Recombinant AAV Virus Production - Packaging & Purification

Triple Transfection of HEK293T Cells

1. 24 hours before transfection, seed HEK cells into 8 x 150-mm dishes to aim for 80%-90% confluency for next day transfection (normal housing - 37ºC in 5% CO2)
2. For transfection (for 1 dish – for 5 multiply times 5)
    a. Make PEI + Opti-MEM (Gibco) AND DNA + Opti-MEM
    b. 120 uL PEI (should be 3x total transfected DNA; concentration is 1ug/uL) + 4 mL Opti-MEM and mix well
    c. DNA (40 ug per dish, see recombinant AAV production in plasmid production section) + 4 mL Opti-MEM and mix well
        i. For DNA pAAV : PUCmini-iCAP-PHP-B (PHP-B):pHelper at ratio of 1:1:2 respectively, specifically 10 ug: 10 ug: 20 ug for each dish (total 40 ug)
    d. Mix PEI solution and DNA solution (add DNA to PEI)
    e. Cap tube and mix thoroughly
    f. Allow to rest for 20-30 minutes (transfection solution should be somewhat cloudy)
    g. Add 8 mL per dish to 12 mL supplemented DMEM
    h. 8 hours later change media
        i. 20 mL of supplemented DMEM (Gently change, preheat at 37ºC in incubator)
        ii. Throw away transfection media

Daily Media Harvest (When media is turning towards yellow, will increase over 5 days as cells increase)

1. Warm supplemented DMEM
2. Gently extract media and store in 1-liter bottle
3. Even more gently add 20 mL supplemented DMEM, using side of dish to aid speed

Cell Harvest (When cells are falling off bottom of dish, around 4-6 days)

1. Resuspend cells into dish with existing media until all cells are off the bottom
2. Put resuspended cells into 50 mL tubes and centrifuge at 2,000g for 15 minutes at -4ºC

a. Pour off supernatant to media extraction bottle

i. Allow excess media to drip back down and extract

b. Take pellet to step 3

3. Resuspend pellet in 5mL SAN buffer + 8 uL SAN enzyme. Add 100 U of SAN (4µl of 25 U/µl SAN) per milliliter of SAN buffer
4. Incubate in a 37ºC waterbath for 1 hour
5. Dry-ice ethanol freeze and keep at 20ºC overnight

PEG Precipitation of Supernatant | Media

1. Aliquot 40 mL into 50 mL tubes
2. Add 10 mL 40% PEG (PEG should be added at 1/5 final concentration for final PEG concentration of 8% (wt/vol))
3. Tighten cap and mix by inversion of tube 10 times
4. Incubate overnight at 4ºC (must be overnight to allow PEG to bind AAV, especially for HEK293T that throws a lot of AAV into the media)

PEG Pellet and Cell Harvest Resuspension (consult Hongying to get advice on tips)

1. Centrifuge the PEG + media at 4,000g for 30 min at 4°C
2. Pour off the supernatant (into bleach)
    a. Allow excess media to drip back and aspirate off
3. PEG pellet resuspension
    a. Add 1 mL SAN buffer + 4 uL SAN to resuspend PEG pellet
4. Thaw cell harvest in 37°C and add PEG pellet resuspension
5. Add 1 mL SAN Buffer + 4 uL SAN to combined solution
6. Incubate in 37°C waterbath for 1 hour
7. Prepare iodixanol density gradient (below)
8. After incubation centrifuge at 2,000g for 10 minutes at room temperature
    a. Take off supernatant and spin down again at 3,000g for 15 minutes
9. Load supernatant to the top of iodixanol gradient

Iodixanol Gradient

1. Prepare solutions for iodixanol gradient from reagents section
2. Layer in from 60%-15% (bottom to top) into OptiSeal Polypropylene Tube (Beckman)
3. Add in supernatant and weigh for balance to .01 g with other tube
    a. Avoid bubbles or tube will collapse
    b. Add SAN buffer to full seal so that top is touching
4. Seal with black top
5. Load in tube into ultra centrifuge rotor – Type 70Ti – 361625 (32.4 mL)
6. Add spacer to the top, if not sample will be crushed
7. Spin at 350,000g (58,400 r.p.m.) for 2 hours and 25 minutes at 18°C with slow acceleration
8. Gather 5 or 10 mL syringe and 16 or 18 gauge needle
9. Using needle, puncture right at the 60% and 40% layer and allow fluid to flow out
    a. If flow is low attach needle and slowly pull out
10. Collect around 5 mL, but avoid the 40%/25% interface layer is this is highly proteinaceous
11. Add 10ml PBS to mix

