Large scale Adenovirus production, purification and amplification

Following protocol is designed to provide ~ 3-5X10E11purified virus particles.

All adenovirus infection and processing of infected cells should be done in BL-2 Hood under standard operating conditions required by the law. Use proper decontamination protocol.

1. Seed 5X10E5 HEK 293cells (QBI Cat # AES 0503) in 60x150mm plates using DMEM media (Life tech Cat #12430-054) containing 5% FBS. Incubate @ 37C in a tissue culture incubator until they are 70 to 80% confluent (it is important not to let them grow too much). Usually plate will be ready for infection in 2 to 3 days.

2. We routinely infect the cells @ 1 to 15 M.O.I. Dilute 0.5ul purified virus or 2-3ul of the crude viral lysate (CVL) in 2-ml infection media per plate. We routinely check the CVL for optimal infectivity. The amount virus used for infection depends on the virus titer.

3. Do not let the cells dry during the infection. Therefore aspirate the media only when everything is ready for the infection. The 293 cells are also very sensitive to the temperature always use media pre heated to 37C. Do not add the media right in the middle of the plate to avoid the detachment of cells from the plate. Add the 2-ml infection media-containing virus from step 2 gently at the corner of the plate. Incubate the plate for an hour tilting the plates every 15 min (very gently and so that the media covers all the plate) or setting a rocker in the incubator (PGC cat#75-7355-10 rock @ speed 3). After the incubation add 15 to 20ml of DMEM media containing 5 % FBS to the plates.

4. Incubate the plates until they start showing cyto-pathic effects (CPE). Typically CPE start to be evident after 24hrs and are fully developed after 36hrs. If cells have developed CPE after overnight incubation (< 24hrs) suspect another cause different from adenovirus infection (discard the preparation).

5. We usually harvest the cells between 36 to 48 hours after infection (cells when 20 to 30 % infected? cell population becomes round and start floating). Detach the cells pipetting up and down with the media. Pool the recovered cells along with the supernatant in a 50ml conical tube and spin them for 10 minutes @ 2500 RPM (1000g). Pool the pellet and resuspend the pellet in 20 ml sterile PBS (for total of 60 plates). Store the pellet @-20C.

6. Break open the cells by freezing (-70C, use dry ice / methanol) and thawing (37C water bath) to release the virus for 5 to 6 cycles. After each cycle vortex for 30 seconds. At the end of this step the preparation is called crude viral lysate (CVL). Spin the cell debris @ 2500RPM (1000g) for 10minutes and save the supernatant in a clean 15ml sterile tube and store @ -20C or -70C till further use.

Purification of Adenovirus by gradient Centrifugation:
Buffers and Solutions:
1XTD Buffer: 140mM NaCL, 5mM KCL, 25mM Tris, 0.7mM Na2HPO4 pH 7.4

10X TD Buffer: 80gm NaCL, 3.8gm KCL, 30gm Trizma base, 1gm Na2HPO4, Make up to 1Liter with distilled water and adjust the pH to 7.4 with HCL. Sterilize the solution by filtering

through 0.2-u filter before use. Store at room temperature.
CsCL at density gm/ml:
Density (gm/ml): 1.25 (use 54 gm), 1.33 (use 66.66 gm), 1.4 (use 77.66 gm)
Add 1X TD buffer to 200ml for the specified density. Use the CsCl indicated in parenthesis. Check density of solutions by weighing 1ml. Adjust density by adding 1X td buffer or CsCL powder as necessary. 1ml of solution should be placed into a weigh boat using a P1000 Pipetman. Several aliquots should be weighed and averaged. Adjust the density with powder as needed. Sterilize the solution by filtering through 0.2-u filter before use. Store at room temperature.
Dialysis Buffer: Prepare the dialysis buffer using sterile glassware and autoclaved water. Mix together sterile distilled water (2 liter), 1M Tris HCL pH 7.4 (40 ml), 40ml 1M MgCL2 (40 ml), Glycerol (40 ml). When all components are in solution bring the volume to 4 Liter with sterile distilled water. Refrigerate overnight.

