Production of High Titer Virus (FV3) (7/8/15)

Protocol used for the production of FV3 on baby hamster (Mesocricetus auratus) kidney (BHK-21) cells.

A) Cell Infection with FV3

Cell culture flasks (75 cm^2) containing BHK-21 cells should be seeded 1 day in advance to ensure that the cell monolayer reaches 80-90% confluency at time of infection.

Buffer and medium: - PBS (Dubecco's Phosphate Buffered Saline – GIBCO #14080)
- BHK-21 Growth Medium:

Procedure:

1. Remove and discard the old media from cell culture flasks (75 cm^2).
2. Rinse the cell monolayer with PBS (2 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 then remove and discard the PBS
3. Add 2 mL fresh BHK-21 media into the flask.
4. Absorb by adding a specific amount of virus (0.1 to **0.01 MOI** – used in Robert Lab) into the flask.
   - label flask with MOI
   - rock the flask back and forth a few times to ensure virus is spread evenly over cells

   * Pipette virus directly into cell media – be careful not to disrupt the cell monolayer
5. Wrap flasks in ddH_2O moistened paper towel and aluminum foil to maintain humidity during viral attachment phase.
6. Incubate cells with FV3 by gently rocking for 1 hr at room temperature.
7. Add 10 mL of fresh BHK-21 cell media to the flask.
   * Robert Lab does not remove inoculum – however, other labs do.
8. Incubate cells at 5% CO_2 at 30^oC for 5 days or until cells are completely lysed.
   * FV3 cannot survive at 37^oC.
9. Harvest the virus by scraping surviving cells and re-suspending vigorously with a 10 mL pipette.
10. Transfer cells into a 50 mL Falcon tube.
11. Vortex a few times and store at -80 ^oC until ready to purify

B) Virus Purification Protocol

1 day in advance: Ultra centrifuge metal tubes must be rinsed with 30 mL 70% ethanol and dried under UV light overnight.
Beckman centrifuge plastic tubes must also be rinsed with 70% ethanol and dried in the hood.

Buffers: - PBS (Dubecco's Phosphate Buffered Saline – GIBCO #14080)
- 30% Sucrose Buffer – 500 mL (150 g sucrose + 50 mL 10x PBS + up to 500 mL ddH_2O)

Procedure:

1. Freeze (on dry ice) and thaw (in 37 ^oC water bath) the harvested virus 3 times, vortexing for 10 sec at each step.
2. Warm the 30% sucrose buffer at room temperature before use, and cool the ultra centrifuge to 4 ^oC before use.
3. Spin cells at 2000 rpm (1800g) for 15 min at 4 ^oC and collect the supernatant.
4. In the meantime, add 5 mL of pre-warmed 30% sucrose into the ultra-centrifuge plastic tubes.
5. Add ~ 22 mL of virus lysate slowly on the top of the sucrose cushion. Be careful not to disrupt the sucrose or to go above 1 inch from the top of the tube.
6. Slowly add ~ 2mL of PBS on top of the virus supernatant (use surface tension to maintain the PBS above the virus supernatant).

7. 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
8. Put centrifuge tubes into the 30 mL centrifuge metal tube for ultracentrifugation (tubes 1-4, 2-5, 3-6).
9. 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
10. Slowly decant the supernatant.
11. Re-suspend in 100 – 500 µL PBS depending on the size of the pellet.
12. Leave samples in 4 ^{o}C overnight for pellet to dissociate. Seal tubes with parafilm.
13. Re-suspend the pellet well and store in autoclaved tubes (1 mL per tube).
14. 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 for 2 days to continue dissolving.

- wait time –

Filter the following through a 0.2µm filter.

Reagent	**Volume**
DMEM (Invitrogen Cat # 11965-092)	230 mL
FBS (2.5%)	12.5 mL
1:1 Penicillin-Streptomycin/Glutamine (2%)	10 mL

* Frozen reagents can be thawed/heated to 37^{o}C in a water bath. Vortex to mix.

