Production and characterization of adeno-associated viral vectors

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The adeno-associated virus (AAV) is one of the most promising viral vectors for human gene therapy. As with any potential therapeutic system, a thorough understanding of it at the *in vitro* and *in vivo* levels is required. Over the years, numerous methods have been developed to better characterize AAV vectors. These methods have paved the way to a better understanding of the vector and, ultimately, its use in clinical applications. This review provides an up-to-date, detailed description of essential methods such as production, purification and titering and their application to characterize current AAV vectors for preclinical and clinical use.

INTRODUCTION

Overview of production

Numerous methods have been established to produce and characterize recombinant AAV (rAAV) vectors[1–16]. This manuscript provides detailed methods for laboratory-scale production, purification and characterization of rAAV utilized by our laboratory for preclinical and clinical applications. For example, our laboratory was the first to establish the triple transfection method (**Fig. 1**), which requires the use of (i) the adenovirus helper plasmid (pXX680), (ii) the AAV helper plasmid (pHelper) and (iii) the inverted terminal repeat (ITR) transgene cassette plasmid (pITR), which contains only the ITRs from the wild-type AAV (wtAAV) genome, eliminating 96% of the viral elements[1,4]. The advantage to this system is that coinfection with adenovirus is not needed. pXX680 supplies the adenovirus proteins (E1A, E1B, E4 and E2A) along with the adenovirus virus-associated RNAs required for helper functions[17]. pHelper encodes the wtAAV genome lacking ITRs to circumvent packaging of the WT genome. The replicating rAAV genome from pITR is packaged into preformed empty capsids within the nucleus of producer cells. rAAV is then harvested from the nuclei of transfected cells after 48–72 h, followed by purification from the cell homogenate. The purified vector is then characterized for genome titer, empty particle titer, infectious titer, transducing titer and integrity of the packaged genome before *in vitro* and *in vivo* studies are conducted. This becomes very important as more variants of AAV vectors are generated in the laboratory that have different infectious properties[18,19].

One of the major quandaries of the AAV field is that many of these protocols have not been standardized and used in every laboratory. For example, infectivity data gathered from rAAV purified from CsCl are different from those purified from iodixanol/heparin and column chromatography[20,21]. This becomes important when comparing transduction data from different serotypes both *in vitro* and *in vivo* among laboratories purifying and characterizing rAAV differently. Standardizing protocols and a series of universal vectors to be used as a reference for titering would make comparing data between laboratories possible and may clarify discrepancies in the literature.

Experimental rationale

Purifying rAAV using nuclear fractionation. The triple transfection method leads to the production of rAAV in the nuclei of producer cells. In the absence of infectious adenovirus, rAAV resides in the nucleus because a lytic phase is not initiated. Isolating rAAV from the nuclei increases overall titer and purity of the viral prep.

Choosing a purification scheme. Heparin columns can be used to purify rAAV serotype 2 vectors (rAAV2) from iodixanol owing to its ability to bind heparin[22]. A similar method can be used to purify AAV serotypes 4 and 5 utilizing mucin columns[23,24]. Other methods such as chromatography column purification have recently been established to purify AAV[20,25]. Discontinuous

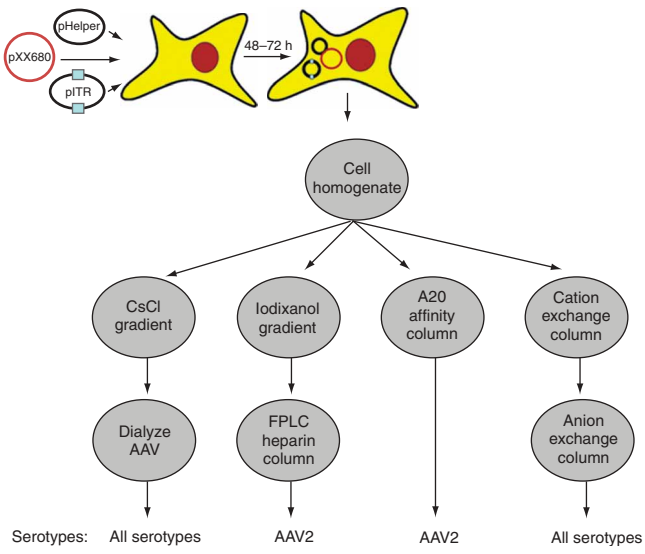


Figure 1 | Schematic diagram summarizing the production and purification of rAAV. rAAV is produced by transfection of three plasmids: transgene plasmid (pITR), the adenovirus plasmid (XX680) and the wild-type helper plasmid (pHelper). Forty-eight to seventy-two hours post-transfection, the cell homogenate is collected and purified utilizing various methods. The CsCl and iodixanol methods are the most commonly used and described in detail in this manuscript.

iodixanol gradients and CsCl can be used to purify all rAAV vectors and capsid mutants.

The main advantages of using CsCl gradient purification are that it enables the physical separation of full particles (AAV containing a genome) from empty particles based on their differences in density, and it is possible to purify all serotypes. Methods such as dot-blot analysis or quantitative PCR, used to assess the presence of viral genomes, are then carried out on each fraction of the gradient to identify the fraction(s) that contain AAV. However, there are disadvantages to using CsCl gradients to purify AAV. For example, this method is time consuming because two to three rounds of CsCl centrifugation must be carried out to get high-purity AAV for *in vivo* studies. In addition, dialysis of CsCl fractions containing AAV against a physiological buffer is necessary before *in vivo* analysis because CsCl can exert toxic affects on animals in the study. This process from start to finish can take up to 2 weeks because dot-blot and quantitative PCR analysis must be done on each fraction to find the peak fractions to place in the next CsCl gradient.

Characterizing the virus according to its physical properties or functionality. Dot blot analysis is used to determine the physical titer of genome-containing particles (per ml) using DNA probes specific for the transgene cassette. A positive signal in this assay indicates that genome-containing rAAV virions were produced. Even though this assay will not indicate if the virus is infectious or if the expression cassette is functional, this method is the most suitable when comparing (i) different serotypes or capsid mutant vectors, or (ii) single-stranded and self-complementary vectors. Establishing titers of the viral samples is crucial for future characterization and utilization of the vectors.

Visualizing the viruses using electron microscopy. The analytical techniques described in this protocol can be used to characterize the viral vectors according to their physical properties and functionalities. Electron microscopy (EM), on the other hand, allows a direct visualization of the viral particles. The glow discharge method described in this protocol is used to make the support film on the grid more hydrophilic. The process of passing electrical current through argon gas produces a glow of ionized argon atoms that bombard the surface of the plastic film, resulting in a clean, negatively charged hydrophilic surface. This process is advantageous in the case of viruses because it discourages artificial aggregation of particles on the surface of the grid and results in a more even stain with fewer gas bubble artifacts upon drying. When using electron microscopy to view AAV, it is important to use purified virus that is stored in low-salt conditions (post-dialysis). The low-salt conditions allow AAV to bind to the glow-discharged grid more efficiently, leading to a greater population of the AAV prep adhering to the grid.

