

Video Article

Production, Purification, and Quality Control for Adeno-associated Virus-based Vectors

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Abstract

Gene delivery tools based on adeno-associated viruses (AAVs) are a popular choice for the delivery of transgenes to the central nervous system (CNS), including gene therapy applications. AAV vectors are nonreplicating, able to infect both dividing and nondividing cells, and provide long-term transgene expression. Importantly, some serotypes, such as the newly described PHP.B, can cross the blood-brain-barrier (BBB) in the animal models, following systemic delivery. AAV vectors can be efficiently produced in the laboratory. However, robust and reproducible protocols are required to obtain AAV vectors with sufficient purity levels and titer values high enough for in vivo applications. This protocol describes an efficient and reproducible strategy for AAV vector production, based on an iodixanol gradient purification strategy. The iodixanol purification method is suitable for obtaining batches of high-titer AAV vectors of high purity when compared to other purification methods. Furthermore, the protocol is generally faster than other methods currently described. In addition, a quantitative polymerase chain reaction (qPCR)-based strategy is described for a fast and accurate determination of the vector titer, as well as a silver staining method to determine the purity of the vector batch. Finally, representative results of the gene delivery to the CNS, following a systemic administration of AAV-PHP.B, are presented. Such results should be possible in all labs using the protocols described in this article.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58960/>

Introduction

Over the past 30 years, wild-type AAVs have been engineered to create recombinant AAV vectors, which have proven to be exceptionally useful tools for gene transfers into the CNS^{1,2,3,4,5,6} and gene therapy approaches to disease (including FDA- and EMA-approved therapies)^{4,7}. Their suitability for use in the CNS largely derives from their ability to infect nondividing, postmitotic cells, typically found in the CNS⁸. However, AAV-based vectors also have the advantage of allowing a long-term expression of any given therapeutic transgene^{4,9} while eliciting a milder immune response compared to other viral vectors^{7,8,10,11,12}.

The main elements of any AAV vector are the genome and the capsid. Wild-type AAVs are single-stranded (ss) DNA viruses with a genome of approximately 5 kilobases (kb)¹³. For the production of recombinant AAV vectors, the *rep* and *cap* genes (necessary for genome replication and the assembly of the viral capsid) are deleted from the genome of the wild-type AAV and provided in trans, leaving room for an expression cassette containing the transgene^{14,15}. The inverted terminal repeat sequences (ITRs) of the original viral genome are the only retained elements in an AAV vector, as these are essential for the replication and packaging^{3,10,14}. AAV vectors can be engineered to enhance transgene expression; a mutation in one of the ITRs leads to the formation of a hairpin loop which effectively allows the generation of a complementary DNA strand^{3,7,15}. The main advantage of this configuration, termed a self-complementary (sc) genome, is that it bypasses the need for the second-strand synthesis typical in the conventional life cycle of AAVs, considerably increasing the speed and levels of transgene expression¹. Nevertheless, using an scAAV genome reduces the cargo capacity of the vector to approximately 2.4 kb. This includes transgene sequences, as well as any regulatory sequences such as promoters or microRNA-binding sites, to limit the expression to specific cell types¹⁶.

The AAV capsid determines the vector-host cell interaction and confers a degree of cell type or tissue tropism for an AAV serotype, which can also be exploited to limit transgene expression to specific locations. Several AAV serotypes are found in nature, whereas others have been produced in laboratories through recombinant approaches (i.e., PHP.B). In addition, some capsids also bestow other useful characteristics, such as the ability to cross the BBB, resulting in the delivery of transgenes throughout the CNS after systemic administration. This has been shown

for adeno-associated virus 9 (AAV9), as well as for the recently described PHP.B capsid¹⁷. As a consequence, these serotypes are proving to be particularly relevant for the new gene therapy approaches of neurodegenerative disorders^{1,17,18}.

The aim of this protocol is to describe a cost-effective method for the small-scale production of AAV vectors with high titer and purity. Although the results presented here use the PHP.B capsid and a scAAV expression cassette, the protocol is suitable for the production of several AAV vector serotypes and genome configurations, allowing maximum experimental flexibility. However, the vector yield and final purity can vary depending on the chosen serotype.

The protocol itself is a variant of the classical tri-transfection method for the viral vector production and incorporates the use of an iodixanol gradient for vector clean-up, which, in comparison to the classical use of cesium chloride (CsCl) gradients, has been reported to produce AAV vectors of higher purity in a more time-efficient manner^{19,20,21}.

The transfection, purification, and concentration steps are intended to be performed according to a good laboratory practice in a tissue culture laboratory rated for viral vector work. Each task needs to be performed in compliance with the relevant local and national legislation concerning viral vector production and use. Work must be carried out under a laminar flow hood and in sterile conditions. Inside the vector facility, it is recommended to wear a lab apron, in addition to the regular tissue culture lab coat. Moreover, a double pair of gloves, as well as plastic overshoes, should be worn at all times.

Before starting the vector production, ensure all necessary equipment and plasmids are available. 1) pCapsid plasmid contains the *rep* gene that encodes four nonstructural proteins necessary for replication, namely Rep78, Rep68, Rep52, and Rep40, and the *cap* gene that encodes three structural capsid proteins, namely VP1, VP2, and VP3. 2) pHelper plasmid contains the genes *E4*, *E2A*, and *VA* from adenovirus, which facilitate the AAV production in HEK293T cells. 3) pTransgene plasmid contains the transgene expression cassette, flanked by two ITRs. These plasmids can be synthesized *de novo* in the laboratory using sequences available online²². For plasmids made *de novo*, especially those containing novel transgenes, sequencing is required, to make sure that the transgene and ITRs are correct. Alternatively, premade plasmids can be directly acquired through online plasmid repositories. When necessary, plasmids can be amplified and purified using standard kits, according to the manufacturer's instructions²³.

Vector titer and purity can adversely affect the transduction ability of the vector. Additional protocols are supplied to evaluate the quality of the produced vector. The final vectors will be useful for studies of the CNS cell function in both in vitro and in vivo applications.

