CENTRO DE INVESTIGACION EN SANIDAD ANIMAL (CISA-INIA)

European Union Reference Laboratory for ASF, (EURL-ASF)
Centro de Investigación en Sanidad Animal
CISA-INIA, Valdeolmos 28130, Madrid, Spain.

Contact people
Dr. Carmina Gallardo
Virginia Pelayo

E-mail: eurl.asf@inia.es
Contact person: Dr. Carmina Gallardo gallardo@inia.es

EU Reference Laboratory for ASF
Animal Health Research Centre
(CISA), INIA
Ctra Algete-El Casar s/n
28130, Valdeolmos, Spain

SOP/CISA/ASF/IB-STRIPS/1/

STANDAR OPERATING PROCEDURE FOR THE PRODUCTION OF INMUNOBLOTTING STRIPS FOR AFRICAN SWINE FEVER ANTIBODY DETECTION

CONTENTS

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 cytoplasmic antigen sample

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:

- 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

ASF REVIEWS:

1. Arias, M., Sánchez-Vizcaíno, J.M. (2012). African swine fever. In: Zimmerman, J., Karriker, L.A., Ramirez, A., Schwartz, K.J, Stevenson, G.W. (Eds), Diseases of swine, 10th Edition. John Wiley and Sons, United States of America, pp. 396-404.
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. DOCUMENTS TO BE USED TOGETHER WITH THIS PROCEDURE

- PROCEDURE TO OBTAIN THE AFRICAN SWINE FEVER VIRUS (ASFV) CYTOPLASMIC SOLUBLE ANTIGEN **(SOP/CISA/ASF/Ag/1).**
- PROCEDURE FOR THE DETECTION OF ANTIBODIES AGAINST AFRICAN SWINE FEVER BY IMMUNOBLOTTING **(SOP/CISA/ASF/IB/1).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

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

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±0.01UpH) and has a low (5.5%) acrylamide concentration to make a porous gel. Under these conditions proteins separate poorly and form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (pH 8.8±0.01UpH), and has a higher polyacrylamide content (in our case, 17%), 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

- Absorbent paper.
- Buffer tank [Ref. 165-1807 (BIORAD) or similar characteristics].
- Casting stand with gaskets [Ref 165-1911 (BIORAD) or similar characteristics].
- Central cooling core with gaskets [Ref 165-1806 (BIORAD) or similar characteristics].
- Combs 1 mm [Ref 165-1897 (BIORAD) or similar characteristics].
- Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml).
- Filter paper Whatman.
- Glass bottles (100 ml, 250 ml, 500 ml).

- Inner Plates 20 cm cell, 20 x 20 (2) [Ref 165-1823 (BIORAD) or similar characteristics].
- Latch Assembly kit Black [Ref 100-5430 (BIORAD) or similar characteristics].
- Lid with power cables [Ref. 165-1808 (BIORAD) or similar characteristics].
- Notched inner plate 20 cm cell [Ref 165-1833 (BIORAD) or similar characteristics].
- Gloves.
- Outer Plates 20 cm cell, 22.23 x 20 cm (2) [Ref 165-1824 (BIORAD) or similar characteristics].
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Pipettes (5 ml, 10 ml, 25 ml).
- Power Pac HC High-current power supply [Ref 164-5052 (BIORAD) or similar characteristics].
- Precision balance.
- Replacement gaskets for central cooling core (2) [Ref 165-1913 (BIORAD) or similar characteristics].
- Replacement gaskets, for casting stand (2) [Ref 165-1912 (BIORAD) or similar characteristics].
- Sandwich clamps 20 cm set (2) [Ref 165-1902 (BIORAD) or similar characteristics].
- Spacers 20 cm, 1mm set 4 [Ref 165-1848 (BIORAD) or similar characteristics].
- Single channel pipette 1-10 µl.
- Single channel pipette 10-200µl.
- Single channel pipette 200-1000 µl.
- Sterile plastic tubes (12ml, 50 ml).
- Sterile disposable tips (1-10 µl, 1-200µl, 100-1000 µl).
- Trans-Blot SD Semidry transfer Cell [Ref 170-3940 (BIORAD) or similar characteristics].
- Thermoblock .
- Vacuum Filter/Storage Bottle Systems 0.45 µm 1000 ml [CORNING Ref 430516 or similar characteristics].
- Vertical Electrophoresis System: PROTEAN II xi CELL 20 cm, 1.0 mm spacers (4), 15 well combs (2) [Ref 165-1813 (BIORAD) or similar characteristics].
- Vortex.