Virus Concentration and Buffer Exchange

1. Prepare the 3 following solutions
    a. Pluronic F68, 10% solution
    b. 0.1% in PBS
        i. 49.5 mL PBS
        ii. 500 uL Pluronic F68
    c. 0.01% in PBS
        i. 45 mL PBS
        ii. 5 mL of (b)
    d. 0.001% in PBS
        i. 45 mL PBS
        ii. 5 mL (b)
        iii. 200mM NaCl
2. Cover the filter (Pierce protein concentrator PES, 100K MWCO 5-20mL, ThermoFisher) with 10 mL of 0.1% Pluronic F68 PBS and incubate for 10 minutes at room temperature
3. Take out the 0.1% Pluronic F68 PBS

4. Add 10 mL of 0.01% Pluronic F68 PBS
5. Spin at 3000 RPM for 5 min at 4°C
6. Discard the flow through and add 10 ml 0.001% Pluronic F68 + 200mM NaCl PBS
7. Spin at 3000 RPM for 5 min at 4°C
8. Discard the flow through
9. Add sample
10. Spin at 3500 RPM for 1 hour at 4°C (if the liquid shows some color add more PBS to wash, then centrifuge again)
    a. Continue spinning at 1hour increments until sample is around 500 uL
11. Titrate sample
12. Store at 4°C for short term (2 weeks) or aliquot at ~15 ml per tube and store at -80°C for long term

Virus Titration (If 1st time, make standards – see below)

1. Treat purified AAV sample with DNase I to eliminate any DNA contaminates (DNase will not penetrate Virus)
    a. 5 uL of sample + 39 uL of H20 + 5 uL 10X DNase buffer + 1uL DNase
    b. Gently mix (no vortexing)
    c. Incubate 30 minutes at 37°C
    d. Inactivate DNase at 95°C for 3 minutes
2. Dilute DNase-treated AAV according to dilution standard:
    a. Dilution standard
3. Create standard
   Should aliquot the standard to different tubes for experiment (do not thaw and refreeze)
    a. Calculate amount of stock to start with:
        i. Size of eGFP: 7563 bp
        ii. Concentration: 1.42 ug/uL
        iii. MW= 7563 bp x 650 daltons/bp (g/mole) = 4.916 x 10^6 grams/mole
        iv. Moles/uL=1.42 ug/ul / 4.916 x 10^6 grams/mole = 2.889 x 10^-13 moles/uL
        v. Molecules/uL=2.889 x 10^-13 moles/uL x 6.022145 x 10^23 molecules/mole=17.395 x 10^10 molecules/uL
    b. To obtain a solution of 2x10^9 molecules/uL
        i. 17.395 x 10^10/2 x 10^9= 86.975 uL
        ii. 100/86.975= 1.15 uL
        iii. So we need to dilute 1.15 uL stock into 98.85 uL H20
    c. Then dilute the following standards:

Volume of 2x109 stock or previous dilution (µL)	**Volume of nuclease-free water (uL)**	**Molecules per µL**
10	90	2x10^8
10 of 2x108 dilution	90	2x10^7
10 of 2x107 dilution	90	2x10^6
10 of 2x106 dilution	90	2x10^5

10 of 2x105 dilution	90	2x104
10 of 2x104 dilution	90	2x103

4. Next dilute samples as it follows:

Dilution Series	**Volume of sample(uL)**	**Volume of nuclease free water (uL)**	**Dilution factor**	**Total dilution**
Dilution 1 (DNase step)	5uL AAV stock	45 uL	10x	10x
Dilution 2	5uL Dil. 1	95 uL	20x	200x
Dilution 3	20uL Dil. 2	80 uL	5x	1000x
Dilution 4	20uL Dil. 3	80 uL	5x	5000x
Dilution 5	20uL Dil. 4	80 uL	5x	25000x
Dilution 6	20uL Dil. 5	80 uL	5x	125000x
Dilution 7	20uL Dil. 6	80 uL	5x	625000x
Dilution 8	20uL Dil. 7	80 uL	5x	3125000x

