AAV Production in HEK293 Cells

-The Han Lab

Materials
HEK293T cells (e.g., ATCC #CRL-11268)
Fetal bovine serum (FBS; e.g., Biological Industries, cat. no. 04-007-19)
10,000 U/ml penicillin-streptomycin (P/S; e.g., Thermo Fisher Scientific, cat. no. 15140-122)
Dulbecco's phosphate-buffered saline (DPBS) with calcium and magnesium (e.g., Sigma-Aldrich, cat. no. D8662)
2.5% (w/v) trypsin (e.g., Thermo Fisher Scientific, cat. no. 15090-046)
pHelper (pPW100) plasmid
Rep/cap plasmid (eg. pPW185, pPW116, pPW126, pXL-930, p150304-2, pZC48)
Transgene of interest

1 mg/ml PEI (see recipe)
OptiPRO (e.g., Thermo Fisher Scientific, cat. no. 12309-019)
Chloroform (e.g., Sigma-Aldrich, cat. no. C2432)
5 M NaCl (e.g., Sigma-Aldrich, cat. no. S3014)
Polyethylene glycol (PEG-8000; see recipe)
HEPES buffer (see recipe)
1M MgCl2 (e.g., Sigma-Aldrich, cat. no. M2670)
DNase I (e.g., New England Biolabs, cat. no. M0303S)
10 mg/ml RNase A (e.g., Thermo Fisher Scientific, cat. no. EN0531)

175-cm2 cell culture flask (e.g., Thermo Fisher Scientific)
Cell scraper (e.g., Fisher Scientific, cat. no. 8100241)
0.5-ml Amicon Ultra Centrifugal Filter, 100 kDa (e.g., Millipore, cat. no. UFC510024)

REAGENTS AND SOLUTIONS
HEPES buffer, 50 mM (pH 8.0)
238.3 g HEPES (free acid; e.g., Sigma-Aldrich, cat. no. H3375)
800 ml Milli-Q water
Adjust pH to 8.0 with 10 N NaOH
Bring volume to 1000 ml with Milli-Q water Store at room temperature for up to 1 year
The solution prepared is a 1 M HEPES stock solution. Before use, dilute 20 in Milli-Q water to prepare a 50 mM solution.
PEG 8000, 50%
250 g PEG 8000 (e.g., Sigma-Aldrich, cat. no. 89510-250G-F)
250 ml Milli-Q water
Heat to 80Â°C to dissolve PEG 8000
Allow solution to cool
Store at room temperature for up to 2 months
PEI, 1 mg/ml
1. Dissolve PEI (e.g., Polysciences, cat. no. 23966-1) by swirling using sterile water heated to ~80 C and using 90% volume required to reach a final concentration of 1 g/L (1 mg/ml).
2. Cool to room temperature.
3. Adjust pH to 7.0 with HCl.

Growth medium
DMEM (Thermo Fisher, 4.5 g/l glucose, L-glutamine, pyruvate),
5% FBS (Atlanta Biologicals),

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1x Glutamax (Thermo Fisher),
1x penicillin/streptomycin (Thermo Fisher).

Production medium
DMEM (Thermo Fisher, 1 g/l glucose, L-glutamine, pyruvate);
1% FBS (Atlanta Biologicals),
1x Glutamax (Thermo Fisher),
1x penicillin/streptomycin (Thermo Fisher),
10 mM HEPES (Thermo Fisher)
Add 0.075% sodium bicarbonate (Thermo Fisher).

Culturing and Splitting cells
1. Grow HEK293T cells in a 175-cm2 flask in Growth medium. Split cells when they reach 75% to 80% confluency (steps 2 to 11), normally two to three times per week.
It is important to never let cells reach 100% confluency.
2. Aspirate medium from flask without touching the cells.
3. Carefully add 10 ml DPBS, and swish over cells to wash off the medium.
4. Aspirate DPBS.
5. Add 3 ml of 2.5% (w/v) trypsin to the flask, and swirl flask to distribute evenly. Then let sit for 3 min.
6. Gently tap flask to detach cells that remain attached to the bottom of the flask.
Cells should easily detach after trypsinization.
7. Add 10 ml DMEM supplemented with 10% (v/v) FBS and 1% (v/v) P/S to inactivate trypsin.
8. Rinse multiple times by pipetting up and down.
9. Collect cells and medium in a 50-ml conical tube.
10. Centrifuge cells 5 min at 500xg, room temperature.
11. Resuspend cells in 5 ml Growth medium, and quantify the number of cells using a hemocytometer.
Seeding cells
12. Seed 11 x 106 HEK293T cells in a 175-cm2 flask using 27 ml growth medium.
Cells should normally be ready for transfection the following day. However, for best result, monitor confluency. Only proceed with the next step once confluence reaches 75% to 80%, as described above (Fig. 2).
For best result, use HEK293T with as low passage number as possible. Thaw new cells on a regular basis (see Critical Parameters).

