PEI transfection of 293 cells

rAAV production by triple transient transfection of 293FT cells

I. 24 hours before transfection, seed cells at $6x10^4$ cells/cm^2. A 150mm dish is 152 cm^2 (~$9x10^6$ cells/dish). The PEI transfection described in the "Standard PEI transfection" protocol typically yields up to 2e12 VGs per 150mm dish (after purification) with a capsid that packages well. Therefore, from 10 plates a good prep of AAV9 or PHP.B will yield about $2x10^{13}$ GCs.

II. See detailed support PEI protocol "Standard PEI Transfection" below. Once familiar with the protocol, use the PEI transfection Calculator to set up the transfection.

AAV Harvest

III. Change the media 12-24 hours post transfection. Add DMEM + 4% FBS.

IV. Harvest media at 72 hours and replace media on cells with fresh DMEM + 4% FBS. Store the harvested media at 4°C.

V. Harvest virus from media and cells at 120 hours post transfection

a. Cell pellet

i. Scrape (gently, away) cells into all 20 mL of media. Prop plates and transfer to 250 or 500 mL conical centrifuge tubes. Combine with media from 72 hour step.

ii. Spin cell, media mixture for 15 min at 2000 x g.

iii. Pour off supernatant into 500 mL vessel (e.g., empty media bottle) and save at 4C for PEG precipitation (below). Pipet off remaining several mls of media.

iv. Add SAN (salt-activated nuclease, Arcticzymes) to SAN digestion buffer. Make enough for 6 ml per virus prep by adding 4ul of 25U/ul SAN per mL.

v. Re-suspend the cell pellet in 5 mL of SAN digestion buffer. Pipet into 50 mL tube and incubate at 37°C for >1 hour.

b. Concentration of culture supernatants

i. Transfer supernatant to 250 mL conical bottles.

ii. Add a 5X stock solution of 40% PEG 8000 (Sigma) in 2.5M NaCl to the supernatant (final concentrations 8% PEG 8000 and 500mM NaCl). Incubate on ice for 2 hours (or overnight). During this step, PEG precipitates proteins. Centrifuge at 4000 x g for 30 minutes in 250 or 500 mL disposable bottles.

iii. Pour out supernatant into used media collection bottle for bleaching. Let the tubes drain of supernatant and aspirate/pipette off remaining media before re-suspending pellets.

iv. Re-suspend in 1 mL of SAN + SAN digestion buffer. This is difficult to do and may take ~5 minutes per prep. Mix resuspension with corresponding cell lysate, vortex briefly, and continue incubation 37°C for an additional 30 minutes.

v. This is a good stopping point if you aren't going to finish the prep in one day. Store at 4ºC overnight.

Iodixanol purification

Background: In the original description of Zolotukhin *et al.* 1999, the authors report the density of rAAV-UF5 to be 1.266 g/mL (genome is only 3400 bp – they state that other genomes of 3-5kb don't substantially alter banding pattern). 1.266 g/mL is equivalent to a 50% solution of iodixanol. The virus should concentrate at the 40-60% interface and within the 40% step. To better define the 40% cushion, they included a phenol red dye (0.01 ug/mL) in the 25% and 60% density steps. Doing this greatly helps visualize the layer. Note: one gradient is sufficient to purify virus from up to 10 – 150 mm dishes. <u>If more than 10 dishes are used to generate virus, divide the lysate into more than one gradient.</u>

VI. Spin lysates at 2000 x g for 10 minutes.

VII. Solutions required:

a. Optiprep (60% w/v iodixanol) from Sigma

b. PBS – low salt
   i. 500 mL PBS w/100mM NaCl (2.92 g), 1mM $MgCl_2$ (2.5 ml from a 200mM stock) and 2.5 mM KCl (93.2 mg KCl).

c. PBS – high salt
   i. 500ml PBS
   ii. 29.22 g NaCl (1M), 1mM $MgCl_2$ (2.5 ml from a 200mM stock) and 2.5 mM KCl (93.2 mg KCl).