Analyzing the integrity of viral genomes. The viral dot blot protocol described below (Steps 13–37) is the method for determining physical viral titer in a vector preparation. However, incomplete genomes that are packaged in virions are also detected, and their presence contributes to the determination of the physical viral titer. In order to determine the purity of the virions that contain the full-size genome, viral genomes should be extracted from virions and analyzed by using alkaline gel electrophoresis under denaturing conditions. This protocol has been slightly modified compared with traditional alkaline electrophoresis so

TABLE 1 | Summary of methods to be used for characterizing rAAV vectors.

AAV helper plasmid	Identified by	Obtaining plasmids encoding capsid sequences	*In vivo* (cell types that are transduced most efficiently by each serotype)
AAV1	R.W. Atchison[33]	R.J. Samulski[24] (UNC vector core facility)	Vascular endothelium[34] Muscle[35]
AAV2	M.D. Hoggan[36]	R.J. Samulski[17,24] (UNC vector core facility); also commercially available from Stratagene (AAV Helper-free System; cat. no. 240071)	Brain[37] Liver[29] Muscle[38]
AAV3	M.D. Hoggan[36]	R.J. Samulski (UNC vector core facility)[24]; S. Muramatsu[59]	Cochlear inner hair cells[39]
AAV4	W.P. Parks[40]	R.J. Samulski[24] (UNC vector core facility); J.A. Chiorini[41]	Ependyma and astrocytes[42]
AAV5	U. Bantel-Schaal[43]	R.J. Samulski[24] (UNC vector core facility); J.A. Chiorini[44]	Lung epithelial cells[45] Retina[46] Brain[47] Arthritic joint tissue[48]
AAV6	E.A. Rutledge[49]	R.J. Samulski (UNC vector core facility); E.A. Rutledge[49]	Muscle[50] Lung[51]
AAV7	G.P. Gao[52,53]	G.P. Gao	Muscle[54]
AAV8	G.P Gao[52,53]	G.P. Gao	Liver[53] Heart[55] Pancreas[56]
AAV9	G.P. Gao[52,53]	G.P. Gao	Heart[57]
AAV10	S. Mori[58]	S. Mori	n/a
AAV11	S. Mori[58]	S. Mori	n/a

Depending on the experimental needs, the best assays to determine rAAV titers should be used. n/a; non applicable.

TABLE 2 | Summary of assays to be used for characterizing AAV.

Assay	Type of characterization	Unit determination
Dot-blot assay and quantitative PCR	Physical characterization for the presence of genomes in the viral particles	Viral genome–containing particles/ml (vg/ml)
Infectious center assay	Functional characterization for the infectivity of the viral particles	Infectious units/ml (IU/ml) (MOI = multiplicity of infection)
Transduction assay	Functional characterization of the ability to transduce an infected cell	Transducing units/ml (TU/ml)
Electron microscopy	Physical observation of the viral particles as a population	Full-to-empty particle ratios
Alkaline electrophoresis	Physical characterization of the size and integrity of genomes	Viral genome–containing particles/ml (vg/ml)
ELISA	Physical characterization of amount of viral capsids	Particles/ml (p/ml)

that the self-complementary AAV (scAAV) vector genome is denatured and analyzed properly.

Characterizing the integrity of assembled virions. Isolating DNA from AAV particles via direct lysis of the virion with the dot blot method described below does not discriminate between incompletely assembled virions and completely assembled virions that can effectively protect their genomes from DNase I/benzonase digestion and carry out a successful infection. Intact AAV particles that have assembled correctly have the topology necessary to successfully bind to their cognate receptor, enter the cell, traffic through the cytoplasm and eventually deliver the genome into the nucleus, whereas incompletely assembled particles may lack this ability. The replication assay described below enables us to assess the genome content and integrity of infectious particles that have entered the cells and released their genomes within the cell nucleus. This assay is of importance when characterizing rAAV packaging larger in size than WT genomes[26]. Because this assay only qualitatively characterizes the infectivity of a viral prep, an infectious center assay must be carried out to quantitatively determine the infectivity of a viral prep.

Quantifying vector infectivity. Unlike dot blot and EM analysis, the infectious center assay is a functional assay to determine the infectivity of a vector preparation. The infectious center assay is distinct from the more common plaque assay. We utilize this method because wtAAV and rAAV do not form plaques, but this method can determine the infectivity of the viral particles.

Application of rAAV vectors. Because different rAAV serotype vectors have different tissue tropisms, it is important to identify the best serotypes to be used in an experiment. It is important to note that the vectors could behave differently when used *in vitro* (cell culture) versus *in vivo* (animal) because the behavior of a cell line could be significantly different from the parental tissue cell type. **Table 1** summarizes cell/tissue types, identified in animal models, that are transduced efficiently by various AAV serotypes.

The infectivity of an rAAV prep is assessed on the C12 cell line (a derivative of HeLa cells)[27]. It is crucial to analyze all virus samples and controls to be used in a given experiment in the same assay. Despite the fact that infectious units are a better representation of viral titer, a drawback of the infectious center assay is that only AAV serotype 2 or cross-packaged serotype capsids with AAV2 ITR can be tested with the C12 cells. Because the infectivity of all serotypes on the C12 cell line is different, viral genome particle numbers, instead of infectious titers, should be used when cross-comparing multiple serotypes in a study. However, infectivity among vector preparations of the same serotype could be cross-compared. If infectious units need to be determined for a viral prep on a particular cell line, similar procedures can be performed, as described in the PROCEDURE, with the addition of wtAAV virus (which provides the corresponding *rep* and *cap* that can recognize the ITR in the rAAV genome) to the cells to allow genome replication. Alternatively, a new cell line with a known copy number of AAV serotype *rep* and *cap* genes integrated into the chromosome can be developed for such an assay.

A summary of techniques described in this protocol is listed in **Table 2**.

