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SOP/CISA/ASFV/TITRATION/1

STANDARD OPERATING PROCEDURE OF GROWING AND TITRATION ADAPTED AFRICAN SWINE FEVER VIRUS (ASFV) ISOLATES (SOP/CISA/ASFV/TITRATION/1).

CONTENTS

1. PURPOSE

This procedure describes the propagation and titration of cell-adaptade African swine fever (ASFV) in established cell lines (VERO or MS)

2. SCOPE

This procedure is applicable to;

- VERO cells infected with the ASFV BA71VR Spanish isolate adapted to VERO cells after 140 passages.
- MS cells infected with the ASFV E70MS48 Spanish isolate adapted to MS cells after 81 passages.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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. Angel L. Carrascosa, M. Jose Bustos, and Patricia de Leon. Methods for Growing and Titrating African Swine Fever Virus: Field and Laboratory Samples. Current Protocols in Cell Biology 26.14.1-26.14.25, December 2011
3. Enjuanes L, Carrascosa AL, Moreno MA, Viñuela E. Titration of African swine fever (ASF) virus. J Gen Virol. 1976 Sep;32(3):471-7. PubMed PMID: 823294.
4. Patricia de León, María J. Bustos, Angel L. Carrascosa. Laboratory methods to study African swine fever virus. Virus Res. 2013 Apr;173(1):168-79. doi: 10.1016/j.virusres.2012.09.013. Epub 2012 Oct 3.
5. Rodriguez F, Alcaraz C, Eiras A, Yáñez RJ, Rodriguez JM, Alonso C, Rodriguez JF, Escribano JM. Characterization and molecular basis of heterogeneity of the African swine fever virus envelope protein p54. J Virol. 1994 Nov;68(11):7244-52. PubMed PMID: 7933107; PubMed Central PMCID: PMC237164.

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). AFRICAN SWINE FEVER: DETECTION AND DIAGNOSIS. A manual for veterinarians. FAO 2017** http://www.fao.org/3/a-i7228e.pdf

3.2. COMPLEMENTARY DOCUMENTS (SOPs) TO BE USED.

- Procedure of Cell Growth and Propagation of African swine fever virus (ASFV) susceptible cells (VERO and MS) **(SOP/CISA/ASFV CELLs/1).**
- Procedure for the detection of antibodies against African swine fever by indirect immunoperoxidase technique **(SOP/CISA/ASF/IPT/1).**

4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
C.E.P: cytophatic effect

IPT: indirect immunoperoxidase technique
m.o.i: multiciplity of infection
MS: kidney epithelial cells extracted from an African green monkey.
VERO: kidney epithelial cells extracted from an African green monkey.

4.2. BACKGROUND

ASFV naturally infected immune-system cells, monocytes-macrophages. While alveolar macrophages and blood monocytes are useful for ASFV isolation and titration, in many cases, it is difficult to obtain these cells in large quantity and reproducibility of results is frequently hindered by variation among different lots of cells. These issues have been partially overcome by the adaptation of some ASFV isolates to grow in different established monkey cell lines like VERO, MS, CV or COS-1. All the established cells lines are cultured according to standard protocols in a 37ºC, 5% CO_2 humidified incubator, grown in plastic tissue culture plates and sub cultured by trypsinization.

The **Spanish strain of ASFV isolated in Badajoz in 1971 (Ba71)** has been adapted to grow in a monkey cell line (VERO). Briefly, the ASFV strain Ba71 was obtained from the spleen of an infected pig in Badajoz (Spain) in 1971. The virus was passaged in porcine macrophages 36 times. VERO cells were infected with virus from passage 36 and virus was propagated 100 times more in those cells. At passage 100 the virus was cloned to obtain Ba71-V, the prototype virus, which was passaged 40 times more.

The **ASFV-E70 virus** was isolated from the spleen of infected pigs in Villagarcía, Pontevedra, Spain, in 1970 and passaged eight times in porcine macrophages before being adapted to monkey stable cells (MS) after 81 consecutive passages. Virus clones generated from E70 MS81 were used as prototype viruses to generate the E70-MS adapted virus.

