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

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TITLE:

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

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Adeno-associated virus (AAV)-based vectors, iodixanol gradient, gene delivery, central nervous system, blood-brain-barrier, PHP.B capsid

SUMMARY:

Here we describe an efficient and reproducible strategy to produce, titer, and quality-control batches of adeno-associated virus vectors. It allows the user to obtain a vector preparation with high-titer, ≥ 1 x 10^{13} vector genomes/mL and a high purity, ready for *in vitro* or *in vivo* use.

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.

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[1-6] 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 CNS[8]. 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[7,8,10-12].

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 expression[1]. 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 types[16].

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 capsid[17]. 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[19-21].

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 online[22]. 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 instructions[23].

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:

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

[Place **Table 1** here]

1. Tri-transfection of HEK293T Cells

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

1.1. 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.

1.2. Seed HEK293T cells at a density of 2 x 10^3 to 6 x 10^3 cells/cm^2 in DMEM10 in a 15 cm-diameter cell culture dishes.

1.3. Grow the cells to 70% - 80% confluence in a standard incubator set at 37 °C, with 95% humidity and 5% CO_2.

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 x 10^3 to 7 x 10^3 cells/cm^2, maintained in 17 - 20 mL of DMEM10 culture medium.

1.4. Prepare polyethylenimine (PEI)/DNA mix at a concentration ratio of 1/3.5 (w/w).

1.4.1. 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.

1.4.2. Distribute the DNA mix over three 50 mL conical tubes (6 mL of DNA mix per conical tube).

1.4.3. 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.

1.4.4. 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.

1.5. 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).

1.6. Ensure the distribution of DPBS over the entire surface by gently tilting the dish.

1.7. 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.

1.8. 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.

1.9. Incubate the transfected cells for 5 h at 37 °C, with 95% humidity and 5% CO_2.

1.10. Add an additional 12 mL of DMEM10 to each culture dish without removing the pre-existing medium (total medium volume = 25 mL).

1.11. Incubate the transfected cells up to 72 h posttransfection at 37 °C, with 95% humidity and 5% CO_2.

[Place **Supplementary Figure 1** here]

1.12. 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.

1.13. Centrifuge the conical tubes at 420 x *g* for 10 min at 4 °C with the acceleration and deceleration of the centrifuge set to maximum.

1.14. 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.

1.15. Resuspend each cell pellet in 2 mL of the lysis buffer directly in the 50 mL conical tube by pipetting up and down 5x - 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).

2.1. 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.

2.2. After the third thawing step, centrifuge at 1,167 x *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.

2.3. 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.

2.4. 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.

2.5. Clarify the supernatant by centrifugation at 13,490 x *g* for 20 min at 4 °C.

2.6. Attach a 0.45 µm 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.

2.7. 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'.

2.8. 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**.

2.9. 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, 4 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.

2.9.1. Pipette 8 mL of 15% iodixanol into each ultracentrifugation tube.

2.9.2. Pipette 5.5 mL of 25% iodixanol solution into a clean 50 mL conical tube (**Figure 1A**).

2.9.3. 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.

2.9.4. 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**).

2.10. 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.

2.11. 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**).

2.12. 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.

2.13. Centrifuge the tubes at 301,580 x *g*, using a fixed-angle titanium rotor, for 1 h and 40 min at 12 °C, using maximum speed of acceleration and deceleration.

2.14. Carefully insert a stainless-steel blunt needle at the 40% and 60% iodixanol interface.

2.14.1. Attach the needle to a 5 mL syringe (**Figure 1E**).

2.14.2. 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.

[Place **Figure 1** here]

2.14.3. Process the collected fraction (desalting and concentration) or store it overnight at 4 °C.

3. Desalting and Concentration of the AAV Vector

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

3.1. 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*.

3.2. 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.

3.3. 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.

3.4. 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 µL.

3.5. 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.

3.6. Rinse the filter with 200 µL 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.

3.7. 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.

3.8. 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.

4.1. Standard curve

4.1.1. Linearize the transgene-expressing plasmid (pTransgene) used for the transfection of HEK-293T cells in section 1.

4.1.2. Prepare the restriction digest mix in a 0.5 mL microcentrifuge tube (refer to **Table 2** for the composition of the restriction digest mix).

[Place **Table 2** here]

NOTE: Adjust the composition of the restriction digest mix according to the enzyme used and the related guidelines from the manufacturer.

4.1.3. Incubate the restriction digest mix for 1 h at 37 °C.

4.1.4. 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.

4.1.5. Purify the linear plasmid DNA using a PCR purification kit, following the manufacturer's instructions[24], 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.

4.1.6. Calculate the molecules (*i.e.*, DNA copies) of the plasmid stock per microliter as follows.

4.1.6.1. 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]

4.1.6.2. 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.

4.1.6.3. 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]

4.1.6.4. 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]

[Place **Table 3** here]

4.1.7. Make serial dilutions of the plasmid stock (1 x 10^9 vg/µL) in triplicates:

10 µL of 1 x 10^9 vg/µL plasmid stock + 90 µL of H_2O = 1 x 10^8 vg/µL solution
10 µL of 1 x 10^8 vg/µL dilution + 90 µL of H_2O = 1 x 10^7 vg/µL solution
10 µL of 1 x 10^7 vg/µL dilution + 90 µL of H_2O = 1 x 10^6 vg/µL solution
10 µL of 1 x 10^6 vg/µL dilution + 90 µL of H_2O = 1 x 10^5 vg/µL solution
Etc.

Continue to obtain a 1x10^1 vg/µL solution.

4.1.8. Keep the serial dilutions of the standard plasmid stock on ice until loading it on the qPCR plate (section 4.3).

4.2. DNA extraction from the AAV vector

4.2.1. 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}'.

4.2.2. 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.

4.2.3. 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.

4.2.4. 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 H_2O = *dil1 x* 10^{-3} dilution
10 µL of *dil1 x* 10^{-3} + 90 µL of H_2O = *dil1 x* 10^{-4} dilution

10 µL o*f dil1 x* 10^{-4} + 90 µL of H_2O = *dil1 x* 10^{-5} dilution

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

4.3. Titration by green-fluorescent detection-based qPCR

4.3.1. 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.

[Place **Table 4** here]

4.3.2. Pipette the qPCR master mix up and down but do not vortex.

4.3.3. 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.

[Place **Figure 2** here]

4.3.4. Seal the plate with a sealing film and briefly centrifuge the qPCR plate at 1,500 x *g* for 30 s at 4 °C.

4.3.5. Run the qPCR reaction on a plate-based real-time PCR amplification and detection instrument, using the conditions suggested in **Table 5**.

[Place **Table 5** here]

4.4. Data analysis to determine the AAV vector titer

[Place **Table 6** here]

4.4.1. 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 must have an efficiency close to 100% and R^2 close to 1.0 (≥ 0.96).

4.4.2. Use the calculated values of *a* and *b* to fill in the corresponding data cells in the spreadsheet (**Table 6C**).

4.4.3. Complete the spreadsheet with the Ct values obtained for the different dilutions of the AAV sample prepared in section 4.2.

4.4.4. Calculate the average AAV vector titer in vector genomes per microliter of the primary fraction by using the following formula:

$$Titer = \frac{Ct - b}{a}\ x\ 400\ x \frac{1}{dil} x\ \frac{1}{2}$$

NOTE: The factor 400 is used for single stranded genomes, while sc genomes use a multiplication factor of 200[25]. 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.

5. Purity Control by SDS-PAGE and Silver Staining

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

5.1. Use 70% (v/v) ethanol to thoroughly clean the glass plates used for casting the gels.

5.2. 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.

5.3. 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.

5.4. Mix gently by swirling the tube containing the gel components. Do not vortex, since excessive oxygenation can impair polymerization.

5.5. 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.

5.6. 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.

5.7. 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 H_2O and, then, dry it using paper towels, taking care not to disturb the surface of the gel.

5.8. 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.

5.9. Place the provided comb in the gel. Perform this action with one steady movement to avoid the formation of air bubbles inside the wells.

5.10. Wait for at least 20 min for the gel to polymerize. Check if the excess gel mix in the conical tube has solidified.

5.11. Prepare the two mixes (**Table 7**) for both the primary and the secondary AAV vector fractions (steps 3.5 and 3.6, respectively).

[Place **Table 7** here]

5.12. Fully denature the sample mixes by heating them for 5 min at 95 °C. Assemble the electrophoresis tank, paying attention to the electrode orientation.

5.13. 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.

5.14. Remove the comb from the gel and clean the wells of the gel with 1x cathode buffer.

5.15. 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.

5.16. Extract the gel carefully from the glass plates and use the silver staining kit (according to the manufacturer's instructions[26]) 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**).

[Place **Figure 3** here]

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 injection[17]. 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 issue[31].

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 2009[2]. To perform a direct comparison of both serotypes, under optimal conditions for transgene expression, we used an sc genome configuration[32]. 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 µm-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**).

[Place **Figure 4** here]

FIGURE AND TABLE LEGENDS:

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.

Figure 2: Plate layout for qPCR-based vector titration. The samples are color-coded: green = standard curve; blue = H_2O control; grey = primary fraction; orange = secondary fraction.

Figure 3: Controlling the vector purity using SDS-PAGE and silver staining. Using a Tricine-SDS gel, 5 µL 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).

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. The 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 ± 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].

Supplementary Figure 1: HEK cells morphology visualized by phase contrast microscopy (left) and confirmation of GFP expression visualized by fluorescence imaging (right 20X). A) Successful transcfection 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.