MATERIALS

REAGENTS

- HEK-293 cells (ATCC, cat. no. CRL-1573) maintained at 37 °C in a 5% CO_2 atmosphere
- DMEM (Cellgro, cat. no. 10-013-CV) supplemented with 10% FBS (Gibco, cat. no. 26140-079) and 1× penicillin-streptomycin (Gibco, cat. no. 15070-063).
- Serum-free DMEM
- AAV and adenovirus plasmids: pXX680 (adenovirus helper plasmid)[1], pHelper (AAV serotype–specific *rep* and *cap* plasmid), pSub201 and pHpa-trs-SK (pITR)[28,29]. These plasmids are available for purchase at the University of North Carolina (UNC) Vector Core Facility (http://www.med.unc.edu/genether/welcome.htm).
- 2.5 M $CaCl_2$ (Fisher, cat. no. C614-500)
- 5 N and 0.5 M NaOH (Malinckrodt, cat. no. 7680-06)
- DNase I (10 mg ml^{-1}) (Sigma, cat. no. AMP-D1)
- Sorbitol (Sigma, cat. no. S6021-1KG)
- Optiprep (60% iodixanol) (Axis-Shield PoC AS, cat. no. 1114542)
- Cesium chloride (CsCl), optical grade (Budenheim Gallard-Schlesinger, cat. no. 612551)
- 0.5 M NaOH (Mallinckrodt, cat. no. 7680-06)
- rAAV plasmid (pITR) used to make recombinant virus (available for purchase at UNC Vector facility)
- 0.4 M Tris-HCl, pH 7.5
- Chloroform (Mallinckrodt, cat. no. 4432)

- Glycogen (Roche, cat. no. 901393)
- 10 M ammonium acetate (Mallinckrodt, cat. no. 3272-04)
- 100% and 70% ethanol (AAPER Alcohol and Chemical Company, cat. no. 010405)
- Radiolabeled probe to transgene prepared according to manufacturer's instructions (Roche Random Primer Labeling Kit (cat. no. 11004760) or RocheDIG-DNA Labeling and Detection Kit (cat. no.11175033910)
- 2% uranyl acetate (ChemAce, cat. no. 541-09-3)
- 3MM Whatmanchromatography paper (cat. no. 3030917)
- 10% SDS (Sigma, cat. no. 71725)
- Proteinase K solution (10 mg ml^{-1}) (Sigma, cat. no. P4850)
- Phenol/choloroform/isoamyl alcohol (24:24:1) (Invitrogen, cat. no. 15593-031)
- Isopropanol (Mallinckrodt, cat. no. 3031)
- 1 M Tris-EDTA supplemented with 50 μg ml^{-1} RNase A (Sigma, cat. no. R6513)
- *Dpn* I (New England Biolabs, cat. no. R0176S)
- Adenovirus (for example, dl309, which is adenovirus serotype 5, available for purchase at the UNC Vector Core facility)
- C12 cell line (HeLa cells with AAV2 *rep* and *cap* genes integrated into the chromosome)[7,27,30]
- Trypsin-EDTA (Gibco, cat. no. 25300-054)

REAGENT SETUP

The following reagents can be stored at 4 °C for up to 1 year:

2× HEPES-buffered saline 0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HPO_4; titrate to pH 7.05 with 5 N NaOH

Polyethylenimine (PEI) linear MW 25,000 (Polysciences, cat. no. 23966) Dissolve 1 mg of PEI in 1 ml of 1× PBS adjusted to a pH between 4 and 5 with 12 N HCl

High-salt buffer (500 ml) 20 mM HEPES, 1.5 mM $MgCl_2$, 1.2 M KCl, 25% (vol/vol) glycerol; filter-sterilize

Low-salt buffer (500 ml) 20 mM HEPES, 1.5 mM $MgCl_2$, 20 mM KCl, 25% glycerol (vol/vol); filter-sterilize

Hypotonic buffer (500 ml) 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 175 mg of spermine; filter-sterilize

10× restore buffer (200 ml) 62.5% sucrose (wt/vol) in hypotonic buffer; filter-sterilize

Low-salt buffer 2.5 mM KCl and 1 mM $MgCl_2$ in 1× PBS

High-salt buffer 2.5 mM KCl, 1 mM $MgCl_2$ and 1 M NaCl in 1× PBS

The following reagents can be stored at 4 °C for up to 2 weeks:

Discontinuous iodixanol gradient[21] Consists of a 17% layer (50 ml 10× PBS, 0.5 ml 1 M $MgCl_2$, 0.5 ml 2.5 M KCl, 100 ml 5 M NaCl, 125 ml Optiprep (iodixanol) and H_2O to 500 ml), a 25% layer (50 ml 10× PBS, 0.5 ml 1 M $MgCl_2$, 0.5 ml 2.5 M KCl, 200 ml Optiprep, 1 ml of 0.5% (wt/vol) phenol red and H_2O to 500 ml), a 40% layer (50 ml 10× PBS, 0.5 ml 1 M $MgCl_2$, 0.5 ml 2.5 M KCl, 333 ml Optiprep and H_2O to 500 ml), and a 60% layer (500 ml Optiprep, 0.25 ml 0.5% phenol red, 0.5 ml 1 M $MgCl_2$ and 0.5 ml 2.5 M KCl).

The following reagents can be stored at room temperature (25 °C) for up to 1 year:

Church buffer 1% BSA (wt/vol), 1 mM EDTA, 0.5 M $NaPO_4$ pH 7.5, 7% SDS (wt/vol)

Low-stringency wash buffer 2× SSC, 0.1% SDS (wt/vol)

High-stringency wash buffer 0.1× SSC, 0.1% SDS (wt/vol)

6× SSC buffer 0.9M NaCl, 90 mM sodium citrate

Denaturation solution 1.5 M NaCl, 0.5 M NaOH

Neutralization solution 0.5 M NaCl, 0.5 M Tris-HCl pH 7.5

DMEM with 10% FBS + penicillin/streptomycin + 0.5 mg ml^{-1} G418 (Gibco, cat. no. 10131-027)

The following reagents must be made fresh each time:

DNase digestion buffer 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 U ml^{-1} DNase I

Proteinase solution 1 M NaCl, 1% (wt/vol) *N*-laurylsarcosine (Sarkosyl), 100 μg ml^{-1} proteinase K

Agarose gel 1% agar (percentage varies), 50 mM NaCl, 1 mM EDTA

Alkaline electrophoresis buffer 50 mM NaOH, 1 mM EDTA

Alkaline transfer buffer 0.4 M NaOH

Hirt solution 0.01 M Tris-HCl pH 7.5 and 0.1 M EDTA

6× loading dye for alkaline electrophoresis 0.4 M NaOH, 5 mM EDTA, 18% Ficoll (wt/vol), xylene cyanol (enough to add color for easy loading). ▲ CRITICAL This buffer must be made fresh immediately before use.

