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AAV Production and Purification V.2

In 1 collection

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Abstract

This protocol is for the production and purification of Adeno-Associated Virus. The protocol contains the necessary steps to produce AAVs from HEK293T cell cultures.

Materials

Reagents/Equipment

· **Media-DMEM+ 10% FCS** (Cellgro Product #10-013-CV containing glutamine+sodium pyruvate)

· **AAV-PHP.eB_capsid: Addgene Plasmid #103005**; RRID:Addgene_103005

· **pAD-ΔF6: Addgene Plasmid #112867**; RRID:Addgene_112867

· **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

· **OptiPrep (60% iodixanol sol)**, Sigma; cat # D1556

· **Beckman Ti70 rotor**

· **Beckman preparative ultracentrifuge**

NaCl/PBS-MK Buffer-1M

	1X PBS=100ml	1X PBS=500ml
NaCl	5.84g	29.2
MgCl2	26.3mg	131.5
KCl	14.91mg	74.55

Dissolve and sterilize through 0.22µm Filter; Store@4^{0}C

PBS-MK Buffer

	1X PBS=100ml	1X PBS=500ml
MgCl2	26.3mg	131.5
KCl	14.91mg	74.55

Dissolve and sterilize through 0.22mm Filter; Store@4^{0}C

Safety warnings

- HEK293T cells and AAVs are biohazardous materials and must be handled according to governmental and institutional regulations

- Experiments involving AAVs were performed using biosafety level 2 practices

Growing HEK 293T Cells: Day 1 -Morning

1 Make Media for HEK293T Cells

1.1
1. 500ml DMEM
2. 5ml 100x Pyruvate
3. 5ml 100x Pen/Strep
4. 5ml 100x L-Glutamine
5. 50ml Fetal Bovine Serum

2 Thaw HEK293T Cells in 10cm Dish with 10 ml HEK Medium (One Vial into three 10cm Dish)

Day 3 -Morning

3 Split HEK293T Cells (Usually become confluent on Day 3) with 3ml Trypsin.

4 Use serum to block Trypsin (100ul for 1ml trypsin)

5 Split one full confluent 10cm dish into five 10cm plates (So Total 15 Plates).

Day 4 - Evening

6 Split all plates of HEK293T Cells (15 10 cm dish confluent plates)

7 Plate 15 million cells/T175 Flask with 20 ml Medium

Transfection: Day 5 - Evening

8 Make sure all plasmids are ready on day of Transfection

8.1
1. pAd-DeltaF (**Order from AddGene**; Glycerol Stock-Cultivate at 30°C and do Maxi)

2. PHP.eB Capsid (**Order from AddGene**; Glycerol Stock-Cultivate at 37°C and do Maxi)
3. ITR-Gene of interest plasmid (i.e. GEARBOCS with gRNA for gene of interest)

9 In a sterile tube, dilute total plasmid DNA (ug) in 6ml Opti-MEM.

9.1
1. 30 ug pAd-DeltaF
2. 15 ug PHP.eB
3. 15 ug ITR-Gene of interest (pAAV2ITR-gfaABC1D-XXX)

10 In another sterile tube, dilute 1.33ml PEI (7.5mM) in 4.66ml Opti-MEM.

11 Incubate 10 minutes at RT.

12 Combine the two tubes, mix well and incubate 20 minutes at RT.

13 Add 2ml of DNA/PEI mixture to each plate of cells, mix well and return to incubator.

Day 6 - Morning

14 Change media (Usually 6-18 hours later)

15 Add 20ml fresh media and culture for 72hrs

Collecting Cell Lysate: Day 8 - Morning or Noon

16 Collect the cells and medium by scraping the cells off the dish with a cell scraper and transferring it to a 50-ml conical tube.

17 Rinse dishes with 2 ml of 1× PBS and transfer it to the same conical tube.

18 Harvest two dishes at a time into the same 50-ml tube

19 Centrifuge at 1300 rpm for 8 min in a tabletop centrifuge.

20 Aspirate the medium from the conical tube and repeat cell collection steps until cells are pelleted from all 6 dishes into one tube. The same tube can be used for pelleting cells from all the dishes.

21 Resuspend the final pellet from all 6 dishes in 4 ml of cell lysis buffer (In TC Room Fridge-50 ml Tube) (4 ml for one virus).

22 Prepare dry ice/ethanol bath during the last spin

23 Take dry ice in ice box and add 100% 190 proof ethanol to that to make dry ice/ethanol bath.

24 Freeze the pellet in the dry ice/ethanol bath and thaw in a 37 °C water bath three times (Almost 10-minute incubation each).

25 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.

