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STANDARD OPERATING PROCEDURE FOR PROCEDURE OF CELL GROWTH AND PROPAGATION OF ASFV SUSCEPTIBLE CELLS (VERO AND MS).

CONTENTS

1. PURPOSE

The main goal of this procedure is to describe the cell growth and propagation of ASFV susceptible established green monkey cell lines, VERO and MS, but can also be used in other cell lines, considering that each cell line will be somewhat different, and line-specific procedures should be followed.

2. SCOPE

This procedure is applicable to established cell lines VERO and MS. All of these protocols can be applied, with minor modifications, to other virus and cell models.

3. REFERENCES

3.1. DOCUMENTS USED IN THE PROCEDURE REDACTION

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3. ATCC® ANIMAL CELL CULTURE GUIDE tips and techniques for continuous cell lines

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]
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4. BACKGROUND INFORMATION

4.1. ABBREVIATION

ASF: African swine fever
ASFV: African swine fever virus
MS: kidney epithelial cells extracted from an African green monkey.
VERO: kidney epithelial cells extracted from an African green monkey.
rpmi; revolution per minute.

4.2. BACKGROUND

ASFV naturally infected immune-system cells, monocytes-macrophages. The persistence of ASFV experimentally induced in green monkey established cell lines has been described through the action of $CINH_4$ 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 **green monkey established cell lines such as VERO, MS or COS cells, can be used for ASFV virus propagation, titration, purification or for the preparation of ASFV the preparation of ASFV-Coated 96-well plates to be used as an antigen in the indirect immunoperoxidase technique (IPT) for ASFV specific antibody detection.**

The cell lines described in this procedure, both VERO and MS, are cultured according to standard protocols in a 37ºC, 5% CO_2 humidified incubator. Established cell lines are grown in plastic tissue culture plates (obtained from several providers as Falcon, Nunc, and Costar) and subcultured by trypsinization.

Most cell cultures can be stored for many years, at temperatures below –130°C **(cryopreservation)**. As the cell suspension is cooled below the freezing point, ice crystals form and the concentration of the solutes in the suspension increases. Intracellular ice can be minimized if water within the cell is allowed to escape by osmosis during the cooling process. A slow cooling rate, generally –1°C per minute, facilitates this process. However, as the cells lose water, they shrink in size and will quickly lose viability if they go beyond a minimum volume. The addition of cryoprotectant agents such as glycerol or dimethylsulfoxide (DMSO) will mitigate these effects.

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.
- Micropipette disposable sterile tips of 1-20, 20-200 and 200-1000 µl.
- Phase Contrast Inverted cell Culture Microscope.
- Ph meter (0.01 UpH).
- Pipetboy acu or equivalent.
- Plastic cryopreservation vials.
- 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

- **DMEM -**Dulbecco's Modified Eagle Medium- with 4.5g/L Glucose [BW12-741F (BioWhittaker) or similar characteristics] →VERO cell culture medium**.**
- **DMSO (Dimethyl Sulphoxide)** (Ref. D2650 (Sigma) or similar characteristics)
- **EMEM** -Eagle Medium- [Ref BF-12-136F (BioWhittaker) or similar characteristics] →Monkey stable cells (MS) cell culture medium.
- **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).
- **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) [Ref.: BE17-516Q (BioWhittaker) or similar characteristics]

5.2. REAGENTS PREPARATION.

- **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. CELL DEFROST.

1. Thaw the vial by gentle agitation in a water bath at 37°C. Thawing should be rapid, approximately 2 minutes or until ice crystals have melted.
2. Remove the vial from the water bath and decontaminate it by dipping in or spraying with 70% ethanol.
3. Follow strict aseptic conditions in a laminar flow tissue culture hood for all further manipulations
4. Unscrew the top of the vial and transfer the contents to a sterile centrifuge tube containing 9 mL of the recommended medium.
    - MS-cell culture medium → EMEM
    - VERO-cell culture medium → DMEM
5. Remove the cryoprotectant agent (DMSO) by gentle centrifugation (5 minutes at 1500 rpm). Discard the supernatant, and resuspend the cells in 1 or 2 mL of complete growth medium.
6. Transfer the cell suspension into a T25 culture flask containing the complete growth medium and mix thoroughly by gentle rocking

7. Examine the cell cultures after 24 hours and subculture as needed (or change medium if necessary) according is described in section 5.3.1.