EQUIPMENT

- Sonicator (Branson Sonifier 250, VWR Scientific)
- Refractometer (Leica Mark II, Abbe Refractometer)
- Beckman Quick-Seal polyallomer (16 × 76 mm) centrifugetubes (cat. no. 342413)
- Ultracentrifuge (for example, Sorvall Discovery 90SE or Beckman Optima TLX)
- Tabletop centrifuge (for example, Sorvall RT 6000D)
- Sorvall NVT65 rotor(or equivalent)
- Sorvall TH-641 rotor (or equivalent)
- Beckman SN434 TLA 120.2 rotor for ultracentrifuge (or equivalent)
- Beckman Tube Sealer
- Gradient maker (PumpPro, Watson Marlow)
- UNO-S1 heparin columns (Amersham, cat. no. 17-0406-01)
- FPLC (fast performance liquid chromatography)
- 15-cm tissue culture plates
- 1.7-ml polystyrene microfuge tubes
- Slide-A-Lyzer Dialysis Cassette with 7,000 molecular weight cut-off (MWCO) and 0.5–3 ml capacity (Pierce, cat. no. 66370)
- Untreated 96-well plate (e.g., Fisherbrand Clear Polystyrene 96-well plates, Fisher cat. no. 12-565-501)
- 50 °C water bath or incubator
- Dot-blot apparatus (Schleicher and Schuell, cat. no. 10-447-900)
- 0.45-μm nylon membrane (Hybond-XL, Amersham, cat. no. RPN 222S)
- STORM PhosphorImager (Amersham, STORM 860) and ImageQUANT densitometry software
- Scintillation counter (Beckman LS 6500)
- Microsoft Excel
- Zeiss EM 910 electron microscope
- 400-mesh carbon grid
- Argon glow discharge machine (Technics Hummer X Sputter Coater)
- Electrophoresis apparatus
- Piece of glass (the size of the gel tray)
- 10-cm Petri dishes
- Rubber policeman (cell scraper)
- Polycarbonate centrifuge tubes
- 48-well plate
- 37 °C tissue culture incubator
- Infectious center assay apparatus

PROCEDURE

AAV production and purification

1| Twenty-four hours before transfection, split confluent 15-cm plates of HEK-293 cells 1:3 into 20 15-cm plates[31].

2| Three to four hours before transfection, remove the medium from the cells and replace with fresh medium.

3| At this stage, transfections can be performed using either calcium phosphate (A) or polyethylenimine (B).

(A) Production of rAAV utilizing calcium phosphate transfection

(i) Set up transfection mix in the following order in 15-ml polystyrene tubes (the following volume is used to transfect four 15-cm plates):

Reagent	Amount
pXX680	90 μg
pHelper	30 μg
pITR	30 μg
2.5 M $CaCl_2$	0.4 ml
ddH_2O	Add to 4 ml
2× HEPES	4 ml
Total	8 ml

(ii) Pipette the transfection mix up and down approximately three times. Incubate at room temperature until precipitate forms.
(iii) To determine the presence of precipitate, remove 10–20 μl of the transfection mix and view under a microscope at 10×. When precipitate has formed, pipette 2 ml dropwise into each of the four 15-cm plates of HEK-293 cells.
(iv) Place cells in an incubator set at 37 °C with 5% CO_2 atmosphere. When using freshly prepared transfection reagents, transfecting a plasmid encoding GFP as opposed to the pITR cassette is necessary to test if the reagents will transfect cells efficiently (50%–70%).
(v) Twenty-four hours post-transfection, remove the medium from the transfected cells and replace with fresh medium.
(vi) Forty-eight to seventy-two hours post-transfection, harvest virus from the HEK-293 cells as described in Steps 4–11.

(B) Polyethylenimine (PEI) transfection

(i) Set up the transfection mix by adding the following into a 1.7-ml microcentrifuge tube (the medium will turn yellow–orange in color owing to the acidity of the PEI added to the transfection mix).

Reagent	Amount
pXX680	12 μg
pHelper	10 μg
pITR	6 μg
Serum-free medium	500 μl
PEI (1 mg ml^{-1})	110 μl

(ii) Vortex the mixture briefly and let sit at room temperature for 5 min. Add the transfection mix to a single 15-cm plate of 293 cells and distribute throughout the medium by briefly swirling the plate. The table above gives the calculated amounts for a single 15-cm plate of HEK-293 cells.
(iii) Forty-eight to seventy-two hours post-transfection, harvest the virus from the HEK-293 cells as described in Steps 4–11.

Isolation of rAAV from the nuclei of producer HEK-293 cells

4| Using a rubber policeman or a cell scraper, scrape the transfected HEK-293 cells from each 15-cm plate and place in centrifuge tubes. Size of centrifuge tube depends on the number of plates used.

5| Pellet the HEK-293 cells at 200*g* for 5 min at 4 °C (using, for example, the Sorvall RT 6000D).

6| Decant the medium from the HEK-293 cells and wash the cells with 1× PBS followed by centrifugation at 200*g* at 4 °C.

7| Decant the 1× PBS from the cell pellet.

8| Gently resuspend the cell pellet in a volume of hypotonic buffer (see REAGENT SETUP) five times the packed cell volume, and incubate on ice for 10 min.

9| Add 0.11 volumes of 10× restore buffer (see REAGENT SETUP) and mix gently via pipetting.

10| Transfer the contents to a 40-ml Kontes homogenizer and homogenize for 12 strokes (gentle on the downstroke).

11| Centrifuge the nuclei down at 500*g* for 10 min in a 50-ml conical tube. The nuclear pellet should be approximately 1 ml per ten 15-cm plates.

Gradient purification of rAAV

12| At this point, rAAV can be purified using a discontinuous iodixanol gradient (A) or by using a CsCl gradient (B).

(A) Purification using discontinuous iodixanol gradients

(i) Completely resuspend the nuclei in 1 ml of low-salt buffer (see REAGENT SETUP) per ten 15-ml plates (20 plates = 2 ml).

(ii) Transfer the contents to a 15-ml conical tube.

(iii) Quickly pipette 6 ml of high-salt buffer (see REAGENT SETUP) into the 15-ml conical tubes containing the nuclei in low-salt buffer. DO NOT INVERT or mix because the viscous lysate will stick to the sides of the tube.

(iv) Incubate for 5 min on ice.

(v) Sonicate each for 1 min to break up the genomic DNA. The Branson Sonifier should be set to duty cycle 40 and output 5.

(vi) Pour the sonicated contents into an ultra-clear (14 × 89 mm) Beckman centrifuge tube.

(vii) Clarify the lysate by centrifuging at 25,000*g* (12,000 rpm in a TH461 rotor, for example) for 45 min (in, for example, a Sorvall Discovery 90SE).