VIII. Gradients – iodixanol is costly, make only what is needed. We've provided a chart which details calculations for the following steps:

a. 15% (w/v) iodixanol
b. 25% (w/v) iodixanol + phenol red
c. 40% (w/v) iodixanol
d. 60% iodixanol (100% Optiprep)+ phenol red stock

			number of gradients				
volume (ml)	step (%)		1	2	4	6	8
4	15	PBS + high salt	3.3	6.6	13.2	19.8	26.4
		Optiprep	1.1	2.2	4.4	6.6	8.8
6	25	PBS + K/MgCl	3.9	7.7	15.4	23.1	30.8
		Optiprep	2.8	5.5	11.0	16.5	22.0
		phenol red	0.1	0.1	0.2	0.3	0.4
7	40	PBS + K/MgCl	2.6	5.1	10.3	15.4	20.5
		Optiprep	5.1	10.3	20.5	30.8	41.1
4	60	Optiprep	4.4	8.8	17.6	26.4	35.2
		phenol red	0.1	0.1	0.2	0.3	0.4

IX. Pour gradient - <u>Start by adding the 15%</u> to Optiseal tubes (Beckman 361625), carefully add layers with increasing density under the previous layer.

a. Form step gradient
   i. 4 ml of 15%
   ii. 6 mL of 25%
   iii. 7 mL of 40%
   iv. 4 mL of 60%

X. Load cell supernatant above the 15% step. Fill remaining void with SAN digestion buffer (buffer only, no SAN). Add small black cap (one-time use) and bigger cap (will bleach later).

XI. Weigh tubes and balance accordingly by adding SAN digestion buffer. Balance tubes to within 0.01g.

XII. In hood, load gradients/lysates into ultracentrifuge rotor *(70Ti)*. Spin at 350,000 x g *(58,400 rpm)* for 2 hours 15 minutes at 18°C. with a slow acceleration (3 minutes)/deceleration (9 minutes) program (optional, we haven't tested whether the slow acceleration/deceleration makes a difference).

XIII. In TC hood, open rotor lid and slowly pull out tube. Clamp tubes one at a time on stand above a large container of 10% bleach.

XIV. Clean side of tube with 70% ethanol.

XV. Collect virus by inserting a 16 G needle with a 5 mL syringe about 2 mm below the 42/60 interface. Once inserted, take off black cap. Collect virus (~4.0 mL). Take care not to collect ANY of the visible protein-rich band at the 25/42% interface. There will NOT be any visible virus band unless something has gone wrong. Before removing the needle, reinsert the black cap.

Sterile filtration and concentration of virus

XVI. Add the virus in iodixanol to a 10 mL syringe attached to a 0.2 um PES (low protein binding) filter containing 10 mL with PBS.

XVII. Sterile filter through 0.2 um PES filter into Amicon Ultra filter. *We add the PBS first, then layer the virus in the iodixanol below the PBS, so the remaining PBS at the top washes the filter after the virus goes through – let the PBS begin dripping through before adding the virus. This filtration step reduces the likelihood of clogging the Amicon filter.*

XVIII. Dialysis and concentration of the virus using Amicon Ultra-15 with a 100 kDa cutoff (UFC810024).

a. After loading virus into the Amicon filters, pipet to mix and spin at 3000 x g for ~5-8 minutes until the volume of the solution remaining in the top chamber is <1500 uL (>10x concentration). *This takes longer initially as the iodixanol greatly slows down the passage of the buffer through the filter. Diluting the virus samples after the iodixanol purification by at least 2-fold dramatically decreases spin time.*

b. Discard flow-through. To virus stock, add dPBS to 14 mL and mix.

   i. *The dialysis should be performed with a buffer that is both isotonic and has high ionic strength. AAV2 aggregates in low ionic strength buffers, and high titer stocks (>1e13 vg/ml) can be maintained in high ionic strength buffers (Wright et al Mol Therapy vol 12 171-8, 2005). They found that 100 mM sodium citrate, 10 mM Tris, pH 8.0 (315 mOsm) allowed for recovery of 93-96% of expected VGs. This aggregation problem may be specific to AAV2. We have been able to maintain AAV9 and AAV-PHP.B at >1e13 vg/ml in PBS at 4C for months.*

c. Spin 3000 x g for 2 minutes and repeat 2 more times (3 total washes, 4 total buffer exchanges).

