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

SOP/CISA/ASF/VI/1

PROCEDURE FOR AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATION ON PORCINE LEUCOCYTES AND HEMADSORPTION TEST.

CONTENTS

1. PURPOSE

The main goal of this procedure is to describe the African swine fever virus (ASFV) virus isolation in porcine leukocytes and the hemadsorption.

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

2. SCOPE

This procedure is applicable to porcine clinical samples such as blood with anticoagulant, sera and ASF target porcine tissues, as well as to homogenate soft ticks.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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

1. AFRICAN SWINE FEVER. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees). CHAPTER 2.8.1. OIE, 2012 [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.01_ASF.pdf]
2. Carnero R., Larenaudie, B., Ruiz-Gonzalvo, F. y Haag, J. (1967). "Peste porcine africaine. Etudes sur la reaction d'hemadsorption et son inhibition par des anticorps specifiques ". Rec. Vet. Med. 143, 49-59.
3. Carrasco L., de Lara FC, Martin de las Mulas J, Gomez-Villamandos JC, Hervas J, Wilkinson PJ, Sierra MA . (1996a). "Virus association with lymphocytes in acute African swine fever".Vet Res;27(3):305-12
4. Carrascosa AL, Bustos MJ, de Leon P. "Methods for growing and titrating African swine fever virus: field and laboratory samples". Curr Protoc Cell Biol. 2011 Dec;Chapter 26: Unit 26.14.
5. Galindo I, Almazan F, Bustos MJ, Viñuela E, Carrascosa AL. (2000). "African swine fever virus EP153R open reading frame encodes a glycoprotein involved in the hemadsorption of infected cells". Virology Jan 20;266(2):340-51.
6. Malmquist, W. y Hay, D. (1960). "Hemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures". Am. J. Vet. Res. 21, 104-108.

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. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of samples processing for African swine fever (ASF) diagnosis **(SOP/CISA/SAMPLE/1).**
- Procedure for the extraction of African Swine Fever Virus (ASFV) DNA (**SOP/CISA/ASF/DNA EXTRACTION/1).**
- Procedure for the detection of African Swine Fever Virus (ASFV) by conventional polymerase chain reaction (PCR) **(SOP/CISA/ASF/PCR/1).**
- Procedure for the detection of African Swine Fever Virus (ASFV) by real time polymerase chain reaction (PCR) **(SOP/CISA/ASF/PCR/2).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever

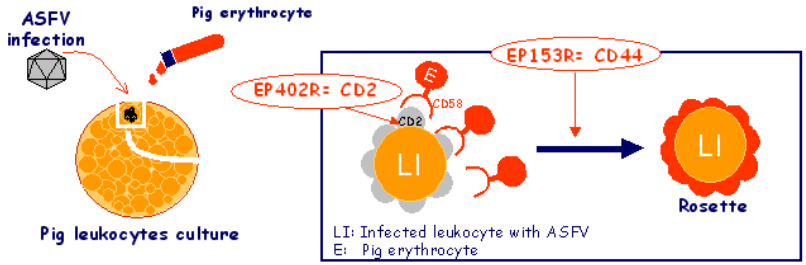
ASFV: African swine fever virus
H.A.D: haemadsorption
C.E.P: cytophatic effect
PC: ASFV isolate positive control
NC: Negative control
r.p.m: revolutions per minute
PCR: Polymerase chain reaction
DIFT: Direct immunofluorescence tests.

4.2. BACKGROUND

Malmquist and Hay made one of the most important advances in the study of African swine fever virus (ASFV) in 1960 demonstrating that ASFV was able to infect and replicates in primary leukocyte cultures from pig peripheral blood. Virus isolation is based on the inoculation of sample material on susceptible primary cell cultures of porcine origin, monocytes and macrophages cells. If the ASF virus is present in the sample, it will replicate in the cells and the **cytopathic effect (C.P.E)** will be produced in the infected cells.