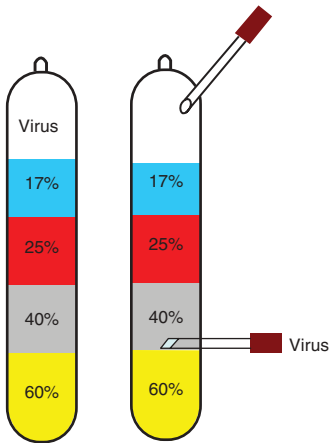


Figure 2 | Illustration of iodixanol gradients before and after centrifugation. rAAV is isolated from the 40%–60% interface utilizing a syringe. A hole is placed at the top of both gradients to enable extraction.

(viii) During the 45-min centrifugation, begin setting up the discontinuous iodixanol gradients using the gradient maker (PumpPro, Watson Marlow). At a very low pump speed or 10 rpm (to avoid mixing of the layers), begin pumping 2 ml of the 17% layer into Beckman Quick-Seal 16 × 76 mm ultracentrifuge tubes followed in turn by the 25%, 40% and the 60% iodixanol layers (2 ml each). This should leave ~3 ml above the 17% layer for the cellular lysate.

(ix) Using a 3-ml or 5-ml syringe with an 18-gauge needle, drip the centrifuged lysate from Step 14 onto the top of the 17% layer of the discontinuous iodixanol gradients very slowly and gently.

(x) Weigh the tubes and be sure that they are balanced. Use high-salt buffer or 1× PBS to balance the tubes.

(xi) Seal the Beckman Quick-Seal 16 × 76 mm ultracentrifuge tubes containing the discontinuous gradients with the sample using the Beckman Tube Sealer.

(xii) Centrifuge the samples for 1 h at 402,000*g* (for example, 65,000 rpm in a Sorvall Discovery 90SE using an NVT65 rotor). After centrifugation, the rAAV will collect or band at the interface of the 40%–60% interface.

? TROUBLESHOOTING

(xiii) Pierce the tube near the top of the sealed tube containing the sample with a needle to allow air to flow in. Using a 3-ml syringe and a 21-gauge needle, collect 1.5 ml of the 40%–60% interface (**Fig. 2**). (Do this by placing the needle through the side of the sealed tube at the interface and collecting into the syringe.)

? TROUBLESHOOTING

(xiv) To purify rAAV2 using FPLC, inject 10 ml of virus in iodixanol into the FPLC over a heparin column. Use a preset program to control the FPLC that allows the low-salt buffer to be pumped through the column, followed by a gradual increase in high-salt buffer in a linear relationship. The virus is eluted from the heparin column when the salt concentration reaches 0.4 M NaCl, and the virus is collected in tubes via a fraction collector.

(xv) Identify the peak fractions containing the rAAV2 vector using the UV detector in the FPLC.

(xvi) Dialyze the peak fractions (three times every 3 h in 1,000 times the volume of the virus volume) against 1× PBS + 5% Sorbitol-in Slide-A-Lyzer dialysis cassettes.

(xix) Remove the sample from the dialysis cassettes and aliquot into several tubes for storage at −80 °C.

(B) CsCl gradient purification. This method can also be used to purify all AAV serotypes. The buoyant density of AAV in CsCl, when packaging a WT-size genome of 4.7 kb, is 1.41 g cm^{-3}.

(i) Resuspend the nuclear pellet in 10 ml of ddH_2O and keep on ice.

(ii) Sonicate each sample for 25 pulses with a Branson Sonifier set at duty cycle 50 and output 5.

(iii) Add 100 µl of DNase (10 mg ml^{-1}) to the sonicated samples and incubate at 37 °C for 30 min to 1 h.

(iv) Bring the sample volume up to 11 ml with ddH_2O.

(v) Add approximately 6.5 g of CsCl to the sample and vortex into solution.

(vi) Remove approximately 20 µl from the sample to determine the density of the CsCl gradient using a densitometer/refractometer. The density of the CsCl gradient should be approximately 1.41 g cm^{-3} based on refractive index (f_r = 1.3722).

(vii) Load the sample into Beckman Quick-Seal polyallomer (16 × 76 mm) centrifuge tubes and use pre-made CsCl solution with a density of 1.41 g cm^{-3} to bring the sample volume to the top of the centrifuge tube.

(viii) Balance all samples before sealing the Quick-Seal tubes using the Beckman Quick Sealer.

(ix) Centrifuge sealed tubes at 402,000*g* for at least 5 h (for example, 65,000 rpm in a Beckman NVT65 rotor in a Sorvall Discovery 90SE centrifuge).

(x) Place a 21-gauge needle through the bottom side of the centrifuge tube and then pierce a hole in the top of the centrifuge tube using an 18-gauge needle (**Fig. 3**). The contents of the CsCl gradient will begin to flow through the 21-gauge needle. Collect 1-ml fractions in 1.7-ml microcentrifuge tubes. Approximately 12 fractions will be collected when isolating samples from Beckman Quick-Seal polyallomer (16 × 76 mm) centrifuge tubes.

(xi) The peak fractions (of which there are usually two) are found when each fraction is probed for the transgene of interest using the dot-blot method described below. If you intend to use the viral preparation for *in vivo* analysis, a second round of CsCl purification will be needed. Load peak fractions identified by dot blot into a CsCl gradient (density 1.41 g cm^{-3}) and centrifuge them as described in Step ix.

(xii) Dialyze the peak fractions, as identified by dot blot, against 1× PBS + 5% Sorbitol in Slide-A-Lyzer dialysis cassettes.

(xiii) Remove the sample from the dialysis cassettes and aliquot into several tubes for storage at –80 °C.

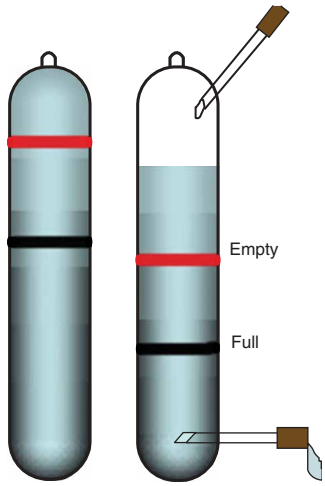


Figure 3 | Illustration of CsCl gradients immediately after centrifugation and the fractionation process used to isolate virus from the gradient.

Dot blot for titering rAAV viral genome–containing units

13| Prepare an experimental plan or template for the position of single-stranded AAV (ssAAV) and/or scAAV samples, controls (positive and negative) and DNA standards in duplicate in a 96-well format. (The samples will initially be prepared in microcentrifuge tubes, but the samples will be loaded onto a 96-well dot-blot apparatus at the end of the procedure.)