XIX. Sterile filter the virus through a 0.2um PES membrane syringe filter fitted on a 3 or 5 ml syringe.
   a. Pre-wet the filter with a small volume of PBS before adding virus (this works best if the PBS is added directly to the filter prior to inserting the syringe.
   b. If yield is more important than achieving a high titer prep, a small volume of PBS can also be pushed through the filter after the virus stock.

XX. Aliquot and titer the virus (see support protocol below).
   a. For long-term storage virus can be aliquoted and stored at -20ºC or -80ºC.
   b. For short-term use (up to 6 months) the virus is quite stable at 4ºC.

SUPPORT PROTOCOL 1

Standard Polyethylenimine (PEI) Transfection

Background

Use of the cationic polymer polyethylenimine (PEI) as a vector for gene and oligonucleotide transfer into cells in culture was first described by Boussif and colleagues (1995). In particular, 25-kDa PEI is well characterized and has been widely used to achieve efficient recombinant protein expression. Polyethylenimine undergoes ionic interactions with DNA, forming polyplexes that can be taken up by cells, traffic through endosomes and cytoplasm, and finally reach the nucleus through an incompletely understood mechanism (Merdan *et al*., 2002). In contrast to the calcium phosphate method, PEI can mediate efficient DNA transfection to mammalian cells grown in suspension in serum-free medium, features that enhance process scalability and the safety of biological products generated by this approach. However, PEI is nonbiodegradable and moderately cytotoxic (Wright, 2009).

Linear PEI Stock Solution, 25-kDa (1L, 0.323g/L)

1. Materials
    a. Cell culture grade water
    b. Linear 25kD PEI (Polysciences, CAT 23966-2).
    c. 1M HCl in cell culture grade water
2. Combine 0.323g of PEI and 950 mL of water.
3. pH to 5 with 1M HCl
4. Heat and mix to dissolve PEI.
5. Sterile filter, 0.22 micrometer.
6. Aliquot and store at -20.

The efficacy of PEI as a transfection reagent may increase with more freeze/thaw cycles, up to 8 cycles. Once thawed, PEI aliquots may be stored at 4°C while in use (Reed *et al*., 2006).

Transfection Procedure

15cm dish (152 cm^2), 20mL media. 90% confluent, HEK293FT
40 µg total DNA.
Dulbecco's PBS (DPBS)
PEI Stock Solution.

PEI	PBS[1]	DNA (µl)	PBS[2]	Total	DNA (µg)	N:P Ratio
439.5	560.5	34.0	966.0	2000	40	25

Total volume added is $1/10^{th}$ of cell culture media in the container, 2mL into 20mL of a 15cm dish, which may or may not be optimal. Total transfected DNA and nitrogen:phosphate (N:P) ratio may vary depending on the application and cell line. For AAV9 production in HEK293FT, the values above generate ≈1e10 DNase resistant genome copies(GC)/mL, as assessed by qPCR, in the media at 72 hours post-transfection, with a media change at 24 hours post-transfection. Increasing the N:P ratio generally increases the cytotoxicity. Passage number (>15) at the time of transfection was not seen to significantly lower AAV yields.

1. Thaw PEI stock in 37°C water bath until clear.
2. Bring DPBS and PEI stock to room temperature.
3. Combine PEI+PBS[1] and DNA+PBS[2] in separate containers.
4. Vortex briefly (2 seconds).
5. Add PEI solution to DNA solution.
6. Vortex briefly (2 seconds).

7. Incubate at room temperature 10min.
8. Add the complete solution uniformly to the dish.
    a. Add dropwise with swirling to mix.
    b. If a media change is performed at the same time, add the complete solution to fresh media and use that to change the media.
9. (Optional) Change the media 6-24 hours after transfection.

Notes: Cell death of ≈ 30% is common. Low confluency at the time of transfection generally resulted in poor cell performance and health therefore higher confluency (approaching 95%) is desirable. In producing a Tet-On system based in AAV9, GFP expression was noted to poorly correlate to final yields.

Bibliography

Grieger, J. C., Choi, V. W., & Samulski, R. J. (2006). Production and characterization of adeno-associated viral vectors. *Nature Protocols , 1* (3).

Kuroda, H., Kutner, R. H., Bazan, N. G., & Reiser, J. (2009). Simplified lentivirus vector production in protein-free media using polyethylenimine-mediated transfection. *Journal of Virological Methods , 157*.

