1. Purpose

1.1. The purpose of this protocol is to amplify an adenoviral vector from a crude lysate (final product in SOP: VDL105.2 Preparation of Adenoviral Vector Lysate) for production of high titer adenoviral vector stock.

1.2. The purpose of this protocol is to purify adenoviral vectors.

1.3. This procedure is routinely performed in the Vector Development Laboratory (VDL) following Good Laboratory Practices (GLP).

2. Abbreviations and Definitions

2.1. SOP Standard Operating Procedure
2.2. VDL Vector Development Laboratory
2.3. GLP Good Laboratory Practice
2.4. BSC Biological Safety Cabinet
2.5. CPE Cytopathic Effect
2.6. Antibiotics Penicillin/Streptomycin/anti-mycotic
2.7. FBS Fetal Bovine Serum
2.8. DMEM Dulbecco's Modified Eagle's Medium with 1% Antibiotics
2.9. DMEM10 DMEM with 10% FBS
2.10. Trypsin 0.25% Trypsin-EDTA
2.11. PBS Phosphate Buffer Saline
2.12. Column buffer 20 mM HEPES pH 7.8, 150 mM NaCl
2.13. Dilution buffer 20 mM HEPES pH 7.8, 150 mM NaCl, 10% glycerol

3. Equipment, Materials, and Reagents

NOTE: All materials in contact with cells must be sterile, pyrogen-free and used according to the manufacturer’s directions unless stated otherwise. Equivalent materials and equipment may be used but all changes must be recorded.

3.1. Equipment

3.1.1. BSC
3.1.2. 37°C/5% CO₂ incubator
3.1.3. Water bath set at 37°C
3.1.4. Pipette aide
3.1.5. Inverted microscope with 20X objective
3.1.6. Table top low-speed centrifuge
3.1.7. Ultracentrifuge
3.1.8. High-speed centrifuge
3.1.9. SureSpin 630 rotor and buckets
3.1.10. TH-641 rotor and buckets
3.1.11. Balance
3.1.12. Ring stand
3.1.13. Spectrophotometer with cuvettes

3.2. Materials
3.2.1. 50 mL conical tubes Corning
3.2.2. 15 mL conical tubes Corning
3.2.3. Cell stack Corning
3.2.4. Serological pipets Corning
3.2.5. Sterile pipet tips VWR
3.2.6. 1 L glass bottle (sterile) Corning
3.2.7. 250 mL glass beaker Corning
3.2.8. 500 mL centrifuge bottles Sorvall
3.2.9. Ultracentrifuge tubes 12 mL Sorvall
3.2.10. Ultracentrifuge tubes 36 mL Seton
3.2.11. 10 mL syringes BD
3.2.12. 18 gauge needles BD
3.2.13. PD-10 desalting columns GE Healthcare
3.2.14. Cryotubes Sorenson
3.2.15. Labels ISC Bioexpress

3.3. Reagents
3.3.1. Trypsin Cellgro
3.3.2. PBS Invitrogen
3.3.3. DMEM Hyclone
3.3.4. DMEM10 VDL
3.3.5. FBS GIBCO
3.3.6. Antibiotics Cellgro
3.3.7. Cesium Chloride EMD Chemicals
3.3.8. Light cesium chloride, density = 1.25 g/mL VDL
3.3.9. Heavy cesium chloride, density = 1.45 g/mL VDL
3.3.10. 10 mM Tris-HCl, pH 8.1 VDL
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3.3.11. 50 mm Tris-HCl, pH 8.0 VDL
3.3.12. Bleach Clorox
3.3.13. Glycerol Sigma
3.3.14. Tris-HCl Sigma
3.3.15. NaCl Sigma
3.3.16. Dilution buffer VDL
3.3.17. HEPES Sigma
3.3.18. Column buffer VDL

3.4. Starting Materials
3.4.1. 5 mL crude virus (final product in SOP105.2: Preparation of Adenoviral Vector Lysate).
3.4.2. Eleven, 150 mm dishes of 100% confluent 293 cells.

3.5. Test Sample Identification
3.5.1. The bar code on the viral lysate will be scanned and compared to the computer database to ensure the correct sample is being processed.
3.5.2. One copy of the barcode will be printed and applied to a copy of this SOP for the final record.
3.5.3. A barcoded label that also contains virus name will be applied to all tubes, dishes, and Cell Stack used in the production of the virus.