5.3.2. CELL GROWTH AND PROPAGATION

1. Bring the trypsin-EDTA solution balanced and complete growth medium to the appropriate temperature for the cell line (for Vero and MS cell lines, this is the temperature used to grow the cells (37ºC).

2. From **90% confluent T25 flask** (growth area 25 cm^2/~2.5x 10^6 cells) remove and discard the cell culture medium.

3. 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.

4. Add **3 ±1 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.

5. Once the cells appear to be detached **discard the trypsin-EDTA** and **add ~5ml of complete** DMEM (VERO) or EMEM (MS) cell culture medium supplemented with 10% of SFBi to the cell suspension to inactivate the trypsin.

6. **Resuspend the cells** by pipetting up and down to disaggregate cells 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.

7. **Subculture the cell line** according to a routine split ratio to maintain the cell culture.

   Examples:

   - **1 T25 flask subcultured to 2 T25 (1:2 split ratio)** → considering a final volume of 10ml/T25 distribute 2.5 ml of cells in 7.5 ml of cell culture medium supplemented with 10% of SFBi in each T25.
   - **1 T25 flask subcultured to 1 T75 (1:3 split ratio)** → considering a final volume of 20ml/T75 distribute 5 ml of cells in 15 ml of cell culture medium supplemented with 10% of SFBi in each T75.
   - **1 T75 flask subcultured to 1 T150 (1:2 split ratio)** → considering a final volume of 50ml/T150 distribute 5 ml of cells in 45 ml of cell culture medium supplemented with 10% of SFBi in each T75.

8. Place the flask/flasks back into **37±3 ºC in 5% CO_2 humidified incubator**. Examine the culture the following day to ensure the cells have reattached and are actively growing. Change the medium as needed or make passages to maintain the cell line.

To ensure viability cell lines need to be subcultured on a regular basis before they enter the stationary growth phase, before a monolayer becomes 100% confluent. After a few passages, it is possible to freeze cells (see 5.3.3).

5.3.3. CELLS CRYOPRESERVATION.

The standard procedure for cryopreservation is to freeze cells slowly until they reach a temperature below –70°C in medium that includes a cryoprotectant (DMSO). Vials are transferred to a liquid-nitrogen freezer to maintain them at temperatures below –130°C.

1. **Trypsinize cells** in the T25 falcon, by adding first 2-3 ml and discarding it (this step is to throw out died cells) and add 2-3 ml of trypsine. Mix cells with complete growth medium.

2. **Centrifuge at 1500 rpm 5 minutes**.

3. **Resuspend cells (pellet) in SFB with 1%DMSO**.

   Example:

   - 1 vial (1ml/vial)= 900µl SFB + 100 µl DMSO (add DMSO drop to drop)
   - 1T25 (1-5x10^6 cells) = 1 vial (90% SFB,10% DMSO)

Important: move the vial inside an ice box during DMSO addition.

4. Allow cells to **equilibrate in the freeze medium** at room temperature for a minimum of **15 minutes** but no longer than 30. This time is usually taken up in dispensing aliquots of the cell suspension into the vials.

5. Place the vials into a mechanical freezer at <–70°C (or colder) for at least 24 hours. After, transfer the vials to a liquid nitrogen or <–130°C freezer.

5.4. CRITICAL POINTS

- **Cells form clumps after dissociation**:
  - The dissociation procedure was too harsh and genomic DNA was released from lysed cells. Either the pipetting was too vigorous or the dissociating solution was too strong or too toxic (i.e., the pH or osmolality of the buffer was incorrect). In the future, treat the cells more gently during pipetting, shorten the incubation period, use a weaker dissociation solution (lower the enzyme concentration or remove the EDTA), or incubate at a lower temperature.
  - The cells aggregated before dilution and dispersion into the medium. Hold the cell suspension on ice if there will be a delay between removing the cells from the flask growth surface and seeding a new flask.
- **Cells have difficulty reattaching to the flask**:
  - The dissociation procedure was too long and stripped away necessary attachment proteins from the cell membrane.
  - Insufficient serum or attachment factors were present in the medium.
- **Viability is lower than expected:**
  - The dissociating procedure was too harsh.
  - The medium was faulty. Use the recommended formulation and make sure it contains all of the required additives.

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