Reed, S. E., Staley, E. M., Mayginnes, J. P., Pintel, D. J., & Tullis, G. E. (2006). Journal of Virological Methods. *138*.

Wright, J. F. (2009). Transient transfection methods for clinical adeno-associated viral vector production. *Human Gene Therapy , 20*.

DETERMINATION OF rAAV TITERS BY THE QUANTITATIVE PCR ASSAY

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This approach works very well to titer single-stranded rAAV, but in some cases underestimates the titer of self-complementary rAAV by as much as 5-10 fold. This is likely due to competition between the presence of 2 complementary halves of the target sequence on the same viral genome, which would anneal together and compete against primers to bind and amplify the DNA. Therefore, self-complementary rAAV preparations should be titered by dot blot in parallel with qPCR, at least initially, to determine the reliability of qPCR to accurately titer the self-complementary rAAV preparations. Just as with the dot blot, this method does not provide any information about the infectivity of the rAAV particles.

Materials

Virus fractions or final virus preparation (e.g., from Basic Protocol 1)
DNase digestion buffer (see recipe)
0.5 M EDTA
Proteinase solution (see recipe)
DNase/RNase-free PCR-grade water
Primers corresponding to the target sequence
96-well qPCR reaction plate or individual reaction tubes (appropriate for your qPCR instrument)
Quantitative PCR instrument
Quantitative PCR reagents (follow the manufacturer's instructions for reaction setup)

Digest virus particles to release DNA

1. Prepare an experimental plan for the position of samples, controls, and DNA standards in duplicate in a 96-well format.

   The samples are prepared in microcentrifuge tubes, but will be loaded onto a 96-well dot-blot apparatus at the end of the procedure.

2. Place 2 µL of virus into microcentrifuge tubes. Add 100 µl DNase digestion buffer to each sample and incubate 1 hr at 37°C.

   This step digests any DNA that may be present but has not been packaged into virions. Be aware that the CsCl salt in the samples collected from basic Protocol 1, step 24, can interfere with the DNase reaction and may affect the accuracy of titering the viral genomes. If non-dialyzed rAAV (still in CsCl) is assayed, use only 1 µL. For obtaining a final titer on dialyzed rAAV, start with 10 µL that has already been frozen once and re-thawed.

3. Inactivate the DNase by adding 5 µl of 0.5 M EDTA. Mix well and incubate 10 minutes at 70°C. Release virion DNA by adding 120 µl of proteinase solution. Incubate a minimum of 2 hr (or up to overnight) at 50°C.

   This step liberates the encapsidated DNA. Failure to mix well after the addition of EDTA and heat-inactivate the DNase will decrease the apparent titer. EDTA inactivates the DNase, which would otherwise degrade the virion DNA when it is released from the virus particle.

4. Boil the rAAV sample for 10 minutes at 95 to inactivate the Proteinase K.

   This step is necessary to ensure that the Proteinase K doesn't degrade the PCR polymerase during reaction setup.

5. Take 3uL of solution, dilute with 897ul of PCR-grade water.

This gives a final dilution of 1: 34,050. At these dilutions the rAAV genomic DNA copies should still be within the dynamic range for qPCR detection and the components of the DNase and Proteinase K digestions will be diluted so that they don't interfere with the qPCR reaction.

Prepare the DNA Standards

1. Preferably, the plasmid used for transfection of each virus should be used to make the standard. Alternatively, another plasmid that contains the same target sequence can be used. The plasmid should be linearized (preferably in the vector backbone) and run over a PCR prep clean up column. Dilute the linearized plasmid DNA with EB or PCR-grade water to a concentration of ~5-10 ng/ul. Make a set of (7) 1:10 dilutions in PCR-grade water. The final standard set should range from ~$5x10^8$ to $5x10^1$ single-stranded DNA molecules per µL.

   At these low concentrations of DNA in water, the DNA is subject to degradation and/or sticking to the walls of the tube, which can significantly affect the reliability of the standard quantification. For these reasons, standards should be prepared fresh just before use.

   We prepare linearized standards that are aliquoted and frozen. We quantify each aliquot at the time of titering by Qubit – nanodrop quantification for this purpose has proven unreliable. The concentration in ng/ul is used to calculate the number of molecules. This calculation also requires the molecular weight of the standard plasmid being used. The molecular weights of our commonly used standards are provided in the titer calculator spreadsheet.