4. Procedure

Seeding of cell stack with 293 cells
4.1. Remove media from 11, 150 mm dishes of 293 cells which are 100% confluent, as determined microscopically.
4.2. Add 10 mL of PBS to each dish to wash cells. Aspirate PBS.
4.3. Add 3 mL of trypsin to each dish and rock dish gently back and forth to ensure that the entire cell surface of the dish is coated with the solution. Incubate for 2 minutes at room temperature then remove trypsin.
4.4. Add 8 mL of DMEM10 to each dish and pipet media up and down over cells to detach residual cells and produce a single-cell suspension.
4.5. Pool the cell suspensions into a 1 L bottle and Q.S. to 1 L with DMEM10.
4.6. Invert bottle with DMEM10/cell suspension several times to distribute the cells evenly throughout the media.
4.7. Obtain 5, 150 mm dishes and one Cell Stack and transfer to the BSC.
4.8. Transfer 20 mL of cell suspension to each 150 mm dish.
4.9. Remove the right, front cap of the Cell Stack and loosen the right, back cap to provide a vent.

4.10. Tilt the Cell Stack so that the only part of the Cell Stack touching the BSC surface is the bottom, right, front corner.

4.11. Transfer the remaining cell suspension to the Cell Stack through the port on the front, right corner. Replace cap and tighten.

4.12. Equalize all fluid levels by holding the Cell Stack on its front, right corner. Slowly stand the Cell Stack on its left side. Gently lay Cell Stack flat on the BSC surface. Repeat this step one more time.

4.13. Tighten both vent caps and return Cell Stack and 150 mm dishes to the 37°C/5% CO₂ incubator.

4.14. Loosen the caps on the Cell Stack to allow air to enter the system.

4.15. Discard any remaining 293 cells.

**NOTE:** When cells are 80-90% confluent as determined microscopically, which should take one overnight incubation, they are ready to be infected with the adenoviral vector.

4.16. When the cells are 80-90% confluent, remove the 15 mL conical tube, containing the cell suspension from the -80°C freezer.

4.17. Subject the cell suspension to three freeze/thaw cycles. This entails freezing the cell suspension by placing the tube in a dry ice/ethanol bath and when it is completely frozen placing the tube in a 37°C water bath to thaw. The initial placing of the cell suspension in the -80°C freezer for storage can be considered the first freeze.

**NOTE:** Adenoviral vectors have a finite half-life and will therefore inactivate after prolonged incubation at 37°C. Therefore take care to remove the tube from the 37°C water bath as soon as the cell suspension has thawed.

4.18. Pellet cellular debris by centrifugation at 1,500 x g for 10 minutes and hold on ice until use.

4.19. Add the cleared viral lysate to a 1 L bottle and Q.S. to 1 L with DMEM2.

4.20. In the BSC, aspirate the medium from the 5, 150 mm dishes.

4.21. Add 20 mL of the virus/media mixture to each 150 mm dish.

4.22. In the BSC, open the front vent cap on the Cell Stack and aspirate the media.

4.23. Transfer the DMEM2/cleared viral lysate to the Cell Stack as described in steps 4.9-4.12

4.24. Place the Cell Stack in the 37°C/5%CO₂ incubator and loosen caps.
NOTE: Using the 150 mm dishes, microscopically inspect the cells every day in order to determine the degree of CPE. When 95-100% CPE is observed continue with the procedure. It will take 2-3 days before this stage of CPE is reached.

Harvesting of adenoviral vector infected 293 cells from cell stack

4.25. When 95-100% CPE is observed, rock and agitate the Cell Stack to collect cells. All cells should be detached at this stage.

4.26. In the BSC, remove the vent cap from the Cell Stack and transfer the cell suspension from the Cell Stack to 3, 500 mL centrifuge bottles. Transfer the media and cells from the 5, 150 mm dishes to the centrifuge bottles.

4.27. Wash the 150 mm dishes with 10 mL of PBS and wash the Cell Stack with 300 mL of PBS to recover residual cells. Collect the wash solution in the same 500 mL centrifuge bottles that contains the cell suspension.

4.28. Pellet cells by centrifugation at 1,500 x g for 30 minutes.

4.29. Aspirate all of the media and resuspend each cell pellet in 10 mL of 50 mM Tris-HCl, pH 8.0 to obtain a final cell suspension of 40 mL.

