Chicken Embryo Fibroblast Cell Culture and Plaque Assay Protocol

Purpose: This protocol describes the methods used to passage chicken embryo fibroblast cells for increasing cell stocks and to perform plaque assays on La Sota Strain B1 lentogenic Newcastle Disease live virion samples to calculate viral titers and observe their infectivity. The protocol was adapted from McGinnes et al. (2006).

Materials:

- Cell Culture Medium: 442.5mL 1X Advanced DMEM, 2.5mL 10mg/mL Pen/Strep, 5mL 200mM L-glutamine, 50mL FBS
- Overlay Medium
    - Media: 38.5mL 1X Advanced DMEM, 500µL Pen/Strep, 1mL 200mM L-glutamine, 10mL stock tryptose phosphate broth warmed to 46˚C
    - Agar: 1.5% Nobel agar autoclaved and cooled to 46˚C
    - Final Overlay: 20µL of 0.05% Trypsin + 1580µL of media + 400µL of agar, kept at 46˚C
- 0.05% Trypsin
- PBS
- Virion Stock Solution
- Trypan Blue
- Centrifuge
- Inverting light microscope
- Hemacytometer
- Biosafety Cabinet
- Serological pipets and pipet aid
- Micropipet and tips
- 70% Ethanol
- Methanol
- Giemsa Stain
- 6-well tissue culture-treated plates

Procedures:

Procedures should be done using aseptic technique in a biosafety cabinet and in biosafety level two laboratory.

1. Cell Seeding
    a. Thaw 1mL vial of about 15 million cells in 37.5˚C water bath until there is only a small ice crystal left
    b. Mix the thawed cells and move the cell suspension into a 50mL conical tube
    c. Add 4mL of 37.5˚C cell culture medium dropwise and mix
    d. Centrifuge the cell suspension at 450 RCF for 5min.
    e. Aspirate the supernatant and resuspend the cells in 15mL of cell culture medium

f. Take a 100µL aliquot of the cell suspension and dilute in 100µL of Trypan Blue to count on a hemocytometer
g. Calculate the cell concentration and add the appropriate volume to a 6-well plate to seed at 300,000 cells per well
    i. 300,000 cells * [Average cell count for each quadrant * dilution factor (2)] * $10^4 mL^{-1}$]
h. Add cell culture media so the total volume is 2-3mL
i. Rock the plate to evenly distribute the cells
    i. avoid swirling
j. Place 6-well plate in a 5% CO_2 and 37.5˚C incubator
    i. cells should adhere within 4hr of seeding
k. Change the media every 2 to 3 days until 80% to 90% confluent with 2mL of media
l. Once 80% to 90% confluence is reached the cells are ready for a plaque assay or for a passage

2. Plaque Assay
    a. Make a 10-fold serial dilution of your virion sample from 10^{-2} to 10^{-8} on ice in cold cell culture medium
    b. Aspirate spent media from 6-well plates
    c. Wash each well with 1mL of PBS by gently rocking the plates
    d. Aspirate the PBS and add 200µL or virion dilution in duplicate to your 6-well plates
        i. Include a positive control of HA titered virus and negative control of only cell culture medium
    e. Rock the plates and incubate at 37.5˚C for 45min
    f. Aspirate off the virion dilutions and controls, then add 2mL of 46˚C overlay medium down the side of each well
    g. Let the overlay set for 30min at room temperature and place the 6-well plates in a 5% CO_2 and 37.5˚C incubator
    h. Let the plates incubated for 72hr or until plaques form
    i. Remove the overlay medium with a spatula
        i. Be careful not to scratch the cell growth surface
    j. Fix the cells with 2mL of Methanol for 5min
    k. Stain the cells with 1:20 dilution of stock Giemsa stain solution
    l. Visualize plaques and calculate the plaque-forming units
        i. [# of plaques * dilution] / 200µL
3. Cell Passaging
    a. Aspirate spent media
    b. Wash each well with 1mL of PBS
    c. Aspirate PBS and add 250µL of 0.05% Trypsin for trypsinization
    d. Rock the plate and incubate at 37.5˚C for 5min
    e. Quench each well with 500µL of cell culture medium and wash the plate several times

f. Move the cell suspension to a 50mL conical tube and centrifuge it at 450 RCF for 5min
g. Aspirate supernatant and resuspend the pellet in 2mL of cell culture media per harvested well
h. Take an aliquot of cell suspension for a 1:2 dilution in Trypan blue and count on a hemacytometer
i. Proceed to step d. of Cell Seeding

References

McGinnes, L.W., Pantua, H., Reitter, J., and Morrison, T.G. (2006). *Newcastle Disease Virus: Propagation, Quantification, and Storage*. In Current Protocols in Microbiology, R. Coico, A. McBride, J.M. Quarles, B. Stevenson, and R.K. Taylor, eds. (Hoboken, NJ, USA: John Wiley & Sons, Inc.), pp. 15F.2.1-15F.2.18.