Day 0: Transfection
1. Prepare a 1:1:1 molar ratio for all plas mids (calculations per 175-cm2 flask):
17.7 ug pHelper (HGT1) (17,800 bp)
7.9 ug rep/cap (8000 bp)
5.9 ug transgene (6000 bp)
94.5 ul of1 ug/ul PEI (3 Î¼g PEI per 1 ug DNA)
Bring to 3 ml with DMEM supplemented with 1% (v/v) P/S.
For this protocol, 1.6 pmol of each plasmid is used for transfection.
2. Before transfection (2-4 hours), replace the culture medium with fresh growth medium. And warm DNA, PEI, and DMEM supplemented with 1% (v/v) P/S to room temperature before mixing.
3. Dilute all plasmid DNA in PBS and mix well- A (or DMEM supplemented with 1% (v/v) P/S).
4. Dilute the PEI solution in PBS and mix well- B (94.5 ul PEI, and mix by vortexing for 10 s).
5. Combine A and B, gently vortex to mix; incubate at room temperature for >= 20 min.
6. Add the transfection mix to the cell culture in a dropwise manner, and swirl flask gently three times to mix

transfection mix with the medium.
Day 1: Change to the Production Medium
7. Replace the transfection medium with Production medium 24 hr post transfection: [Keep 3 ml old medium, and add 27 ml OptiPRO supplemented with 1% (v/v) P/S.]

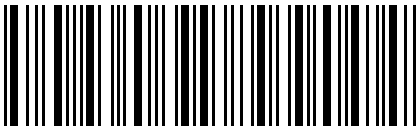
Day 3: First collection of AAV-containing medium
8. Harvest first AAV-containing medium 48 hr after medium change into 50-ml tubes (~27 ml each).
9. Add fresh production medium to the cells and continue culturing.
10. Add 1/10 volume Chloroform to each tube containing the harvested medium (eg. 3ml in 30 ml).
11. Vortex for 5 min.
12. Add 7.6 ml 5M NaCl to each tube and vortex for 10 s.
13. Place tubes into precooled centrifuge, and centrifuge 5 min at 3000 x g, 4Â°C.
14. Using a 25-ml serological pipette, carefully collect the aqueous phase into a new 50-ml conical tube (Fig. 3A). Avoid including any chloroform when transferring supernatant.
15. Add 9.4 ml of 50% (w/v) PEG 8000 to each tube and vortex for 10 s.
16. Incubate overnight at 4C. (AAVs are stable up to 2 days in this solution)
17. Centrifuge for 1hr at 3000 x g, 4Â°C (Fig. 3B).
18. Gently pour off supernatant, and place tubes upside down on paper towels for 10 min to dry. Dry residual liquid at the edge of the tube with paper towel if necessary (Fig. 3C).
19. Resuspend the AAV pellet in 1.4 ml HEPES buffer by vortexing. Do not resuspend the pellet by pipetting because this may shear the viral particles and decrease the final AAV yield. Let the pellet sit at 4 Â°C and mix every so often until it is entirely suspended. Make sure that the pellet is completely resuspended before commencing with the next step. Save at 4C to continue with the final medium/cell collection.

Figure 3 Key steps during AAV puriï¬•cation. (A) The three fractions after centrifugation with NaCl and chloroform added to the cells. (B) Pellet after PEG 8000 precipitation. (C) Dried pellet after PEG 8000 precipitation. (D) The three phases after chloroform extraction. (E) Clear and evaporated sample after chloroform extraction. (F) Sample ready for eluting after ï¬•ve washes in an Amicon Ultra Centrifugal Filter.

