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AAV Production Protocol

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We use this protocol and it's working

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Abstract

AAV Production methods used in Falasconi & Kanodia et al 2025 and is the general protocol for Arber Lab.

Guidelines

Important: Keep insert size under 5kb between ITR's. If necessary remove b-globin intron from construct. The iodixanol purification method will not change the overall titer as compared to CsCl if you follow the purification method described here including step 19 for CsCl purification. However, **iodixanol** results in an **increased infectivity of 5-10 fold**.

Ideal case: Construct between ITRs <5kb, cell type specific promoter, iodixanol preparation.

Expected titers: With CMV promoter: 10^{12} - 10^{13} (usually around 5×10^{12})

With cell type specific promoter: 5×10^{13}

You will not be able to package more than 5kb. If you have to use a construct that is larger you can do so but you need a high titer such that multiple particle can recombine after infection to generate the full-length transcript. If you plan double infections you will be advised to have construct smaller than 5kb. If they are larger and have partially the same sequence (e.g. GFP as marker, IRES for expression of multiple proteins) you may get a mismatch recombination between the two viruses.

Before generating the virus **ALWAYS** check for ITR recombination with SmaI (cuts at 25°C) or XmaI (cuts at 37°C). Ratio of linearized plasmid to plasmid+ insert band should be as low as possible. 20% of recombination is acceptable, but less is better. Note, it is almost impossible to obtain a plasmid prep with no recombination. Use XL1blue or Stable2 cells to amplify the plasmid. If recombination ratio is still bad grow plasmid at 30°C. Additionally, growing 2 Liters of culture and not exceeding cell density of OD=0.7 helps. As a general rule set up at least 10 mini-preps (3ml cultures) overnight at 30°C and perform next day the mini-preps with 2ml of the culture. Inoculate the maxi-prep with the remaining 1ml of culture from the miniprep that had the least recombination.

Safety warnings

 Wear appropriate PPE as required by your institution.

Before start

Read through the entire protocol before starting.

Cell Culture, transfection and first step of viral purification:

- 1 **20-24h before transfection:** seed 293T cells into 10 dishes (15cm) for 1 viral prep. If you do 1 viral prep do ~1:3 dilution of your cells, meaning split 3 confluent 15cm plates into 10 15cm plates. If you do 2 viral preps split 5 plates into 20 plates. Use standard media for 293T cells (DMEM, +10%FCS and Pen/Strep). Transfect cells at ~80% confluency.
- 2 **For PEI transfection:**
 - 70ug AAV helper plasmid (Rep/Cap)
 - 70ug AAV vector (ie. pAAV-mGFP)
 - 200ug pHGTI-adeno1 (provide adenoviral helper function)
 - add DMEM (without FCS or Pen/Strep) up to 48mL in falcon tube with above DNA
 - add 1360 uL of PEI (assumed 1:4 ratio of DNA:PEI; needs to be tested prior for each batch of PEI)
 - fill to 50mL with DMEM and mix by inverting tube
 - let sit RT for 15 minutes at RT
 - meanwhile replace media in cells with 20mL/plate of fresh media (DMEM without FCS or Pen/Strep)
 - add 5mL transformation mix per plate.
 - change media the day after with standard 293T media, DMEM, +10%FCS and Pen/Strep, (24 hour later is ideal)
- 3 48-60 hours (closer to 60h) after transfection, dislodge cells by pipetting up and down with the culture media. Collect all cells of 1 prep (10 plates) by centrifugation (depending on your centrifuge around 2000 rpm) and remove supernatant. The yield of cells should be around 5ml.
- 3.1 **Optional:** wash cell pellet with 15ml of PBS re-centrifuge and discard supernatant. The pellet can be stored at -80°C for extended periods of time.
- 4 Re-suspend the cell pellet in 15mL (for 10 15cm plates of transfected cells) lysis buffer (150mM NaCl, 20mM tris pH 8.0, filter sterilized and stored at 4°C).
- 5 Freeze-thaw the cells three times between dry ice/ethanol bath and 37°C water bath. Make sure it freezes well in the dry ice and thaws completely.
- 6 Dounce homogenize (tight-fitting) the cell lysates 20 times to destroy cell nuclei. Put in fresh falcon tube. Do not bring dounce homogenizer all the way out of the solution. After use, clean with kordolex, pull up and down a few times, shake it, then 70% ethanol and then dH₂O.



- 7 Add MgCl_2 to a final concentration of 1mM (add 15uL of 1M MgCl_2) to the cell lysates.
- 8 Add Benzonase (Sigma E8263-25K) to a final concentration of 250U/mL (15ul of 25,000U/ml stock). Mix and incubate at 37°C for 10-15 min. The genomic DNA/protein aggregates that formed from the thaw/freezing cycle should dissolve.
- 9 Spin down the cell debris at 4,000 rpm, 4°C for 20min (in Eppendorf 5810R, recalculate rpm if using a smaller rotor). Virus is in supernatant, but contains still lots cellular debris. Purify further with either CsCl or Iodixanol method.