Protocol

Solution	Composition	
Cell culture and transfection		
DMEM1	DMEM 1x	
	1% FBS (v/v)	
	1% GlutaMAX (v/v)	
DMEM10	DMEM 1x	
	10% FBS (v/v)	
	1% GlutaMAX (v/v)	
150 mM NaCl	4.380 g NaCl	
	Up to 500 mL Ultrapure water	
AAV purification and desalting		
5 M NaCl	146.1 g NaCl	
	Up to 500 mL Ultrapure water	
1 M Tris HCl (pH 8.5)	12.11 g Tris base	CAUTION
	Up to 100 mL Ultrapure water	
	Add 1 M HCl using a Pasteur pipette to reduce the pH to 8.5	
Lysis buffer	15 ml of 5 M NaCl	
	25 ml of 1 M Tris HCl (pH 8.5)	CAUTION
	Up to 500 mL Ultrapure water	
10x Phosphate-buffered saline (PBS)	80 g NaCl	
	2 g KCl	CAUTION
	14.4 g Na ₂ HPO ₄	
	2.4 g KH ₂ PO ₄	
	Up to 1 l _{dd} water	
1 M MgCl ₂	20.33 g MgCl ₂ ·6H ₂ O	
	Up to 100 ml Ultrapure water	
1 M KCl	7.45 g KCl	CAUTION
	Up to 100 mL Ultrapure water	
5x PBS Magnesium-Potassium (PBS-MK) stock solution	250 mL of 10x PBS	
	2.5 mL of 1 M MgCl ₂	
	6.25 mL of 1 M KCl	CAUTION
	Up to 500 mL Ultrapure water H ₂ O	
15% Iodixanol	12.5 mL of Optiprep density gradient medium	CAUTION
	10 mL of 5 M NaCl	
	10 mL of 5x PBS-MK	
	17.5 mL of Ultrapure water	
25% Iodixanol	20.8 mL of Optiprep density gradient medium	CAUTION
	10 mL of 5x PBS-MK	
	19.2 mL of Ultrapure water	
	100 µL of phenol red	
40% Iodixanol	33.3 mL of Optiprep density gradient medium	CAUTION
	10 mL of 5x PBS-MK	

	6.7 mL of Ultrapure water	
60% Iodixanol	50 mL of Optiprep density gradient medium	CAUTION
	100 µL of phenol red	CAUTION
AAV purity control		
10x Tris acetate EDTA (TEA) buffer	44.8 g Tris base (121.14 g/mol)	CAUTION
	11.4 mL glacial acetic acid (17.4M)	
	3.7 g EDTA	
	Up to 1 L Ultrapure water	
Agarose gel	0.8 g Ultrapure agarose	
	Up to 100 mL 1x TEA buffer	
Gel buffer	181.7 g Tris base (121.14 g/mol)	CAUTION
	1.5 g SDS	CAUTION
	Adjust pH to 8.45 with 1 M HCl	CAUTION
	Up to 500 mL Ultrapure water	
Cathode buffer 10x	121.14 g Tris base	CAUTION
	179.2 g Tricine	CAUTION
	1% SDS (w/w)	CAUTION
	Up to 1 l Ultrapure water	
Anode buffer 10x	242.3 g Tris base	CAUTION
	Up to 1 l Ultrapure water	
	Adjust pH to 8.9 with 1 M HCl	CAUTION
Sample buffer 5x	For 20 mL:	
	605 mg Tris base	CAUTION
	4 g SDS	CAUTION
	10 mg Serva Blue G	
	12 g Glycerol	
	Adjust pH to 6.8 with 1 M HCl, aliquot and store at -20 °C	CAUTION
Stacking gel	For 2 gels:	
	400 µL acrylamide	CAUTION
	750 µL gel buffer	
	1.85 mL Ultrapure water	
	4 µL TEMED	CAUTION
	20 µL 10% APS (v/v)	CAUTION
	Add TEMED and 10% APS immediately before pouring the gel. Use both chemicals under a chemical hood.	
Resolving gel	For 2 gels:	
	3.32 mL acrylamide	CAUTION
	3.35 mL gel buffer	
	1.14 mL Ultrapure water	
	2.12 mL 50% glycerol	
	6 µL TEMED	CAUTION
	50 µL 10% APS (w/v)	CAUTION
	Add TEMED and 10% APS immediately before pouring the gel. Use both chemicals under a chemical hood.	

Water-saturated butanol	10 mL n-butanol	CAUTION
	1 mL Ultrapure water	
CAUTION: Refer to the Materials Table for guidelines on the use of dangerous chemicals.		

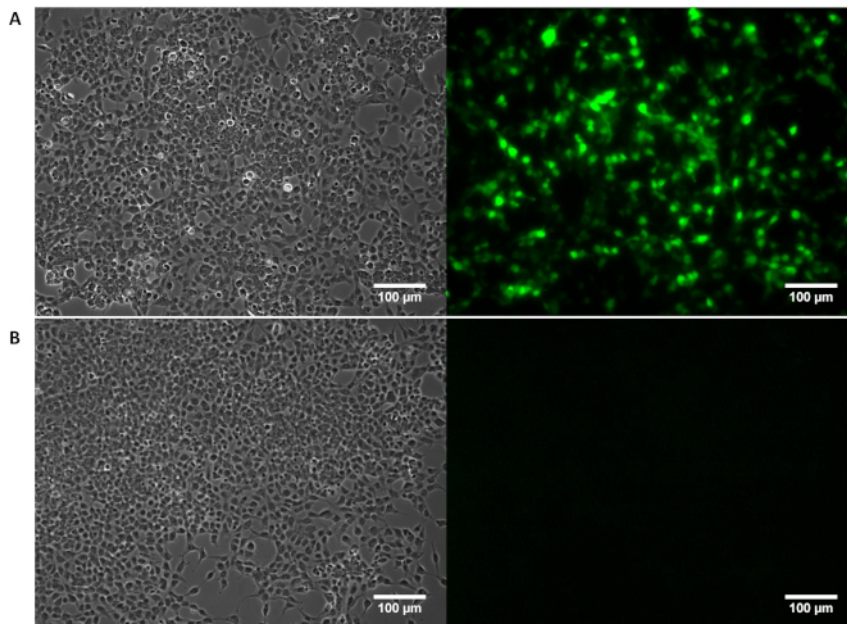
Table 1: Composition of the required solutions.

1. Tri-transfection of HEK293T Cells

NOTE: Please refer to **Table 1** for the composition of the buffers and solutions used in the protocol.

NOTE: The performance of this section of the protocol takes approximately 4 days.

- Thaw a vial of human embryonic kidney (HEK) 293T cells in a water bath set at 37 °C.
NOTE: Use only cells that have been passaged less than 20x to guarantee optimal transfection efficiency.
- Seed HEK293T cells at a density of 2×10^3 to 6×10^3 cells/cm² in DMEM10 in a 15 cm diameter cell culture dishes.
- Grow the cells to 70–80% confluence in a standard incubator set at 37 °C, with 95% humidity and 5% CO₂.
NOTE: The production of one batch of AAV vectors using this protocol requires 18 cell culture dishes of 15 cm in diameter. A cell confluence of 70% - 80% corresponds to 6×10^3 to 7×10^3 cells/cm², maintained in 17–20 mL of DMEM10 culture medium.
- Prepare polyethylenimine (PEI)/DNA mix at a concentration ratio of 1/3.5 (w/w).**
 - Prepare the DNA mix for 18 cell culture dishes in one 50 mL conical tube by mixing 360 µg of pΔF6, 180 µg of pCapsid, and 180 µg of pTransgene in 18 mL of 150 mM NaCl.
 - Distribute the DNA mix over three 50 mL conical tubes (6 mL of DNA mix per conical tube).
 - Prepare the PEI mix for six cell culture dishes in a new 50 mL conical tube by mixing 840 µg of PEI (1 µg/µL) in 6 mL of 150 mM NaCl.
 - Prepare the PEI/DNA mix by adding 6 mL of the PEI mix (prepared in step 1.4.3), drop by drop, to one of the conical tubes containing the DNA mix (prepared in step 1.4.2) and incubate for 20 min at room temperature.
NOTE: After 20 min of incubation, the PEI/DNA mix will become slightly turbid.
- Take six cell culture dishes out of the incubator and completely aspirate the medium from each culture dish in a laminar flow hood. Remove the traces of the medium by rinsing the dishes with 5 mL of prewarmed Dulbecco's phosphate-buffered saline (DPBS).
- Ensure the distribution of DPBS over the entire surface by gently tilting the dish.
- Gently aspirate the DPBS and add 12 mL of DMEM1 to each dish.
NOTE: Avoid detaching the cells by adding the medium slowly, from a pipette placed at the edge of the dish.
- Mix the PEI/DNA by pipetting up and down 3x - 5x. Add 2 mL of the PEI/DNA mix to each of the six cell culture dishes in a drop-by-drop fashion, carefully distributing it over the entire surface. Once the mix is added to each dish, place the dishes back in the incubator. Repeat steps 1.4.3– 1.8 for the remaining culture dishes.
- Incubate the transfected cells for 5 h at 37 °C, with 95% humidity and 5% CO₂.
- Add an additional 12 mL of DMEM10 to each culture dish without removing the pre-existing medium (total medium volume = 25 mL).
- Incubate the transfected cells up to 72 h posttransfection at 37 °C, with 95% humidity and 5% CO₂.