When the virus replicates in these cultures, the majority of the ASFV strains produced the **haemadsorption reaction (HAD)** due to adsorption of pig red blood cells on ASFV infected leukocytes. Cell lysis and C.P.E. usually occurs after 48-49 hours of haemadsorption. The importance of this finding relies on its specificity because none of the other pig viruses are capable of haemoadsorbing in leukocyte cultures.

The **phenomenon of HAD** is linked to two different genes of the ASFV genome. The ORF *EP402R* and ORF *EP153R* of Spanish isolate BA71VR ASFV isolate (*GenBank accession no. ASU18466*). The first gene encodes a protein homologous to CD2, the cell adhesion receptor of T cells and an immune response modulator, and the second one encodes a protein homologous to CD44 molecules, involved in cellular adhesion and T-cell activation. In the case of the *EP402R* gene is responsible for the adhesion of swine erythrocytes to infected cells, and the *EP153R* is as a stabilizer of this adhesion.



Virus isolation and identification by HAD are recommended as a **reference test for the confirmation of positive results of a prior antigen ELISA, Polymerase chain reaction (PCR) or Direct immunofluorescence tests (DIFT).** They are also recommended when ASF has already been confirmed by other methods, particularly in **case of a primary outbreak or case of ASF**.

5. DESCRIPTION

5.1. EQUIPMENT AND MATERIALS

MATERIALS.

- Analytical Balance.
- Adsorbent paper.
- Chronometer.
- Centrifuge tubes.
- Cell cultures flasks, T25, T75 and T150 [Falcon].
- CO_2 (±0.5%) Incubator/ 37±3ºC.

- Counter chamber [THOMA or NEUBAHUER or similar characteristics].
- 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.
- Latex or nitrile gloves.
- Laminar flow cabin class II.
- 96 well cell culture plate's bottom flat [NUCLONTM Surface, NUNC or similar characteristics].
- Mechanical defibrinator.
- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.
- Multichannel pipette 5-50µl.
- Multichannel pipette 50-300 µl.
- Phase Contrast Inverted cell Culture Microscope.
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Reagent reservoir Polystyrene 50 ml.
- Shaker incubator 37±2ºC.
- Sterile conic plastic tubes 12 ml and 50 ml.
- Sterile glass bottle 250ml and 500 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 Megafuge 1.0R [rotor Heraeus #7570 or similar characteristics].
- Vortex.

REAGENTS.

- **Ammonium Chloride** NH_4Cl [Ref.: 1.01145.1000 (Merck) or similar characteristics]. *Store at room temperature.*
- **Dihydrogénophosphate *potassium* ($P0_4H_2K$)** [Ref. 1.04873.1000 (MERCK) or similar characteristics]. *Store at room temperature.*
- **Phosphate buffered saline (PBS 1x) in tablets** [Ref.: 524650-1 (CALBIOCHEM) or similar characteristics]. *Store at room temperature.*
- **Potasium Clhoride (ClK)** [Ref. 1.04936.0500 (MERCK) or similar characteristics]. *Store at room temperature.*
- **Sodium Clhoride (ClNa)** [Ref. 1.06404.1000 (MERCK) or similar characteristics]. *Store at room temperature.*
- **Sodium phosphate ($P0_4HNa_2$)** [Ref. 1.06586.0500 (MERCK) or similar characteristics]. *Store at room temperature.*
- **Türk´s colorant.** [Ref.: 1.09277.0500 (Merck) or similar characteristics]. *Store at room temperature.*
- **PC:** ASFV positive HAD isolate. *Store at ≤ -70º C.*

5.2. PREPARATION.

5.2.1. SAMPLE PREPARATION.

Sample preparation is performed according is described in the sample processing procedure for ASF diagnosis **(SOP/CISA/ASF/SAMPLE/1).**

5.2.2. REAGENTS PREPARATION.

- **Erythrocytes lyses solution:** 0.83% Ammonium Chloride sterile → 8.3gr (±0.1) of NH_4Cl in 1,000 ml of distilled water. *Store at 4±3ºC. Expiry date: 6 months.*
- **Phosphate buffered saline (PBS 1x) pH 7.2** (±0.2 UpH) → The PBS could be obtained in tablets 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)
Distilled water	------	1,000 ml

Store at room temperature. Expiry date: 1 year.