Purification:
· Generate cesium chloride gradient in a 17 ml tube (Beckman polyallomer tubes, Catalogue # 337986) by placing first 2.5ml CsCl solution (density 1.25) and under laying with 2.5ml CsCl (density 1.40) solution. Overlay12-15 ml cleared CVL on top of the gradient and spin 2hr at 26,000 rpm in an SW-28 rotor @ 15C. Insert the tube into SW 28 bucket and balance the weight of the bucket on weighing scale in the BL-2 hood. The buckets must be well balanced to avoid spills and tube collapse during ultra centrifugation.

· Adenovirus will band at the junction of the two step gradients and the immature virus will band in the 1.25gm/ml gradient. Clean the outside of the tube with ETOH. Harvest the virus band using 18g needle in a 5-10cc syringe. Insert the needle just below the live virus band and pull as much of the virus as possible from the interface without disturbing the upper gradient (use proper precaution in handling the needle. Avoid any needle stick to the skin). Pool the virus band in a 15-ml tube (Corning and cat# 430052). Calculate the density of the virus solution and should be around 1.33 gm per ml.

· Transfer the virus band to a fresh 10-mlcentrifuge tube (Beckman, thick wall polycarbonate tube with cap, Catalogue # 355603) and fill the tube with 1.33 gm/ml CsCl solution. Balance the other tube with equal amount of 1.33gm/ml CsCl solution. Centrifuge for 18 hours at 15 C in SW 65 rotor @ 46K rpm.

· Recover opalescent virus band and transfer in to the 15-ml sterile tube.

· Transfer the virus to a Pierce slide-a lyzer cassette(Catalogue # 66425) using syringe fitted with19Gx1.5 needle. Dialyze for 30min (500ml x 2) and for an hour (1Liter X 3) in cold dialysis buffer. Dialyze the virus for total of 4 hours at room temperature.

· At the end of the dialysis remove the virus and aliquot 50 to 100 ul virus in to sterile 0,5ml screw cap tube (VWR cat# 20170-233).

· Store purified virus @ -70C.

· Treat the used buffer as biological waste. Add Clorox bleach to inactivate the virus

· Note about storage conditions: CVL can be stored in PBS @ -20C. Purified virus after dialysis MUST be stored @ -70C.

Titer determination by Plaque Assay:

1. Assays should be set up in duplicates. Plate 293 cells (1X10E5/ml) in COSTAR 3506-well plates (VWR cat #29442-036). Incubate @37C in a tissue culture incubator overnight on flat shelf (do not stack). Check the plates before the assay, cells should be around 60 to 70% confluent.

2. Arrange 10 sterile 5ml Falcon polypropylene tubes in a rack. Dispense 1.98ml infection media (IMEM containing 2%FBS Cat # 10373-017) in the first tube. Dispense 1.80-ml infection media in all the tubes. Carefully put 20ul of purified virus with a pipetman. Mix the virus and the media by pipetting 8 times with a1ml serological pipette. Take 200ul of the mixture with a pipetman and add it to the next tube in the series. Mix and repeat as before until 10E12 dilution.

3. Remove the media from the 6 well plate, being careful not to touch the bottom of the dish, there by damaging the monolayer. Carefully add 1ml of the diluted virus to each well except for the 6th well.(from 10E8-10E11 for purified viruses). Incubate the plate at 37C for 1 to 2hrs, rocking gently every 15 minutes.

4. Make 2% seaplaque agarose(FME cat# 50102) in sterile distilled water. Autoclave for 35 minutes, then equilibrate @ 50C. Similarly place 2XMEM(LTI cat#11935-038) + 15% FBS (Hyclone Cat #SH30070-03) +1%PS (LTI Cat 15140-148) in a50ml conical tube and put the tube @ 37C. Combine equal amount of 2% agar + the media and keep @ 37 C until ready to use.

5. After incubating the 6-well plate with virus. Gently aspirate the media from the plate (from Step 3) and overly with 3ml of the agarose/2xMEM media (Step 4), starting with the higher dilution and working back.

6. Watch the plate after overnight incubation. Watch for the cell shape. If the cells start to round up, cells may not survive. Repeat the plaque assay. If cells look healthy, proceed to step 7.

7. Overly with 2ml of the agarose/2XMEM again after 5 days and 10 days. Plaques are counted at 10-12 days. Read plaques in wells containing plaques at the highest dilution and also at the next lower dilution. Combine the values from both such wells in the two plates to obtain a mean value. The titer is calculated as the number of plaques/well X 10 E dilution.

8. Stain the plaques with Natural red solution (LTI Cat# 15330-079) and take photographs for the record keeping.