Day 5: Final collection of AAV-containing medium and cells
20. 48 hr after the final medium change, Add 0.5 M EDTA (pH 8.0) in a 1:80 ratio of the production.
21. Allow the plates to stand at room temperature for 10 min. (or Use cell scraper to scrape cells from the bottom of the flask).
22. Transfer cells and medium from one flask to a 50-ml conical tube using a 25-ml serological pipette. If needed, rinse flask with extra PBS to make a total volume of 30 ml.
23. Add 3 ml chloroform to each tube.
24. Vortex for 5 min.
25. Add 7.6 ml of 5 M NaCl to each tube.
26. Vortex each tube for 10 s.
27. Place tubes into precooled centrifuge, and centrifuge 5 min at 3000 x g, 4Â°C.
28. Using a 25-ml serological pipette, carefully collect the aqueous phase into a new 50-ml conical tube (Fig. 3A). Avoid including any chloroform when transferring supernatant.
29. Add 9.4 ml of 50% (v/v) PEG 8000 to each tube.
30. Vortex each tube for 10 s.
31. Incubate 1 hr on ice.
32. Centrifuge 30 min at 3000 Ã— g, 4Â°C (Fig. 3B).
33. Gently pour off supernatant, and place tubes upside down on paper towels for 10 min to dry. Dry residual liquid at the edge of the tube with paper towel if necessary (Fig. 3C).

34. Resuspend with AAV-containing HEPES from Step 19. Do not resuspend the pellet by pipetting because this may shear the viral particles and decrease the final AAV yield. Let the pellet sit at 4 Â°C and mix every so often until it is entirely suspended. Make sure that the pellet is completely resuspended before commencing with the next step.
35. Vortex tubes for 5 min.
36. To each tube add:
3.5 Î¼l of1M MgCl2
14 Î¼l DNase I
1.4 Î¼l of 10 Î¼g/Î¼l RNase A.
37. Incubate tubes at 37Â°C for 20 min.
38. Split resuspended solution of each tube into two 1.5-ml microcentrifuge tubes with â¼750 Î¼l each.
39. Add chloroform at 1:1 (v:v) proportion to each tube.
40. Vortex tubes for 10 s.
41. Centrifuge tubes 5 min at 3000 x g, 4Â°C.
42. Carefully collect aqueous phase using a 1000-ml pipette (Fig. 3D).
43. Repeat steps 39 to 42 two to three times (until aqueous phase is clear; Fig. 3E).
44. Leave tubes with the lid open in a biosafety cabinet, and evaporate chloroform for 30 min.
Note: After complete evaporation of the chloroform, the AAV-containing supernatant can be used for in vitro studies. Aliquot the AAVs and store at -80 Â°C.
Day 6: Aqueous two-phase partitioning and Amicon Ultra Centrifugation
45. Weigh the AAV-containing supernatant from Step 44.
46. Per 1 g of the AAV-containing supernatant, add 5 g of 20% (NH4)2SO4 solution and 1.5 g 50% PEG 8000 solution. IMPORTANT: use weight/weight ratios.
47. Vigorously vortex the mixture for 2 min and let sit for 20-30 min at room temperature.
48. Centrifuge the two-phase mixture at 2400 x g for 15 min at room temperature.
49. Remove the top layer and interphase by aspiration and transfer the clear bottom phase (AAV-containing (NH4)2SO4 solution) into a new tube.
Note: Some proteins salt out and form a precipitate at the bottom. The unprecipitated virus resides in the bottom soluble phase. All other proteins partition into inter- and top PEG phase.
50. Rinse an Amicon 100 kDa Ultra Centrifugal Filter with 400 ul DPBS+0.01% Pluronic F68, remove the solution.
51. Add up to 400 Î¼l AAV-containing solution from Step 49 to an Amicon 100 kDa Ultra Centrifugal Filter.
52. Centrifuge tubes 5 min at 14,000 x g, room temperature. Discard flow-through.
53. Add 400 Î¼l DPBS+0.01% Pluronic F68, and pipette carefully three to four times to mix.
54. Centrifuge tubes 5 min at 14,000 x g, room temperature. Discard eluate.
55. Repeat steps 53 and 54 three times.
56. After the last centrifuge (This usually results in 25 Î¼l AAV), place column upside down in a new tube (provided with Amicon Ultra Centrifugal Filter; Fig. 3F).
57. Centrifuge 2 min at 1000 x g, room temperature.
58. Adjust total volume to 25 Î¼l by adding DPBS+0.01% Pluronic F68.

(The end)



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