Table 1: Composition of the required solutions.

Table 2: Restriction digest mix composition.

Table 3: Stock plasmid volume calculator.

Table 4: Primer sequences designed against the CBA promoter.

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

Table 6: Template for qPCR data analysis.

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

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 yield[33]. 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 transfection[21]. 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* endosomes[34]. PEI-based transfection is easy and fast to perform, in contrast to other widely used methods, such as the coprecipitation of DNA with calcium phosphate[35]. Also, PEI-based transfection is much cheaper when compared to other newly introduced methods, such as the usage of cationic lipids and magnet-mediated transfection[36].

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 reported[37]. 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 capsids[38]. 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 mix[20]. 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 overconcentration[33]. 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 observed[41].

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 injection[32]. 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 response[42]. 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 disease[18].

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Daya, S., Berns, K.I. Gene Therapy Using Adeno-Associated Virus Vectors. *Clinical Microbiology Reviews*. **21** (4), 583-593, doi: 10.1128/CMR.00008-08 (2008).

2. Duque, S. *et al.* Intravenous Administration of Self-complementary AAV9 Enables Transgene Delivery to Adult Motor Neurons. *Molecular Therapy*. **17** (7), 1187-1196, doi: 10.1038/mt.2009.71 (2009).

3. Bourdenx, M., Dutheil, N., Bezard, E., Dehay, B. Systemic gene delivery to the central nervous system using Adeno-associated virus. *Frontiers in Molecular Neuroscience*. **7**, doi: 10.3389/fnmol.2014.00050 (2014).

4. Kantor, B., McCown, T., Leone, P., Gray, S.J. Clinical Applications Involving CNS Gene Transfer. *Advances in Genetics*. **87**, 71-124, doi: 10.1016/B978-0-12-800149-3.00002-0 (2014).

5. Crystal, R.G. Adenovirus: The First Effective In Vivo Gene Delivery Vector. *Human Gene Therapy*. **25** (1), 3-11, doi: 10.1089/hum.2013.2527 (2014).

6. Weinberg, M.S., Samulski, R.J., McCown, T.J. Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology*. **69**, 82-88, doi: 10.1016/j.neuropharm.2012.03.004 (2013).

7. Henckaerts, E., Linden, R.M. Adeno-associated virus: a key to the human genome? *Future Virology*. **5** (5), 555-574, doi: 10.2217/fvl.10.48 (2010).

8. Howarth, J.L., Lee, Y.B., Uney, J.B. Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). *Cell Biology and Toxicology*. **26** (1), 1-20, doi: 10.1007/s10565-009-9139-5 (2010).

9. Nathwani, A.C. *et al.* Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B. *The New England Journal of Medicine*. **365** (25), 2357-2365, doi: 10.1056/NEJMoa1108046 (2011).

10. Carter, B.J. Adeno-associated virus and the development of adeno-associated virus vectors: a historical perspective. *Molecular Therapy*. **10** (6), 981-989, doi: 10.1016/j.ymthe.2004.09.011 (2004).
11. Schambach, A., Zychlinski, D., Ehrnstroem, B., Baum, C. Biosafety Features of Lentiviral Vectors. *Human Gene Therapy*. **24** (2), 132-142, doi: 10.1089/hum.2012.229 (2013).
12. Van Vliet, K.M., Blouin, V., Brument, N., Agbandje-McKenna, M., Snyder, R.O. The Role of the Adeno-Associated Virus Capsid in Gene Transfer. *Methods in Molecular Biology*. **437**, 51-91, doi: 10.1007/978-1-59745-210-6_2 (2008).
13. Rincon, M.Y., VandenDriessche, T., Chuah, M.K. Gene therapy for cardiovascular disease: advances in vector development, targeting, and delivery for clinical translation. *Cardiovascular Research*. **108** (1), 4-20, doi: 10.1093/cvr/cvv205 (2015).
14. Samulski, R.J., Muzyczka, N. AAV-Mediated Gene Therapy for Research and Therapeutic Purposes. *Annual Review of Virology*. **1** (1), 427-451, doi: 10.1146/annurev-virology-031413-085355 (2014).
15. McCarty, D.M. Self-complementary AAV Vectors; Advances and Applications. *Molecular Therapy*. **16** (10), 1648-1656, doi: 10.1038/mt.2008.171 (2008).
16. Choi, J.-H. *et al.* Optimization of AAV expression cassettes to improve packaging capacity and transgene expression in neurons. *Molecular Brain*. **7**, 17, doi: 10.1186/1756-6606-7-17 (2014).
17. Deverman, B.E. *et al.* Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. *Nature Biotechnology*. **34** (2), 204-209, doi: 10.1038/nbt.3440 (2016).
18. Jackson, K.L., Dayton, R.D., Deverman, B.E., Klein, R.L. Better Targeting, Better Efficiency for Wide-Scale Neuronal Transduction with the Synapsin Promoter and AAV-PHP.B. *Frontiers in Molecular Neuroscience*. **9**, doi: 10.3389/fnmol.2016.00116 (2016).
19. Lock, M. *et al.* Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale. *Human Gene Therapy*. **21** (10), 1259-1271, doi: 10.1089/hum.2010.055 (2010).
20. Strobel, B., Miller, F.D., Rist, W., LamLa, T. Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. *Human Gene Therapy Methods*. **26** (4), 147-157, doi: 10.1089/hgtb.2015.051 (2015).
21. Jungmann, A., Leuchs, B., Katus, H.A., Rommelaere, J., Müller, O.J. Protocol for efficient generation and characterization of adeno-associated viral (AAV) vectors. *Human Gene Therapy Methods*. doi: 10.1089/hum.2017.192 (2017).
22. Tolmachov, O. Designing Plasmid Vectors. *Methods in Molecular Biology*. **542**, 117-129, doi: 10.1007/978-1-59745-561-9_6 (2009).
23. Qiagen. QIAGEN Plasmid Purification Handbook. https://www.qiagen.com/us/resources/download.aspx?id=46205595-0440
24. QIAquick_Spin_Handbook.pdf. QIAquick PCR Purification Kit Protocol. http://2012.igem.org/wiki/images/a/a3/QIAquick_PCR-purification.pdf (2008).
25. Lock, M., Alvira, M.R., Chen, S.-J., Wilson, J.M. Absolute Determination of Single-Stranded and Self-Complementary Adeno-Associated Viral Vector Genome Titers by Droplet Digital PCR. *Human Gene Therapy Methods*. **25** (2), 115-125, doi: 10.1089/hgtb.2013.131 (2014).

26. SIGMA. ProteoSilver Silver Stain Kit (PROTSIL1) - Bulletin. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/protsil1bul.pdf.
27. Machholz, E. *et al.* Manual Restraint and Common Compound Administration Routes in Mice and Rats. *Journal of Visualized Experiments*. (67), e2771 (2012).
28. Gage, G.J., Kipke, D.R., Shain, W. Whole Animal Perfusion Fixation for Rodents. *Journal of Visualized Experiments*. (65), e3564 (2012).
29. Puntel, M. *et al.* Gene transfer into rat brain using adenoviral vectors. *Current Protocols in Neuroscience*. **50** (1), doi: 10.1002/0471142301.ns0424s50 (2010).
30. Bachman, J. Immunohistochemistry on Freely Floating Fixed Tissue Sections. *Methods in Enzymology*. **533**, 207-215, doi: 10.1016/B978-0-12-420067-8.00013-1 (2013).
31. Hordeaux, J. *et al.* The Neurotropic Properties of AAV-PHP.B Are Limited to C57BL/6J Mice. *Molecular Therapy.* **26** (3), 664-669 (2018).
32. Rincon, M.Y. *et al.* Widespread transduction of astrocytes and neurons in the mouse central nervous system after systemic delivery of a self-complementary AAV-PHP.B vector. *Gene Therapy*. **25** (2), 83-92, doi: 10.1038/s41434-018-0005-z (2018).
33. Crosson, S.M., Dib, P., Smith, J.K., Zolotukhin, S. Helper-free Production of Laboratory Grade AAV and Purification by Iodixanol Density Gradient Centrifugation. *Molecular Therapy. Methods & Clinical Development*. **10**, 1-7, doi: 10.1016/j.omtm.2018.05.001 (2018).
34. Merdan, T., Kunath, K., Fischer, D., Kopecek, J., Kissel, T. Intracellular processing of poly(ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments. *Pharmaceutical Research*. **19** (2), 140-146 (2002).
35. Meissner, P. *et al.* Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnology and Bioengineering*. **75** (2), 197-203 (2001).
36. Reed, S.E., Staley, E.M., Mayginnes, J.P., Pintel, D.J., Tullis, G.E. Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *Journal of Virological Methods*. **138** (1-2), 85-98, doi: 10.1016/j.jviromet.2006.07.024 (2006).
37. Zolotukhin, S. *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy*. **6** (6), 973-985, doi: 10.1038/sj.gt.3300938 (1999).
38. Kaludov, N., Handelman, B., Chiorini, J.A. Scalable Purification of Adeno-Associated Virus Type 2, 4, or 5 Using Ion-Exchange Chromatography. *Human Gene Therapy*. **13** (10), 1235-1243, doi: 10.1089/104303402320139014 (2002).
39. Nass, S.A. *et al.* Universal Method for the Purification of Recombinant AAV Vectors of Differing Serotypes. *Molecular Therapy. Methods & Clinical Development*. **9**, 33-46, doi: 10.1016/j.omtm.2017.12.004 (2017).
40. Qu, G. *et al.* Separation of adeno-associated virus type 2 empty particles from genome containing vectors by anion-exchange column chromatography. *Journal of Virological Methods*. **140** (1-2), 183-192, doi: 10.1016/j.jviromet.2006.11.019 (2007).

