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SOP/CISA/ASF/IPT-PLATES/1

STANDARD OPERATING PROCEDURE FOR THE PREPARATION OF AFRICAN SWINE FEVER VIRUS-COATED 96-WELL PLATES FOR INDIRECT IMMUNOPEROXIDASE TEST (IPT)

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1. PURPOSE

The main goal of this procedure is to describe the preparation of African swine fever virus (ASFV)-Coated 96-well plates to be used as an antigen in the indirect immunoperoxidase technique (IPT) for ASFV specific antibody detection.

2. SCOPE

This procedure is applicable to established green monkey cell lines infected with adapted ASF viruses.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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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 of Growing and Titration adapted African swine fever virus (ASFV) isolates **(SOP/CISA/ASFV/TITRATION/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. The persistence of ASFV experimentally induced in VERO and MS cells has been described through the action of CINH4 and 5-iodo-2'-desoxiuridina. The ASFV multiplies in the cytoplasm of the cell requiring the cell nucleus to do it. The entry in the cell is by endocytosis with the formation of vesicles in which several virions are enclosed fused their envelopes with the membrane endosome releasing to the cytoplasm cell. In Vero cell cultures this step seems to be associated with a receptor. The complete intracellular virus migrates to the membrane cell and it is released from the cell with a new cell envelope with viral proteins.

The **immunoperoxidase technique (IPT)** is an immune-cytochemistry technique on fixed cells to determine the antibody-antigen complex formation through the action of the peroxidase enzyme. In this procedure, VERO or MS cells are infected with ASFV adapted isolates to these cell cultures. The infected cells are fixed and are used as antigens to determine the presence of the specific antibodies against ASF.

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.
- 96 well cell culture plate's bottom flat [NUCLONTM "Surface, NUNC or similar characteristics].
- 96 Well (1 x 8 Stripwell™) Clear Flat Bottom Polystyrene [REF 9102 CORNING or similar characteristics].

- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.
- 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.
- Water bath
- Vortex.

REAGENTS

- **Acetone** [Ref. 1.00014.1000 (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. **Monkey stable cells (MS)** [ATCC/ECACC 91070510]**.**.
- **Gentamicin sulfate 50mg/ml** [Ref.: 17-518Z (BioWhittaker) or similar characteristics].
- **Glacial acetic acid** [Ref.: 141008.1611 (PANREAC) 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]
- **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) [Ref.: BE17-516Q (BioWhittaker) or similar characteristics]

5.2. REAGENTS PREPARATION.

- **Fixed solution;** cold solution acetone 30%-methanol 70%. *Store at <-10°C.*
- **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. PREPARATION OF ASFV-Coated 96-well

A) Growth of monkey stable (MS or VERO) cells in 96 well cell culture plate's bottom flat.

1. From 90% confluent T150 flask [growth area 150 cm^2/~1.5x 10^7 cells] remove and discard the cell culture medium

2. Add 2-3 ml of trypsin-EDTA solution, spread the liquid onto the entire surface by tilting the flask, and then immediately remove as much liquid as possible.

3. Add 5 ±2 ml of trypsin-EDTA to cover the entire surface, incubate 5-10 minutes at 37◦C in 5% CO_2 humidified incubator until the cells appear to be detached (they will appear rounded and retractile under the microscope). Check the progress of cell dissociation by microscopy. To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for them to detach.

4. Once the cells appear to be detached discard the trypsin-EDTA and add 10 ml of complete DMEM (VERO) or EMEM (MS) cell culture medium supplemented with 10% of SFBi to the cell suspension to inactivate the trypsin.

5. Resuspend the cells by pipetting up and down to disaggregate cell clumps. Check the cells with the microscope to be sure that most (>95%) are single cells. If cell clusters are apparent, continue to disperse the cells with gentle pipetting.

6. Subculture the line at a 1:2 split ratio (split the culture in half) and distribute the diluted cell suspension into the required number of 96-microwell plates [growth area/well 0.32 cm^2] to be prepared.

