

## Small and Micro-Scale Recombinant Adeno-Associated Virus Production and Purification for Ocular Gene Therapy Applications

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### Abstract

Over the past two decades recombinant adeno-associated virus (rAAV) vectors have emerged as the gold standard for transferring genetic material to cells of the retina. The ability to effectively produce small batches of rAAV vector at high enough purity for in vitro and in vivo applications in a cost-effective manner is paramount. This is particularly the case when conducting preclinical experiments to screen novel serotypes, promoters or transgenes, where production of numerous vector batches is required. Current vector production methods often produce large quantities of vector, limiting the cost-effectiveness and practicality of such screening experiments, which often require only small volumes of vector to carry out. Herein, we describe a method to produce high titer ( $10^{12}$ – $10^{13}$  vector genomes (vg)/mL) rAAV vector on small (~100  $\mu$ L) or micro (~15  $\mu$ L) scale for in vitro and in vivo applications.

**Key words** AAV, Manufacture, Purification, Virus vector, Gene therapy, Retina

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### 1 Introduction

Recombinant vectors based upon adeno-associated virus (rAAV), a small, nonpathogenic dependovirus, have demonstrated to be highly effective in transducing cells of the retina in numerous preclinical and clinical studies [1, 2]. Numerous methods have been developed for generating rAAV [3–5], with the most common approach involving triple transfection of adherent HEK293T cells with an adenovirus-derived helper plasmid, a plasmid encoding the AAV *rep* and *cap* genes, and a plasmid containing the transgene cassette flanked by inverted terminal repeats (ITR) [6–8]. Typically, rAAV production protocols produce large vector volumes (~300–500  $\mu$ L) of high titer ( $10^{12}$ – $10^{13}$  vg/mL) vector, which are appropriate for systemic administration or injection in large animal models, but are excessive for in vitro tissue culture experiments or intraocular administration in rodents. Furthermore,

when conducting screening experiments to establish the tropism or specificity of novel vector serotypes or promoters, it is typically necessary to carry out only a small number of injections, leading to vector waste and increased research cost. Here, we describe two methods to produce small quantities of high titer ( $10^{12}$ - $10^{13}$  vg/mL) virus in a cost-effective manner (<\$1000): A small-scale preparation which produces ~100  $\mu$ L of highly pure vector suitable for preclinical in vivo gene therapy applications, and a micro-scale preparation which produces ~15  $\mu$ L of crudely purified (minimal gradient purification) vector suitable for rapid screening of novel cell-specific promoters or capsid mutants in difficult to transfect cell lines or primary ocular tissue (e.g., retinal explants).

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## 2 Materials

### 2.1 *Small- and Micro-Scale General Materials*

1. pHelper plasmid encoding adenovirus helper genes E2A, E4 and Viral Associated RNA.
2. pRep-Cap plasmid encoding rAAV *Rep* and *Cap* genes.
3. pAAV construct containing the transgene cassette consisting of promoter, transgene, and regulatory elements flanked by inverted terminal repeats.
4. A ubiquitously expressing fluorescent reporter plasmid (i.e., CBA-eGFP).
5. Growth Media: Dulbecco's Modified Eagle Medium-DMEM + GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution.
6. Transfection Media: DMEM + GlutaMAX supplemented with 2% FBS and 1% antibiotic/antimycotic solution.
7. 0.01 M phosphate buffered saline (PBS).
8. HEK293T cells (ATCC, #3216).
9. 1  $\mu$ g/ $\mu$ L Polyethylenimine (PEI) transfection reagent: Linear, molecular weight—25,000 (PolySciences) (*see Note 1*).
10. 150 mM NaCl.
11. 10 M HCl.
12. 1 $\times$  TrypLE or other trypsin-EDTA.
13. Lysis buffer: 150 mM NaCl, 50 mM Tris-HCl, pH 8.5. Filter-sterilize, do not autoclave. Store at room temperature.
14. 100% ethanol.
15. Dry ice.
16. 250 U/ $\mu$ L Ultra-Pure Benzonase nuclease.
17. HBSS/Tween 20: Hanks balanced salt solution (HBSS), no phenol red, containing 0.014% Tween 20.