41. Holehonnur, R. *et al.* Adeno-associated viral serotypes produce differing titers and differentially transduce neurons within the rat basal and lateral amygdala. *BMC Neuroscience*. **15**, 28, doi: 10.1186/1471-2202-15-28 (2014).
42. Wang, H. *et al.* Widespread spinal cord transduction by intrathecal injection of rAAV delivers efficacious RNAi therapy for amyotrophic lateral sclerosis. *Human Molecular Genetics*. **23** (3), 668-681, doi: 10.1093/hmg/ddt454 (2014).

Figure 1

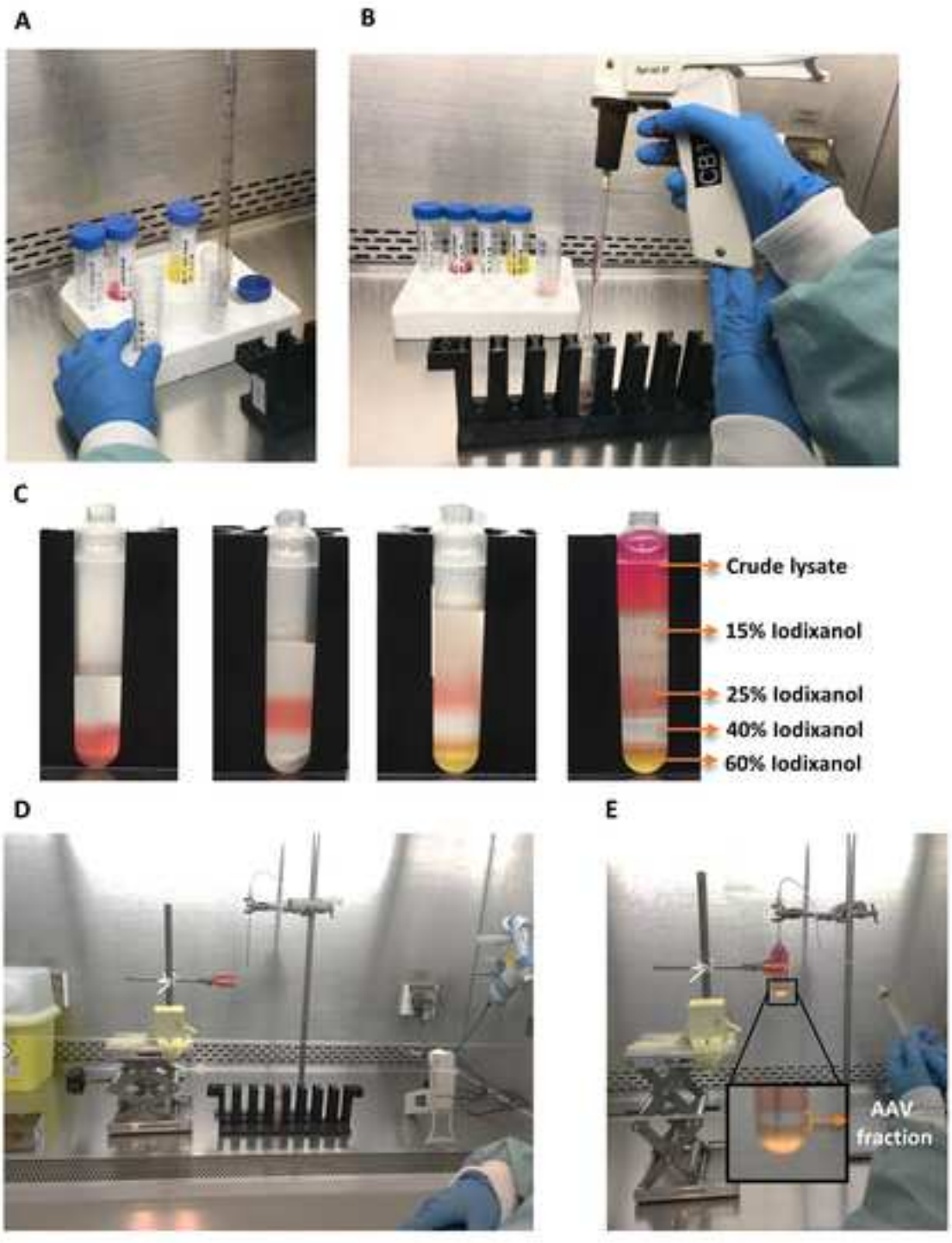


Figure 2

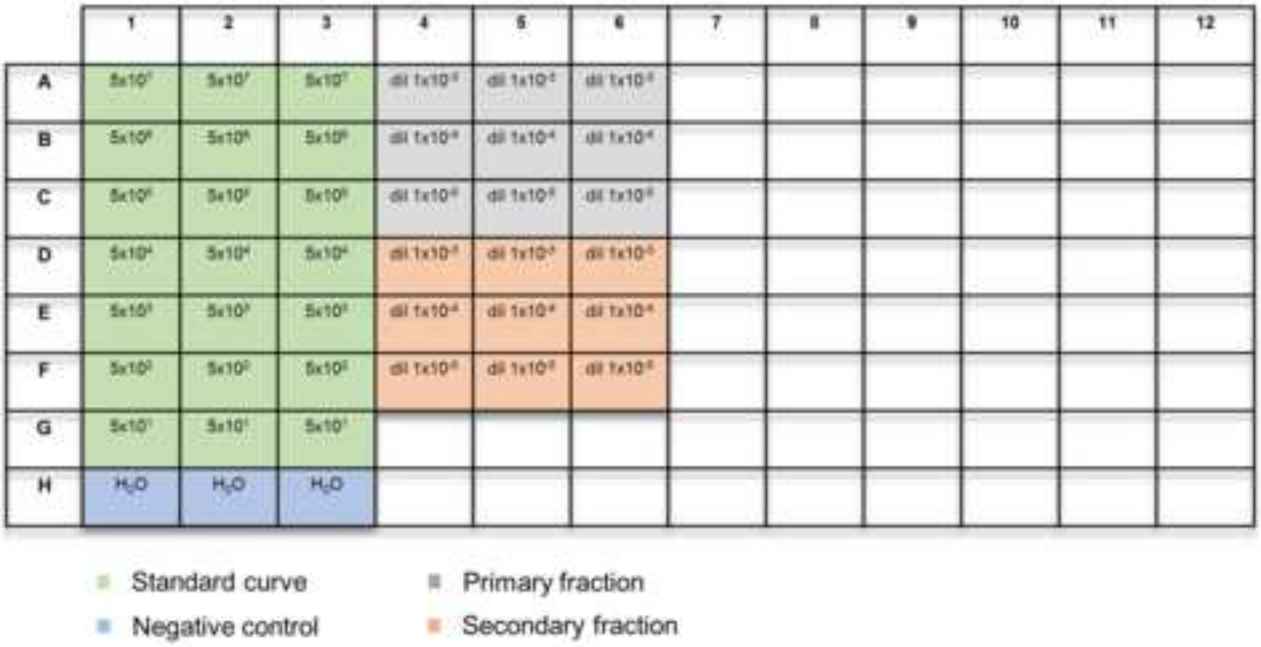


Figure 3

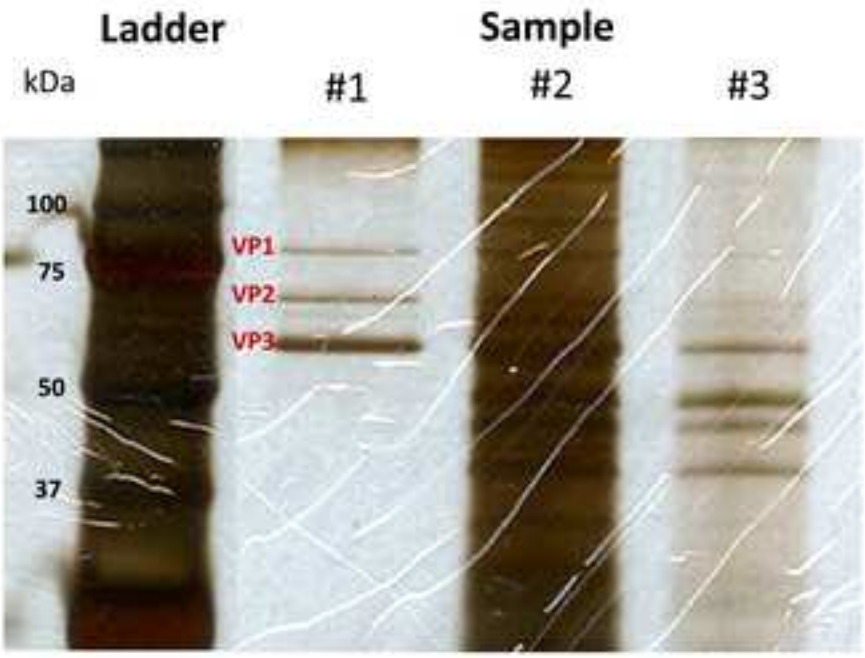


Figure 4

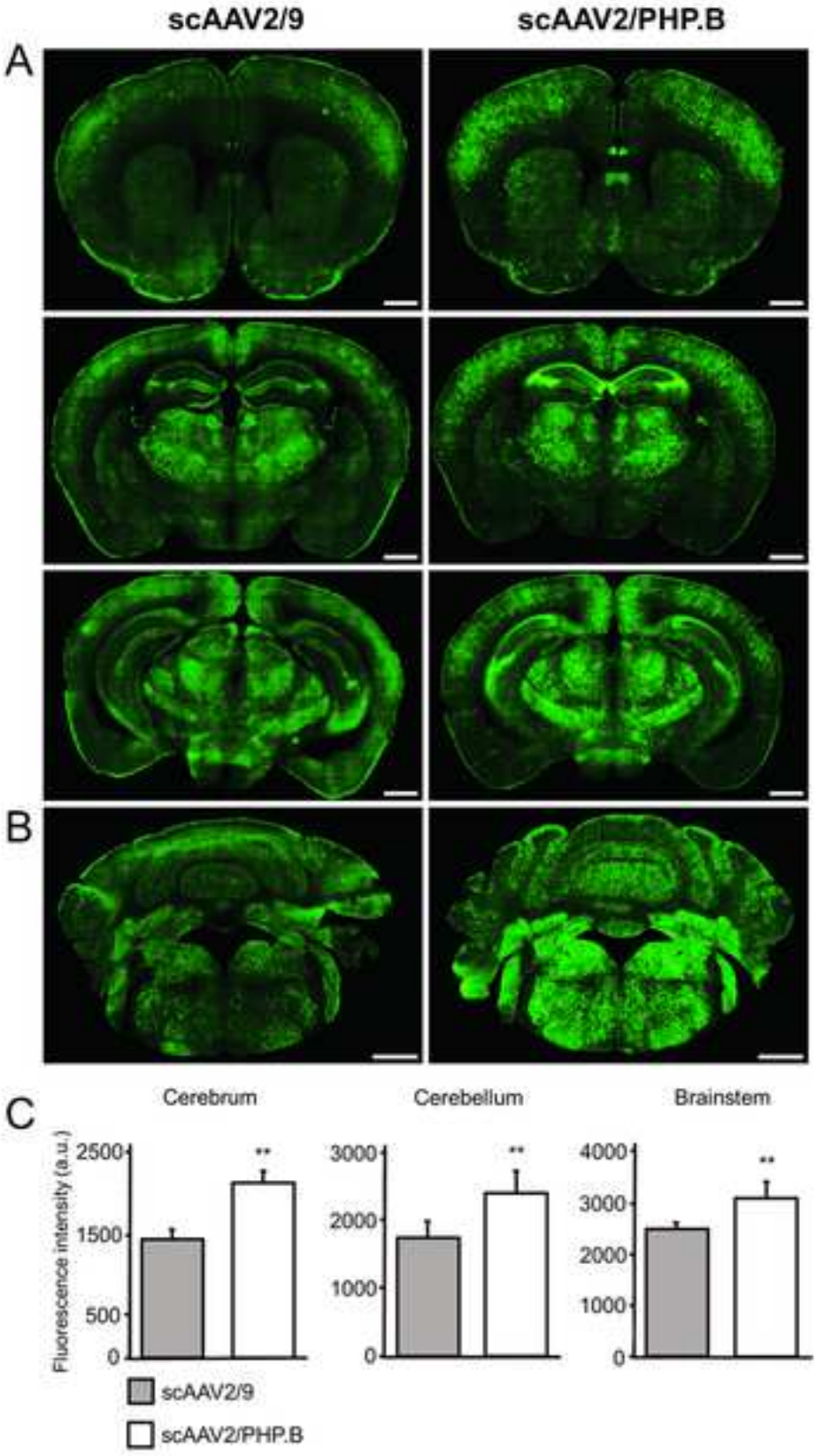


Table 1

Click here to access/download;Table;Table 1. Composition of the required solutions.xlsx

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	0.438 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_2HPO_4 2.4 g KH_2PO_4 Up to 1 l $_{dd}$water

1 M $MgCl_2$	20.33 g $MgCl_2$-$6H_2O$ Up to 100 ml Ultrapure water	
1 M KCl	7.45 g KCl Up to 100 mL Ultrapure water	CAUTION
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 Up to 500 ml Ultrapure water H_2O	CAUTION
15% Iodixanol	12.5 ml of Optiprep density gradient medium 10 ml of 5 M NaCl 10 ml of 5x PBS-MK 17.5 ml of Ultrapure water	CAUTION
25% Iodixanol	20.8 ml of Optiprep density gradient medium 10 ml of 5x PBS-MK 19.2 ml of Ultrapure water 100 µl of phenol red	CAUTION
40% Iodixanol	33.3 ml of Optiprep density gradient medium 10 ml of 5x PBS-MK 6.7 ml of Ultrapure water	CAUTION
60% Iodixanol	50 ml of Optiprep density gradient medium 100 µl of phenol red	CAUTION CAUTION
AAV purity control		
	44.8 g Tris base (121.14 g/mol) 11.4 ml glacial acetic acid (17.4M)	CAUTION

10 x Tris acetate EDTA (TEA) buffer	3.7 g EDTA	
	Up to 1 L Ultrpure water	
Agarose gel	0.8g 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
	For 2 gels:	
	400 µl acrylamide	CAUTION

Stacking gel	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 2

Component	Amount
10x Restriction enzyme buffer	5 µl
Restriction enzyme	2,5 µl
plasmid	5 µg
H_2O	up to 50 µl

Table 2

Component	Amount
10x Restriction enzyme buffer	5 µl
Restriction enzyme	2,5 µl
plasmid	5 µg
H_2O	up to 50 µl

