**SOP: Clean Cell Culture**

One Health Center of Excellence

Emerging Pathogen Institute

University of Florida

1. **PURPOSE:**

This SOP provides details and background for maintaining cultured cells and preparing cells for infection with viruses. This protocol will describe how to grow VERO cells from frozen stock.

*Consult your supervisor or University of Florida* ***before*** *varying any part of this protocol.*

1. **PRECAUTION:**
2. Biosafety:

Although, clean cell culture has a requirement of being pathogen free, some cell lines are carcinoma-like and must be treated as infected. In this SOP we will only deal with VERO cell line which is not associated with cancers. Treat all cells and media as though they are infectious, i.e. wear gloves, autoclave all spent material, etc.

1. Other Safety:

Avoid looking into the hood when the UV-light is turned on.

1. During Assay:
2. Use sterile technique at all times.
3. Wash your hands and arms with soap before touching tissue culture flasks and reagents.
4. Turn on the UV light and air 10 min prior to working in the hood.
5. Immediately before working and after working, wipe the working surface of the hood with 70% freshly prepared ethanol or SaniWipes®.
6. Do not place anything into the tissue culture hood unless you wipe the surface with 70% ethanol first.
7. Keep the incubator very clean, if your incubator has a self-cleaning system, make sure to run it at least once per week, if not, then clean the interior surface with alcohol and avoid having any drops of media or contaminated water in it. Make sure to only use distilled water to fill the incubator gaskets. Any contamination within the hood will affect culture growth and would be very difficult to get rid of once accumulated.
8. Place cells in clean bags when transferring from one room to the other. Avoid them being thawed during transferring process.
9. Media bottles should never be open outside the hood. Make sure to close it tightly and seal the rim with additional paraffin to prevent introduction to contamination.
10. Cells must not be allowed to overgrow.
11. **BACKGROUND:**

Vero cells are derived from African green monkey kidney cells (Cercopithecus aethiops). They are the most commonly used mammalian cell line used in virology laboratories. There are multiple lines of the cells commercially available (Vero, Vero 76, Vero-E6) all are derived from the same source and all follow the same protocol for propagation.

1. **PROCEDURE:**
2. Preparing first cell passage from ATCC viral stock:
   1. Prepare all flasks, pipettes and equipment inside the hood after wiping with alcohol before you thaw cell vial.
   2. In a 37⁰C clean water bath quickly thaw the vial of Vero cells by gently swirling until fully thawed.
   3. Decontaminate the vial before bringing inside a laminar flow hood.
   4. Transfer cell suspension from the cryovial into a 15mL conical tube containing 10 mL of DMEM supplemented with FBS.
   5. Frozen cell stocks contains cryopreservant such as dimethyl sulfoxide (DMSO), which has negative and harmful effect on cells, therefore it is important to wash the cells from them before you propagate them.
   6. Centrifuge the tube at 200 x g for 5 minutes at room temperature (high speed may have negative effect on the cells)
   7. Remove and discard supernatant; resuspend cells in 5-10mL of DMEM supplemented with 10% FBS/
   8. Transfer Vero cell suspension to tissue culture flask with vented cap.
   9. Incubate flasks in 37⁰C incubator with 5% CO2.
   10. Observe cells daily under an inverted microscope, change media every 3-4 days. When cells reach >90% confluent monolayer, pass cells into a new tissue culture flask as described below.
   11. Note that Vero cells recover slowly after freezing; therefore it may take up to a week before the cells are ready to be passaged. It may take 2-3 passages before the cells reach their normal growth rate.
3. Maintenance of Vero Cell Culture:
4. Use a T-75cm2 culture flask of more than 90% confluency for splitting. *Note: The cells would act like normal cells when pressured with crowding signaled by touching effect; meaning cells would stop growing or would highly slow down when touched by neighboring cells and eventually die. Hence it is crucial to observe cells properly and split immediately after confluency is attained.*
5. Inside a laminar-flow hood, remove the growth media by decanting the flask or with a pipet adhered to the upper side of the flask (the side that has no cells).
6. Wash cells with (pre-warmed to 37⁰C) 10mL of 1X DPBS by gently rocking the flask to allow the solution to wash all cells. Growth media containing serum which would inhibit the effect of trypsin and prevent cells from dislodging of the flask surface.
7. Add 5mL of DMEM of 1X trypsin-EDTA and incubate cells at 37⁰C for 2-3 minutes, until cells start to streak as they detach from the flask.
8. You may tap the flask gently or shake it multiple times to facilitate detachment.
9. When most cells have fallen off, add 5mL of DMEM with 10% FBS to inactivate the trypsin-EDTA.
10. Gently pipette the 10mL using a 10mL serological pipette, press the tip of the pipet to the bottom of the flask and allow the fluid to pass through helping cells to separate individually and prevent clumping.
11. Transfer cell solution in a 15mL conical tube.
12. Centrifuge at 200 X g for 5 min at room temperature.
13. Remove and discard supernatant; re-suspend cells in 10mL DMEM with 10% FBS.
14. Prepare the desired dilution of cells in a total of 12-20mL DMEM with 10% FBS and add to 75cm2 cell culture tube with vented caps.
15. Cryo-preservation of cell stocks:
16. When culturing it is crucial to store some of your passaged cells for future usage to avoid wasting cells of lower passages. Also the number of passages for cells should not exceed 12-15 passage past the ATCC number; hence you need to store much of your early passages to use them in the future for virus isolation.
17. In a 15mL conical tube add 9mL of DMEM with 10% FBS.
18. Add 1mL of DMSO solution to the above tube to have a final concentration of 10% DMSO. Open the vial of DMSO under sterile conditions only.
19. Remove growth medium from confluent monolayer of Vero cells.
20. Wash cells with 10mL 1X DPBS. Serum will inhibit trypsin effect; therefore it is important to wash cells with sterile PBS to remove traces of serum.
21. Add 5mL of 1X trypsin-EDTA and incubate for 2-3 minutes, until cells start to streak as they detach from flask. A gentle shake or spank to the tube would help cells detach.
22. Add 5mL of DMEM with 20% FBS to inactivate the trypsin-EDTA.
23. Wash down cells in media, pipetting gently to break up any clumps of cells.
24. Remove cell suspension from the flask into a 15mL conical tube.
25. Centrifuge at 200 X g for 5 min at room temp.
26. Under sterile conditions discard supernatant; resuspend cells in 10mL DMEM with 20% FBS and 10%DMSO.
27. Aliquote 1mL of cell suspension into well labeled cryovials carrying the following information: [cell line- passage- date cryopreserved- initials of technician working].
28. Place cryovials in slow freezing container (Mr. Frosty) with a cooling rate of -1⁰C/min.
29. Place Mr. Frosty in -80⁰C for 24hrs, then transfer in liquid nitrogen for long term storage.