4.30. Transfer the cell suspension to a 50 mL conical tube and transfer to the –80°C freezer until required.

Purification of adenoviral vector

4.31. Subject the cell suspension to 3 freeze-thaw cycles as described in step 4.17.

4.32. After the last thaw, pellet the cellular debris by centrifugation at 1,500 x g for 20 minutes.

Cesium chloride purification of adenoviral vector

4.33. Pipet 10 mL of light CsCl solution into the 36 mL ultracentrifuge tube.

4.34. Take up 10 mL of heavy CsCl solution into a pipet and insert the tip of the pipet to the bottom of the ultracentrifuge tube and carefully dispense the heavy cesium chloride. **Note:** The light CsCl will float atop the heavy layer – the interface between the layers should be very sharp.

4.35. Carefully layer the viral supernatant on top of the cesium gradient. A maximum of 18 mL of supernatant can be loaded per ultracentrifuge tube. **Note:** The volume of the viral supernatant can be adjusted with 10 mM Tris-HCl, pH 8.1 to fill up the tube.

4.36. Load the tubes into the buckets of the TH-641 rotor.

4.37. Balance the tubes with 10 mM Tris-HCl, pH 8.1, if needed.

4.38. Load the buckets onto the rotor and insert rotor into centrifuge.

4.39. Spin at 72,000 x g at 4°C for 2 hours.
4.40. Remove tubes from the centrifuge and clamp a tube onto a ring stand above a beaker of bleach.

Note: Virus will appear as a narrow opaque white band 2/3 down the heavy/light cesium gradient.

4.41. With an 18G needle attached to a 5 mL syringe, puncture the tube immediately under the band and pull it into the syringe barrel. Do not collect top band or any other bands.

4.42. Dilute the collected band with equal volume of 10 mM Tris-HCl, pH 8.1.

4.43. Pipet 4 mL of light CsCl solution into a 12 mL ultracentrifuge tube and pipet 4 mL of the heavy CsCl solution beneath it.

4.44. Carefully layer the diluted viral band on top of the gradient. A maximum of 4 mL can be loaded on this small gradient.

4.45. Load the tubes into the buckets of the SureSpin 630 rotor.

4.46. Balance the tubes with 10 mM Tris-HCl, pH 8.1, if needed.

4.47. Load the buckets onto the rotor and insert rotor into centrifuge.

4.48. Spin at 72,000 x g at 4°C overnight.

4.49. Remove tubes from the centrifuge and clamp a tube onto a ring stand above a beaker of bleach.

4.50. Collect the band by puncturing the side of the tube with a needle and syringe.

4.51. Transfer the virus to 15 mL conical tube and place on ice.

Desalting column purification

4.52. Clamp the desalting column onto a ring stand, remove the tip.

4.53. Wash the resin bed 5x with 5 mL column buffer.

4.54. When the last column buffer has reached the end of the resin, add the virus to the column.

4.55. Allow the virus to pass through the column.

4.56. When the drops falling from the tip appear milky/cloudy begin collection in a 15 mL conical tube.

4.57. Add 5 mL of column buffer.

4.58. Measure the volume of virus collected.

Determine total particles produced

4.59. Dilute the virus 1:50 and take an OD260 reading.

4.60. To determine the particles/mL perform the following calculation:

\[
\text{OD260} \times \text{dilution} \times 50 \times 10^{12} = \text{particles/mL}
\]

4.61. Dilute to 5x10^{12} particles/mL in dilution buffer.
4.62. Make 0.5 mL aliquots of the virus in cryotubes pre-labeled with the virus name, particle concentration, and date.
4.63. Store the adenoviral vector in the -80°C freeze until use.

5. Data Collection and Management

5.1. All data will be collected in a laboratory notebook.
5.2. Deviations of the protocol will be recorded in the laboratory notebook and on the Lab Meeting Sheet.

6. Review and Revisions

Written by: ______________________________
Director, VDL
Reviewed by: ______________________________
Director, Vector Production
Reviewed by: ______________________________
Director, QA/QC
Date Issued: 12/1/2006 Replaces VDL 106.1

Annual Review:

2011
Reviewed without changes Changed and this version archived
Reviewed by: ______________________________
QA/QC by: ______________________________
Date Issued: 6/1/2011 Replaces VDL 106.2

2012
Reviewed without changes Changed and this version archived
Reviewed by: ______________________________
QA/QC by: ______________________________
Date: ______________________________
VDL106.3 LARGE-SCALE AMPLIFICATION AND PURIFICATION OF ADENOVIRAL VECTOR

2013

Reviewed without changes

Reviewed by: ____________________________

QA/QC by: ____________________________

Date: ____________________________