Table 3

Click here to access/download;Table;Table 3. Stock plasmid volume calculator.xlsx

Plasmid name	pssAAV-CBA-B9
Concentration (ng/µl)	173.8
Plasmid size (bp)	5850

Copy number/µl	2.75E+10	*

	1X10E9 copies/µl in 100 ul
Volume of plasmid	3.63
H_2O	96.37

Replace values in the highlighted cells with user obtained data

*Corresponds to the formula in step 6.1

Table 3

Click here to access/download;Table;Table 3. Stock plasmid volume calculator.xlsx

Plasmid name	pssAAV-CBA-B9
Concentration (ng/µl)	173.8
Plasmid size (bp)	5850

Copy number/µl	2.75E+10	*

	1X10E9 copies/µl in 100 ul
Volume of plasmid	3.63
H_2O	96.37

Replace values in the highlighted cells with user obtained data

*Corresponds to the formula in step 6.1

Table 4

Click here to access/download;Table;Table 4. Primer sequences.xlsx

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

Table 4

Click here to access/download;Table;Table 4. Primer sequences.xlsx

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

Table 5

Click here to access/download;Table;Table 5. Thermal cycling protocol.xlsx

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 6

Click here to access/download;Table;Table 6. qPCR data template.xlsx

A. Raw data

Replace the provided example data with the user's Ct value in the highlighted rows.

Well	Well	Ct value
1	A1	11.2
2	A2	11.5
3	A3	11.3
4	A4	10.1
5	A5	10.5
6	A6	10.6
7	A7	
8	A8	
9	A9	
10	A10	
11	A11	
12	A12	
13	B1	14.1
14	B2	14.2
15	B3	14.3
16	B4	13.7
17	B5	14.4
18	B6	14.2
19	B7	
20	B8	
21	B9	
22	B10	
23	B11	
24	B12	
25	C1	17.5
26	C2	17.6
27	C3	17.4
28	C4	17.5
29	C5	17.3
30	C6	17.4
31	C7	
32	C8	
33	C9	
34	C10	
35	C11	
36	C12	
37	D1	20.9
38	D2	21.2
39	D3	21.3
40	D4	11.5
41	D5	11.9
42	D6	11.8
43	D7	
44	D8	
45	D9	
46	D10	
47	D11	
48	D12	
49	E1	24.5
50	E2	24.1

Well	Well	Ct value
51	E3	24.3
52	E4	15.1
53	E5	14.9
54	E6	14.8
55	E7	
56	E8	
57	E9	
58	E10	
59	E11	
60	E12	
61	F1	27.5
62	F2	27.9
63	F3	28.0
64	F4	17.8
65	F5	17.8
66	F6	17.8
67	F7	
68	F8	
69	F9	
70	F10	
71	F11	
72	F12	
73	G1	30.8
74	G2	31.0
75	G3	30.7
76	G4	
77	G5	
78	G6	
79	G7	
80	G8	
81	G9	
82	G10	
83	G11	
84	G12	
85	H1	35.6
86	H2	35.7
87	H3	36.1
88	H4	
89	H5	
90	H6	
91	H7	
92	H8	
93	H9	
94	H10	
95	H11	
96	H12	