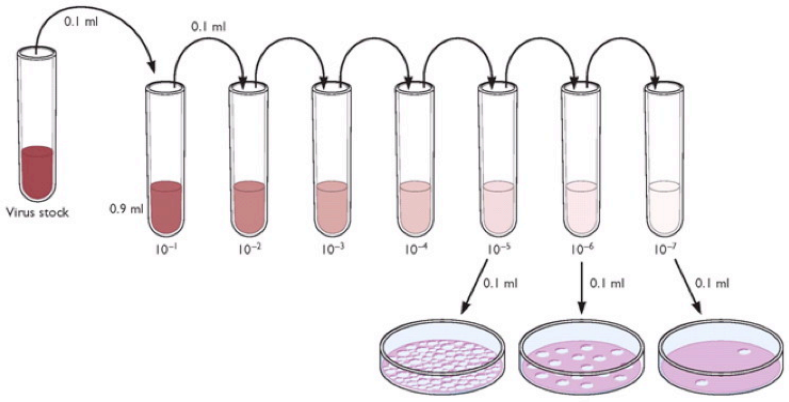
Add the filtered DMEM mixture into the methylcellulose solution and mix well.

1. Add 2 mL of BHK-21 growth media into each well of a 6-well plate.
2. Seed BHK-21 cells into 6-well plates at a density of 4.0 x 10^5 cells per well.
    - rock the plate back and forth a few times to evenly distribute cells
    - make sure to pipette cells directly into media
    - between seeding each well, make sure cells are kept in suspension by mixing up and down with pipette
3. Incubate cells overnight at 5% CO_2 at 37 ^{o}C. They should reach about 80-90% confluence.

- wait time –

*** Warm the 1% methylcellulose overlay media and BHK-21 growth media to 37°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. Make 10-fold serial dilutions (10^{-1} to 10^{-7}) of FV3 stock virus in BHK-21 growth media. Use a fresh pipette tip for each dilution, mixing well.



8. Label the 6-well plates with dilution concentrations.
9. Remove and discard the old media from the prepared 6-well plates.
10. Rinse the cell monolayer with PBS (1 mL per well).
    - rock the plate back and forth a few times, making sure the solution covers the entire surface area of each well
    - remove and discard the PBS
11. Infect cells in duplicate wells with 500 µL/well of the 10^{-5} to 10^{-7} FV3 dilutions.
    * Pipette virus along the sides of the well – be careful not to disrupt the cell monolayer
12. Wrap plates in ddH_2O moistened paper towel and aluminum foil to maintain humidity during the viral attachment phase.
13. Inoculate for 1-2 hrs at room temperature with constant gentle rocking to spread virus uniformly.
14. Remove and discard the inoculum, using a fresh pipette tip for each dilution.
15. Gently and slowly add 3 mL of the overlay medium to each well.
    * Add along the sides of the well – be careful not to disrupt the cell monolayer
16. Incubate cells at 5% CO_2 at 30 °C for 5-7 days.

- wait time –

17. Remove and discard the overlay medium (use a 5mL pipette).
18. Add 500 µL of 0.1% crystal violet (dissolved in 20% ethanol and stored indefinitely at room temperature) to each well.
19. Incubate 5-10 min at room temperature.
20. Remove and discard the crystal violet, wash with 3 mL/well of ddH_2O water 1-2 times and allow well to dry.
21. Count the number of isolated plaques in each well on an illuminated surface, and then use the following formula to determine the titer (PFU/mL) of the FV3 stock.
    * The most accurate results are obtained from wells with 20 to 100 plaques. Plaques can be overlapping – count accordingly.

    Number of plaques / (d x V) = PFU/mL
    d = dilution factor (10^{-5} to 10^{-7})
    V = volume of diluted virus added to the well (e.g. 500 µL)

Mammalian BHK-21 cell culture

The following protocol is used for the culture of BHK-21 baby hamster (Mesocricetus auratus) kidney cells.

A) BHK-21 Growth Medium Composition

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 µL

* 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:

1. BHK-21 cells should be expanded into vented sterile cell culture flasks (75 cm^2).
2. Inspect cells for confluency and lack of contamination.
3. Remove and discard the old media.
4. 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.