Production of High Titer Virus (FV3) (/ /1)

P

Be sure that the plastic tubes weigh the same

- use a balance for this step and add PBS using a glass pipette until all the tubes weigh the same

Put centrifuge tubes into the 30 mL centrifuge metal tube for ultracentrifugation (tubes 1-4, 2-5, 3-6).
Ultracentrifuge samples at 28000 rpm for 90 min at 4 ^{o}C.

- Rotor type: SW28 #3766
- Temperature: 4 ^{o}C
- RPM: 28K
- Run length: 1 hr 30 min
- w^2t: 4.64
- rad^2/sec:10

Slowly decant the supernatant.
Re-suspend in 100 – 500 µL PBS depending on the size of the pellet.
Leave samples in 4 ^{o}C overnight for pellet to dissociate. Seal tubes with parafilm.
Re-suspend the pellet well and store in autoclaved tubes (1 mL per tube).
Quantify by plaque assay or TCID50.

C) <u>Determination of virus load by Plaque Assay</u>

***Prepare 1% methylcellulose overlay medium at least two days in advance.*

Reagent	**Volume**
Methyl cellulose (Invitrogen Cat # 11965-092)	5 g
DMEM (Invitrogen Cat # 11965-092)	250 mL

Weigh out methylcellulose into a 500 mL bottle with a magnetic stir bar and autoclave. When cooled, add the DMEM, and dissolve with stirring at room temperature for ~ 2hr. Store at 4 ^{o}C

*** Warm the 1% methylcellulose overlay media and BHK-21 growth media to 37^{o}C.**

4. Thaw FV3 stock virus to be titrated on ice. Vortex when thawed.
5. Prepare and label micro-tubes for viral dilutions.
6. For plaque assay in duplicates add 1mL BHK-21 growth media to each micro-tube (except the last dilution, add only 900 mL BHK-21 growth media).
7.

Mammalian BHK-21 cell culture

BHK-21 (M) .

) - 1

Filter through 0.2µm filter. Once growth media is mixed, it can be stored at 4°C for 1 month (as long as care is taken to prevent contamination & maintain sterility). Label with date.

Reagent	**Volume**
DMEM (Invitrogen Cat # 11965-092)	200 mL
FBS (10%)	20 mL
1:1 Penicillin-Streptomycin/Glutamine (2%)	4.2 mL
Kanamycin (FMD # 80502-840 5g) (10mg/mL)	200 !L

* Frozen reagents can be thawed/heated to 37°C in a water bath. Vortex to mix.

Sub-culturing Protocol:

Cell line should be passaged every 2 days.
Cells are incubated in a humidified atmosphere of 5% CO_2 at 37°C.

Solutions: PBS (Dubecco's Phosphate Buffered Saline – GIBCO #14080)
BHK-21 Growth Medium
Trypsin

Procedure:

BHK-21 cells should be expanded into vented sterile cell culture flasks (75 cm^2).
Inspect cells for confluency and lack of contamination.
Remove and discard the old media.
Rinse the cell monolayer with PBS (5 mL per 75 cm^2 flask)

- rock the flask back and forth a few times, making sure the solution covers the entire surface area of the flask
- remove and discard the PBS

5. Add trypsin (1 mL per 75 cm^2 flask)
   - rock the flask back and forth a few times, making sure the solution covers the entire surface area of the flask
   - incubate at 37 °C for ~ 5 mins, until cells are detached
6. Add 20 mL BHK-21 growth media to the flask, creating a cell suspension. When pipetting growth media into the flask, "wash" the sides of the flask to ensure all cells become suspended. The same cell suspension solution can be used to wash two flasks (75 cm^2).
   * If more than two flasks are being sub-cultured, growth media added to create cell suspension should be increased proportionally; otherwise, cells will be too dense when counting in a hemacytometer.
7. Transfer the cell suspension into a Falcon tube (50 mL).
8. Centrifuge for 10 min, at 1000 rpm, at 4 °C.

9. Meanwhile, add fresh BHK-21 growth media into new flasks (10 mL per 75 cm^2 flask). Label the flask with the cell line identifier, date, and cell passage number.
10. Pour of the supernatant from the centrifuged cell suspension and re-suspend cells in 20 mL fresh BHK-21 growth media.
11. Using the new cell suspension, determine the appropriate inoculum for sub-culturing.
    - seeding density may be determined by performing growth rate studies, or by counting
    - see Excel file (worksheet "Cell Count") for hemacytometer protocol

    * When simply passing a culture, when tracking exact cell densities is not necessary, a split according to a suspension ratio is commonly used.
12. Dispense the appropriate amount of cell suspension (~ 4 x 10^6 BHK-21 cells per 75 cm^2 flask) into the new culture flask
    - add the cells directly into the growth medium and mix well by pipetting up and down a few times
    - rock the flask back and forth a few times
    - between seeding each flask, make sure cells are kept in suspension by mixing up and down with pipette
13. Incubate cells at 5% CO_2 at 37 ^oC.
14. After 24 hrs, observe culture for reattachment and active growth.