STEP CASE

CsCl Purification Method

30 steps

Concentration and Purification with CsCl

- 10 Collect supernatant in 50mL Falcon tube and add 10g CsCl. Wait until CsCl is completely dissolved then add lysis buffer to get final volume of 20mL.
- 11 Transfer lysate to a 36mL Optiseal tube (Beckman 362183). Top off the tube with 1.37 g/ml CsCl solution (25g CsCl in final volume of 50ml lysis buffer).
- 12 Load the tubes onto a Beckman VTi50 rotor for a Beckman ultracentrifuge (get adaptor for Optiseal tubes) and spin at 50,000 rpm. 15°C for 12-16 hours.
- 13 Place the centrifuged tube in a Beckman Fraction Collector (or self made fraction collector). Collect approximately 9-10 fractions from the bottom (4ml each). Store fractions at 4°C.
- 14 Determine the rAAV containing fractions by real-time PCR (see Real Time PCR for viral titration section for protocol for running RT-PCR).
- 15 Add the two fractions with the highest titer (this should be between fraction 4-7; mostly fraction 4, 5, 6) to an 11.2 OptiSeal tube.
- 16 Underlay with 1mL of 1.5g CsCl/lysis buffer solution (67.5g CsCl in 100mL final lysis buffer). Do this by needle and syringe injecting solution via a tube with tapered extended to the bottom of the optiseal tube. Top tube with 1.37g/ml CsCl in lysis buffer.
- 17 Load the tubes onto Beckman NVT65 rotor and spin at 65,000 rpm, 15°C for 12-16 hours.



- 18 Collect 10 1mL fractions into 1.5ml eppendorf tubes. Store fractions at 4°C while determining the fraction with the highest titer by RT-PCR.
- 19 Dialyze the positive fraction in MWCO 10,000 cassettes (Pierce 66810) against 3 changes of 1L PBS for 1 hour each. Pre-wet the dialysis bags briefly before putting virus in.
- 20 Recuperate the virus (~1ml) and determine titer by RT-PCR. Your titer should be between 10^{11} - 5×10^{12} depending on the size of the insert and if the CMV promoter or a cell specific promoter was used.
- 21 **OPTINAL BUT IMPORTANT:**
To obtain a higher titer pool the 2 fractions with the highest titer of step of step 17. Do step 18 with ~2ml of virus and after dialysis you can further concentrate the virus another 5-10 fold with a single spin.

Place virus solution in a Amacon 100K columns (UFC910008) and spin for 20 min at 3000 rpm (R3CB Sorval). After spin Recuperate virus and determine titter by RT-PCR. You should have 150-250ul of virus solution with a titter of 5×10^{12} - 5×10^{13} .

Real-Time PCR for viral titration

- 22 Prepare standards:
 - 22.1 Identify an efficient primer pair and probe for the TaqMan RT-PCR (Primer express software from Applied Biosystems).
 - 22.2 Test ideal PCR conditions on your target sequence.
 - 22.3 Determine DNA concentration of the linearized plasmid that you want to use for the standard curve.
 - 22.4 Perform dilution series of this (1:10), 8 dilutions.
 - 22.5 For RT-PCR use at least 6 of the 8 dilutions. Start with the dilution that has a concentration below 1ng/ul.



22.6 Calculate the copy number of DNA strands in your dilution series. (For GFP product length is 248bp, meaning the MW is $\sim 248 \times 2 \times 310 = 153760$. If you have 1ng/ul of DNA fragment you have 1ug/ml of DNA. Thus you can employ the following calculation: $X \times 10^{-6} [\text{ug/ml}] / Y [\text{g/mol}] \times 6.23 \times 10^{23} \text{mol} = \text{DNA fragments/ml}$ (where: X is DNA concentration in ug/ml; Y is molecular weight in gram; the result corresponds to your titer in ml)

23 Prepare virus:

23.1 Take 5uL of the purified virus into a tube.

23.2 Add 5uL 10x DNase buffer, 1uL DNase (Roche 10,000 U/ml), 39uL dH₂O (make master mix before putting into tubes).

23.3 Incubate at 37°C for 15 minutes to remove residual plasmid DNA from purification.

23.4 Incubate at 95°C for 10 minutes to denature DNase.

23.5 Add to each tube 50uL proteinase K solution (5ul DNase buffer, 44ul dH₂O, 1ul Proteinase K (10mg/ml)).

23.6 Incubate at 37°C for 15 minutes.

23.7 Denature ProteinaseK at 95°C for 10 minutes.

23.8 Use 1ul of this solution for RT-PCR (make a 1:5 dilution to decrease pipetting error).

23.9 RT-PCR reaction mix per tube

	A	B	C
	Component	Volume (ul)	Final Concentration
	Water	6.25	



	A	B	C
	Primer Forward	0.50	400 nM
	Primer Reverse	0.50	400 nM
	Dual-labeled probe	0.25	100 nM
	TaqMan Master Mix 2x	12.50	
		20.00	

Add 5 ul of prepared virus (final volume 25 ul).

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NOTE: Your titer (GC/ml) will be 40'000 fold higher than what is obtained from the RT-PCR due to the dilution (1:20) of the virus, the conversion from ul to ml and the fact that it's a single stranded virus.