5. DESCRIPTION

5.1. EQUIPMENT, MATERIALS AND REAGENTS

MATERIAL

- Adsorbent paper.
- Cell cultures flasks:

Description	Growth area (cm2)	Recommended working volume (ml)	Cell yield (based upon a density of $1x10^6$ cell/cm^2)
T-25	25	5-10	$2.5 x10^6$
T-75	75	15-25	$7.5 x10^6$
T-150	150	30-50	$15 x10^6$
T-175	175	35-60	$17.5 x10^6$
T-225	225	45-75	$22.5 x10^6$

- CO_2 (±0.5%) Incubator/ 37±3ºC.
- 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.
- Liquid nitrogen tank.

- 96 well cell culture plate's bottom flat [NUCLONTM "Surface, NUNC or similar characteristics].
- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.
- MiniSart filtre 0.45 µm (Ref. Sartorius 16555).
- 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.
- Sterile glass bottle 250ml and 500 ml.
- Sterile plastic tubes 12 ml and 50 ml.
- Single channel pipettes 200-1000µl.
- Tabletop centrifuge.
- Water bath
- Vortex.

REAGENTS

- **Acetone** [Ref. 1.00014.1000 (Merck) or similar characteristics].
- **Crystal violet** [Ref.:1.1059400100-169 (MERCK) (or similar characteristics]
- **DMEM** -Dulbecco's Modified Eagle Medium- with 4.5g/L Glucose [BW12-741F (BioWhittaker) or similar characteristics] →VERO cell culture medium**.**
- **EMEM** -Eagle Medium- [Ref BF-12-136F (BioWhittaker) or similar characteristics] →Monkey stable cells (MS) cell culture medium.
- **Formaldehyde** [Ref.: 13-128 (PANREAC) or similar characteristics].
- **Gentamicin sulfate 50mg/ml** [Ref.: 17-518Z (BioWhittaker) or similar characteristics].
- **Glutamine [4mM]** [Ref.: BE17-605E (BioWhittaker)] or similar characteristics].
- **Green monkey cells (VERO)** (ATCC, CCL 81).
- **Methanol** [Ref.: 1.06009.1000 (Merck) or similar characteristics]
- **Monkey stable cells (MS)** [ATCC/ECACC 91070510].
- **Na Pyruvate** [Ref.: BE13-115E (BioWhittaker) or similar characteristics]
- **Non-essential amino acid solution 100x** [Ref.: BE13-114E (BioWhittaker) or similar characteristics].
- **Nystatine** [10.000 U/ml] [Ref.: 15340029 (Gibco) or similar characteristics].
- **Serum Fetal Bovine** (SFB) [Ref.: 91S1810-500 Linus or similar characteristics].
- **Trypsin-EDTA** [Ref. BE17-161F (Lonza] or similar characteristics)
- **Phosphate buffered saline (PBS 1x) pH 7.2**(±0.2 UpH) The PBS could be obtained in tablets [Ref.: 524650-1 (CALBIOCHEM) or similar characteristics], liquid [Ref.: BE17-516Q (BioWhittaker) or similar characteristics] or could be prepared as follows:

ClNa [Ref. 1.06404.1000 (MERCK) or similar characteristics]	------	8.0 gr (±0.1)
ClK [Ref. 1.04936.0500 (MERCK) or similar characteristics]	------	0.2 gr (±0.01)
PO_4H_2K [Ref. 1.04873.1000 (MERCK) or similar characteristics]	------	0.2 gr (±0.01)
PO_4HNa_2 [Ref. 1.06586.0500 (MERCK) or similar characteristics]	------	1.15 gr(±0.05)
Distilled water	------	1,000 ml

Store at room temperature. Expiry date: 1 year.