18. Picogreen lysis buffer: 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.2% SDS. Do not autoclave. Store at room temperature.
19. Quant-iT Picogreen dsDNA Assay Kit: 20× TE, Picogreen Reagent, Bacteriophage Lambda DNA Standard (Thermo Fisher).
20. ddH<sub>2</sub>O: Autoclaved double distilled H<sub>2</sub>O.
21. 96-well solid black microtiter plate.
22. Fluorescent plate reader capable of exciting at 485 nm and reading emission at 535 nm.
23. 1% solution of Virkon disinfectant (DuPont).
24. 25% solution of Virkon disinfectant.
25. Inverted light microscope.
26. Vortex tube mixer allowing mixing at high and slow speed, e.g., range 250–2500 rpm.
27. PCR machine/thermal cycler.

## **2.2 Small-Scale Specific Materials**

1. CellBIND Surface Hyperflask Cell Culture Vessel (Corning).
2. 175 cm<sup>2</sup> angled neck cell culture flask with vent cap.
3. 500 mL centrifuge bottles (Nalgene).
4. 50 mL conical bottom “falcon” centrifuge tubes.
5. 25 × 89 mm Quick-Seal Ultra Clear centrifuge tubes (Beckman Coulter).
6. Amicon Ultra-15 100 kDa molecular weight cutoff centrifugal filter units (Millipore).
7. 250 mL glass bottles.
8. 10 mL syringes.
9. 5 mL syringes.
10. 1-in. regular bevel 18-G needles.
11. Microcapillary pipettes, disposable soda-lime glass, 100 µL blue (Kimble).
12. Syringe-microcapillary “funnel”: 10 mL syringe, 1-in. regular bevel 18-G needle, 100 µL blue Kimble disposable soda-lime glass microcapillary pipettes.
13. 5× PBS-MK: 685 mM NaCl, 26 mM KCl, 40 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM MgCl<sub>2</sub>. Do not autoclave. Store at room temperature.
14. Opti-Prep density gradient medium (iodixanol).
15. 0.5% Phenol red indicator solution in Dulbecco’s Phosphate Buffered Saline.
16. Beckman sealer/tube topper (Beckman Coulter).

17. Type T70 Ti fixed angle rotor (Beckman Coulter).
18. Red aluminum spacer for T70 Ti fixed angle rotor (Beckman Coulter).
19. Optima XE (or “preparation” certified) Ultracentrifuge (Beckman Coulter).
20. Retort stand and clamp.
21. 500 mL plastic or glass beaker.

### 2.3 Micro-Scale Specific Materials

1. 6-well cell culture plates.
2. 15 mL conical bottom “falcon” centrifuge tubes.
3. 1.5 mL microcentrifuge tubes.
4. Amicon Ultra-0.5 Centrifugal Filter Units Ultracel-100 (Millipore).
5. 1.8 cm blade Cell Scrapers.

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## 3 Methods

All steps should be conducted at room temperature and in a biological safety cabinet, unless otherwise specified. It is recommended that liquid and solid waste produced throughout this protocol be disposed of by bleaching in a 1% solution of Virkon disinfectant (DuPont), which effectively destroys rAAV particles. Due to the necessity to centrifuge vector preparations at several stages during production, it is recommended that an even number (2, 4, 6, or 8) of preparations are made at any one time to facilitate rotor balancing.

### 3.1 Seeding and Transfection

*3.1.1 Small-Scale, One  
Hyperflask Yields  
Approximately 100  $\mu$ L  
Highly Purified rAAV Vector*

1. Seed one T-175 flask with approximately  $2.0 \times 10^6$  HEK293T cells and allow 2 days for the flask to reach confluency.
2. Once the flask has reached 90–100% confluency, wash the flask with 15 mL of PBS.
3. Detach adherent cells using 10 mL 1 $\times$  trypsin-EDTA (3–5 min, 37 °C).
4. Transfer cell suspension directly into a 500 mL bottle of complete Growth Media.
5. Slowly pour the bottle of Growth Media containing suspended HEK293T cells into a sterile Hyperflask, being careful not to introduce bubbles.
6. Check cell confluency within the Hyperflask daily using an inverted light microscope daily (*see Note 2*); cells are ready for transfection when they reach ~70% confluency, which usually take 2–3 days.

7. Pipette 9.0 mL sterile 150 mM NaCl and 1 mL 1  $\mu\text{g}/\mu\text{L}$  polyethylenimine (PEI) (*see Note 1*) into a 50 mL conical bottom “falcon” tube labeled “PEI.”
8. Pipette 9.5 mL 150 mM NaCl and 500  $\mu\text{g}$  of plasmid DNA consisting of equimolar amounts of the pHelper, pRep-Cap and transgene plasmids (*see Table 1*) in a 50 mL conical bottom falcon tube labeled “plasmid DNA” (*see Note 3*).
9. Add the contents of the “PEI” tube to the “Plasmid DNA” tube in a dropwise manner at a rate of 1 mL/min. It is *critical* during this step to gently agitate the “DNA tube” by swirling throughout the addition of PEI (*see Note 4*).
10. Incubate the PEI–DNA complex for 20 min without agitation. During incubation, place one bottle of complete Transfection Media in to a 37 °C water bath to warm.
11. Following incubation, pour the PEI–DNA complex directly into the warmed 500 mL bottle of complete Transfection Media.
12. Remove the Growth Media from the Hyperflask by pouring into a beaker with ~25% Virkon and replace immediately with the Transfection Media containing the incubated PEI–DNA complex being careful not to introduce bubbles.
13. Incubate the flask for 72 h at 37 °C in 5% CO<sub>2</sub> to allow for viral production.

**3.1.2 Micro-Scale, One  
6-Well Yields**  
Approximately 15  $\mu\text{L}$   
Crudely Purified  
rAAV Vector

1. In Growth Media, seed a single well of 6-well plate with approximately  $3.0 \times 10^5$  HEK293T cells.
2. Check the plate with an inverted light microscope daily to determine confluency. Cells are ready for transfection when plates reach 70% confluency, which usually take 1–2 days.
3. Pipette 500  $\mu\text{L}$  of prewarmed Transfection Media and 2.7  $\mu\text{g}$  of plasmid DNA (consisting equimolar amounts of pHelper, pRep-Cap, and the transgene plasmid as described in Table 1) into a 15 mL conical bottom “falcon” tube and label “DNA.” Pipette 500  $\mu\text{L}$  of prewarmed Transfection Media and 5.4  $\mu\text{g}$  of PEI (*see Note 2*) in to a second 15 mL tube and label “PEI.” Pipette the contents of the “PEI” tube into the “DNA” tube dropwise and then gently vortex mixing (250 rpm for 15 s) the solution and incubate for 20 min at room temperature.
4. Dilute the DNA-PEI mixture (~1 mL) with Transfection Media to a final volume of 6 mL.
5. Replace the Growth Media in the well with 1 mL of the diluted DNA-PEI mixture and incubate for 72 h at 37 °C in 5% CO<sub>2</sub> to allow for viral production.

**Table 1**  
**Plasmid DNA calculation**

Plasmid	Plasmid size (kb)	Plasmid DNA ( $\mu\text{g}$ )
pHelper	11.6	$(11.6) \times (B)$
pRep-Cap	8.1	$(8.1) \times (B)$
Transgene	X	$(X) \times (B)$
Total size (A)	$19.7 + X$	
Ratio (B)	$500 \mu\text{g}/A$	

The total size (base pairs) of each plasmid is summed ("A") and divided by the total amount of plasmid DNA ( $500 \mu\text{g}$  for small-scale and  $2.7 \mu\text{g}$  for micro-scale) per prep to produce the ratio of DNA-Base Pair ("B"). The ratio "B" value is multiplied by the size of each plasmid to determine the quantity of each plasmid to add during the transfection

## 3.2 Purification

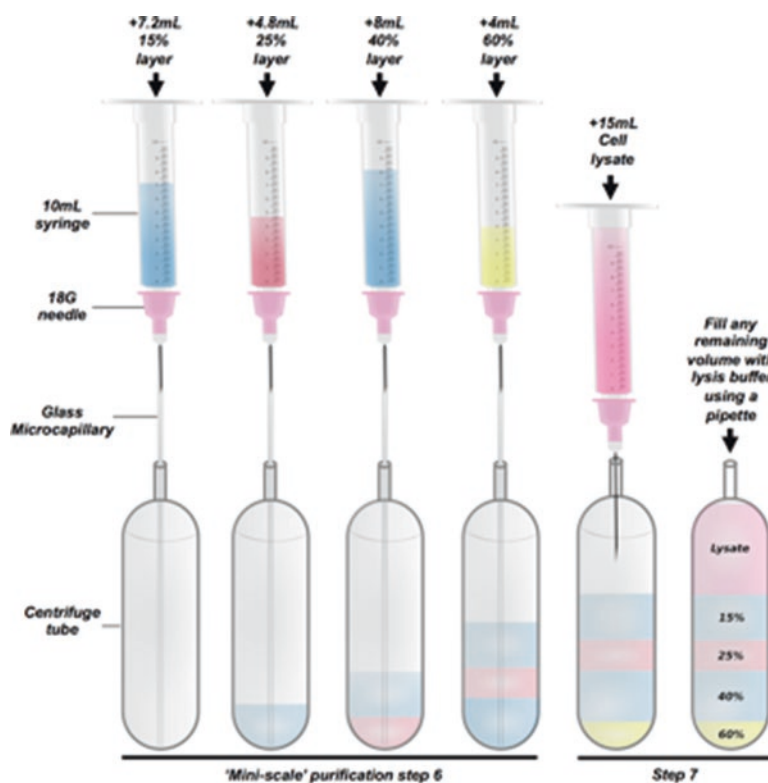
### 3.2.1 Small-Scale Preps

1. Pour out approximately 35% of the media from the Hyperflask into the waste.
2. Replace the cap and vigorously shake (*see Note 5*) the remaining media in the Hyperflask to detach the cells.
3. Pour the cell suspension (300–350 mL) into a 500 mL centrifuge bottle.
4. Pellet the cells by centrifugation at  $\sim 400 \times g$  for 15 min.
5. Pour off the supernatant and resuspend the cell pellet in 15 mL of lysis buffer by pipetting.
6. Transfer the cell suspension to a new 50 mL round bottom centrifuge tube.
7. Tightly seal the tube and immediately freeze the suspension by placing the tube in a slurry of dry ice and 100% ethanol (*see Note 6*) for a period of approximately 30 min (*see Note 7*). In order to lyse the cells it is necessary to carry out four freeze-thaw cycles. This process is where the cell suspension is rapidly heated and frozen repetitively in order to fracture the cell membranes.
8. Following freezing, place the tube in a  $37^\circ\text{C}$  water bath until all the contents are thawed. This step should take 15–25 min and should be monitored closely (*see Note 8*).
9. Immediately following thawing, the cell suspension should be vortex mixed at maximum speed (2500 rpm) for 30 s.
10. Replaced in the dry ice–ethanol slurry for 30 min to refreeze. Repeat this step until the cells have been frozen and thawed four times.

**Table 2**  
**Preparation of iodixanol gradient layers**

Master mix (mL)						
	Iodixanol	5 M NaCl	5× PBS-MK	H <sub>2</sub> O	Phenol red	Total volume
15%	40	32	32	56	0	160
25%	50	0	24	46	0.4	120.4
40%	67	0	20	13	0	100
60%	100	0	0	0	0.4	100.4

11. When the cell lysate has been thawed for the fourth time, add 3  $\mu$ L of a 250 U/ $\mu$ L stock of Benzonase Nuclease to the lysate to generate a working concentration of 50 U/mL.
12. Incubate for 1 h at 37 °C to degrade cellular RNA and DNA.
13. Pellet the cellular debris by centrifugation at  $\sim 24,000 \times g$  for 20 min at room temperature.
14. Whilst the cell lysate is centrifuging, prepare the master mix for each iodixanol layer in four autoclaved 250 mL glass bottles, as described in Table 2 (*see Note 9*).
15. Set up the syringe-microcapillary “funnel” by placing a quick-seal ultra-clear centrifuge tube into an appropriate test tube rack and insert a 100- $\mu$ m microcapillary glass pipette into the top until the microcapillary reaches the bottom of the tube. Remove the plunger from a new 10 mL syringe, attach an 18-G needle and insert it into the lumen of the microcapillary tube, as shown in Fig. 1. This forms the syringe-microcapillary “funnel” through which the layers of the density gradient are poured. The density gradient used for purification of the rAAV is poured from the least dense (15%) to the most dense (60%) layer using the syringe-microcapillary “funnel,” which allows layers to flow into the tube *underneath* the previous layer (*see Fig. 1*).
16. To pour the gradient, first pipette 7.2 mL of the 15% layer into syringe-microcapillary “funnel” and allow it to run through by gravity flow into the bottom of the centrifuge tube.
17. Once the 15% layer has completely run through the syringe-microcapillary “funnel,” the process is repeated by flowing 4.8 mL of the 25% layer, 8 mL of 40% layer, and lastly 4 mL of the 60% layer through the “funnel.” The result is discontinuous density gradient consisting of four layers.
18. Remove the syringe and the glass microcapillary tube, taking care not to disturb the gradient (*see Note 10*).



**Fig. 1** Preparation of iodixanol gradients. A 10 mL syringe with an 18 G needle is placed in a 100  $\mu$ m microcapillary glass pipette and placed in an ultra-centrifuge tube. Create the iodixanol gradient by sequentially adding each layer from the lowest percent of iodixanol (15%) to highest (60%). Remove the glass pipette and slowly layer the lysate onto the gradient with a fresh 10 mL syringe and 18 G needle

19. Using a new sterile 18-gauge needle and 10 mL syringe (with plunger), aspirate the supernatant of the lysate from **step 3** and slowly layer the fluid on top of the iodixanol gradient in a dropwise manner. Fill any remaining volume within the centrifuge tube with lysis buffer (*see Note 11*).
20. Seal the tube with the tube sealer (*see Note 12*).
21. Carefully insert the sealed tube in a T70 Ti fixed rotor, ensuring that the tube cap is in place.
22. Centrifuge at a maximum of  $\sim 360,000 \times g$  (we recommend 59,000 rpm when using the T70 Ti rotor) for 1 h and 30 min using a certified "preparatory" ultracentrifuge. Due to the high speed of centrifugation, it is essential that the rotor be properly balanced.
23. Following centrifugation, carefully remove the tube making sure not to disturb the gradient layers and place the tube in a sterilized biological safety cabinet.



24. Secure the centrifuge tube directly above a 500 mL plastic or glass beaker using a retort stand and clamp.
25. Pierce the centrifuge tube at the top (near the neck) with an 18-G needle.
26. Insert a 5 mL sterile syringe attached to an 18-G needle horizontally through the sidewall of the centrifuge tube approximately 2 mm below the 40–60% gradient layer interface.
27. Aspirate ~4–5 mL of the 40% gradient layer being careful not to disturb the interface of the 25% layer (*see Note 13*).
28. Inject the aspirated liquid directly into an autoclaved 250 mL glass bottle and dilute with 100 mL sterile room temperature HBSS/Tween-20 (*see Note 14*).
29. Pour 15 mL of diluted virus in an Amicon Ultra-15 centrifuge tube with a molecular weight cutoff of 100 kDa and centrifuge at  $\sim 4000 \times g$  for 15 min using a centrifuge with a swinging bucket rotor.
30. Discard flow through and refill the Amicon Ultra-15 centrifuge tube with vector and repeat the centrifugation until all the vector solution has been added. Following the final centrifugation step, approximately 200–300  $\mu\text{L}$  of vector suspension should remain in the top of the concentrator.
31. Wash the concentrated virus twice with 10 mL of room temperature HBSS/Tween 20.
32. After the second wash, spin the virus for an additional 40 min in order to achieve the highest possible concentration. There should be 120–175  $\mu\text{L}$  of fluid containing the purified rAAV vector.
33. Using a 200  $\mu\text{L}$  pipette, aspirate the concentrated virus and use it to rinse the sides of the concentrator's filter 20 times. Aspirate the final product and place in a 2.0 mL centrifuge tube and store virus at 4 °C until virus is titered.

### 3.2.2 Micro-Scale Preps

1. Detach the cells from the 6-well using a cell scraper and transfer the cell suspension to a 1.5 mL microcentrifuge tube using a sterile pipette.
2. Centrifuge the tube at  $\sim 200 \times g$  for 15 min to pellet the cells.
3. Discard the supernatant and resuspend the cell pellet in 500  $\mu\text{L}$  of lysis buffer.
4. Lyse the cells by carrying out four rounds of freeze–thaw as described above.
5. Add 1  $\mu\text{L}$  of Benzonase Nuclease to the tube (500 U/mL final concentration) and incubate the samples at 37 °C for 1 h.
6. Pellet cell debris by centrifuging at  $\sim 18,000 \times g$  for 20 min.

7. Place 500  $\mu\text{L}$  of 40% iodixanol (*see* Table 2) in a 1.5 mL microcentrifuge tube. In a dropwise manner, carefully pipette the supernatant on top of the iodixanol layer.
8. Centrifuge for 90 min at  $\sim 18,000 \times g$ .
9. Carefully pipette 80% of the 40% iodixanol layer into an Amicon Ultra-0.5 centrifugal filter unit, ensuring not to disturb the supernatant-iodixanol interface.
10. Centrifuge the tube for 15 min at  $\sim 18,000 \times g$ .
11. Discard the flow-through and add 400  $\mu\text{L}$  of HBSS/Tween-20 to the centrifuge filter unit.
12. Centrifuge again at  $\sim 18,000 \times g$  for 15 min.
13. Repeat this wash/buffer exchange step eight times. Following the final concentration step, the final volume should be 15–20  $\mu\text{L}$ .
14. To elute the virus, invert the centrifugal filter unit into a sterile 2 mL microcentrifuge tube and centrifuge at  $\sim 2300 \times g$  for 5 min. Store virus at 4 °C until titered.

### 3.3 Viral Titering (Small and Micro- Scale Preps)

Titering protocol adapted from Piedra et al. [9]

1. In a 100  $\mu\text{L}$  PCR tube, add 10  $\mu\text{L}$  of Picogreen lysis buffer, 8  $\mu\text{L}$  of autoclaved ddH<sub>2</sub>O and 2  $\mu\text{L}$  of vector purified using either the small- or micro-scale preparation methods outlined above.
2. Using a PCR machine/thermal cycler, heat the sample to 70 °C for 1 h and then slow cool the mixture down at a rate of 5 °C/min until it reaches 25 °C.
3. In a separate PCR tube, add 10  $\mu\text{L}$  of 1 $\times$  TE buffer (20 $\times$  TE buffer stock is provided with picogreen kit), 8  $\mu\text{L}$  of autoclaved ddH<sub>2</sub>O and 2  $\mu\text{L}$  of purified virus and incubate at room temperature for 1 h; this sample will act as an unlysed control.
4. In parallel, produce a six-point standard curve using Bacteriophage  $\lambda$  DNA (provided with picogreen kit). This is accomplished by diluting 6  $\mu\text{L}$  of 100 ng/ $\mu\text{L}$   $\lambda$  DNA in 94  $\mu\text{L}$  of 1 $\times$  TE buffer to produce a 6 ng/ $\mu\text{L}$  stock followed by one-third serial dilutions. This produces samples for a standard curve ranging from 120 to 0.49 ng per 20  $\mu\text{L}$  of sample.
5. Load a black 96-well plate with 20  $\mu\text{L}$  of each standard in duplicate, as well as the lysed sample, unlysed control and a blank control (20  $\mu\text{L}$  of 1 $\times$  TE buffer).
6. Dilute Quant-iT Picogreen reagent 1:200 in 1 $\times$  TE buffer and add 180  $\mu\text{L}$  of the diluted reagent to each well.
7. Incubate the samples for 5 min in the dark and quantify the fluorescence using a plate reader with an excitation of 485 nm and an emission of 535 nm.

8. Subtract the blank control from all samples and generate a line of best fit from the six-point standard using fluorescence values as the  $Y$ -axis coordinates and the amount of DNA as the  $X$ -axis coordinates. Using the equation of the line of best fit, calculate the rAAV genome concentration. Using the equation below calculate the rAAV titer (*see* **Note 15**).

$$\text{rAAV Titer} \left( \frac{\text{vg}}{\text{mL}} \right) = \frac{\text{rAAV genome concentration} \left( \frac{\text{ng}}{\text{mL}} \right) \times 1.82\text{E} + 12 \left( \frac{\text{bp}}{\text{ng}} \right)}{\text{Length of rAAV genome} \left( \frac{\text{bp}}{\text{vg}} \right)}.$$

9. Aliquot 15  $\mu\text{L}$  of virus each into sterile 2.0 mL centrifuge tubes and store at  $-80^\circ\text{C}$ .

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## 4 Notes

1. To prepare 1  $\mu\text{g}/\mu\text{L}$  solution of PEI, dissolve 100 mg of linear PEI in 90 mL of  $\text{ddH}_2\text{O}$  in a 250 mL beaker with a magnetic stir bar by constant stirring and heating to  $\sim 50^\circ\text{C}$  (This process can take 2–4 h). Bring solution to pH 7 by adding 10 M HCl drop-by-drop. Adjust final volume to 100 mL with  $\text{ddH}_2\text{O}$  and in a sterile biological safety cabinet, filter sterilize through a 0.22  $\mu\text{m}$  membrane. Aliquot 1.0 mL of sterile PEI into 1.5 mL centrifuge tubes and store at  $-20^\circ\text{C}$ . Once aliquots are thawed, do not refreeze; use a fresh aliquot each time.
2. Only the bottom 1–2 layers of a Hyperflask can be visualized using an inverted microscope, even when using low power (e.g., 4 $\times$  or 10 $\times$ ) objectives. As cells were introduced into the Hyperflask in suspension, and cells cannot move between layers, the confluency of the bottom layer can be considered representative of all ten layers.
3. The optimal ratio of plasmid DNA to PEI must be optimized for each batch of PEI manufactured. To this end, in a 12-well plate transfect 1  $\mu\text{g}$  of a ubiquitously expressing fluorescent reporter plasmid (i.e., CBA-eGFP) with varying concentrations of PEI (0.5, 1, 2, and 3  $\mu\text{g}$ ). The concentration of PEI that produces the most fluorescent positive cells without causing toxicity is the ratio of DNA: PEI that will be used for transfections.
4. PEI is a cationic polymer that binds DNA to form positively charged complexes capable of entering cells via endocytosis. Excessive agitation of the PEI through vigorous rocking/swirling while adding the plasmid DNA mixture can lead to the formation of toxic precipitates, which can reduce transfection efficiency and lead to cell death.

5. “Vigorous” in this context entails roughly shaking the Hyperflask up and down using both hands, in the manner of a bartender mixing a cocktail. We recommend shaking for at least 1 min in order to dislodge all of the cells. During the shaking process, pause intermittently (approximately every 15 s) to strike the flask firmly on the end with an open palm in order to maximize cell displacement.
6. To make the dry ice/ethanol slurry for rapid freezing, place an 50 mL test tube rack into a white polystyrene-shipping box, fill the box with dry ice to a depth of 2–3 in., and then pour in 100% ethanol until the dry ice is completely submerged. The ethanol will initially produce substantial amounts of white vapor (carbon dioxide) when added to the dry ice—this is normal and will subside.
7. Once frozen, the cell suspension/lysate can be stored in the  $-80^{\circ}\text{C}$  freezer indefinitely.
8. The cell suspension should be incubated at  $37^{\circ}\text{C}$  only for as long as it takes for the contents to thaw completely. This is to prevent breakdown of the rAAV vector by proteases released during cell lysis.
9. Gradient layers can be stored indefinitely at room temperature. When adding phenol red, the 60% layer should turn a yellow color, while the 25% layer should appear red. The 15% and 40% layers do not have phenol red and should be transparent/clear.
10. If any of the iodixanol layers stop flowing through the syringe, *carefully* insert the plunger into the syringe in order to displace any air bubbles in the microcapillary tube. Remove the plunger and allow the layer to proceed through the glass microcapillary tube by gravity flow.
11. It is imperative that there is no air in the centrifuge tube as this may cause the tube to collapse during ultracentrifugation. Centrifuge tubes should be precisely balanced—this can be checked using a three decimal place electronic balance.
12. It is important to ensure that the centrifuge tube is completely sealed. To check, forcefully squeeze the sealed tube and confirm that no liquid is coming out of the top of the tube.
13. It is better to aspirate less volume than to remove volume from the 25% layer, as this can detrimentally affect downstream buffer exchange. Once aspirated, the vector can be stored in the syringe overnight at  $4^{\circ}\text{C}$ .
14. It is important to use a glass bottle, as AAV is prone to sticking to plastics.
15. When calculating the titer of a virus encapsulating a self-complimentary genome, the genome length is doubled.

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