REAGENTS

- **ASF-Ag:** ASFV soluble cytoplasmatic antigen.
- Acetic acid (glacial) [Ref 141008.1611 (PANREAC) or similar characteristics].
- Acetone [Ref 1.00014.1000.050 (MERK) or similar characteristics].
- Acrylamide [Ref 10675 (SERVA) or similar characteristics].
- Ammonium Persulfate (PA) [Ref 161-0700 (BIORAD) or similar characteristics].
- Ethanol [Ref 1.00983.100 (MERCK) or similar characteristics].
- Glycine [Ref 1.04201.1000 (MERCK) or similar characteristics].
- Hidroximetil amino metano (TRIS) [Ref. 1.08387.2500 (MERCK) or similar characteristics].
- Hydrocloric acid fuming (HCl) [Ref. 1.00317.1000 (MERCK) or similar characteristics].
- Loading sample buffer 4X (TR4X) [XT Sample Buffer,4X (BIORAD) Ref 161-0791 or similar characteristics].
- Methanol [Ref. 1.06009.1000 (MERCK) or similar characteristics].
- N, N´-diallyltartar diamine (DATD) [Ref 161-0620 (BIORAD) or similar characteristics].
- N,N,N,N'-tetrametilnediamina (TEMED) [Ref 161-0800 (BIORAD) or similar characteristics].
- Nitrocelulose [Ref 162-0115 (BIORAD) or similar characteristics].
- Potassium Chloride (ClK) [Ref. 1.04936.0500 (MERCK) or similar characteristics].
- Potassium phosphate (P04H2K) [Ref. 1.04873.1000 (MERCK) or similar characteristics].
- Red Ponceau [Ref. P7170-1L (SIGMA)].
- Sodium Chloride (ClNa) [Ref. 1.06404.1000 (MERCK) or similar characteristics].

- Sodium dodecyl sulfate (Electrophoresis Purity reagent) (SDS) [Ref. 161-0301 (BIORAD) or similar characteristics].
- Sodium phosphate ($P0_4HNa_2$) [Ref. 1.06586.0500 (MERCK) or similar characteristics].

5.2. PREPARATION

A) REAGENTS PREPARATION

- **Acrylamide 30%**

		V_F 1000 ml	V_F 500 ml
Acrilamide	------	280 gr (±1)	140 gr(±1)
N, N´-diallyltartar diamine (DATD)	------	7.35gr(±0.1)	3.675gr(±0.1)
H_2O (MilliQ) (q.s.)	------	600 ml	300 ml

**Heat at 37º±2ºC and add H_2O up to 1000 ml or 500 ml.*
**Filter with millipore 0.45µm.*
**Store at 4ºC* (±3ºC) *in dark conditions. Expiry date 1 month.*
CAUTION! Weigh acrylamide and DATD with gloves and face pack

- **Ammonium Persulfate (PA) 10 %**

PA	------	100 mg (±0.01)
H_2O (MilliQ)	------	1ml

Store at <-20ºC in aliquots. Expiry date 12 months.

- **Electrophoresis Buffer TRIS-Glicine 10X**

Tris	------	30 gr (±0.5)
Glicine	------	144 gr(±1)
SDS 20%	------	50 ml
H_2O (MilliQ) (q.s)	------	500 ml

Once dissolved, complete with water up to 1000 ml carefully once all the foam disappear.
Store at room temperature. Expiry date 1 month.

- **Phosphate buffered saline (PBS 1x) pH 7.2→** The PBS could be obtained in tablets [Ref.: 524650-1 (CALBIOCHEM) or similar characteristics] or could be prepared as follows:

ClNa	------	8.0 gr (±0.1)
ClK	-----	0.2 gr (±0.01)
$P0_4H_2K$	------	0.2 gr (±0.01)
$P0_4HNa_2$	------	1.15 gr(±0.05)
H_2O (milliQ)	------	1,000 ml

Store at room temperature. Expiry date: 1 year.