5.3. METHODS

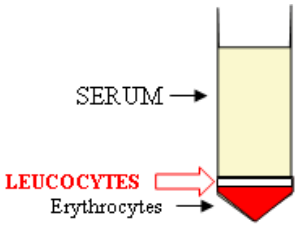
A) LEUCOCYTES HARVESTING AND CULTURE.

1. Collect the required volume of un-clotted fresh blood from pigs over three months of age using a mechanical defibrinator or using anticoagulant such as EDTA. The blood is collected by puncture in cava vein to the mechanical defibrinator and is shaking during 15-30 minutes. *The recommended volume is 20ml/per 1Kg of pig.*

2. The defibrinate blood is dispensed in aliquots of 50 ml tubes.

3. Centrifuge at **2,500 r.p.m** in a Megafuge 1.0R rotor Heraeus #7570 during **30 minutes** (~1,300g) without brake.

4. The blood is separated in **three fractions**; the serum (culture medium for leucocytes), a fine white layer (leucocytes) and the third fraction are the erythrocytes. The three fractions are colleted:



- ***The serum*** is collected in a bottle of 250-500 ml for being used as culture medium of the leukocytes.
- ***The fine white layer*** (leucocytes) is collected by capillarity avoiding collecting the red cells (erythrocytes).
- Collect ***the erythrocytes*** diluted 1/10 in PBS 1x.

NOTE: for the specificity of the test is necessary to use the serum and the erythrocytes of the pig from which the leucocytes has been obtained (homologous serum). This fact avoids unspecific haemadsortion reactions.

5. Place the leukocytes in a sterile conic tube of 50 ml. Add three volumes of 0.83% of **erythrocytes lyses solution** and mix it briefly. Incubate **on ice for 15 minutes**. *The ammonium chloride allows the lyses of the erythrocytes remained.*

6. Centrifuge at **2,000 r.p.m** in a Megafuge 1.0R rotor Heraeus #7570 (~1,050g) for **15 minutes**.

7. Carefully remove the supernatant and add three volumes of 0.83% **erythrocytes lyses solution**. Briefly mix it and incubate **on ice for 15 minutes**.

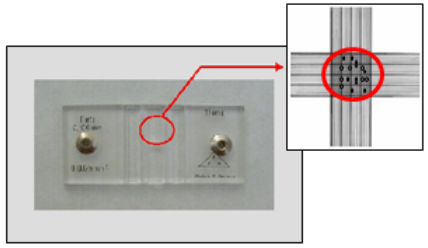
8. Centrifuge at **2,000 r.p.m** in aMegafuge 1.0R rotor Heraeus #7570 (~1,050g) for **15 minutes**.

9. Remove the supernatant and **collect the pellet (clean leucocytes)** with 10ml of homologous serum collected in step 4.

10. Immediately count the leukocytes and adjust the concentration of the suspension to a **final concentration of 8-10x10^6 leukocytes per ml**.

Example:

- 5 µl of leukocytes resuspend with 10 ml of homologous porcine serum + 495 µl of Turk colorant.
- Vortex
- 25-30 µl are put in the THOMA or NEUBAUER counter chamber



Thoma counter chamber

Resuspend leukocytes with 10 ml of serum

Count; 80

Dilution ; 1/100

Concentration of leukocytes/ml $10x10^6$

$$\frac{80x100x10.000\ x10}{10.000.000} = \textbf{80 ml serum}$$

11. Finally, distribute the leukocytes culture in 96 well cell culture plate's bottom flat adding 200 µl per well **(300,000 cells/well)** and **incubate at 37±3ºC in CO_2 (±0.5%) for 3-4 days** allowing the maturation of the leukocytes.

B) SAMPLE INOCULATION.

1. After 3-4 days inoculate the leucocytes culture with **1/10 dilution (20 µl/well) of treated sterile sample** as is described in the sample processing procedure for ASF diagnosis (SOP/CISA/ASF/SAMPLE/1). It is recommended to inoculate at least four wells per sample.