14| Place 5 µl of each sample into a microcentrifuge tube. Add 100 µl of DNase digestion buffer to each sample and incubate 1 h at 37 °C. This step digests the input DNA and any replicated DNA that may not have been packaged into virions.
▲ **CRITICAL STEP** If viruses are in CsCl salt, volume of viruses should be kept minimal because a high concentration of CsCl salt can interfere with the DNase reaction and affect the accuracy of titering the viral genomes.

15| Terminate the digestion by adding 4 µl of 0.5 M EDTA. Mix well, because the DNase activity must be completely inhibited before adding the proteinase solution; otherwise, the viral genomes inside the capsids will be digested, and the titer determined will be inaccurate.

16| Release virion DNA by adding 120 µl of proteinase solution. Incubate a minimum of 2 h at 50 °C. This step liberates the DNA that is encapsidated.
■ **PAUSE POINT** Alternatively, incubate the samples overnight at 50 °C.

17| To purify the DNA from the DNA–protein mixture, add 250 µl phenol/chloroform (24:1) to the digestion mixture. Mix well and spin down at maximum speed on tabletop centrifuge for 5 min at 4 °C.
▲ **CRITICAL STEP** Failure to remove protein contamination will interfere with DNA binding to the positively charged nylon membrane and will affect the accuracy of titer.

18| Carefully transfer the aqueous phase of the samples into a clean microcentrifuge tube.

19| Add 1 µl of 20 mg ml^{-1} glycogen and one-quarter volume of 10 M ammonium acetate to the samples. Then add 2.5 volumes of 100% ethanol to precipitate the viral DNA. Incubate samples at –80 °C for 2 h. Alternatively, incubate samples on dry ice for 30 min.

20| Centrifuge samples for 20 min at 4 °C at maximum speed in a tabletop centrifuge. Discard the supernatant carefully. The DNA carrier glycogen should precipitate the viral DNA to a visible pellet. Remove the last droplet of solution by pipette if necessary to avoid disturbance of the DNA pellet.

21| Wash the pellet with 500 µl of 70% ethanol and then centrifuge at maximum speed on tabletop centrifuge for 5 min at 4 °C. Air-dry the DNA pellet. Alternatively, dry the DNA pellet using a speed-vac for 5 min.

22| Dissolve the DNA pellet with 200 μl of TE and evenly split each sample into two tubes.

23| Perform six twofold serial dilutions of each tube of sample with TE. Each diluted sample should yield a final volume of 50 μl.

24| Denature viral DNA by adding 120 μl of 0.5 M NaOH to each tube and incubating 10 min at room temperature. Transfer sample to a 96-well plate according to the positions planned.
▲ **CRITICAL STEP** Failure to denature DNA completely will affect the efficiency of DNA probe binding to the viral genomes. This step is especially important for analyzing scAAV vectors.

Prepare standards

25| Prepare 1 μg of linearized plasmid (pITR) by restriction digest. Dilute the digested DNA into 100 μl TE at a concentration of 10 ng/μl to use for DNA concentration standards. Make a twofold dilution series in TE buffer (pH 7.5) by adding 10 μl DNA to 90 μl TE buffer into the first tube of the dilution series (D1). Add 50 μl TE to a second tube (D2), and transfer 50 μl of DNA from D1 into D2. Successively dilute the rest of the series to yield a total of seven dilutions. Remove 50 μl of diluted DNA from D7.

26| Denature DNA standards by adding 100 μl of 0.5 M NaOH/1 M NaCl to each tube and incubating at room temperature for 10 min. For each dilution, 30 μl of denatured standard will be loaded into the dot-blot apparatus in duplicates (10 ng, 5 ng, 2.5 ng, 1.25 ng, 625 pg, 312.5 pg and 156.3 pg) in Step 29.

Perform hybridization

27| Equilibrate nylon membrane in 0.4 M Tris-Cl, pH 7.5.

28| Prepare a dot-blot manifold apparatus with two pieces of pre-wet Whatman paper and a pre-wet nylon membrane.

29| Add the denatured DNA samples (170 μl volume for each sample) and DNA standards (30 μl of each serial dilution (D1 to D7), which yields standards of 10 ng, 5 ng, 2.5 ng, 1.25 ng, 625 pg, 312.5 pg and 156.3 pg) to the assembled dot-blot apparatus. Allow the samples and standard to incubate on the membrane for 5 min before applying the vacuum for 15 min.

30| Disassemble the apparatus, remove the membrane and wash for 5 min with gentle shaking in 10 ml of 0.5 M NaCl/ 0.5 M Tris-Cl, pH 7.5.

31| UV cross-link the membrane.
■ **PAUSE POINT** Membrane can be stored indefinitely at this point.

32| Hybridize the membrane with hybridization buffer. Then, probe the membrane with a radiolabeled probe (Roche Random Primer Kit) or digoxin-labeled probe (Roche DIG DNA Labeling and Detection Kit) specific for the rAAV sequences using Church buffer (see REAGENT SETUP).
! **CAUTION** The probe should be limited to the transgene cassette and should not include the plasmid backbone or ITR sequences. See Steps 36–37 for further explanations.

33| Probe the DNA membrane overnight (12 h) with a ^{32}P-labeled probe specific for the transgene cassette in Church hybridization buffer.

34| Wash the membrane three times with low-stringency wash buffer (8 min each wash) and twice with high-stringency wash buffer (20 min each wash).

35| For radioactive hybridization, place the membrane against the film and expose. After developing the film, align the spots, excise the regions of the membrane and quantify in a scintillation counter. Alternatively, place the membrane into a PhosphorImager cassette for 30 min (longer if signal is weak) (**Fig. 4**), analyze the exposed cassette by STORM and ImageQUANT densitometry software and export the numerical values to an Excel file for further quantifications. Chemiluminescence can also be used to detect signals for the digoxin-labeled hybridization method.

Quantification of dot-blot analysis

36| Calculate the number of molecules represented in the serially diluted plasmid standards as shown below. This calculation method is used to determine the number of genome-containing particles in a viral preparation. The detailed explanation can be applied to both conventional single-stranded rAAV (rAAV) and scAAV vectors. The most important parameter in this calculation is to know the size of the terminal repeat (TR) plasmid used in generating the rAAV vector.
1 μg of 1,000-bp double-stranded (ds) DNA $= 9.1 \times 10^{11}$ molecules (according to New England Biolabs), so 1 μg of 1,000-base single-stranded (ss) DNA $= 1.82 \times 10^{12}$ molecules. For example, if the TR plasmid is 7,200 bases, 1 μg of 7,200-base DNA $= 2.5278 \times 10^{11}$ molecules, and 1 ng of 7,200-base DNA $= 2.5278 \times 10^{8}$ molecules.

