Purification of Virus on Dextran Gradients

Obj. Provides virion fraction separated from infected cell membranes

Materials

Hep 2 cells in either as designated
2 RB (2) or 24 × 150 cm2
Virus stock in milk or serum
Dextran T-10
0.1 M phosphate buffer stock pH 7.4
tissue douncer
gradient maker
centrifuge tubes (clear)
large centrifuge bottles (GSA)
tabletop centrifuge - refrigerated
phase contrast scope
slides & coverslips
goggles
needles (pack)
Nunc tubes to store virus
cannula & syringe
light source

Dextran T10
Code # 17-0250-02
Pharmacia

Time Required:

Cell infection → Harvest ~ 3 days

Infection ~ 2½ hrs total
Gradient production ~ 1 hr.
dextran preparation ~ 2 hr
Harvest & purification ~ 4 hr
Titer of virus ~ 3 hr.

For original reference. See Fuller + Spear J. Vir. 55(2): 475-482 1985

FLOWCHART FOR VIRUS PURIFICATION

Infect cells 3 pfu/cell, 2 hr 37°C

↓

Remove inoculum + overlay

↓

Grow virus for 2-3 days at 34°C

↓

Harvest infected cells → Some medium + harvest virus for some strains 20K, 1 hr, add pellet to douncer supernatant

↓

Centrifuge cells 2K, 15 min

↓

Break up cells, but not nuclei in hypotonic buffer + douncer

↓

Spin out nuclei in tabletop centrifuge

↓

Layer in equal amounts final supernatant onto dextran gradients

≤ 2 RB/grad.

↓

Centrifuge 1 hr, 20K at 4°C w/o break

↓

Carefully remove gradients + examine for virus bands

↓

Remove lipid layer + discard
Remove viral band w/ cannula

↓

Aliquot virus + store in Nunc tubes at -70°C

↓

Titer one virus fraction after freezing
≥ 10^9 pfu/ml

Procedure

1. Infect cells at 3 pfu/cell. Allow 2 hrs for adsorption. Remove inoculum & add medium + 1% + 5% CS. Gas & incubate 34°C for 2-3 days or until extensive CPE - (cells rounded up & just about to detach.)

If radiolabeling virus, add label in deficient medium at ~ 4-5 hr p.i. ~10-20 cpm/mL → Add back 20% leucine to leucine media

Temperature may differ for viral ts mutants.

2. Prepare dextran & gradients the day of or before harvest

(a) **0.1M phosphate buffer, pH 7.4** (1.8 wt/vol better)

Sol. A. 1.42 g Na_2HPO_4 / 100 mL dH_2O

Sol. B. 1.38 g NaH_2PO_4 / 100 mL dH_2O

hi pH / low pH | check to be sure

Add Solution B to Solution A (hi pH) until desired pH is reached.

(b) **.001M phosphate buffer, pH 7.4**

Add 1 part 0.1M PO_4 to 99 parts dH_2O.
Check pH at 4°C

(c) **Dextran T-10 ρ = 1.09 g/mL**

(Filtering can take a long time.)

place 65 g dextran in 200 mL 1mM PO_4 pH 7.4

Dissolve dextran & either autoclave to melt
Sterilize & filter thru whatman to remove aggregates OR prefilter & sterilize w/ 0.45 μ millipore filter

Refractive index should be 1.372
Store at 4°C for several days ≤ week

3. Prepare dextran gradients

Heavy dextran - stock dextran from step (2) 65g/200ml

(a) Light dextran 40 ml of heavy (ρ=1.09)
50 ml of 1mM PO4 — (pH 7.8 or 8)
refractive index = 1.351

(b) Prepare an even # of gradients ≈ 1/grad/2 RB

use 18.4 ml of light solution ρ=1.04
17.6 ml of heavy solution ρ=1.09

(c) Place heavy in gradient chamber above outlet with stirbar. Drip dextran down side of clear centrifuge tube (~40 ml tube) being careful not to disrupt flow of gradient.

(d) Store gradients securely at 4°C covered with parafilm. Can store overnite if needed.

4. Harvest infected cells in head. Keep cold.

(a) Scrape or shake cells into medium and pour into cold centrifuge GSA tubes. Spin 2K for 15 min with refrigeration to pellet cells.

(b) Pour off medium. Spin at 20K for 1-2 hr to pellet virus if required for virus strain. Otherwise, disinfect & discard medium. If radioactive, disinfect & discard as radioactive liquid waste.

this step is needed for (F). Not needed for (HFEM).