Example:

- **1 T150 flask** [growth area 150 cm^2/~1.5x 10^7 cells] **equivalent (1:1 proportion) to five 96-microwell plates** [growth area 32 cm^2/~3.2 x 10^6 cells]
- **1 T150 flask** subculture at a 1:2 split ratio → 10ml of cells in 90 ml of cell culture medium supplemented with 10% of SFBi to seed 10 plates (100 µl/well)

7. Incubate **24 (VERO) or 48 (MS) hours** at **37±3°C in 5% CO_2 humidified incubator** to get an **80-90% confluent** plate

B) Infection with adapted ASF virus.

1. After **24 or 48 hours of incubation** carefully decant the medium of the cell cultures grown in the 96-microwell plates.

2. In a separate bottle prepare the appropriated dilution (in culture medium without SFB) of the **adapted ASFV to inoculate with a m.o.i between 0.025-0.05**

The dilution factor is calculated using the following formula;

$$\textbf{Dilution factor} = \frac{0.7\text{x Virus titer* x Volume}}{\text{nº cells x m.o.i}}$$

**NOTE; for virus titration see the Procedure of Growing and Titration adapted African swine fever virus (ASFV) isolates (SOP/CISA/ASFV/TITRATION/1).*

Example (m.o.i of 0.03)

- Our virus stock contains 1.5 x 10^6 TCDI50/ml.
- The number of infectious virus particles in 1ml stock is thus 0.7x1.5 x 10^6 TCDI50/ml infectious particles.
- We add 0.1 ml of virus solution to a well that has 0.32 x 10^5 cells.
- **Final MOI = 0.03.**

$$\textbf{Dilution factor} = \frac{0.7 \times 1.5 \times 10^6 \times 0.1}{0.32 \times 10^5 \times 0.03} = 109.4$$

If you want to add diluted virus solution to 10 plates, the total volume you need is 100wells×10platesx100µl and therefore a volume of **914µl** of the virus stock (100.000/dilution factor = 100000/109.4 = 914 µl) **should be diluted to 100ml** in appropriate medium

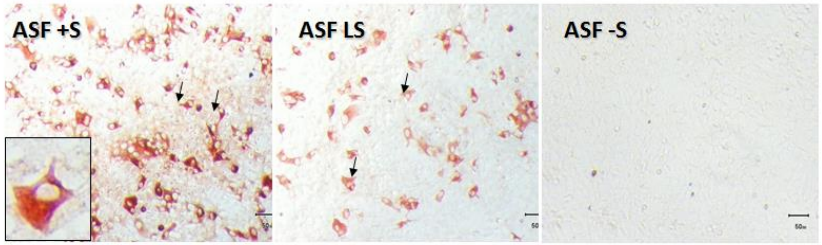
3. **Inoculate the plates** with 100 µl/well of the inoculum and **incubate** at 37±3 ºC in 5% CO_2 humidified incubator.
   a. **18±1 hours** in case of VERO infected cell plates**.**
   b. **24±1 hours** in case of MS infected cell plates: **In this case, after two hours** at 37±3ºC, complete the volume to 200µl with medium +4% SFBi (final concentration of 2%SFBi).

4. **Fix the cells**:
   - Remove the inoculum by aspirating.
   - Add 50 µl/well of fixed solution.
   - Incubate between 8±2 minutes at room temperature.
   - Wash the fixed plates 20 minutes with sterile PBS1x in continuous agitation.
   - Dry the plates at room temperature

5. **The fixed and dry IPT plates can be used directly or stored at <-10°C for 6 months.**

5.4. QUALITY CONTROL

The **suitability of each batch** of the coated 96-well plates fixed with ASFV adapted viruses for the **IPT** (SOP/CISA/ASF/IPT/1) must be demonstrated by *checkerboard titration* of the ASF reference positive, limit and negative sera (PC, LC and NC) performing the procedure as is described in SOP/CISA/ASF/IPT/1. A new batch of ASFV-IPT plates is consider as **optimal** when at **1:40 dilution of sera** we observe red cytoplasmic coloration in the ASF PC (intensive) and in the LC (mild). Negative control must appear without staining as is showed in the following figure.



NOTE; positive, limit and negative reference control sera for IPT can be supplied by the EURL (order at the eurl.asf@inia.es)

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