Purifying Virus: Day of convenience - Morning

26 **Hydrolyze nucleic acids**

26.1
1. Thaw cell lysate in a 37 °C water bath
2. Add 8ul of Benzonase to the 4 ml of thawed cell lysate at a final concentration of 50 U/ml (Benzonase stock conc. 25U/ul).
3. Incubate at 37 °C in a water bath for 30 min.
4. Centrifuge lysed cells at 4500 rcf for 30 min at 4 °C in a tabletop centrifuge

27 **Prepare the iodixanol gradient during the Centrifugation.**

27.1 The following volumes are for two gradients (or two viruses) using OptiSeal tubes. (Note: Always keep one extra. i.e if you have three viruses make gradient solutions for four)

27.2 Take four 50ml Falcon Tubes and label well each concentration of iodixanol

27.3 Add each component carefully to each tube and mix well by vortexing:

Iodixanol Gradient for two Viruses (Make one extra if you have 3 or more viruses)

	A	B	C	D	E
		60% Iodixanol (OptiPrep Density Gradient-Sigma-D1556) # Don't dilute	1 M NaCl/PBS - MK buffer	PBS-MK buffer	Phenol Red
	15% Iodixanol	4.5mL	13.5mL	No	No
	25% Iodixanol	5mL	No	7mL	30uL
	40% Iodixanol	6.7mL	No	3.3mL	No
	60% Iodixanol	10mL	No	No	45uL

27.4 Use a 10 or 12 ml syringe with an 18 G needle to add these solutions to each labelled centrifuge Optiseal tube in the order below.
(Notes before you add: Add very slowly along the sides of the tube and take care to avoid bubbles. The same needle can be used for loading all steps. Take exact volume otherwise 4ml virus cannot be accommodated over the top in last step)

i. 5 ml of 60% iodixanol (Bottom)
ii. 5 ml of 40% iodixanol
iii. 6 ml of 25% iodixanol
iv. 9 ml of 15% iodixanol step (Top)

27.5 Collect vector-containing supernatant from centrifuged tube using 10 or 12- ml syringe with 18G needle. The volume of the supernatant is approximately 4-4.5 ml.

27.6 Load the vector-containing supernatant over the iodixanol density gradient prepared before

27.7 Fill to the very top until the hinge of the tube. If you have space left over, top off the tube with cell lysis buffer but only until the hinge.

27.8 Close with the dark plug provided along with the Optiseal tube without anair bubble. If your tube does collapse and is hard to remove from the rotor, then drip acetone between the tube and rotor wall and extract with tweezers

28 **Ultracentrifugation** (67,000 rpm for 1 hour at 18°C)

28.1 1. Bring Beckman Ti70 rotor

2. Put aerocap over the Optiseal Tubes
3. Keep tubes inside the rotor and press down
4. Tighten the lid and bring to the Beckman ultracentrifuge and keep inside
5. Close the lid and press vaccum (Green light blinks)
6. Press Speed-Press 67000-Press Enter
7. Press Temp- Press 18°C-Press Enter
8. Press Time- Press 1.00hr- Press Enter
9. After the vacuum green light is constant without blinking-Press Enter-Press START
10. Wait until it reaches the maximum speed before leaving centrifuge

Virus Collection: Same Day as Virus Purification

29 Set up ring stand and clamp inside the hood to hold tube

30 Prepare bleach in bottle and keep inside the hood to discard tips and tubes

31 Once the centrifugation is done, bring rotor to TC room and open

32 Remove the aerocap from each tube using a pair of pair pliers

33 Remove black plug from each tube and discard into bleach bottle

34 Take out the Optiseal Tube with pliers by clipping the topside and place inside the hood

35 Spray down 70% alcohol to player, aerocap and inside rotor; and keep for 10 minute and wipe with tissue paper.

36 Keep ready the Millipore Amicon filter unit (UFC910008, 100K MWCO) with proper label

37 Keep the optiseal tube in the holder and tight well.

38 Lay a bunch of tissue paper down and keep the bleach bottle.

39 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.

40 Slowly draw the 2-3 ml viral solution out from clear zone from each centrifuge tube by aspiration using the needle and put into the filter unit.

41 ***CRITICAL STEP****: Avoid the proteinaceous material near the 40–25% interface.*

Virus Concentration: Same Day as Virus Purification

42 Add 1ml of DPBS (#14190-144-Ca/MgCl2 Free) to filter unit and mix well using P1000

43 Spin 4000g for 10 minutes.

44 Repeat steps 43 and 44 five times.

45 In last step, concentrate up to 150-200ul

46 Collect virus from filter unit using P200 into a 1.6 ml Eppendorf tube

47 Aliquot the virus in 10ul amount to tubes and freeze in liquid N2

48 Store at −80 °C indefinitely