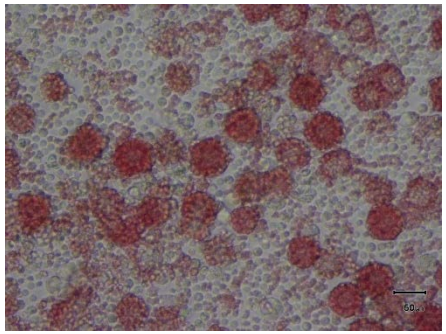
2. Inoculate four wells with PC and leave four wells without inoculum as negative control (NC) to check the possibility of nonspecific haemadsorption.

3. Add **20 µl per well of a fresh preparation of 1% pig erythrocytes** in buffered salt solution to each well (final dilution 1/100 in PBS sterile buffer).

4. Incubate at 37±2ºC in CO_2 (±0.5%) incubator. **Read the plates every day for 7 days to check the presence of HAD or CPE.**

5.4. ANALYSIS AND INTERPRETATION OF THE RESULTS.

NOTE: At the moment of reading results, each well is analyzed as individual well comparing with the PC and the results obtained in the wells without inoculated. In this way, samples will be analyzed respect to the controls of its plate.



The inoculated well should be read every day in the microscope to check the presence of **a positive HAD and/or CPE.** The first read can be performed at 14-16 hours post inoculation.

To check the presence of the HAD positive results in the microscope the plates must be gentile shaken allowing the read of the HAD positive results. The read period must be extended until check the presence of HAD and/or CPE positive results until 7 days.

Haemadsorption consists of the attachment of large numbers of pig erythrocytes to the surface of infected cells (rosette). A CPE consists in a reduction of the number of adherent cells in the absence of haemadsorption due to the cytotoxicity of the inoculum, Aujeszky's disease virus or non-haemadsorbing ASFV, which can be detected by the FAT on the cell sediment or by use of PCR. If no change is observed, or if the results of the immunofluorescence and PCR tests are negative, subinoculate the supernatant into fresh leukocyte cultures.

CPE+/PCR +/ HA - → Nonhaemadsorbing ASFV
CPE+/PCR -/ HA - → Cytotoxic (no ASFV)

5.4. CRITICAL POINTS

In the last years has been analyzed a lot of sample by VI, with good results of specifity and sensitivity for virological diagnosis of ASF. But there are some critical points:

1. **The procedure is long and laborius.** To give a correct ASF diagnosis it is required between 5-10 days if HAD pattern if observed in the first passage. If not can be delay until 15-30 days. It is not recommended as choice technique for a primary diagnosis. It is used as confirmatory technique for PCR or DIFT positive results.

2. The presence of CPE without HAD can be due to the presence of additional viruses or to **a cytotoxic effect**. This issue makes more difficult a correct diagnosis of the disease and requires a late PCR confirmation.

3. It is very important to use **erythrocytes and serum from the same animal** as the leukocytes because this avoids non-specific HAD reactions.

4. Previous studies have demonstrated that **badly conserved sample could origin false reaction avoiding the isolation of the ASFV.** The samples must be kept in a cold chain during the storage and transport.

5. Previous studies have demonstrated the **influence** of lyophilized samples **in the HAD technique decreasing the** effectiveness

6. This technique requires a Laboratory with cell culture conditions and animal facilities department.

5.5. SAFETY CAUTIONS

- Read the protocol previously.
- Work in sterile conditions to avoid the cell culture contamination.
- Avoid any reagent contamination.
- Do not eat, smoke or drink while the manipulation of reagents.
- Do not pipette by mouth.
- Use a new tip for each sample.
- Always include PC and NC.

Worksheet CISA/ASF/VI/1

ID REGISTER:
DATE:
TECHNICIAN:
CELLS:
DATE CELL CULTURE:
DATE CELL INOCULATION:

	1	2	3	4	5	6	7	8	9	10	11	12
A												PC
B												PC
C												PC
D												PC
E												NC
F												NC
G												NC
H												NC

COMMENTS: