AAV production

Day 0 – Day before Transfection:

1. Plate 12.1 x10^6 293T cells/ 15 cm dish one day before transfection (plate a total of 6 dishes each containing 25 ml of DMEM+ 10% FCS).

Day 1 – Day of Transfection

- In a sterile tube, dilute total plasmid DNA (ug) in 1 ml diluent*. Use Adenoviral helper HgT1-Adeno: serotype helper (pLTAAV2.5 for serotype 2.5, pRepCap6 for serotype 6): vector plasmid at 2:1.6: 1 ratio (12:10:6 ug respectively).
- Add 112 ul of PEI (1ug/uL) to the diluted DNA. Mix immediately by pipeting up and down/ vortexing. The volume of PEI used is based on a 4:1 ratio of PEI (ug): total DNA (ug).
- Incubate 10-15 minutes at RT (don't go over 20 min). Add 1ml of DNA/PEI mixture to each plate of cells and incubate for 48 hrs.

Day 3

- Prepare dry ice/ethanol bath by placing a centrifuge tube rack in a styrofoam container. Add ~2 lbs of dry ice, pour 2 liters of ethanol over it and cover the container.
- Collect the cells and medium by scraping the cells off the dish with a rubber policeman and transferring it to a 50-ml conical tube. We use Corning 50-ml conical tubes (see details in Reagent list), as they are more resistant to the subsequent freeze-thaw cycles. Rinse dishes with 2 ml of 1× PBS and transfer it to the same conical tube. Harvest two dishes at a time into the same 50-ml tube.
- Centrifuge at 1200 rpm for 5 min at 20 °C in a tabletop centrifuge
- Aspirate the medium from the conical tube and repeat Steps 14–15 until cells are pelleted from all 6 dishes. The same tube can be used for pelleting cells from all the dishes.
  Resuspend the final pellet from all 6 dishes in 4 ml of cell lysis buffer.
- Freeze the pellet in the dry ice/ethanol bath and thaw in a 37 °C water bath three times. Freeze again in dry ice/ethanol bath and store at -80 °C. The pellet can be stored at − 80 °C indefinitely and thawed at your convenience.

Day convenient

- Thaw cell lysate in a 37 °C water bath.
- Add Benzonase to the cell lysate at a final concentration of 50 U/ml (Benzonase stock conc. 25U/ul-add 8 ul for 4 ml).
- Incubate at 37 °C in a water bath for 30 min. Prepare iodixanol gradient (**below**) during the incubation.

The following volumes are for two gradients using OptiSeal tubes. The 1.27 mm × 89 mm spinal needle holds ~0.4 ml.
Prepare the following iodixanol solutions:
***15% Iodixanol step*: Mix 4.5 ml of 60% iodixanol and 13.5 ml of 1 M NaCl/PBS-MK buffer.**
***25% Iodixanol step*: Mix 5 ml of 60% iodixanol, 7 ml of PBS-MK buffer and 30 µl of Phenol red.**
***40% Iodixanol step*: Mix 6.7 ml of 60% iodixanol and 3.3 ml of PBS-MK buffer.**
***60% Iodixanol step*: Mix 10 ml of 60% iodixanol and 45 µl of Phenol red.**

Overlay each centrifuge tube with these solutions in the order below using a 10-ml syringe and 1.27 mm × 89 mm spinal needle, taking care to avoid bubbles. The same needle can be used for loading all steps.
5 ml of 60% iodixanol step
5 ml of 40% iodixanol step
6 ml of 25% iodixanol step
9 ml of 15% iodixanol step

- Centrifuge at 4500 rpm for 30 min at 4 °C in a tabletop centrifuge.
- Collect vector-containing supernatant. The volume of the supernatant is approximately 5 ml.
- Load the 5 ml of vector-containing supernatant over the iodixanol density gradient prepared before. Top off the tube with cell lysis buffer.
- Centrifuge at 67,000 rpm for 1 h at 18 °C with maximum acceleration and deceleration in a Beckman Ti70 rotor on a Beckman ultracentrifuge; **use proper spacers for tubes.**
- Puncture the tube on the side slightly below (3–5 mm) the 60–40% interface with an 18-gauge needle (bevel up) attached to a 10 ml syringe.
- Collect 3–4 ml from each centrifuge tube by aspiration using the same needle.
  CRITICAL STEP: Avoid the proteinaceous material near the 40–25% interface.
- The vector stocks can be aliquoted and stored at –80 °C indefinitely. Store stocks in small aliquots and avoid repeated freezing and thawing. Alternatively, Serotype 2 vector stocks can be further purified over a HiTrap heparin affinity column, followed by desalting over a HiTrap desalting column

Reagents/Equipment

- Media-DMEM+ 10% FCS (Cellgro Product #10-013-CV containing glutamine+sodium pyruvate)
- NaCl/PBS-MK buffer (1 M): Dissolve 5.84 g of NaCl, 26.3 mg of MgCl2 and 14.91 mg of KCl in 1× PBS in a final volume of 100 ml. Sterilize by passing through a 0.22-µm filter and store at 4 °C.
- PBS-MK buffer: Dissolve 26.3 mg of MgCl2 and 14.91 mg of KCl in 1× PBS in a final volume of 100 ml. Sterilize by passing through a 0.22-µm filter and store at 4 °C.
- Cell lysis buffer: Add 3 ml of 5 M NaCl and 5 ml of 1 M Tris-HCl (pH 8.5) to 80 ml of dH2O. Adjust the pH to 8.5 with NaOH and adjust the volume to 100 ml with dH2O. Sterilize by passing through a 0.22-µm filter and store at 4 °C.
- Benzonase, Novagen cat # 70664
- Centrifugation tubes for freeze thawing: Corning with centristar cap; cat # 430828
- Ultracentrifuge tubes, Beckman Optiseal polyallomer cfg tubes; cat # 361625
- Beckman Ti70 rotor
- Beckman preparative ultracentrifuge
- Pipetting needles from Cadence Sciences , blunt end 10 x 4" Ref# 9872
- **Diluent**: 10 mM HEPES/ 150mM NaCl (pH 7.05) – No $NaCO_3$ buffering. Use 1L TC grade water (commercial), and add HEPES/NaCl, and wrap up the bottle with foil to prevent light. Store at room temp.