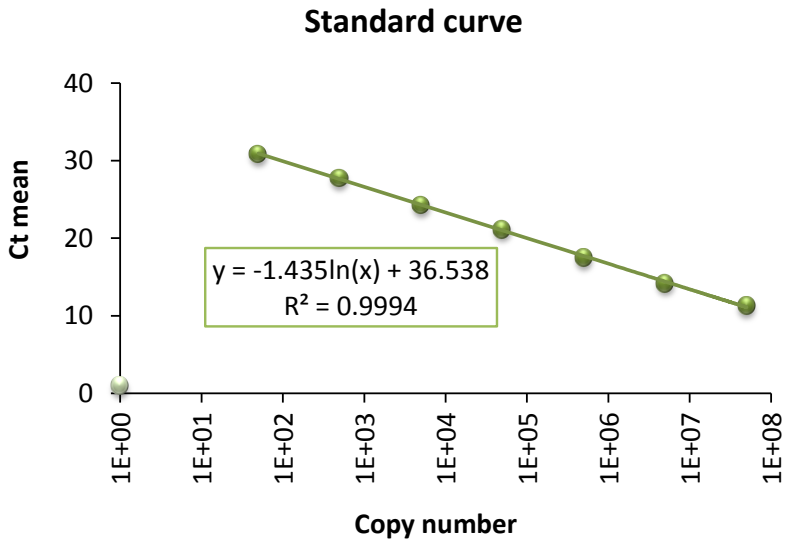
B. Ct values overview and standard curve calculation

Schematic view of the 96 well qPCR multiwell plate and Ct values:

	1	2	3	4	5	6
A	11.2	11.5	11.3	10.1	10.5	10.6
B	14.1	14.2	14.3	13.7	14.4	14.2
C	17.5	17.6	17.4	17.5	17.3	17.4
D	20.9	21.2	21.3	11.5	11.9	11.8
E	24.5	24.1	24.3	15.1	14.9	14.8
F	27.5	27.9	28.0	17.8	17.8	17.8
G	30.8	31.0	30.7			
H	35.6	35.7	36.1			

Standard curve plot:

Copy Number (CN)	ln (CN)	Standard Curve			
		Ct1	Ct2	Ct3	Ct mean
5E+07	17.73	11.2	11.5	11.3	**11.33**
5E+06	15.42	14.1	14.2	14.3	**14.2**
5E+05	13.12	17.5	17.6	17.4	**17.5**
5E+04	10.82	20.9	21.2	21.3	**21.1**
5E+03	8.52	24.5	24.1	24.3	**24.3**
5E+02	6.21	27.5	27.9	28.0	**27.8**
5E+01	3.91	30.8	31.0	30.7	**30.8**
5E+00	H2O	35.6	35.7	36.1	35.8



C. Titer calculation

Fill the highlighted cells with the equation fitting the standard curve y = **-a** ln(x) + **b**

y=-1,435ln(x)+36,538

a=	b=
-1.435	36.538

Sample titer calculation:

Sample 1

Dilution	Ct1	Ct2	Ct3	Ct mean	CN	Titer VG/ml
1.00E-03	10.1	10.5	10.6	**10.4**	8.14E+07	3.26E+13
1.00E-04	13.7	14.4	14.2	**14.1**	6.18E+06	2.47E+13
1.00E-05	17.5	17.3	17.4	**17.4**	6.19E+05	2.48E+13
					Average:	**2.73E+13**
					Std dev:	4.51E+12

Sample 2

Dilution	Ct1	Ct2	Ct3	Ct mean	CN	Titer VG/ml
1.00E-03	11.5	11.9	11.8	**11.7**	3.21E+07	1.29E+13
1.00E-04	15.1	14.9	14.8	**14.9**	3.46E+06	1.38E+13
1.00E-05	17.8	17.8	17.8	**17.8**	4.69E+05	1.88E+13
					Average:	**1.51E+13**
					Std dev:	3.16E+12

2.73E+13 Titer of the AAV vector (Primary fraction)

1.51E+13 Titer of the AAV vector (Secondary fraction)

Table 7

Click here to access/download;Table;Table 7. Silver staining mix.xlsx

	High Amount	Low Amount
AAV vector stock	5 µL	1 µL
5x Sample buffer	3 µL	3 µL
H_2O	7 µL	11 µL

The concentration of the vector is the same in both instances. Only the toal loaded is different

Table 7

Click here to access/download;Table;Table 7. Silver staining mix.xlsx

	High Amount	Low Amount
AAV vector stock	5 µL	1 µL
5x Sample buffer	3 µL	3 µL
H_2O	7 µL	11 µL

The concentration of the vector is the same in both instances. Only the toal loaded is different

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Plasmid production			
pTransgene plasmid	*De novo* design or obtained from a plasmid repository	N/A	See step 1 of main protocol for further details
pCapsid			
pHelper	Agilent	240071	
Plasmid *Plus* maxi kit	Qiagen	12963	
QIAquick PCR purification Kit	Qiagen	28104	
AAV Helper-Free System	Agilent	240071	
Cell culture and transfection			
Dulbecco's Modified Eagle Medium (DMEM), high glucose, no glutamine	Life technologies	11960-044	Supplement DMEM with FBS (1% or 10% v/v) and GlutaMAX 200 mM (1% v/v) then filter sterilize the medium using a 0.22 μm filter
Fetal bovine serum (FBS)	GIBCO	10500-064	
GlutaMAX supplement (200 mM)	GIBCO	35050038	
Corning bottle-top vacuum filter system	Sigma-Aldrich	CLS430769	
Dulbecco's Phosphate Buffered Saline (DPBS) with no calcium and no magnesium	GIBCO	14190094	
HEK293T cells	American Tissue Culture Collection	CRL3216	Upon receipt, thaw the cells and culture as described in the protocol. After a minimal number of passages, freeze a subfraction for future in aliquots. Always use cells below passage number 20. Once cultured cells have been passaged more than 20 times, restart a culture from the stored aliquots
Cell culture dishes	Greiner Cellstar	639160	15 cm diameter culture dishes

Cell scrapers	VWR	10062-904	
Polyethylenimine (PEI)	Polyscience	23966-2	PEI in powder form is dissolved at 1 µg/µL in deionized water ($_{dd}$water) at pH=2 (use HCl). Prepare in a beaker and stir for 2-3 h. When dissolved, bring the pH back to 7 with NaOH. Filter sterilize and store the resuspended stock solution in 1 ml aliquots at -20 °C. PEI aliquots can freeze/thawed multiple times.
Virkon solution	Fisher Scientific	NC9821357	Disinfect any material that has been in contact with assembled viral particles with Virkon solution
Mutexi long-sleeve aprons	Fisher Scientific	11735423	Wear an apron over the top of a regular lab coat
Fisherbrand maximum protection disposable overshoes	Fisher Scientific	15401952	
AAV Purification and desalting			
Optiprep density gradient medium	Sigma-Aldrich	D1556	Optiprep is a 60% (w/v) solution of iodixanol in water (sterile). CAUTION. Use under a laminar flow hood. Wear gloves
Phenol red	Sigma-Aldrich	P0290	CAUTION. Use under a laminar flow hood
Pasteur pipette	Sigma-Aldrich	Z627992	Sterilize before use
OptiSeal Polypropylene tubes	Beckman	361625	
Benzonase (250 U/µL)	Sigma-Aldrich	E1014	Supplied as a ready-to-use solution
Acrodisc syringe filter	Pall corporation	4614	
Omnifix syringe (5mL)	Braun	4617053V	
Blunt syringe needle	Sigma Aldrich	Z261378	Stainless steel 316 syringe needle, pipetting blunt 90° tip gauge 16, L 4 in. Referred to in the text as a blunt-end needle
Aqua Ecotainer	B. Braun	0082479E	Sterile endotoxin-free water. Referred to as 'Ultrapure water'

Amicon ultra-15 centrifugal filter unit	Millipore	UFC910024	These filters concentrate the final product by collecting the viral particles in consecutive centrifugation steps
Pluronic F68 (100X)	Thermo Fisher	24040032	Non-ionic surfactant. Dilute in sterile PBS to use at 0,01% (v/v)
Fisherbrand Sterile Microcentrifuge Tubes with Screw Caps (2 mL)	Fisher Scientific	02-681-374	Use skirted tubes for easy handling
AAV Titration			
Restriction enzyme: StuI (10 U/µL)	Promega	R6421	
DNAse I (1 U/µL)	Fisher scientific	EN0521	
Proteinase K	Sigma-Aldrich	3115852001	Reconstitute in ultrapure water and use at a final concentration of 10 mg/ml. Solution can be stored at -20°C
EasyStrip Plus Tube Strips (with attached caps)	Fisher scientific	AB2000	
Eppendorf microtube 3810x	Sigma-Aldrich	Z606340-1000EA	
LightCycler 480 SYBR Green I Master Mix	Roche	4707516001	
LightCycler Multiwell Plates, 96 wells	Roche	4729692001	White polypropylene plate (with unique identifying barcode)
Microseal 'A' PCR Plate and PCR Tube Sealing Film	Bio-Rad	msa5001	
AAV Purity control			
Ammonium persulfate (APS)	Sigma-Aldrich	A3678	Reconstitute in ultrapure water to 10% (v/v). CAUTION. Use under laminar flow hood. Wear gloves
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281	CAUTION. Use under a laminar flow hood. Wear gloves