**Appendix I:**

Equipment:

* + - Incubator with CO2 gas chamber 37⁰C
    - Biosafety Cabinet
    - Water Bath
    - Refrigerator
    - Tissue Culture Plates (Polypropylene)
    - CO2 cylinder
    - Pipet aid
    - Ice Maker machine
    - Mr. Frosty

**Appendix II:**

Materials:

* Vero Cell Stock, frozen in liquid nitrogen or -800C ( commonly purchased from ATCC or ECC)
* Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% inactivated fetal bovine serum (FBS)
* 1X Trypsin-EDTA in DPBS without calcium or magnesium, filter-sterilized.
* Tissue culture flasks of different sizes (T25-75-150 cm2)
* 15mL conical tubes, sterile (polypropylene)
* Serological pipets, sterile.
* Dimethyl sulfoxide (DMSO)

Cell medium volume requirements:

|  |  |
| --- | --- |
| **Vessel** | **total medium volume** |
| T-25 Flask | 4-6 mL |
| T-50 Flask | 7-10 mL |
| T-75 Flask | 12-20 mL |
| T-150 FkasK | 24-40 mL |
| 12 well plate | 1.5mL/well (18mL/plate) |
| 24 well plate | 750uL/well (18mL/plate) |
| 96-well plate | 200uL/well (19mL/plate) |
| 1cm2 tube | 1mL/tube |

Cell Culture tube request form:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | | Split for TC tubes | | | | |
|  | % Confl | Dilution |  | # of tubes | Conf | Ready in N days |
| Cell line | Split\* | +1d |
| VERO | 90-95% | 25 to100ml |  |  |  | 1 d |
| 5 to 100mL |  |  |  | 2d |
|
| 4 to 100mL |  |  |  | 3d |
|
| 0.8mL to 100mL |  |  |  | 6d |
|

\*Indicate the desired volume of cells + the volume of media to add. E.g. if you want 90 tubes of Vero cells ready in 2 days, ask for “5 + 95” (5 ml of cells + 95 ml of media = 100 ml – so you will be sure to have enough volume for 90 tubes).

##### **Clean Cell Culture**

Worksheet

Cell \_\_\_\_\_\_ TCCRXG\_\_\_\_\_ Maximum passages\_\_\_\_\_

Observations (%Confluence/temp) Viability/

Pass Date Tues Thurs Sat Sun Split Observations

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Abbreviations: Cl=clumped; O.G.=overgrown; d=detached; 28 or 37=moved to the 28°C (or 37) incubator; B.U.=backup flask (kept at 28°C)

##### **Clean Cell Culture Reagents**

Worksheet

LOTS

Date DMEM FBS Gln P/S Trypsin

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