Q-PCR Reaction Setup and Analysis

2. Set up the reactions using 2 µL of sample or standard per reaction tube. Add the primers and qPCR reagents specific for your instrument, following the manufacturer's instructions.

   For SyBR Green-based detection systems, the following primers can be used with GFP: Forward 5'- AGCAGCACGACTTCTTCAAGTCC; Reverse 5'-TGTAGTTGTACTCCAGCTTGTGC. In general, primers should amplify a target sequence of approximately 150-250 bp.

3. Following the reaction, generate a standard curve based on the number of single-stranded molecules per tube and the crossing point. Using the crossing point of each rAAV sample, the vector genome copies can be determined. Most quantitative PCR instruments use software that will calculate these values automatically.

   Similarly, the dilution factor of rAAV DNA in each tube will be 1:34,050 (for 2 µL of starting rAAV). Multiply the quantity of rAAV DNA in each tube by the dilution factor to get the titer of the original rAAV stock (in vector genomes per µL).

4. Additional Considerations. When using a new primer set, the final PCR products following the qPCR reaction can be assayed by agarose gel electrophoresis to determine the purity of the PCR product. Non-specific PCR products can adversely affect the precision of the qPCR method. Also, the quantitation is based on the assumption that the standard and samples amplify with the same efficiency. This can be tested by making serial dilutions of the sample DNA (e.g., 1:1, 1:5, 1:25, 1:125) and running them as normal for qPCR. When accounting for the dilution factor, these should give the same value. If there is a clear trend of change for the values, this indicates that the PCR conditions and/or primers are not optimal.

SUPPORT Document 1

REAGENTS

SAN Digest Buffer (500 mL) *(Prepare fresh)*

- ❖ *Add 100U(4ul)/ml SAN (salt-active nuclease) just before use*
- 40 mM Tris base
- 10 mM $MgCl_2$
- 500mM NaCl
- Fine adjust pH 8.5 with concentrated HCl
- *Add 100U(4ul)/ml SAN (salt-active nuclease) just before use*

5x PEG 8000-NaCl (500 mL)

- 2.5M NaCl
- 40% PEG 8000
- TC grade water
    - Dissolve NaCl in 250 mL H20 and dissolve fully before adding PEG. Add PEG and then bring water up to just under 500 mL. Leave at 37C for several hours to overnight. Shake occasionally. If you are in a hurry, heat to 65C until PEG is dissolved. It will look turbid, but there should be no more flakes of PEG. Upon cooling the PEG solution will become clear. Make up to 500 mL and filter sterilize. <u>It is necessary to pre-wet the filter with a small amount of water prior to adding the PEG/NaCl solution</u>. This solution is extremely viscous and will take several hours to filter (always remember to make ahead of time).

Linear PEI Stock Solution, 25-kDa (1 L, 0.323g/L)

- 0.323g of PEI (25kD (Polysciences, CAT 23966-2)
- 950 mL TC grade water
- pH to 2-3 with 1M HCl.
    - Heat in a 37C water bath for several hours (mix several times to dissolve PEI).
    - Bring volume up to 1L
    - Sterile filter, 0.22 micrometer.
    - Aliquot and store at -20.

The efficacy of PEI as a transfection reagent may increase with more freeze/thaw cycles, up to 8 cycles. Once thawed, PEI aliquots may be stored at 4°C while in use (Reed *et al*., 2006).

DNase digestion buffer (250 mL)

- ❖ *Add 50 U/ml DNase I just before use*
- 10 mM Tris·Cl, pH 7.5
- 10 mM $MgCl_2$
- 2 mM $CaCl_2$

Proteinase solution (250 mL) ***(****Prepare fresh)*

- ❖ *Add 100 µg/ml proteinase K (Sigma) just before use*
  - 1 M NaCl
  - 1% (w/v) *N*-lauroylsarcosine (Sarkosyl)

PBS-low salt (500 mL)

- PBS +
- 100mM NaCl
- 1mM $MgCl_2$
- 2.5 mM KCl
- sterile filter

PBS-high salt (500 mL)

- PBS +
- 1M NaCl
- 1mM $MgCl_2$
- 2.5 mM KCl
- sterile filter