Tris Base ULTROL Grade	Merck	648311	CAUTION. Use under a laminar flow hood. Wear gloves
UltraPure Agarose	Thermo Fisher	16500-500	
Rotiphorese® Gel 30 (37,5:1)	Carl Roth	3029.3	Aqueous 30 % acrylamide and bisacrylamide stock solution at a ratio of 37.5:1. CAUTION. Use under laminar flow hood. Wear gloves
Serva Blue G	Sigma-Aldrich	6104-58-1	
Precision Plus prestained marker	Bio-Rad	1610374edu	
1-Butanol	Sigma-Aldrich	B7906	CAUTION. Use under a laminar flow hood. Wear gloves
Immunohistochemistry			
Rabbit anti-GFP	Synaptic System	132002	1:300 dilution
Anti-rabbit Alexa Fluor 488	Invitrogen	A21206	1:1000 dilution
Equipment	**Company**	**Catalog number**	**Comments**
Vector production lab			
Rotina 380 bench-top centrifuge *	Hettich	1701	
Optima XPN 80 ultracentrifuge *	Beckmann Coulter	A95765	
Type 50.2 Ti fixed-angle titanium rotor *	Beckmann Coulter	337901	
Entris digital scale *	Sartorius	2202-1S	
Warm water bath *			Set at 37°C
Ice bucket *	VWR	10146-290	Keep material used in the vector production lab separate from that used in standard lab areas
Pipetboy pro *	Integra	156,400	
Graduated pipettes: Cell star *	Greiner bio-one	606180 607180 760180	Capacity of 5 ml, 10 ml and 25 ml
Co_2 incubator CB150 *	Binder	9040-0038	Set at 37°C, 5% CO_2 and 95% humidity

Nuaire safety cabinet NU 437-400E *	Labexchange	31324	Clean all the surfaces with 70% ethanol and Virkon before and after use
Conventional lab			
T100 thermal cycler *	Bio-Rad	1861096	
LightCycler 480 Instrument II *	Roche	5015278001	
ThermoMixer *	Eppendorf	C 5382000015	
Nanodrop *	ThermoFisher Scientific	ND 2000	
Mini-Protean Tetra Cell *	Bio-Rad	1658001FC	For use with handmade or precast gels
ProteoSilver silver stain kit	Sigma-Aldrich	PROTSIL1	High sensitivity protein detection with low background
Centrifuge 5804 R *	Eppendorf	B1_022628045	High speed centrifuge for medium capacity needs (up to 250 ml)
Graduated pipettes Cell star *	Greiner bio-one	606180 607180 760180	5 ml, 10 ml and 25 ml
Filter tips *	Greiner bio-one	750257 738257 771257	2 µl, 20 µl, 200 µl
Ice bucket with lid *	VWR	10146-290	
Mini diaphragm vacuum pump, VP 86 *	VWR	181-0065	
Pipetman P2, P20, P100, P200, P1000	Gilson	F144801 F123600 F123615 F123601 F123602	

* Materials marked with an asterisk are expensive vpieces of equipment and are usually central infrastructure items shared between multiple labs. These items can also be replaced by equivalents if available. Note, when a different ultracentrifuge is used, care must be taken to select the correct rotor and centrifuge tubes.

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Author(s): Shelly Fripont, Catherine Rouillé, Nanika Nanino, Melvin Y. Zinnon, Matthew G. Holt

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A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

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Signature:	
Date:	21-09-2018

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2. Fax the document to +1.866.381.2236
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JoVE58960

VIB-KU LEUVEN CENTER FOR BRAIN & DISEASE RESEARCH

KU LEUVEN

To:
Vineeta Bajaj, Ph.D.
Review Editor JoVE

Leuven, October, 2018

Dear Doctor Bajaj,

Ref: Manuscript JoVE58960

On behalf of all the authors, I would like to thank you for handling our manuscript 'Efficient and Flexible Protocols for Production of Adeno-Associated Virus-Based Vectors, Purification and Quality Control' that we submitted.

We have read the comments raised by the editorial committee and found their critiques to be both considered and fair. Therefore, we have applied changes in the manuscript and addressed these modifications in a point-by-point response letter for the benefit of the editors. All the comments were addressed accordingly, specific steps modified into notes.

We hope the manuscript is now suitable for publication in *JoVE* and remain available to address any additional concerns.

Sincerely,

Matthew G. Holt
Corresponding author
VIB-KU Leuven Center for Brain & Disease research.

The **editorial comments** have been addressed by the authors as follows:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same. We preserved the formatting done by the editor and included in the new sub steps of the protocol.
2. Please have the title in alignment with the highlighted section of the protocol. The title was modified following the recommendation of the editor and the reviewer comment.

.

3. Please address all the specific comments marked in the manuscript. All the comments marked in the manuscript were addressed. Please find the detailed response to each comment as follows:
   - This part is not explained in the protocol section. The title should reflect the highlighted portion of the protocol. Either change the title to reflect the protocol or include a subsection/ step that the transgene is delivered to the mouse and the expression is studied. This can reference already published protocols. Perform transgene delivery to xx mice as shown previously. The title was modified accordingly to reflect the protocol.
   - Results for this section is missing. Show cells with GFP and without GFP AAV transfection. How does it look? We thank the editor for bringing this point to our attention. A supplementary figure has been added to the protocol to show HEK293T cells expressing GFP after transfection.
   - Please use imperative tense throughout. Changed here, please check. For how many hours are the cells grown?. The text has been revised to only include actions written in imperative tense. The amount of hours during which cells are grown is variable and an exact time window is difficult to define. Therefore, we suggest monitoring the cells until they reach a confluency of 70-80%, at which point they are ready to be transfected.
   - This is not an action step. Please convert this to a note and renumber the steps accordingly. The step has been converted to a note (please, check note after step 1.3) and the rest of the protocol modified accordingly.
   - Please remove redundancy and write exactly how you perform your experiment in a step wise manner. The protocol has been revised to remove redundancy and all unnecessary remarks (redundancy was corrected specifically in step 1.4.1).
   - Dilute to what concentration? Also 10 ml of 150 mM NaCl? The text of step 1.4.1 has been corrected. The word “dilute” has been replaced by “mix” and the rest of the section modified accordingly.
   - Again, this is not a step but note instead. Alternatively this can be moved to the discussion section. The step has been modified accordingly and replaced for a note.

- Are the cells out at this stage? Yes, the cells are out of the incubator at this stage and handle under a flow laminar cabinet. The text was modified to clarify this comment and corresponds to step 1.5.
- How much per tube? The specific step has been modified to state "6 ml of DNA mix per conical tube". Please, check step 1.4.2
- We cannot have paragraph of text in the protocol section so converted to a different step. Please check. Thank you for bringing this point to our attention. The protocol has been revised to include more frequent use of short steps, such as 1.7.
- Check the step number. The mistake was corrected. Please check step 1.8.
- Where is the result for this part? Test for different fractions. The proper preparation of the different iodixanol fractions can be ensured by visual confirmation. Since the fractions all have different densities, they should not intermix during the layering step (please check Figure 1C). A note after step 2.9.4 was added to clarify the issue.
- Again, this is not a step and can be converted to a note. Steps should contain only action items that direct the reader to do something. Also notes cannot be filmed so please remove the highlight. The section has been converted to a note and the highlight removed, as requested.
- What solution do you use to make different percentages of iodixanol? The solutions used to prepare the different iodixanol fractions are described in Table 1. Moreover, a note directing the reader to Table 1 was added at the beginning of the protocol section.
- Write a one liner caution here concerning iodixanol. The description of safety measures is now included after step 2.9.
- Where did you add the phenol red to the gradient? Please write exactly as you perform your experiment. Also if this needs to be filmed please convert to imperative tense. A new step (number 2.8) has been added to the text, which refers to the preparation of the iodixanol solutions as described in Table 1, in which the phenol red is mentioned.
- We cannot have a paragraph of text in the protocol section so converted to substeps. Please check. We thank the editor for the comment and the remodelling of the section.
- We cannot have steps which are not numbered. Converted to a substep instead. The step was changed to a substep and numbered accordingly. Please check substep 2.14.3
- Please refer to the table 1 where ever you are listing the solution for the first time. Thank you for the comment. To reduce the amount of text not strictly related to the protocol, we added have added a note at the beginning of the protocol directing the reader to Table 1 for details of all solutions used during the protocol.
- Please use imperative tense and please do not used phrases like should be, would be etc. The text in step 3.2 was modified as requested.

- Imperative tense please. The text in step 3.6 was modified accordingly.
- Result for this part is missing. We thank the editor for bringing this point to our attention. A note has been added which directs the readers to Table 6, where the results of an exemplary qPCR run can be found..
- Some of the details can be moved to supplemental material. We believe that the calculations provided in the text are necessary for the user to accurately understand how the attached qPCR template is organised, and how to use it properly. Hence, we have left this text unaltered.
- Please remove the redundancy from the protocol by removing the repetition. The protocol should only contain action steps which direct the reader to something and should be written in imperative tense. Any discussion about the protocol should be placed in the discussion section and not the protocol. The protocol has been modified accordingly. Please, check steps from 4.1.2 to 4.1.6.
- Please make it substeps or convert this to a table in .xls/xlsx format and upload it separately to your editorial manager account. We cannot have bullet or dashes in the protocol section. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. The bullet point list has been replaced by Table 2. As requested, the numbering has been adjusted throughout the section in question.
- Citation for the manual to follow. Citations of the manuals to follow to perform steps such as the gel purification have been added to the text. Please, check references number 23 and 24.
- Please reword. Instead can be written as.. Now add 1 microlitre of DNase I to each tube. Step 4.2.1 has been reworded, according to the Editor's suggestion.
- Please provide step number instead. In step 4.2.3, the text has been adjusted. Reference to step 4.2.1 is now made.
- Few details can be moved to the discussion. Also this is not an action, hence converted to a note instead. The text was converted into substep 4.2.3
- This step is not needed as you would recommend the user to run the qPCR instead. Please remove the redundancy. The protocol was adjusted accordingly and the unnecessary step removed.
- Please number it as supplemental table. Upload each table in this case individually as .xls/.xlsx file. A supplementary table (Table 6), containing the template for the qPCR-based titration of the AAV vector, has been added to the manuscript.
- Details about the gel preparation are irrelevant and can be removed. We thank the editor for the comment. However, we believe that, self-made gels (made according to the details provided in our protocol) work better and offer greater flexibility for Silver Staining, compared to precast gels. Hence we have left this section in the protocol.