5.2. REAGENTS PREPARATION.

- **Fixed solution** *(in case of IPT staining);* cold solution acetone 30%-methanol 70%. *Store at <-10°C.*
- **Staining solution** *(in case of crystal violet staining);* 2% of crystal violet+ 10% formaldehyde in PBS1x buffer. *Store at room temperature.*
- **MS-cell culture medium → EMEM** supplemented with 10% of inactivated Serum Fetal Bovine (SFBi), 1% non-essential amino acid solution 100x, 1% glutamine [4mM], Gentamicin sulfate 50mg/ml. *Store at 4 ±3°C.*
- **VERO-cell culture medium → DMEM** supplemented with 10% of inactivated Serum Fetal Bovine (SFBi), 1% non-essential amino acid solution 100x, 1% Na Pyruvate, 1% glutamine [4mM], Gentamicin sulfate 50mg/ml. and Nystatine [10.000 U/ml]. *Store at 4 ±3°C.*

- **Heat Inactivated Serum Fetal Bovine** (SFBi) → Heating for 30 minutes at 56°C with mixing to inactivate complement in a water bath. *Store in aliquots at <-10°C.*

5.3. METHODS

5.3.1. VIRUS PRODUCTION

1. **Infect 70-80% confluent MS or VERO cells (**~$1.5x10^7$ cells) with a m.o.i of 10 or 0.2 using the adapted ASFV Spanish isolate Ba71V or E70MS respectively - The cells are infected with:
   - **VERO cells**: 1.5ml of ASVF Ba71VR with titer of 10^{7-8} $TDCI_{50}$+ 1.5ml of medium DMEM.
     Example: in T75 cm2 flask with 80% confluent cells the virus infection is performed by adding 1.5ml of ASFV with estimate titer of 10^8 $TDCI_{50}$ and 1.5ml of medium
   - **MS cells**: 4.3ml of ASVF E70 with titer of 10^6 $TDCI_{50}$+ 4.3ml of medium EMEM.
     Example: in T150 cm2 flask with 80% confluent cells the virus infection is performed by adding 4.3ml of ASFV with estimate titer of 10^6 $TDCI_{50}$ and 4.3ml of medium
2. **Incubate for two hours at 37±2ºC** in continuous agitation (adsorption of the virus). After two hours complete the corresponding flask volume with **medium containing 2% SFBi.**
3. **Incubate at 37±3ºC** in CO_2 atmosphere until an extensive cytopathic effect is observed under a (non-inverted) microscope (**usually at 4-5 days after infections in VERO and MS cells)**
4. Previous to collect cells, **freeze and thaw the flask three times** to assure cell rupture. Then, collect cells and medium by decanting into a 50ml centrifuge tube and clarify by **centrifuging at low-speed 10min 2000 x g, 4ºC** to sediment most of cellular debris.
5. Decant the supernatant into a clear centrifuge tube. The **supernatant contains virus particles** released from the infected cells, in addition to cellular contaminants like vesicles, membrane fragments and subcellular organelles.
6. **Filtrate the supernatant with MINISART filters** of 0.45 microns to remove cellular contaminants. The filtrate supernatant corresponds to the **BA71VR or E70MS STOCK VIRUS** which must be titrated according is described in point 5.3.2

   After this step and in order to increase the titer, **the virus can be concentrate (optionally)** as is described below:

   1. Centrifuge at high-speed 6 hours at 15,000 x g, 4ºC, to concentrate the particulate material, and remove the supernatant.
   2. Consistent sediment must appear in a base corner and along the lower side of the wall near the pellet.
   3. Resuspend the sediment in a small volume (1/300th of the initial volume before centrifugation) of saline (PBS, for further purification) or in culture medium (for concentrated virus to produce virus infections at high m.o.i)

5.3.2. VIRUS TITRATION BY END POINT DILUTION.

1. Seed the cells in a **96-well plate** and incubate the plates at 37±3ºC in CO_2 atmosphere:
   - **VERO cells:** add 100µl of cells per well and incubate for **24 hours** in CO_2 atmosphere**.**
   - **MS cells:** add 100µl of cells per well and incubate for **48 hours** in CO_2 atmosphere.

2. Gently aspirate the medium and **infect the cells with ten-fold dilutions** (100µl final volume per well) **of the virus in culture medium without SFB.** Use a 12-channel mechanical pipettor for multiple titrations.