The conversion of amount of standard DNA (in ng) to number of molecules is crucial. Because the full sequence of the TR plasmid is used as a standard, DNA sequence from the bacterial backbone (**Fig. 5**, black) also contributes to the total weight of the DNA. Therefore, by converting the weight of the standards to number of molecules, a direct comparison between the number of molecules in the standard DNA and the unknown samples can be performed. It is important to take into consideration that the plasmid standards are double-stranded, whereas the rAAV virions harbor only a single strand.

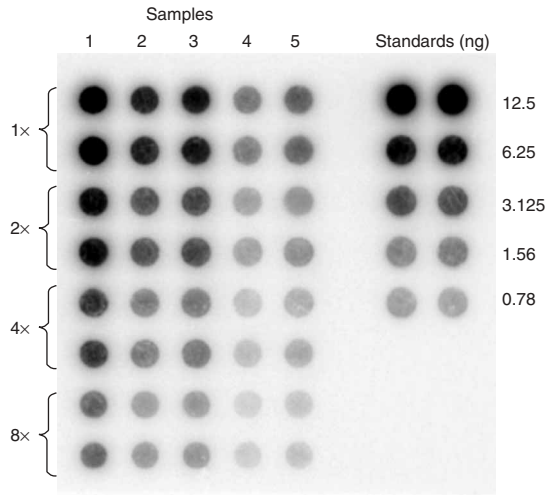


Figure 4 | Dot blot of five rAAV preparations. Serial twofold dilutions (1×, 2×, 4×, 8×, etc.) of the isolated viral genomes are done to ensure that at least two of the dilutions fall into the range of the linearized transgene standards. Viral titers are determined by densitometry.

37| Determine the viral sample titer. A standard curve can be generated by plotting the radioactive signal (y-axis) and the number of molecules from the standards (x-axis) (**Fig. 6**). Because the standards were serially diluted twofold, the radioactive signal should yield a twofold difference, and thus their relationship should be linear. A linear best-fit line (or trendline in Excel) should be generated, and the equation $y = mx + b$ is the mathematical representation of the relationship between signal and number of molecules. The correlation coefficient r (or R in Excel) gives the measurement of the reliability of the linear relationship between the x and y values. Because the numbers outside of the range of the standards may not lie on this straight line, the radioactive signal of the viral samples must fall on this standard curve. The titer of such samples can be calculated using the linear relationship equation. The final unit of the viral sample is represented as number of viral genomes ml^{-1} (vg ml^{-1}).

With the increasing use of the scAAV vector owing to its improved transduction efficiency when compared with the ssAAV vector[29], it is important to understand the differences in the calculation of dot-blot analysis. Each scAAV genome contains two copies of the transgene expression cassette (both the coding sequence and complementary sequence are packaged in the same genome) as shown in **Figure 7**. Therefore, the amount of radioactive probe bound to a molecule of scAAV genome is twice as much as is bound to each single-stranded molecule of the standard DNA and to the conventional ssAAV (**Fig. 7**). If this factor of two were not taken into consideration, then the titer of the scAAV vector would be twice the actual amount.

Electron microscopy analysis to visualize rAAV particles

38| Place the grids into the Argon discharge machine.

? TROUBLESHOOTING

39| Place the glow-discharged grids onto a ~20-μl drop of purified AAV for 5 min.

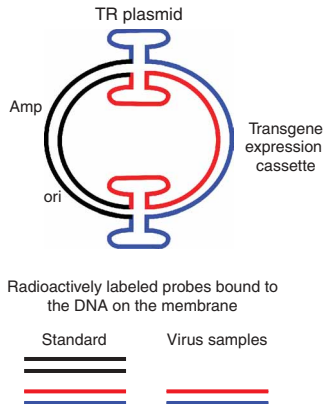


Figure 5 | Graphical representation of the suggested portion of the transgene plasmid to be used as the template of the probe for dot-blot analysis. The black lines represent sequences in the bacterial backbone; the blue and red lines represent sequences of the coding and complementary sequence of the transgene expression cassette, respectively.

40| Wash the grid three times with 1× PBS by placing the grid onto a ~20-μl drop of 1× PBS for a few seconds and then moving the grid onto the next drop.

41| Stain the virus for 1 min with 2% uranyl acetate by placing the grid onto a drop of 2% uranyl acetate.

42| Place the stained grid onto a 20-μl drop of ddH_2O to remove excess 2% uranyl acetate.

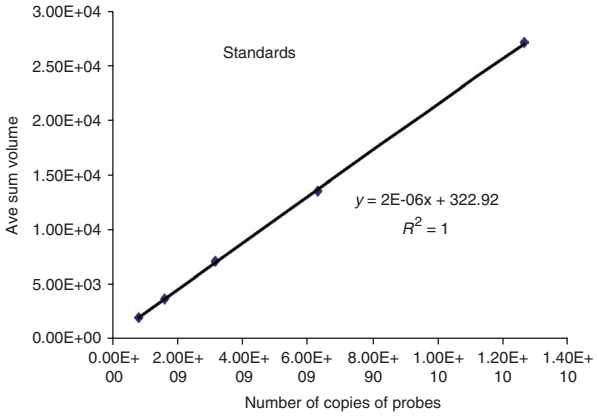
43| Remove the excess ddH_2O by touching the side of the grid to filter paper.

44| Place the grids into a Zeiss EM 910 electron microscope to view the purified AAV that adhered to the grid.

45| Randomly select a number of fields and count both full and empty particles. From this, it is possible to calculate an accurate ratio of full to empty particles in each preparation of AAV (**Fig. 8**).

Determine the viral genome integrity by alkaline gel electrophoresis

46| Prepare samples for alkaline electrophoresis. Perform viral DNA (vDNA) extraction using the same procedure as described above (Steps 14–21). Use 20 µl instead of 200 µl of TE to resuspend the DNA pellet. If restriction digest will be performed on the extracted vDNA, a larger volume of virus can be used in the extraction. For gel loading quantity, 5 µl of virus should be visible, but the volume used depends on the viral titer. As a marker/control, prepare plasmid DNA using the same construct as for making the virus. Perform restriction digests on the construct so that fragments of rAAV sequence with known sizes can be purified by gel extraction and can be used as a size marker. A sequence used for making a probe should be able to anneal to a common sequence in all sizes of fragments. Resuspend marker DNA fragments in 1× alkaline loading buffer.



Standard curve
1. Both *x*- and *y*-axis should be in linear scale.
2. Unknown samples must fall into the range of the standards.
3. Value of volume should correspond to the fold of dilution performed.