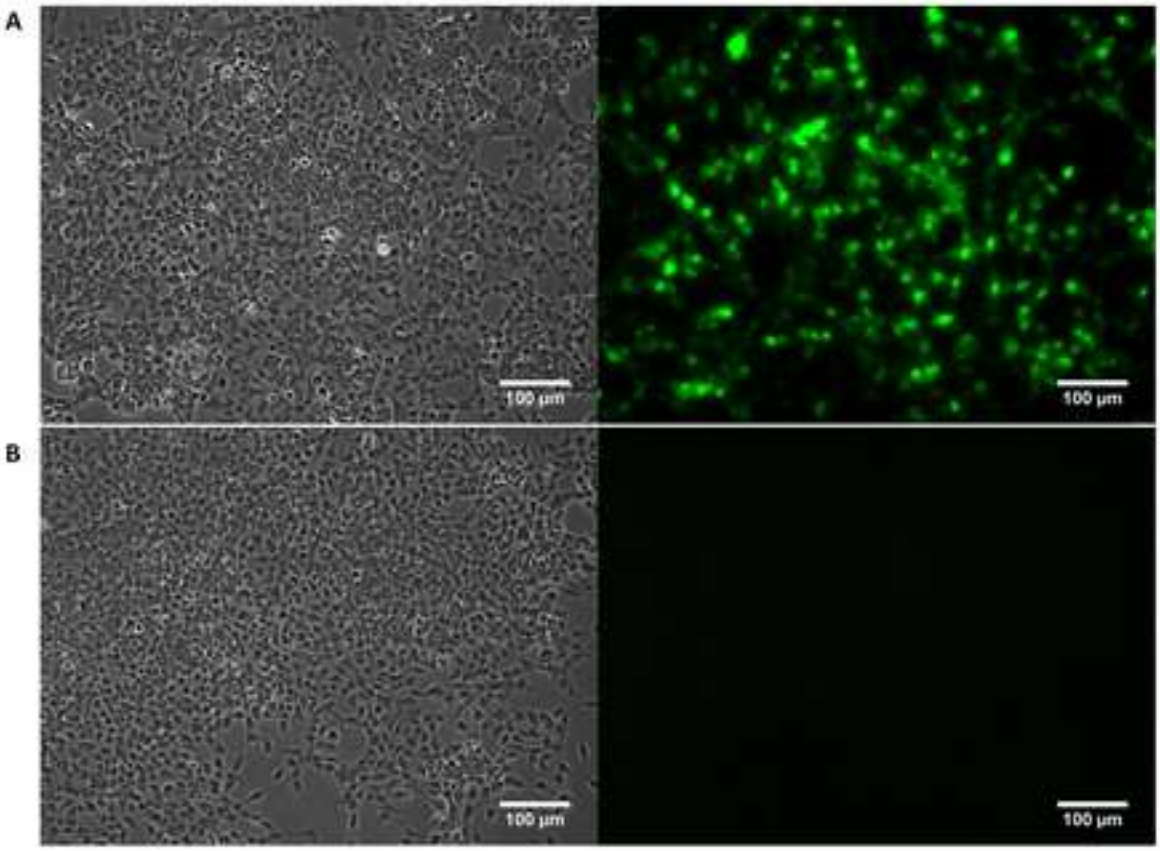
- Either convert to a one liner note, move the discussion to the discussion section or convert to imperative tense. The text has been modified to reduce redundancy and include only direct instructions in the imperative tense. Please, check step 5.3.
- Convert this to a note. Or use imperative tense. Another option is to remove the redundancy and bring out clarity by: Mix the gel components by swirling the tube by hand. Do not vortex since… The text has been modified to implement the suggestion. Please, check step 5.4.
- What is caution here. Include the caution statement after the step. Thank you for bringing this point to our attention. A caution statement has been added, as requested, after step 5.6.
- Please convert this to substeps and do not use dashes. Use complete sentences. We thank the editor for the feedback. The bullet point list was substituted by an additional Table (Table 7).
- Please see my comment for the title of the protocol. How do you perform the transduction in the mice? What concentration of the AAV is used? Controls if any? All these steps are relevant here. For how long you leave the mouse? When do you dissect the tissues and perform IHC.. Following the recommendation of the reviewer, the title of the protocol was modified to reflect the protocol.
- We cannot have commercial terms in the manuscript. Please move it to the Materials table. Any use of a commercial name has been removed from the text. Reference to the generic and commercial names, plus the relevant suppliers, is now made in the Materials Table.
- How are you sure that the protein obtain is viral protein only? Western blot etc data? We are grateful to the editor for the question. However, we would like to point out that using Silver Stain (or Colloidal Coomassie) to establish vector purity is widely used amongst vector labs. AAV capsids are known to be composed of three proteins, with distinct sizes, that give a characteristic pattern when run in SDS-PAGE. Thus, any additional bands are considered contamination, irrespective of the identity of the proteins. By using a Western Blot, which is more time consuming and expensive (due to the antibody steps) gives no significant advantage over the protocol described. Hence, we have left the text largely unaltered.

4. We have a limit of 10 pages for the protocol section. Please consider making some of the standard details as a supplementary materials section. In the actual version the revised protocol does not exceed 10 pages (including notes)
5. Please ensure that the protocol is in imperative tense throughout. we have ensured that the imperative tense is used throughout the manuscript.
6. Please ensure that there is no redundancy and the action steps are as crisp as possible. We have ensured that any redundant text has been removed o modified into additional steps or notes if necessary.
7. Please ensure that the highlighted section of the protocol has an associated representative result. The highlighted section has associated a representative result as

requested. Section 1 results are show in the Supplementary Figure 1, section 2 results are explained in the note after step 2.9.4. and step 2.9.14.

8. Once done please ensure that the protocol is no more than 10 pages and highlighted section is no more than 2.75 pages in length including heading and spacing The highlighted portion of the protocol is no longer than 2.75 pages (including heading and spaces, after excluding notes).

Supplementary Figure 1



HEK293T cell morphology visualized by phase contrast microscopy (left) and confirmation 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.

JoVE58960

VIB-KU LEUVEN CENTER FOR BRAIN & DISEASE RESEARCH

KU LEUVEN

To:
Vineeta Bajaj, Ph.D.
Review Editor JoVE

Leuven, September, 2018

Dear Doctor Bajaj,

Ref: Manuscript JoVE58960

On behalf of all the authors, I would like to thank you for handling out the manuscript 'Production of adeno-associated virus-based vectors for transgene expression in the central nervous system of adult mice after systemic delivery' that we submitted.

We have read the points that were raised by both reviewers and the editorial committee and found their critiques to be both considered and fair. Therefore, we have applied several changes in the manuscript and indicated these changes in a point-by-point response letter for the benefit of the reviewers.

We hope the manuscript is now suitable for publication in *JoVE* and remain available to address any additional concerns.

Sincerely,

Matthew G. Holt
Corresponding author
VIB-KU Leuven Center for Brain & Disease research.

The **editorial comments** have been addressed by the authors as follows:

-Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

All the authors have thoroughly proofread the manuscript to correct spelling and grammar issues.

-As the authors already paid for Open Access, please print and sign the attached Author License Agreement (ALA) with the Open Access checkbox checked.

The Author License Agreement (ALA) with the Open Access Checkbox checked has been uploaded.

-Please revise lines 426-428, 665-667 to avoid previously published text.

Lines 426-428, 665-667 have been modified to avoid previously published text.
In the revised manuscript the lines originally indicated as 426-428 are now lines 326-328.
Lines originally indicated as 665-667 are now lines 485-487.

-Please define all abbreviations before use.

All abbreviations have been defined prior to their use in the manuscript.

-JoVE cannot publish manuscripts containing commercial language.

All commercial language and company names have been removed from the manuscript.

-Please revise the protocol text to avoid the use of any personal pronouns.

The protocol text has been revised to avoid the use of personal pronouns.

-Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.).

In the manuscript are now included only actions to direct the reader. Actions are now described in imperative tense.

-Lines 188-227, 558-566, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the introduction/discussion about the protocol to the Introduction/Discussion. Please move the solutions, materials and equipment information to the Materials Table.

The protocol has been revised in order to contain exclusively discrete steps. Introduction/Discussion paragraphs, as well as solutions, materials and equipment information have been moved according to the reviewer suggestion. Therefore, new paragraphs have been added to introduction and discussion and have been highlighted.

-Please revise the Protocol steps (1.1, 1.9, etc.) so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

The protocol has been revised to describe only discrete steps in a maximum of 4 sentences per step. Sub-steps have been included when necessary.

-Lines 319-320, 523-524, 607-608: Please remove the embedded tables from the manuscript. Please upload tables individually to your Editorial Manager account as an .xls or .xlsx file and reference them in the protocol.

Embedded tables have been removed from the manuscript and individually uploaded in an .xls or .xlsx format. Tables have been properly referenced in the protocol.

-In the JoVE Protocol format, "Notes" (Tip in this manuscript) should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

The number of notes in the manuscript has been reduced. They have been only used when necessary to provide extraneous details, optional steps and recommendations that are not critical to the development of the methodology.