3. **Incubate for two hours at 37±2ºC in continuous agitation** (adsoption of the virus)

4. After two hours **complete the volume to 200µl with medium to contain a final concentration of 2%SFBi**.

5. **Incubate** at 37±3ºC in CO_2 atmosphere:
   - **5.1** Until **extensive cytopathic effect is** observed: **5 days after infection** in **VERO cells and 3 days after infection** in **MS cells**. If the **TCID CALCULATION** are going to be done **by crystal violet staining see step 7.1**
   - **5.2 For 18h or 24h for Vero and MS cells respectively:** if the **TCID CALCULATION** are going to be done **by IPT staining see step 7.2**

6. Remove the medium (containing the detached cells and cellular debris). *This can be done by holding the plate without the lid and generating a quick 180º flick over a wide waste receptacle*

7. Prepare the plates for **TCID CALCULATION** that can be done;

   7.1 Crystal violet staining→ stain the cell cultures by adding 50µl of 2% crystal violet in 10% formaldehyde to each well. Incubate for 20±2 minutes at room temperature and remove the liquid under a moderate flow of tap water not directed against the cell monolayer. Remove lids from the plates to dry over filter paper to identify positive wells versus non-infected wells.

   7.2 IPT staining → add 50 µl/well of fixed solution. Incubate between 8±2 minutes at room temperature and wash the fixed plate's 20±2 minutes with sterile PBS1x in continuous agitation. Identify positive wells versus non-infected wells by indirect immunoperoxidase technique (SOP/CISA/ASF/IPT/1) using the ASF reference positive sera.

5.4. ANALYSIS AND INTERPRETATION OF THE RESULTS

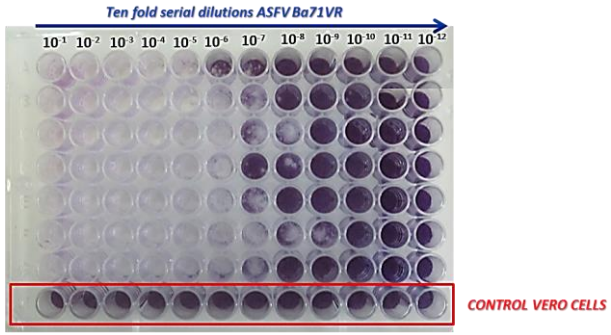
If the **TCID CALCULATION** has been done by crystal violet staining a visual examination of the plates will define the **positive wells in which massive cytopatic effect** lead to the **extinction of the cell monolayer**, while the **non-infected wells will appear heavily stained in blue** (crystal violet stain)

If the **TCID CALCULATION** has been done by IPT staining the plates should be read in a microscope. Those wells in which **≥5** cells show an **intensive red cytoplasmic**

coloration will be defined as positive wells. Wells with less than 5 dyed cells or without any, will be define as negative wells.

TCID CALCULATION

Score the data as percentage of positive wells for each virus sample and calculate the titer in tissue culture infective dose yielding 50% of infection (TCID50) per milliliter using the method of Reed&Muench according is described in the following example.



Reed & Muench Calculator

1. Enter the starting dilution	initial dilution:	0,1												
2. Enter the dilution factor	dilution factor:	10												
3. Enter the volume tested per well	ml:	0,2												
This is your calculated dilution series:		1,00E-01	1,00E-02	1,00E-03	1,00E-04	1,00E-05	1,00E-06	1,00E-07	1,00E-08	1,00E-09	1,00E-10	1,00E-11	1,00E-12	
4. Enter the total # of wells examined per dilution	total wells:	7	7	7	7	7	7	7	7	7	7	7	7	
5. Enter the # of positive wells for each dilution	positive wells:	7	7	7	7	7	7	5	2	1	1	0	0	
These values are calculated automatically:	negative wells:	0	0	0	0	0	0	2	5	6	6	7	7	
	cum pos:	42	35	28	21	14	7	5	2	1	1	0	0	
	cum neg:	0	0	0	0	0	0	2	7	13	19	26	33	
	% infected:	100,00	100,00	100,00	100,00	100,00	100,00	71,43	22,22	7,14	5,00	0,00	0,00	
	prop dist:	0	0	0	0	0	0	0,43548387	-1,84210526	-20	-9	0	0	
6. This is your TCID50	TCID50:							3,67E-08						
7. This is your TCID50/ml	TCID50/ml:							1,36E+08						

TITER 1, 36X10^{8} TCDI$_{50}$/ML

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