5. Samples expected to have a titer <1x10^12GC/mL, use Dilutions 3-6
6. Samples expected to have a titer >3.6x10^13GC/ml, use Dilutions 5-8
7. Run triplicate qPCR
    a. Create master mix
        i. 7.5 uL 2X SYBR Green Master Mix
        ii. 1.5 uL Primers
        iii. 5 uL H20
        iv. 1 uL Dilution DNA
    b. Run triplicates:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10^9	10^9	10^9	10^8	10^8	10^8	10^7	10^7	10^7	10^6	10^6	10^6
B	10^5	10^5	10^5	10^4	10^4	10^4	NTC	NTC	NTC			
C	D-3	D-3	D-3	D-4	D-4	D-4	D-5	D-5	D-5	D-6	D-6	D-6

8. Analyze data according to standard curve (will add later according to addgene titer calculator)
    a. _____

Recombinant AAV production (Plasmid production)

Split Cells (Could be more thorough)

1. Suction off old media
2. Wash with Dulbecco's Phosphate Buffered Saline (1-2 mL per dish)
3. Add 1ml .25% Typrsin
    a. Put back in the incubator
    b. Wait 5-10 minutes
4. Once cells are falling off, add 1 mL of fortified DMEM
    a. Resuspend cells until they are mostly off the dish

5. Centrifuge 5 minutes at 900 RPM
6. Add 8-10 mL DMEM to dish
7. Suction off supernatant (with trypsin)
8. Gently, gently and thoroughly resuspend cells in 8-10 mL of fortified DMEM
9. Aliquot cells in dish according to desired density

Reagents (not common elsewhere; specific to AAV production/packaging)

Supplemented DMEM (DMEM 1x + 10% FBS + 1x Pen-Strep)

1. Add 50 mL FBS (Gemini) to DMEM 1x (Gibco)
2. Add 5 mL of 100x Pen-Strep (Gibco)

SAN Digestion Buffer

1. 29.22 g NaCl
2. 4.85 g Tris Base
3. 2.03 g MgCl2-6H20
4. Add to 1 Liter DiW
5. Procedure
   a. Mix
   b. Filter-sterilize
   c. pH should be around 10
   d. Can store at room temperature for several months

SAN + SAN Digestion Buffer

1. 4 ul of 25 units/uL of SAN per milleter of SAN digestion buffer (4uL SAN/1mL SAN buffer)
2. Pipette to mix

40% (wt/vol) PEG Stock Solution

1. Add 500mL DiW into sterile bottle a
2. Add another 500mL DiW into bottle b
3. Add 146.1 g NaCl to bottle b and mix until dissolved
4. Add 400 g PEG (polyethylene glycol)
   a. Heat and spin at 37 C for overnight/until dissolved
      i. Can expedite by heating until 65 C until no flecks are dissolved (solution might be turbid)
5. Add bottle a to bottle b and filter-sterilize into bottle a
   a. Filter by pre-wetting the filter minimally with DiW
   b. Filter should take around 1-2 hours due to the viscosity of the solution

DPBS + high salt (1M NaCl/PBS-mK buffer)

1. 29.2 g NaCl
2. .07455 g KCl
3. .2807 g MgCl2-6H20
4. 500m mL filtered PBS
5. Mix all together (Mix in order of list)

DPBS + low salt (1 X PBS-mK Buffer)

1. .07455 g KCl
2. .2807 MgCl2-6H20
3. 500 mL filtered PBS
4. Mix all together (Mix in order of list)

Iodixanol density gradient solutions (15%, 25%, 40%, 60% (wt/vol) iodixanol)

1. Must make fresh every time

		1 Rxn 5 mL per		
	60% iodixanol stock	1 x PBS-MK buffer	1 M NaCl|PBS- Mk buffer	Phenol red/ul
60% iodixanol	5 mL			45 uL
40% iodixanol	3.3 mL	1.7 mL		
25% iodixanol	2 mL	3 mL		30 uL
15% iodixanol	1.25 mL		3.75 mL	
		2 Rxn 10 mL per		
60% iodixanol	10 mL			90 uL
40% iodixanol	6.6 mL	3.4 mL		
25% iodixanol	4 mL	6 mL		60 uL
15% iodixanol	2.5 mL		7.5 mL	

2. Using glass tube and dropper add 60% (bottom), 40% (middle-bottom), 25% (middle-top), 15% (top) slowly drop by drop. Ensure not to disturb solution
3. Add virus sample on top