Supplementary Figure 1: HEK 293T cells morphology visualized by phase contrast microscopy (left) and confirmation of GFP expression visualized by fluorescence imaging (right 20x). (A) Successful transfection of HEK293T cells with a GFP-encoding pTransgene is confirmed by fluorescence imaging. (B) HEK293T cells treated solely with transfection reagents show no GFP expression. [Please click here to view a larger version of this figure.](#)

12. Harvest the medium and the cells 72 h posttransfection. Use a cell scraper to carefully detach the cells from the culture dish. Collect the medium and the cells in a 50 mL conical tube kept on ice.
NOTE: The contents of two cell culture dishes can be collected into a single 50 mL conical tube. At the end of this step, each of the nine tubes will contain approximately 50 mL of medium.
13. Centrifuge the conical tubes at $420 \times g$ for 10 min at 4°C with the acceleration and deceleration of the centrifuge set to maximum.
14. Carefully discard the supernatant from each tube. Do not use pipettes to prevent the loss of the cell pellet. Instead, gently pour the supernatant into a waste disposal container and place the tubes containing the cell pellets back on the ice.
15. Resuspend each cell pellet in 2 mL of the lysis buffer directly in the 50 mL conical tube by pipetting up and down 5–10x. Do not vortex. Pool the lysates from three tubes together.
NOTE: At this point, there will be three 50 mL conical tubes, each one containing 6 mL of the resuspended cells in lysis buffer.

2. AAV Vector Purification

NOTE: The performance of this section of the protocol takes approximately 1 day. Perform the following steps simultaneously on each of the three 50 mL conical tubes containing the cells resuspended in lysis buffer (see the previous note).

1. Freeze and thaw the resuspended cells 3x to lyse them and release the AAV particles. Perform the freezing step by placing the tubes in a bucket containing dry ice mixed with ethanol. Perform thawing by immediately placing the cells in a water bath set at 37°C .
2. After the third thawing step, centrifuge at $1,167 \times g$ for 15 min at 4°C .
NOTE: A noticeable pellet composed of cell debris will be formed. When handling the tubes, avoid sudden movements as these can cause the detachment of the pellet into the supernatant, which will compromise the purity of the final vector.
3. Carefully transfer the supernatants to clean 50 mL conical tubes and, then, add nuclease to each tube, to a final concentration of 50 U/mL supernatant.
4. Incubate for 30 min at 37°C . Swirl the 50 mL conical tubes by hand every 10 min to ensure that the nuclease is thoroughly mixed with the supernatant.
5. Clarify the supernatant by centrifugation at $13,490 \times g$ for 20 min at 4°C .
6. Attach a $0.45 \mu\text{m}$ filter to a 10 mL syringe and place it on top of a clean 50 mL conical tube. Carefully remove the plunger and fill the syringe with the supernatant from step 2.5.
7. Use the plunger to force the lysate through the filter. Use a new filter and syringe for each of the tubes of the supernatant obtained in step 2.5.
NOTE: The obtained fraction is known as 'crude lysate'.
8. Prepare each of the 15%, 25%, 40%, and 60% iodixanol fractions in four separate 50 mL conical tubes, according to the instructions in **Table 1**.
9. **Prepare the iodixanol gradients in three ultracentrifugation tubes using the following order of iodixanol fractions: 8 mL of 15% iodixanol, 5.5 mL of 25% iodixanol, 5 mL of 40% iodixanol, and 4.5 mL of 60% iodixanol.**
CAUTION: Iodixanol can cause irritation to eyes, skin, and the gastrointestinal and respiratory tracts. When handling iodixanol gradients, wear gloves and work under a laminar flow hood.
 1. Pipette 8 mL of 15% iodixanol into each ultracentrifugation tube.
 2. Pipette 5.5 mL of 25% iodixanol solution into a clean 50 mL conical tube (**Figure 1A**).

3. Use a nongraduated Pasteur pipette (as the neck of the ultracentrifugation tube is too narrow for conventional graduated pipettes) to carefully layer 5.5 mL of the 25% iodixanol solution below the 15% iodixanol solution (**Figure 1B**).
NOTE: This can be successfully achieved by adding the iodixanol in three steps since the Pasteur pipette can only hold around 2 mL.
4. Add the 40% and 60% iodixanol solutions as described in step 2.9.3. Do not disturb the different iodixanol interfaces during layering.
NOTE: The proper preparation of the different iodixanol fractions can be ensured by visual confirmation thanks to the phenol red added to the iodixanol fractions (see the instructions in **Table 1**). Since each fraction has a specific density, they will not intermix during the layering step, if it is performed properly (check **Figure 1C**).
10. Layer the crude lysate on top of the 15% iodixanol gradient with a Pasteur pipette. Proceed drop by drop to avoid disturbing the interface between the crude lysate and the iodixanol solution.
11. Fill the ultracentrifugation tube up with lysis buffer until the meniscus reaches the base of the tube neck to ensure the tube does not collapse under the very high forces generated during the ultracentrifugation (**Figure 1C**).
12. Close the ultracentrifugation tubes using appropriate lids. Use a digital scale to make sure all three ultracentrifugation tubes have the same weight. Adjust the weight, if necessary, by adding more lysis buffer on top of the 'crude lysate'.
NOTE: The weight difference between the ultracentrifugation tubes must be below 0.1 g to ensure the safe operation of the ultracentrifuge. Ultracentrifuges are potentially dangerous equipment and should only be used by properly trained personnel.
13. Centrifuge the tubes at $301,580 \times g$, using a fixed-angle titanium rotor, for 1 h and 40 min at 12 °C, using maximum speed of acceleration and deceleration.
14. **Carefully insert a stainless-steel blunt needle at the 40% and 60% iodixanol interface.**
 1. Attach the needle to a 5 mL syringe (**Figure 1E**).
 2. Aspirate only the clear fraction, containing the vector particles.
NOTE: The total volume collected is approximately 2.5–3 mL. Avoid the collection of material from the 25–40% interface, since this will increase the level of contamination in the final vector batch due to the presence of nondesirable proteins.
 3. Process the collected fraction (desalting and concentration) or store it overnight at 4 °C.

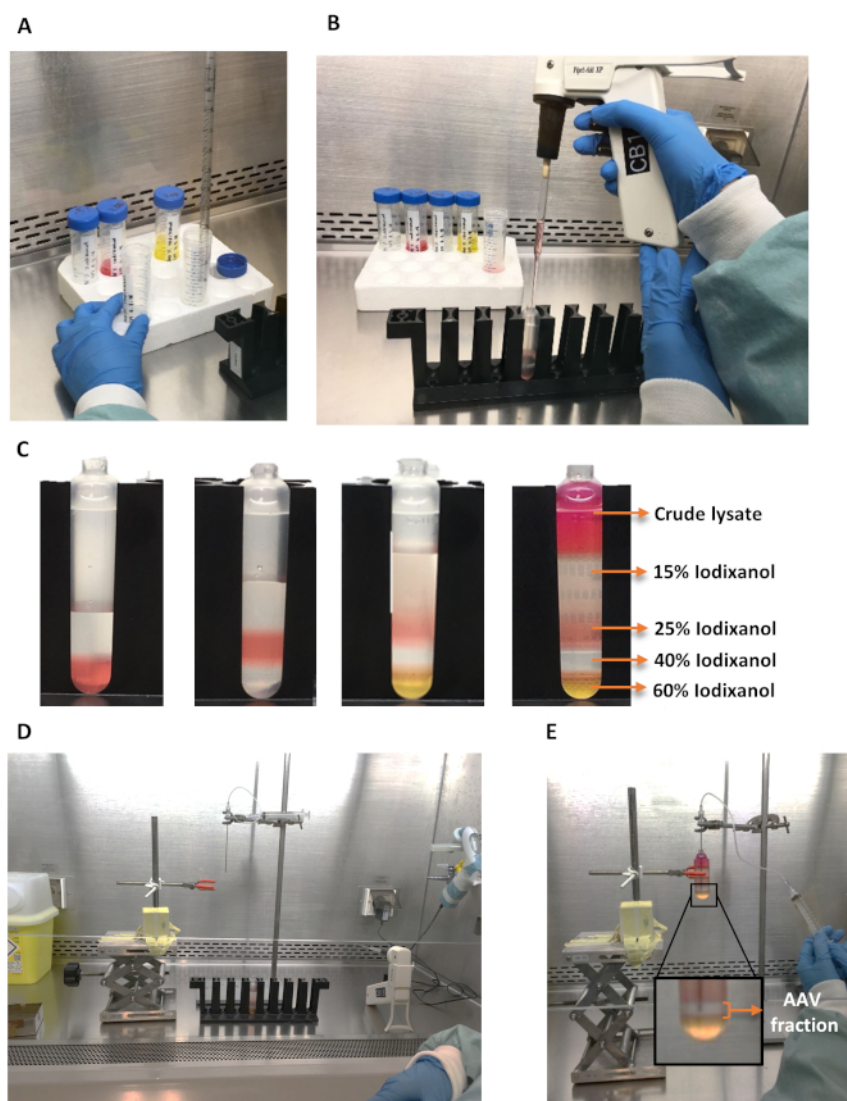


Figure 1: Setup for AAV vector collection following iodixanol gradient purification. (A) Before pipetting the different iodixanol gradients into the ultracentrifugation tube, pipette an adequate volume of each iodixanol solution into a separate conical tube. (B) Then, use a Pasteur pipette to sequentially transfer each iodixanol solution to the ultracentrifugation tube: layers of an increasingly high iodixanol concentration should be added at the bottom of the tube underneath the previous layer(s). (C) Layer the crude vector lysate on top once the gradient has been prepared. This vector collection system does not use sharp needles, which present a risk of 'needlestick' injuries. (D) A stainless-steel 316-syringe needle is inserted through the iodixanol gradient up to the 40%/60% interface. (E) Vector particles are found in the 40% iodixanol phase and are collected. [Please click here to view a larger version of this figure.](#)

3. Desalting and Concentration of the AAV Vector

NOTE: The performance of this section of the protocol takes approximately 2 h.

1. Prerinse the filter membrane of the centrifugal filter by adding 5 mL of 1x PBS-MK and centrifuge for 5 min at 4,000 x g.
2. Add 5 mL of 1x PBS-MK to the collected AAV vector fraction, mix everything, and then add the total volume to the filter. Upon addition of 1x PBS-MK, turbidity is observed. Centrifuge at 4,000 x g until the volume present on the cone-shaped filter has been reduced to 1 mL. Discard the flow-through accumulated in the outer collection tube.
3. Add 13 mL of 1x PBS-MK to the cone-shaped filter and repeat the centrifugation step as many times as necessary (minimum 3x), until there is no more turbidity observed when fresh 1x PBS-MK is added.
4. In the final wash step, add 13 mL of PBS + 0.01% (v/v) non-ionic surfactant and centrifuge at 4,000 x g until the volume is reduced to 300–350 μ L.
5. Collect the remaining fraction present on the filter by pipetting the whole volume into a sterile skirted microcentrifuge tube.
NOTE: This fraction contains the desalted and concentrated AAV vector. In the following steps of this protocol, this fraction is referred to as the 'primary' fraction. Make sure to handle the tube under the hood and store it at 4 °C.
6. Rinse the filter with 200 μ L of 1x PBS-MK to collect the residual vector particles that are attached to the walls of the cone-shaped filter and collect it in a sterile microcentrifuge tube.

NOTE: This is a diluted vector stock, which may be suitable for some applications requiring lower vector titers. In future steps, this fraction is referred to as the 'secondary' fraction.

7. For short-term storage, store the vector fraction(s) at 4 °C (less than 2 weeks). Aliquot and store the vector at #20 °C when long-term storage is required.
8. Perform quality control as described in section 4.

4. Titration of the Vector by Quantitative Polymerase Chain Reaction

NOTE: The performance of this section of the protocol takes approximately 3 h.