- **Ponceau red [final concentration 0.1%]**

Ponceau Red	------	0.5gr (±0.01)
Acetic acid 5% [25ml Acetic acid (glacial)+475ml H2O milliQ (55 v/v)]	------	500ml

Store at room temperature. Expiry date: 1 year.

- **Sodium dodecyl-sulfate**

		SDS 10%	SDS 20%
SDS	------	10 gr(±0.1)	20gr(±0.1)
H_2O (MilliQ)	------	80 ml	80 ml

Once dissolved, complete with water up to 100 ml carefully once all the foam disappear.
Store at room temperature. Expiry date 1 year.

- **Transfer Buffer (10X) (TT 10X)**

Tris	------	30.3 gr(±0.5)

Glicine	------	114 $gr_{(\pm1)}$
H_2O (MilliQ) (q.s.)	------	1 000 ml

Store at room temperature. Expiry date 1month.

- **Transfer Buffer (1X)**

TT 10 X	------	50 ml
Methanol	------	100 ml
H_2O (MilliQ)	------	350 ml

Store at room temperature. Expiry date 1month.

- **TRIS (hidroximetil amino methane) - HCl 1.5 M (pH 8.8±0.1)**

Tris	------	54.5$gr_{(\pm0.5)}$
H_2O (MilliQ) (q.s.)	------	300 ml

*pH must be adjusted to $8.5_{(\pm0.01)}$ with ClH every 15 days.
Store at room Temperature. Expiry date 1month.

- **TRIS (hidroximetil-amino-methane) -HCl 1 M (pH 6.8±0.1)**

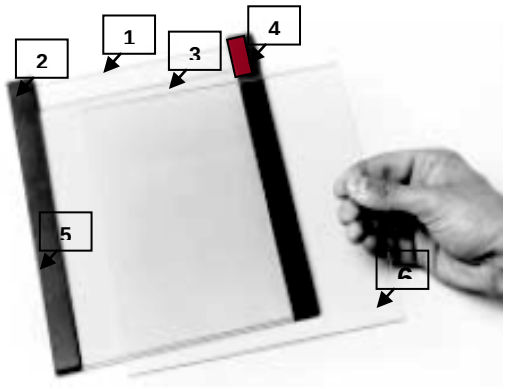
Tris	------	36. 3$gr_{(\pm0.5)}$
H_2O (MilliQ) (q.s.)	------	300 ml

*pH pH must be adjusted to $6.8_{(\pm0.01)}$ with ClH every 15 days
Store at room Temperature. Expiry date 1month.

B) EQUIPMENT PREPARATION

Before beginning, set the thermo block to 100±2ºC

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



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

Fig. 1

- **Place the 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 and avoiding any leak from the crystals.

Fig. 2

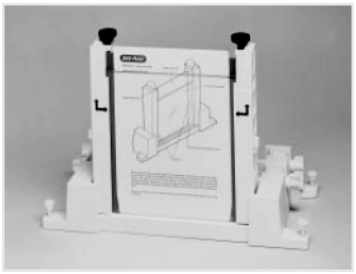


Fig. 3

C) SAMPLE PREPARATION

SAMPLE: soluble cytoplasmic Ag ASFV obtained as is described in the described SOP/CISA/ASF/Ag/1.

Amounts per gel: Mix 500 µl sample (ASF -Ag) + 125 µl TR4X and heat it at 100±2ºC during 2 minutes to allow the denaturation of the proteins.

5.3. METHODS

5.3.1. SDS-PAGE ELECTROFORESIS

Amounts described below are used for *gels system Bio-Rad (Protean II®)* but can be adapted for other systems, with the same capacities.

1. **Resolving Gel 17%, pH 8.8±0.1:** 1 mm thickness: in this gel the ASFV proteins are separated according to the molecular weight.