-Please consider moving some of the notes about the protocol to the discussion section. For example, include details in lines 321-329 in a numbered step.

The protocol section has been modified accordingly to the editorial recommendations.
New discrete steps have been added to the protocol starting from step 2.9.1.

-Please reference different panels of Figure 1 in the protocol where these steps are described.

The protocol has been modified in compliance with the editorial advices.

-Line 508: Table 3 does not exist. Please revise.

The manuscript has been revised to correct the issue.

-Table 1: Please use small x for dilution.

The manuscript has been revised accordingly.

-Please remove the title of tables from the uploaded table files. The information provided in the Figure and Table Legends after the Representative Results is sufficient.

The title of tables has been removed from the uploaded table files.

-Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We confirm that Dr. Melvin Y. Rincon retains copyright on the figures 4A, 4B included in the manuscript "Production of adeno-associated virus-based vectors for transgene expression in the central nervous system of adult mice after systemic delivery". The figures were previously published in "Widespread transduction of astrocytes and neurons in the mouse central nervous system after systemic delivery of a self-complementary AAV-PHP.B vector. Gene Therapy 25(2):83-92, 2018 Apr". The copyright policies of Springer Nature allow Dr. Melvin Y. Rincon to reuse the figures, provided proper referencing of the original work.

-Please provide the composition of the lysis buffer.

As with other solutions, materials and equipment, information on the composition of the lysis buffer are included in Table 1 (AAV purification and desalting).

-After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

As requested, we highlighted 2,75 pages of text. However, we believe that the desalting and concentration of the vector (step 3) would be of great interest to the reader. Therefore, we

would be grateful if it could be added to the video. Moreover, showing how the filters are loaded would be a great visual help to the reader.
Meanwhile, we ensured that the editorial comments "Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense" and "Please include all relevant details that are required to perform the step in the highlighting" have also been properly addressed.

Reviewer #1 comments have been addressed by the authors as follows:

Manuscript Summary:
The Manuscript "Production of adeno-associated virus-based vectors for transgene expression in the central nervous system of adult mice after systemic delivery" describes the production and purification of AAV vectors for in vivo use. The manuscript is well written and contains well-defined instructions for the production of AAV vectors. Overall, this protocol should prove very helpful for laboratories who would like to produce AAVs. However, prior to publishing a few minor points should be addressed.

We are thankful to the reviewer for the positive feedback.

Major Concerns:
no major concerns. The introduction needs some re-modelling tough.
The introduction has been remodeled to assume a clearer methodical cut. Moreover, an introduction to the methods employed in the protocol has been provided.

Minor Concerns:
- The abstract states that PHP.B did not have toxic side effects, however, recent reports by Wilson and group reported safety concerns in non-human primates. Although it is unclear whether the safety concerns were due to the vector or impurities thereof, this issue needs to be acknowledged and thus, maybe this sentence should be removed from the abstract and other parts of the text unless this paper is cited and properly discussed that we currently don't know the origin of the problem.
We thank you for pointing out this problem and we acknowledge the fact that this has been reported. Therefore, following reviewer advice the sentence has been removed.

- Accuracy of qPCRs for titration (as mentioned in the abstract). Although qPCRs can be very accurate, the authors should discuss that there remains an error range that experimenters should keep in mind (this could be part of the discussion)
We are grateful for your feedback. In the discussion, we added a section addressing the issue.

- Introduction: the introduction reads very much like an introduction from a review paper for AAVs rather than an introduction into a methods paper. Thus, the introduction should be altered to include more details (at least a paragraph or more) about how AAV vectors are currently produced, what different methods of transfection, harvesting and purification are used and advantages and disadvantages and major difficulties in AAV production (yields, purity and such) - some other parts of the introduction could be shortened

We revised the introduction and removed the unnecessary information. To comment on the alternative methods, the discussion was expanded. In particular, we added new information concerning the chosen methods of transfection and purification and other options currently available.

- The statement AAVs provoke little to no immune response is misleading. We recommend to use little or milder immune responses compared to other viral vectors.
Thank you for raising the point. We are in agreement with your recommendation and the sentence has been altered accordingly.

- Line 124 and following: If praising PHP.B for crossing of the blood brain barrier, AAV9 should at least be mentioned here...it should not sound like PHP.B is the only vector that crosses the blood brain barrier
Thank you for bringing this point to our attention. We have modified the section by mentioning AAV9 as another serotype which is able to cross the BBB, as recommended.

- Line 128/129: The sentence "In an AAV vector, ..." is confusing at the position where it was entered - it could also be deleted, or alternatively, should be moved to a different position
The introduction has been altered according to your recommendation and the section you indicated has now been removed.

- Line 147: needs to be remembered could be replaced by "should be kept in mind"
Thank you for your feedback. The paragraph has been remodeled.

- Discussion could go in more detail for factors that influence yield of production
Taking into consideration your comment, we have added a paragraph in the discussion section that handles about determining factor that could influence the final yield of the production.

- Line 198: it would be good to mention here the size of the 18 culture dishes here
Information on the size of the 18 culture dishes has been added to the manuscript in line 174.

- Line 224: for someone who never made AAVs, it might be unclear what E4, E2A and VA RNA is for...maybe one could add another sentence explaining this.
Thank you for bringing this point to our attention. Hoping to clarify the concept for the readers, a description of the role of E4, E2A and VA RNA has been added in line 152.

- The step from 1.2 to 1.3 is a bit confusing - from a mix for 18 plates to suddenly having 3 tubes...maybe one could add in brackets something like: 1 tube per 6 plates...
The modification you suggested has been inserted in the manuscript (step 1.5)

- 1.8: maybe it should be specified that the media is not changed
The modification you suggested has been inserted in the manuscript (step 1.10)

- 4.1.1 : the plasmid needs to be linearized is not correct, this sentence should be changed to "in this method, we use linearized plasmid as a standard and therefore, the plasmid needs to be cut..."
Thank you for your feedback. We inserted the recommended modification in line 305.

- 4.1.2: a sentence could be added that the efficiency of the digest should be verified on a gel...also, can the plasmid stock be prepared and then stored and re-used, or is it prepared fresh every time?
The modifications you suggested have been implemented in the step 4.1.3.

- For the qPCR, a tip could also be that if you want to compare two different preparations, you should try and use the same primers (ergo primers in the promoter) as different primers can have different amplification efficiencies.
Thank you for your comment. Hoping to add relevant information for the readers, we have introduced changes in the manuscript (lines 621-634).

- Representative Results: the first paragraph is a bit misleading - it is currently unclear how much better PHP.B is compared to AAV specifically in different mouse strains. It should at least be mentioned that the mouse strain could have an impact on the transduction efficiency with different AAVs...
Thank you for raising this point to our attention. The Representative Results section has been modified to include your suggestions.

- Line 677: the sentences should say "vector administration was PERFORMED via tail vain
Thank you for bringing the issue to our attention (line 504).

Figure and tables:
- Figure 1: we need a closer zoom in for e) and an arrow as to where the band is that needs to be pulled - this will help people to follow the protocol if they know how it looks after the spin
We have added a zoomed-in picture of the fraction that needs to be collected.

Discussion:
- The paragraph 758 - 763 could be in the introduction for example.
Many thanks for your feedback. Hoping to improve the clarity of the manuscript, the introduction and discussion paragraph have been reshaped.

- Discussion about passage number of 293 cells: where were those purchased and what was the passage when they were acquired? or does it only matter how long they were in culture?
Thank you for your comment. Information about the purchasing of HEK293T cells have been provided in the material table.

- Csf delivery should be mentioned as an alternative
Following your suggestions, the discussion has been modified by mentioning CSF as an alternative route of administration.

Reviewer #2 comments have been addressed by the authors as follows:

Manuscript Summary:

The manuscript entitled „Production of adeno-associated virus-based vectors for transgene expression in the central nervous system of adult mice after systemic delivery" by Shelly Fripont et al. Provides a full protocol of production and purification of two AAV serotypes including titration and quality/purity controls, leading all the way to systemic delivery (i.v. injection) of the viral vectors and detection of transgene expression in the CNS.
Although the technologies used are not completely novel, the manuscript displays a very informative and complete walkthrough for the whole procedure.
The steps in the protocol are well described and the list of materials would allow the reader to easily reproduce the AAV-production and utilization.
We are thankful to the reviewer for the positive feedback.

Major Concerns:
None

Minor Concerns:
I only have minor remarks on the manuscript and would suggest discussing a few additional aspects that are not directly related to the AAVs and transgene constructs provided here:

- It would be great to include perspectives for additional AAV serotypes in the discussion to give the reader some hints (where possible) if their AAV-serotype of choice may be suitable at least for production and purification as described here. E.g. AAV2/5 may be tricky on iodixanol gradients.
Thank you for bringing this point to our attention. The discussion has been modified to insert your suggestion (lines 614-620)

- Also for qPCR approaches for tittering it may be useful to include some additional promotor elements, or alternative sequence areas, that would enable titration of viruses with alternative constitutive (e.g. CAG, CMV, TK) or tissue specific propters (in CNS e.g. hSYN, CamKII etc.).
We are grateful for your suggestion. Hoping to provide useful information to the readers, we have added a tip in the discussion section (625-634).

- Also, there are some spelling mistakes in various positions, which easily can be addressed prior to publication
We thank you for pointing this out. The checked manuscript has been proof-read to address these mistakes.