1. Standard curve

1. Linearize the transgene-expressing plasmid (pTransgene) used for the transfection of HEK-293T cells in section 1.
2. Prepare the restriction digest mix in a 0.5 mL microcentrifuge tube (refer to **Table 2** for the composition of the restriction digest mix).
NOTE: Adjust the composition of the restriction digest mix according to the enzyme used and the related guidelines from the manufacturer.
3. Incubate the restriction digest mix for 1 h at 37 °C.
4. Check the efficiency of the restriction digestion by running the linearized plasmid on a 0.8% (w/v) agarose gel at 100 V for 1 h. A unique fragment of expected size shows a complete digestion of the plasmid.
5. Purify the linear plasmid DNA using a PCR purification kit, following the manufacturer's instructions²⁴, and measure the DNA concentration with a spectrophotometer by measuring the absorption at 260 nm. After the titration, store the remaining aliquot of the linear plasmid at #20 °C for further use.
6. Calculate the molecules (i.e., DNA copies) of the plasmid stock per microliter as follows.
 1. First, calculate the molecular weight of the plasmid. Assuming that the average weight of a DNA base pair (bp) is 650 Daltons (Da) and that one mole of a bp weighs 650 g, the molecular weight of any double-stranded DNA template can be estimated by taking the product of its length (in bp) and weight per base pair.
Plasmid molecular weight [g/mol] = plasmid size [bp] x 650 [Da/bp]
 2. Next, calculate the number of moles of plasmid per microliter.
Moles of plasmid per microliter = plasmid concentration [g/μL] / plasmid molecular weight [g/mol]
NOTE: The inverse of the molecular weight is the number of moles of plasmid present in 1 g of the material.
 3. Then, calculate the number of plasmid molecules per microliter, using Avogadro's number (6.022×10^{23} molecules/mole).
Molecules of plasmid per microliter = moles of plasmid per microliter [moles/μL] x Avogadro's number [molecules/mole]
 4. Finally, dilute the plasmid stock (molecules/μL) to obtain a 100 μL solution with the desired concentration of 1×10^9 molecules (vector genomes (vg) per microliter).
Plasmid stock (100 μL) = (desired concentration [molecules/μL] x 100 μL) / plasmid molecules [molecules/μL]
7. Make serial dilutions of the plasmid stock (1×10^9 vg/μL) in triplicates:
 - 10 μL of 1×10^9 vg/μL plasmid stock + 90 μL of H₂O = 1×10^8 vg/μL solution
 - 10 μL of 1×10^8 vg/μL dilution + 90 μL of H₂O = 1×10^7 vg/μL solution
 - 10 μL of 1×10^7 vg/μL dilution + 90 μL of H₂O = 1×10^6 vg/μL solution
 - 10 μL of 1×10^6 vg/μL dilution + 90 μL of H₂O = 1×10^5 vg/μL solution
 Continue to obtain a 1×10^1 vg/μL solution.
8. Keep the serial dilutions of the standard plasmid stock on ice until loading it on the qPCR plate (section 4.3).

Component	Amount
10x Restriction enzyme buffer	5 μL
Restriction enzyme	2,5 μL
plasmid	5 μg
H ₂ O	up to 50 μL

Table 2: Restriction digest mix composition.

Table 3: Stock plasmid volume calculator. [Please click here to download this table.](#)

2. DNA extraction from the AAV vector

1. Mix 2 μL of the AAV vector stock (the primary fraction from step 3.5) with 198 μL of DNase I buffer (1x) in strip tubes (PCR tubes) and add 2 μL of DNase I.
NOTE: DNase I will degrade any genetic material that is not contained inside a viral capsid (which would distort the qPCR results). This solution is referred to as dilution '*dil1* x 10^{-2} '.
2. Incubate for 30 min at 37 °C, followed by 10 min at 95 °C.
NOTE: The protocol can be stopped at this point and the material can be stored indefinitely at 4 °C, to avoid product deterioration.
3. Add 2 μL of proteinase K to the '*dil1* x 10^{-2} ' solution (step 4.2.1) and incubate for 60 min at 50 °C, followed by 20 min at 95 °C.
NOTE: This step will disassemble the AAV vector capsid and release the AAV vector genome into the solution. Add proteinase K in excess as the protein (capsid) content of the sample is not known. Note, it is essential to ensure that all proteinase K activity is removed by denaturation, prior to qPCR, to avoid issues with (partial) polymerase degradation influencing the final product.
4. Prepare 1:10 serial dilutions of the proteinase K treated '*dil1* x 10^{-2} ' solution (step 4.2.3) in 1.5 mL microcentrifuge tubes as follows:
 - 10 μL of '*dil1* x 10^{-2} ' + 90 μL of H₂O = '*dil1* x 10^{-3} ' dilution
 - 10 μL of '*dil1* x 10^{-3} ' + 90 μL of H₂O = '*dil1* x 10^{-4} ' dilution

10 μL of $\text{dil}1 \times 10^{-4} + 90 \mu\text{L}$ of $\text{H}_2\text{O} = \text{dil}1 \times 10^{-5}$ dilution

5. Keep the serial dilutions of DNA extracted from the vector on ice until loading them on the qPCR plate (section 4.3).

3. Titration by green-fluorescent detection-based qPCR

1. Prepare the qPCR master mix in a 1.5 mL microcentrifuge tube, for both the sample and the standards. Use 10 μL of SYBR Green Master Mix, 1 μL of forward primer (10 μM stock), 1 μL of reverse primer (10 μM stock), and 3 μL of H_2O per reaction. See the protocol in **Table 4** for primer sequences.
2. Pipette the qPCR master mix up and down but do not vortex.
3. Add 15 μL of the qPCR master mix, followed by either 5 μL of the standard curve prepared in section 4.1 or the DNA extracted from the AAV vector in section 4.2 into each well. Include three wells containing only the qPCR master mix, as a negative control. Refer to **Figure 2** to follow the plate layout.
4. Seal the plate with a sealing film and briefly centrifuge the qPCR plate at 1,500 x g for 30 s at 4 $^{\circ}\text{C}$.
5. Run the qPCR reaction on a plate-based real-time PCR amplification and detection instrument, using the conditions suggested in **Table 5**.

Primer name	Sequence
Forward primer	5'-CCCACTTGGCAGTACATCAA-3'
Reverse primer	5'-GCCAAGTAGGAAAGTCCCAT-3'

Table 4: Primer sequences designed against the CBA promoter.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5x10 ⁷	5x10 ⁷	5x10 ⁷	dil 1x10 ⁻³	dil 1x10 ⁻³	dil 1x10 ⁻³						
B	5x10 ⁶	5x10 ⁶	5x10 ⁶	dil 1x10 ⁻⁴	dil 1x10 ⁻⁴	dil 1x10 ⁻⁴						
C	5x10 ⁵	5x10 ⁵	5x10 ⁵	dil 1x10 ⁻⁵	dil 1x10 ⁻⁵	dil 1x10 ⁻⁵						
D	5x10 ⁴	5x10 ⁴	5x10 ⁴	dil 1x10 ⁻³	dil 1x10 ⁻³	dil 1x10 ⁻³						
E	5x10 ³	5x10 ³	5x10 ³	dil 1x10 ⁻⁴	dil 1x10 ⁻⁴	dil 1x10 ⁻⁴						
F	5x10 ²	5x10 ²	5x10 ²	dil 1x10 ⁻⁵	dil 1x10 ⁻⁵	dil 1x10 ⁻⁵						
G	5x10 ¹	5x10 ¹	5x10 ¹									
H	H ₂ O	H ₂ O	H ₂ O									

■ Standard curve ■ Primary fraction
■ Negative control ■ Secondary fraction

Figure 2: Plate layout for qPCR-based vector titration. The samples are color-coded: green = standard curve; blue = H₂O control; grey = primary fraction; orange = secondary fraction. [Please click here to view a larger version of this figure.](#)

Step	Time	Temperature	Cycles	Aim
Pre-incubation	5 min	95 °C	x1	DNA denaturation and polymerase activation (hot-start reaction).
Amplification	10 min	95 °C	x1	Amplification of the DNA. Settings may be optimized if alternative primers with different annealing temperature are used.
	10 s	95 °C	x40	
	40 s	60 °C		
	1 s	72 °C		
Cooling	10 s	40 °C	x1	Plate cooling. End of the PCR.

Table 5: Thermal cycling protocol for SYBR green-based qPCR titration.

4. Data analysis to determine the AAV vector titer
 1. Fill the spreadsheet data cells (Table 6A) with the Ct values obtained for the different dilutions of the standard sample prepared in section 4.1 to generate a standard curve.
NOTE: The equation of the standard curve will be shown ($y = a \ln(x) + b$) together with the R^2 efficiency (Table 6B). A qPCR R^2 close to 1.0

2. Use the calculated values of *a* and *b* to fill in the corresponding data cells in the spreadsheet (Table 6C).
3. Complete the spreadsheet with the Ct values obtained for the different dilutions of the AAV sample prepared in section 4.2.
4. Calculate the average AAV vector titer in vector genomes per microliter of the primary fraction by using the following

$$\text{formula: } Titer = \frac{Ct - b}{a} \times 400 \times \frac{1}{dil} \times \frac{1}{2}$$

NOTE: The factor 400 is used for single stranded genomes, while sc genomes use a multiplication factor of 200²⁵. In fact, as DNA quantities double during each qPCR cycle, sc DNA is detected 2x in comparison to an ss genome. The aim of the titration is to calculate the concentration in terms of vector genomes per microliter. A correction is necessary for running the qPCR, using 2 µL of starting material (step 4.2.1). *dil* represents the dilution factors from step 4.2.4. The titer of the secondary AAV vector fraction (step 3.6) can be calculated simultaneously.

Table 6: Template for qPCR data analysis. [Please click here to download this file.](#)

5. Purity Control by SDS-PAGE and Silver Staining

NOTE: The performance of this section of the protocol takes approximately 5 h.

1. Use 70% (v/v) ethanol to thoroughly clean the glass plates used for casting the gels.
2. Assemble the gel casting system. Ensure that the bottoms of the glass plates are not chipped, to prevent leakage of the acrylamide mixture when casting the gel.
3. Prepare the stacking and the resolving gel solutions in two separate 50 mL conical tubes. Omit the tetramethylethylenediamine (TEMED) and the 10% (w/v) ammonium persulfate (APS), as they are responsible for the polymerization of the gel and add them immediately before casting the gel.
NOTE: The final percentage of acrylamide in the gel influences the separation profile of the proteins: typically, a 10% (v/v) final concentration of acrylamide will be sufficient to test the purity of an AAV vector preparation.
4. Mix gently by swirling the tube containing the gel components. Do not vortex, since excessive oxygenation can impair polymerization.
5. Add TEMED and 10% (w/v) APS to the resolving gel solution. Mix it properly and, then, pour it into the gel holder until it reaches 1–2 cm below the top of the small glass.
6. Place a layer of water-saturated butanol on the top of the acrylamide mixture. This will ensure the formation of a flat surface during polymerization. Do not leave the gel under alcohol for longer than 30 min as this will dehydrate the gel and impair its function.
CAUTION: Butanol is hazardous in case of skin contact. It also presents a flammable risk. Handle with care.
7. Wait for the gel to polymerize and, then, pour off the butanol. Tip: Check to see whether the excess gel mix in the tube has solidified. Polymerization occurs in less than 20 min. Wash the surface of the gel with H₂O and, then, dry it using paper towels, taking care not to disturb the surface of the gel.
8. Add TEMED and 10% (w/v) APS to the stacking gel and pour the gel into the gel holder on top of the separating gel.
9. Place the provided comb in the gel. Perform this action with one steady movement to avoid the formation of air bubbles inside the wells.
10. Wait for at least 20 min for the gel to polymerize. Check if the excess gel mix in the conical tube has solidified.
11. Prepare the two mixes (Table 7) for both the primary and the secondary AAV vector fractions (steps 3.5 and 3.6, respectively).

	High Amount	Low Amount
AAV vector stock	5 µL	1 µL
5x Sample buffer	3 µL	3 µL
H ₂ O	7 µL	11 µL

Table 7: Composition of the sample mixes required for silver staining.

12. Fully denature the sample mixes by heating them for 5 min at 95 °C. Assemble the electrophoresis tank, paying attention to the electrode orientation.
13. Fill the tank with 1x cathode buffer on the inside of the electrode assembly and 1x anode buffer on the outside.
NOTE: Anode buffer can be recycled from run-to-run. Fresh cathode buffer must always be used.
14. Remove the comb from the gel and clean the wells of the gel with 1x cathode buffer.
15. Load 1 µL of protein ladder in a well. Load the sample mixes in different wells on the same gel (Figure 3). Run the gel at 50 V until the samples enter the resolving gel. Increase the voltage to 100 V until the dye front reaches the lower limit of the gel.
16. Extract the gel carefully from the glass plates and use the silver staining kit (according to the manufacturer's instructions²⁶) to visualize the viral protein (VP1, VP2, and VP3 subunits) that comprise the AAV capsid, as well as to check for possible protein contamination (Figure 3).

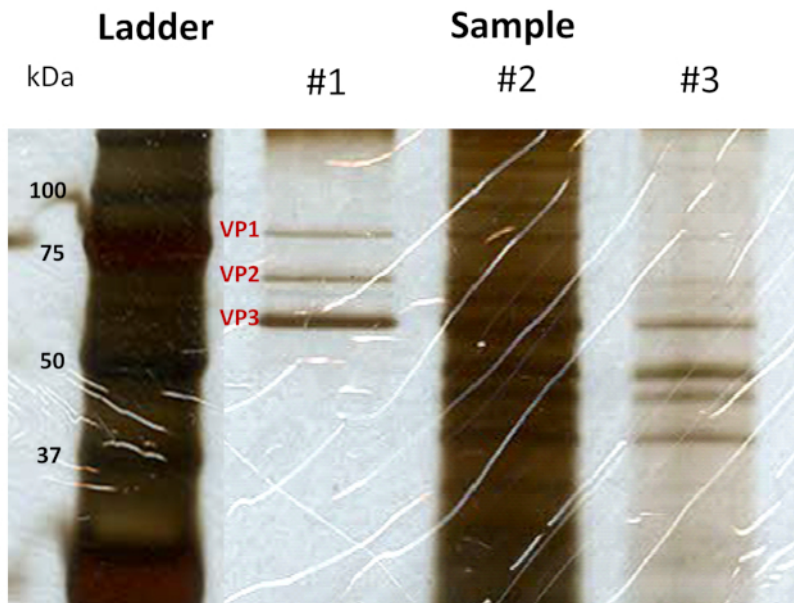


Figure 3: Evaluation of vector purity using SDS-PAGE and silver staining. Using a Tricine-SDS gel, 5 μ L of various vector preparations were separated. Proteins were subsequently detected by silver staining. Vectors are considered pure when VP1 (82 kDa), VP2 (67 kDa), and VP3 (60 kDa) are visible in a 1:1:10 ratio (lane 1), without excessive background (lane 2) or nonspecific bands (lane 3). [Please click here to view a larger version of this figure.](#)

Representative Results

AAV9 was considered, until recently, to be the most effective AAV vector serotype in crossing the BBB and transducing cells of the CNS, following peripheral administration. A significant advance in capsid design was achieved when Deverman et al. reported the use of a capsid selection method called Cre recombination-based AAV-targeted evolution (CREATE)¹⁷. Using this method, they identified a new capsid, named PHP.B, which they reported as able to transduce the majority of astrocytes and neurons in multiple CNS regions, following systemic injection¹⁷. At this point, it should be noted that even though PHP.B provides positive results in C57/Bl6 mice (which was the strain used in the initial isolation experiments), preliminary reports suggest its efficiency may vary in a strain-dependent manner. Further experiments will, no doubt, shed more light on this issue³¹.

However, despite these issues, PHP.B offers exciting possibilities for noninvasive gene manipulation in the CNS of mice, including proof-of-concept gene therapy experiments in disease models. As such, we chose to evaluate the efficiency of transgene expression using PHP.B *versus* AAV9, which has been the 'gold-standard' vector for CNS transduction following peripheral administration since 2009². To perform a direct comparison of both serotypes, under optimal conditions for transgene expression, we used an sc genome configuration³². Both vectors carried the transgene for green fluorescent protein (GFP) under the control of the ubiquitous chicken β -actin (CBA) promoter. Female C57/Bl6 mice at postnatal day 42 (approximately 20 g in weight) received a dose of 1×10^{12} vg per mouse of either scAAV2/PHP.B-CBA-GFP or scAAV2/9-CBA-GFP. Vector administration was performed *via* tail vein injection. The experiments were approved by the Ethical Committee of the KU Leuven.

Three weeks postinjection, the mice underwent transcardial perfusion with ice-cold PBS, followed by 4% (w/v) ice-cold paraformaldehyde (PFA). Their brains were harvested and underwent further postfixation by an overnight incubation in the same fixative, before transferring to 0.01% (w/v) Na-azide/PBS for storage until further analysis. Afterward, the brains were sectioned using a vibrating microtome, and immunohistochemistry was performed on 50 μ m thick sections.

To evaluate the levels of transgene expression, sections were stained with primary antibodies against GFP (rabbit anti-GFP), with detection using secondary antibodies conjugated to a fluorescent dye (anti-rabbit Alexa Fluor 488) (**Figure 4A, B**). Fluorescence intensity measurements (in arbitrary units [au]) confirmed a significant increase in GFP expression when an sc genome and the PHP.B capsid were used relative to AAV9. Increases in GFP were observed in the cerebrum (2105 ± 161 vs. 1441 ± 99 au; $p = 0.0032$), the cerebellum (2601 ± 196 vs. 1737 ± 135 au; $p = 0.0032$), and the brainstem (3082 ± 319 vs. 2485 ± 88 au; $p = 0.0038$) (**Figure 4C**).

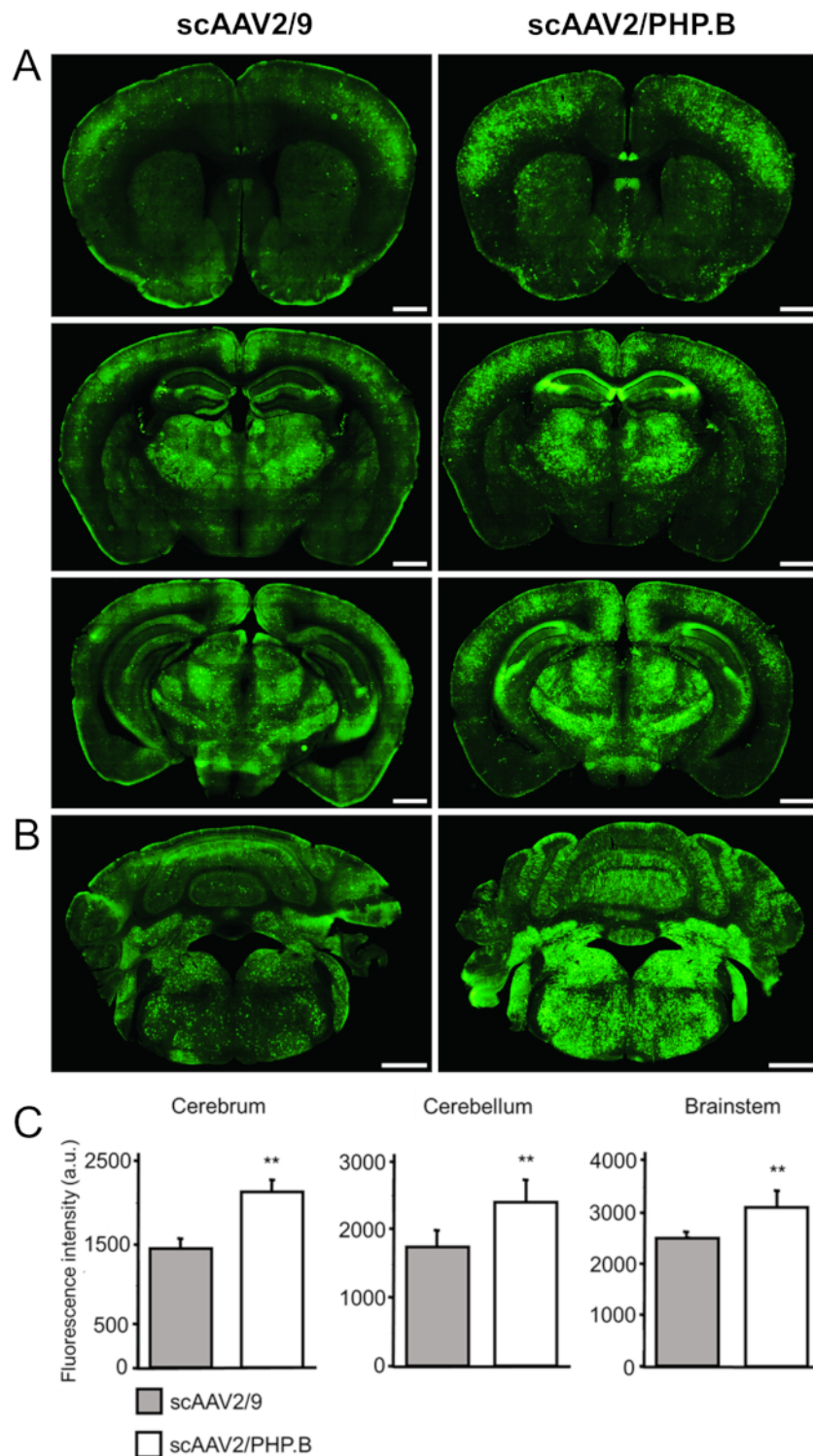


Figure 4: Systemic delivery of scAAV2/PHP.B-CBA-GFP leads to a high GFP expression in the CNS. scAAV2/PHP.B-CBA-GFP or scAAV2/9-CBA-GFP (1×10^{12} vg/mouse) was administered to 6-weeks-old C57/Bl6 mice via tail vein injection. GFP was detected using immunohistochemistry on coronal brain sections 3 weeks postinjection. **(A)** The cerebrum and **(B)** the cerebellum and brainstem are shown. Scale bars = 1 mm. **(C)** The quantification of relative fluorescence intensities was performed to determine the levels of GFP signal achieved with each vector (10 sections per mouse; three mice per vector group). A one-way ANOVA test was performed, followed by a two-tailed Student's *t*-test; the data are expressed as mean $\pm$ standard deviation; ***p* < 0.01; au. arbitrary units. pCapsid, used for AAV vector production, contains the gene *rep* from serotype 2 and the gene *cap* from serotype PHP.B or AAV9, accordingly. This figure has been modified from Rincon et al.³². Please click here to view a larger version of this figure.

Discussion

The production of recombinant AAV vectors described here uses materials and equipment common to most molecular biology labs and cell culture facilities. It allows the user to obtain pure, preclinical grade AAV vectors that can be used to target multiple cell and tissue types across a range of in vitro and in vivo applications. One of the greatest advantages of this protocol, compared to other (i.e., CsCl-based purification), is the shorter working time needed. Ready-to-use AAV vectors are obtainable in a maximum of 6 working days after the initial transfection of HEK293T cells.

Several factors can negatively influence the final yield or the quality of the AAV vector. Poor transfection efficiency is one of the main reasons for a low viral yield³³. A major recommendation is the use of HEK293T cells that have not been passaged for more than 20 times and do not have a cell confluence greater than 90% at the time of transfection²¹. In addition, the transfection method selected has a major impact on the results. This protocol is based on the use of PEI. PEI is a cationic polymer with the ability to deliver exogenous DNA to the cell nucleus through the generation of complexes of polymer and nucleic acid, known as polyplexes, which are up-taken by the cell and trafficked *via* endosomes³⁴. PEI-based transfection is easy and fast to perform, in contrast to other widely used methods, such as the coprecipitation of DNA with calcium phosphate³⁵. Also, PEI-based transfection is much cheaper when compared to other newly introduced methods, such as the usage of cationic lipids and magnet-mediated transfection³⁶.

The purification strategy plays a key role in the protocol. Compared to other methods, iodixanol-based purifications tend to contain a higher percentage of empty viral particles (20%)²⁰. This is offset, to a degree, by the fact that iodixanol-based purification routinely results in AAV vector preparations with a particle-to-infectivity ratio of less than 100. This represents a significant improvement in comparison to conventional CsCl-based procedures, for which substantial loss of particle infectivity is reported³⁷. Another common alternative method to purify AAV vectors is chromatography-based purification. However, this method has the major drawback that a specific column is required for each vector capsid used: for example, while AAV2 is classically isolated using heparin columns, this methodology does not work with AAV4 and AAV5, which do not possess heparin-binding sites on their capsids³⁸. Considering that chromatography purification is also expensive, iodixanol-based purification is generally more suitable for laboratories that wish to produce high-quality batches of AAV vectors on a small scale^{33,39,40}. However, to maximize the final yield and purity of the vector, extreme care is needed when making the iodixanol gradients. The various iodixanol fractions should be transferred to the ultracentrifugation tube using a sterile Pasteur pipette whose tip is touching the wall of the tube: iodixanol should be expelled from the pipette slowly and continuously. As the vector particles accumulate in the 40% iodixanol layer, care needs to be taken to ensure that the gradient interfaces do not mix²⁰. Finally, the fraction containing the vector should be recovered by the insertion of a stainless-steel blunt needle with a gauge not larger than 20 G. To maximize vector recovery, the clear fraction should be retrieved in its entirety. During this step, timing is critical. To avoid compromising the purity of the preparation, it is essential to stop the collection before other (contaminating) phases of the gradient are collected.

Differences in the obtained viral titer can be also attributed to the intrinsic ability of the virus to produce packaged viral particles. A comparison between different AAV serotypes showed that some AAV vectors are more difficult to produce at a higher titer than others (e.g., AAV2)⁴¹. Precipitation of the virus, during the desalting step, can be a possible reason for a lower titer and easily prevented by avoiding overconcentration³³. Moreover, it is also possible that the efficiency of iodixanol gradient-based purification slightly differs between serotypes and, therefore, discrepancies in the viral titer of different serotypes can be observed⁴¹.

Finally, it needs to be pointed out that, even though qPCR is a very accurate method for DNA quantification, some inherent variability in the technique can be observed. Accuracy in the titration primarily depends on the precise pipetting and proper vortexing of all the solutions. To guarantee the most accurate titer reading, the qPCR can be independently repeated, and the obtained values averaged. The choice of primers presented in this protocol is based on the sequence of the CBA promoter located in the pTransgene plasmid used in our laboratory. The CBA promoter is a strong synthetic promoter that is widely used in the vector field to drive expression across multiple cell types. It incorporates multiple elements, including the cytomegalovirus (CMV) early enhancer element; the promoter, first exon, and the first intron of the CBA gene; and the splice acceptor of the rabbit β -globin gene. However, primers can be designed for virtually any element located within the expression cassette (including the promoter, transgene, and regulatory elements). The comparison of titer across batches is also possible, providing primers are used against regions common to the vectors in question.

In conclusion, this protocol can be used to produce AAV vectors with a variety of capsids, genome configurations, promoter types, and transgene cargos. This will allow users to easily adapt the final characteristics of their vectors to best suit experimental needs. In the example presented in the representative results, the use of the PHP.B capsid, which efficiently crosses the BBB, gave a highly efficient gene expression in the CNS, following tail vein injection³². The systemic administration of CNS penetrant vectors has considerable advantages in terms of possible side-effects^{2,17,32}. A possible alternative to peripheral injection, while avoiding the caveats of invasive techniques, is intrathecal delivery, which consists of a delivery of the AAV vector into the cerebrospinal fluid. This delivery route is proven to be effective, showing a widespread expression of transgene across the CNS, less off-target effects in peripheral organs, and low levels of immune response⁴². However, intrathecal injections are much more challenging, as they require higher technical skills than tail vein injection.

Further capsid development to refine this technology will be driven by the opportunities for AAV vector use in gene therapy applications. Such approaches offer attractive possibilities to treat currently incurable CNS disorders, such as amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, and Parkinson's and Alzheimer's disease¹⁸.

Disclosures

The authors have nothing to disclose.

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