2 minutes (denaturalization).

5.3. METHODS

SDS-PAGE ELECTROFORESIS

Amounts described bellow is used for gels system Bio-Rad (Protean II®). These can be adapted for other systems, with the same capacities.

- **Resolving Gel (17%) (pH 8.8)** 1 mm thickness: in this gel proteins are separated according to the molecular weight. It is important to add the reagents in the follow order:

Spacers 1mm	2 gels
H_2O	16, 85 ml
Acrilamide 30%	56, 6 ml
TRIS - HCl 1.5 M pH 8,8	25 ml
SDS 10%	1 ml
Ammonium Persulfate 10%*	500 µl
TEMED	50 µl
FINAL VOLUME	100 ml

* Prepare when is going to be used (100 mg in 1 ml H_2O)

- **Add the Separating Gel** slowly between each glass avoiding bubbles. The volume necessary is approximately 30 ml.
- **Add water** or butanol (to avoid oxygen contact). Take the gel at room temperature during 1– 1:30 hours to polymerize.
- **Remove water** and dry with filter paper.

- **Stacking Gel pH 6.8:** once the top of separating gel is dry

Stacking Gel 1mm	2 geles
H_2O	18, 3 ml
Acrilamide 30%	3, 9 ml
TRIS - HCl 1.5 M pH 6,8	7, 5 ml
SDS 10%	300 µl
Ammonium Persulfate 10%*	150 µl
TEMED	30 µl
FINAL VOLUME	**30 ml**

- **Add Stacking gel** avoiding the formation of bubbles and immediately put combs. Check that there is enough stacking gel, and add more if necessary. Leave the gel at room temperature during 1 – 1:30 hour.
- **When Stacking gel is polymerized,** put the casting stand in the central cooling core as figure 4.
- **Add Electrophoresis buffer 1x** in the top of the central cooling core to cover the electrodes. Check that there isn't any escape of buffer leaving during 15 minutes at room temperature. During this time, prepare samples as indicated in section 5.2

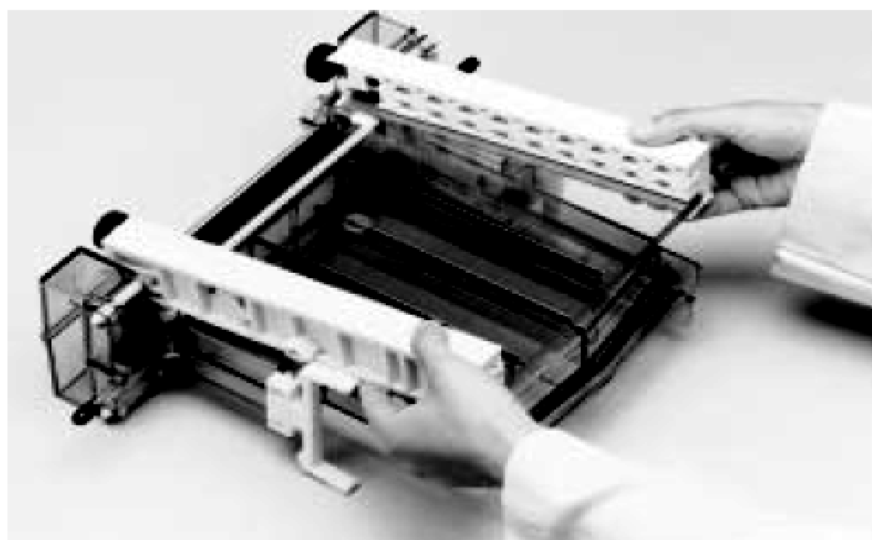


Figure 4

- **Put the gel** in the buffer tank.. Get out combs and add electrophoresis buffer to the glasses
- **Put the samples** with a syringe.
- **Connect up to the power supply.** The electrophoresis runs at constant amperage: **9 mA per gel during 18 hours (maximum voltage 150 V).**
- **Important: turn the tap on** to cool.

SEMIDRY ELECTROTRANSFER

- Once finish electrophoresis (SDS-PAGE), take gels getting out staking gel and put on in transfer buffer 1 x during 10 minutes with Whatman paper and the nitrocellulose (**HANDLE WITH GLOVES)**
- Prepare sandwich with gel. Bands of the gel will be transferred to the nitrocellulose membrane. Assembly transfer Cell. Note that proteins will migrate to the anode. Prepare sandwich as indicate as follow:

- Connect high current supply at constant voltage

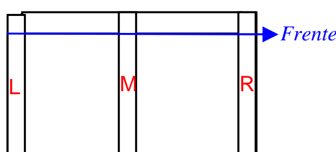
Volt	22 V
Limit	1.2 Ampere
Time	30 minutes

- Wash with PBS 1 x during 10 minutes and dry at room temperature
- The efficiency of transfer can be observed by staining with a solution of Ponceau Red 0.5 % and acetic acid 1%

5.4. RESULTS

If the transfer is good, we must test de nitrocellulose strips by a quality control of each batch through the Immunoblotting technique described in procedure SOP/CISA/ASF/IB/1/2008:

- Mark front of nitrocellulose membrane carefully
- Discard one strip of 0.5 cm of each side (in this area proteins are deformed)
- Cut three strips (left, middle and right) 0.3 wide.



- Strips L, M and R are analyzed with PC by Immmunoblotting (SOP/CISA/ASF/IB/1/2008) and batch is valid if shows specific ASFV pattern.
-

5.5. CRITICAL POINTS

Electrophoresis is a very sensible technique and can be affected by a lot of experimental error. The most important critical points are:

- **Temperature during the polymerization and electrophoresis**: the mobility of proteins varies because of water viscosity is increased at low temperatures. It is important to maintain similar temperature through the gel during electrophoresis.
- **Polymerization speed :** fast polymerization can distort bands, so in this case must reduce TEMED and ammonium persulfate to make the process slower.
- **Reagents purity:** it is necessary to use high quality reagents and deionised water to take reproducible and high resolution gels. The quality of Acrilamida and SDS is very important.
- **Electrophoresis time:** if the electrophoresis is very short, samples run very fast so the separation of them is not correct, but short times of the process minimize sample dispersion.
- **Sample preparation:** is necessary a correct protein denaturalization to avoid double bands.

5.6. SAFETY CAUTIONS

- Read the protocol previously.
- Storage reagents at the adequate temperature
- Avoid any reagent contamination
- Do not use the reagents after the expiry date
- Do not eat, smoke or drink while the manipulation of reagents.