Resolving gel (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

*****It is important to add the reagents in the above described order*

2. **Add the Resolving Gel** gently between each glass avoiding bubbles. The required volume is approximately 30 ml to leave space enough for the stacking gel.
3. **Add water** or buthanol (to avoid oxygen contact) and keep it at room temperature for 1 hour or until the gel is completely polymerized.
4. **Remove water** and dry with filter paper. Once it is dry, prepare the stacking gel.

5. **Stacking Gel, pH 6.8±0. 1:**

Stacking Gel 1mm*	2 gels
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**

**It is important to add the reagents in the above described order*

Add the Stacking gel avoiding the formation of bubbles and immediately put the combs. Check if there is enough stacking gel, and add more if necessary. Leave the gel at room temperature during 1h or until the gel is completely polymerized. Then, place the casting stand in the central cooling core as is indicated in figure 4.

6. **Fill the central cooling core with Electrophoresis buffer 1x** to cover the electrodes. Check the buffer doesn´t leak out the glasses leaving the gels at least 15 minutes at room temperature. Meanwhile, prepare samples as indicated in section 5.2. C

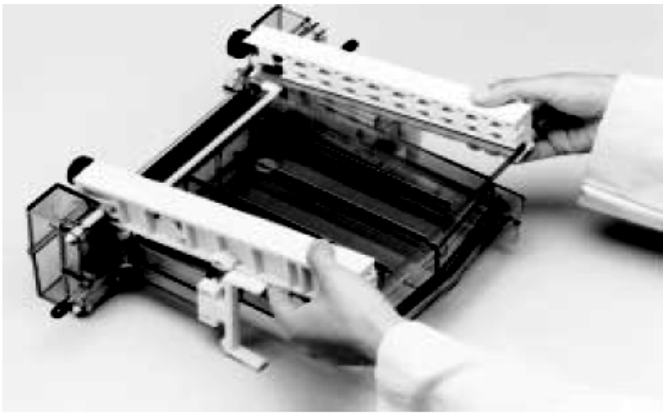


Fig. 4

7. **Place the gel** inside the buffer tank. Remove the combs and add the electrophoresis buffer to the tank until reaches the lower edge of the glasses.

8. **Add the treated ASF-Ag** in the gap left by the combs when removed with a syringe. **Connect to the power supply.** The electrophoresis runs at constant amperage: **9 mA per gel during 18-20 hours (the voltage shouldn´t raise more than 150 V).**
   ***Note: turn the tap on to cool.*

5.3.2. SEMIDRY ELECTROTRANSFER

1. Once the electrophoresis has finished (SDS-PAGE), gently separate the gels from the glasses and remove the staking gel. Swamp the stacking gel in **transfer buffer 1 x during 10 minutes** with the Whatman Paper and the nitrocellulose (**HANDLE WITH GLOVES).**

2. To transfer the ASFV proteins to the nitrocellulose membrane, prepare a "sandwich" with the gel, the Whatman Paper and the nitrocellulose as is indicated:

3. Assembly the Transfer Cell considering the proteins will migrate to the anode (-).
4. Connect high current supply at constant voltage.

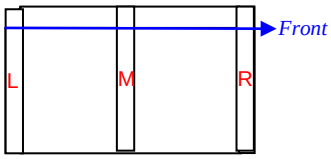
i. **Volt 22 V**
ii. **Limit 1.2 Ampere**
iii. **Time 30 minutes**

2. Finally wash with PBS 1 x during 10 minutes and dry at room temperature.
3. The efficiency of transfer can be observed staining with Ponceau Red 0.1 %.

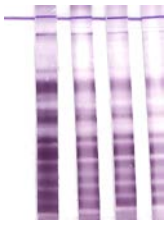
5.4. RESULTS AND INTERPRETATION OF RESULTS.

If the transfer has been successful, we must test de nitrocellulose strips by a quality control of each batch through the Immunoblotting technique described in the procedure **SOP/CISA/ASF/IB/1/**

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



The strips must be checked against the ASF positive reference serum (ASF-PC) 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-IB-STRIPS is consider as **optimal for using in the IB 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. CRITICAL POINTS

Electrophoresis is a very sensitive technique and can be affected by a lot of experimental errors. 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 Acrylamide 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.

5.7. QUALITY CONTROL

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.