ⓒ For all virus strains, dislodge cell pellet with small volume (½ ml / RB) of cold 1mM PBG. Transfer pellet (≤ 2 RB cells) to Dounce homogenizer.

ⓓ Rinse (serially) centrifuge tubes with ~1 ml of buffer and add to homogenizer. Resuspend, gently, cells thoroughly, and let sit on ice ~15 min to swell cells.

5. Disrupt cells in Dounce homogenizer (Wear goggles)

Using a tight fitting pestle, disrupt the cells with as many strokes as required to break cells without breaking nuclei. Monitor cell disruption in phase microscope
~ 10-12 strokes, general # required.
Monitor progress in microscope.
DO NOT BREAK NUCLEI!! (DNA released & ruins gradient)
Thoroughly clean Dounce.

6. Preparation of supernatant for gradient

Add virus pellet from medium.

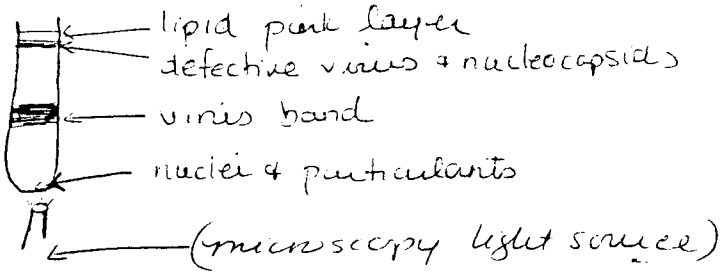
ⓐ Transfer homogenate to centrifuge tube, wash homogenizer with small amount of buffer & add to tube. Pellet nuclei 2.5K ~10 min. Remove supernatant to fresh tube. Leave on ice

ⓑ Can resuspend & wash pellet in small volume of buffer. Spin again & add 2nd supernatant to 1st.

7. Layer 2-3 ml aliquots of the virus-membrane supernatant onto each dextran gradient. Centrifuge in SW27 rotor for 2 hr at 20K rpm at 4°C. No break. Do not allow to sit w/o spinning for prolonged time (> 10 min)

8. Recovery of virus band: Wear goggles

(a) Examine gradient with light source underneath tube. Should clearly see dense/foggy viral band near center of tube.



(b) Remove upper layers with pasteur pipette to disinfectant or radioactive waste.

(c) With syringe and cannula, remove virus band from just underneath virus band. Collect as much as possible without unduly diluting sample. (Some of band will remain)

(d) Collect to blue cap 16 ml tube on ice. Determine total volume. Aliquot to Nunc tubes. Label + store at -80.

9. Store material at ≤ -70°C. Label as: virus strain
date initials

Record in virus stock book including titer.

10. Titer of purified virus.

Thaw a frozen vial, or leave ~300 µl especially for titering. Freeze. Titer on 8 Vero or Hep 2 plaque dishes. Good preparations are

≥ 1×10^9 pfu/ml.

If radiolabeled, determine activity per 10–100 µl of virus. Record in virus book.

Purification of PRV Virions — Fuller lab

(as communicated via phone from T. Ben-Porat) 4/91

Cells can be pig kidney or Vero or ST (any cells that give 10^9 titers reproducibly)

Infect at 3PFU/cell. Let grow 48 hrs or until extensive CPE.

Scrape or shake all cells into medium. Spin at ~5000K? to pellet cells. Remove supernatent which contains virus particles.

Spin out debris from supernatent by another spin at ?K for ? minutes.

Prepare a 1.0 ml 30% sucrose cushion in ? . Using a tube that fits into an SW25 or equivalent rotor, spin at 13,500 for 1.0 hr.

Will see pellet at bottom which has gone through sucrose cushion. Carefully remove sucrose and resuspend pellet in PBS-A (~1.0ml). Sonicate mildly.

Prepare a 15-30% sucrose gradient with PBS-A or PBS-BSA.
w/BSA to get rid of excessive viral proteins (gX)
w/o BSA if viral proteins are desired

Layer sonicated virus over gradient. Spin for 70 minutes at 13,000 in an SW27 or equivalent g force.

Remove gradient and view as with dextran gradient. See a very distinct band of virus about 1/2 way into gradient.

Remove sucrose before use by diluting in buffer and spinning down and resuspending virus in desired volume.

To Do:
-Determine best cell to grow PRV for this SK6 better than Vero.
-Work our centrifuge equivalencies
-Find reference of T. Ben-Porat for this procedure.
-Try procedure and fill in missing details