Figure 6 | An example of standard curve generated using Microsoft Excel. (These are actual data that we recorded, showing that it is possible to achieve an R^2 value of 1.)

Denaturation and electrophoresis

47| Denature the viral DNA in the 6× alkaline loading buffer.

48| Prepare the agarose gel and alkaline electrophoresis buffers. After the gel has solidified, equilibrate in approximately three times the volume of the agarose gel in the electrophoresis buffer for 15–20 min with very gentle rocking. Repeat three times.

49| After buffer equilibration, place the gel into the gel box very carefully. Cut off the part of the gel above the row of wells to reduce the volume of the gel.

50| Load the samples and record the loading sequence. Place a piece of glass on top of the gel in order to prevent the gel from floating during electrophoresis.

51| Set the power supply to 20–25 V (for a big gel box), and run the gel for 9–10 h.
■ **PAUSE POINT** Alternatively, set the power supply to 15–18 V and run the gel overnight (14 h).

Southern hybridization

52| Prepare alkaline transfer buffer (see REAGENT SETUP).

53| When the gel is done, lift up the glass carefully. Cut the gel at the top row of wells to further reduce the size of the gel. If necessary, remove other edges of the gel that are predicted to be unused.

54| Soak the gel into the transfer buffer for 10–20 min.

55| Measure the size of the gel and prepare one piece of DNA membrane (for example, Amersham Hybond-XL) and six pieces of 3MM Whatman paper the same size as the gel.

56| Wet the DNA membrane and the Whatman paper in the transfer buffer. Set up the gel sandwich as usual for Southern

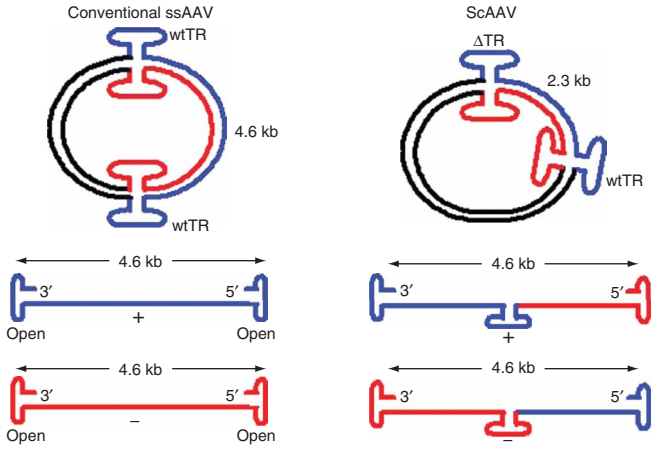


Figure 7 | Graphical representation of the difference between conventional ssAAV and scAAV vectors. For ssAAV vectors, the coding sequence and complementary sequence of the transgene expression cassette are on separate strands and are packaged in separate viral capsids. For scAAV vectors, both the coding and complementary sequence of the transgene expression cassette are present on each plus-and minus-strand genome. The black lines represent sequences in the bacterial backbone; the blue and red lines represent sequences of the coding and complementary sequence of the transgene expression cassette, respectively.

blot transfer. Transfer for at least 6 h. Make sure bubbles are absent between every layer.

■ **PAUSE POINT** Alternatively, transfer can be performed overnight (14 h).

57| When the transfer is completed, neutralize the membrane in 6× SSC quickly, twice. The membrane is ready for probe hybridization, as described in the dot-blot protocol in Steps 27–35 (**Fig. 9**).

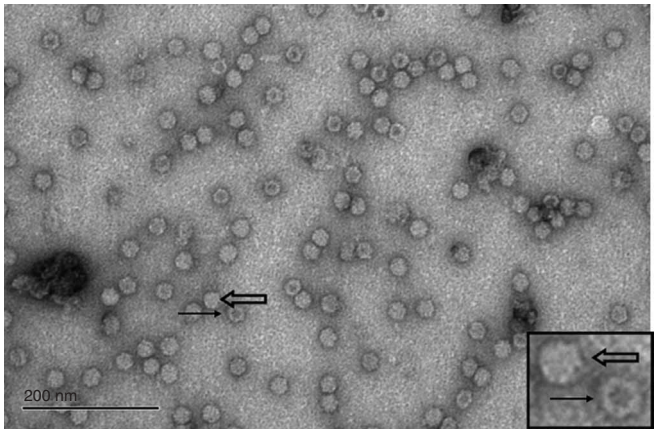


Figure 8 | Transmission electron microscopy of rAAV. It is clear that full particles (open arrow) stain differently from empty particles (darkly stained center; small dark arrow). Viewing numerous fields similar to this will allow determination of the full-to-empty particle ratio.

Viral genome replication

58| Twenty-four hours before transfection, split a confluent 10-cm plate of HEK-293 cells 1:3.

59| Transfect HEK-293 cells with 3.5 μg of pHelper and 10 μg of pXX680 using the calcium phosphate transfection protocol or PEI 6 h before infection.

60| Infect cells with 1,000 viral genomes per cell (as determined by dot blot), and incubate cells for 24 h.

Harvest replicated DNA

61| Harvest cells by using a rubber scraper and place cells into a 15-ml conical tube.

62| Centrifuge the samples at 200*g* for 5 min in a Sorvall RT 6000D centrifuge to pellet the cells.

63| Decant medium from the cells and resuspend the cell pellet in 1× PBS.

64| Centrifuge the samples at 200*g* for 5 min in a Sorvall RT 6000D centrifuge to pellet the cells.

65| Decant PBS from the cells and resuspend the pellet in 1 ml of 1× PBS and transfer the mixture into a 1.7-ml microcentrifuge tube.

66| Pellet cells at 500*g* for 5 min in a microcentrifuge.

67| Resuspend pellet in 740 μl of Hirt solution followed by addition of 50 μl of 10% SDS and mix by inversion. Add 330 μl of 5 M NaCl and mix again by inversion.

68| Store the samples overnight (12 h) at 4 °C.

69| Pour the samples into polycarbonate centrifuge tubes and centrifuge at 355,000*g* for 5 min (for example, 100,000 rpm in a SN434 TLA 120.2 rotor in a Beckman Optima TLX ultracentrifuge).

70| Recover the supernatant and place in a separate 1.7-ml microcentrifuge tube.

71| Add 100 μl of proteinase K solution (10 mg ml^{-1}) to the supernatant and incubate at 55 °C for 1 h.

72| Pipette an equal volume of phenol/choloroform/isoamyl alcohol (24:24:1) onto the supernatant and mix well.

73| Microcentrifuge at maximum speed for 5 min and collect the aqueous phase (top layer).

74| Add an equal volume of chloroform and centrifuge in a microcentrifuge at maximum speed for 5 min.

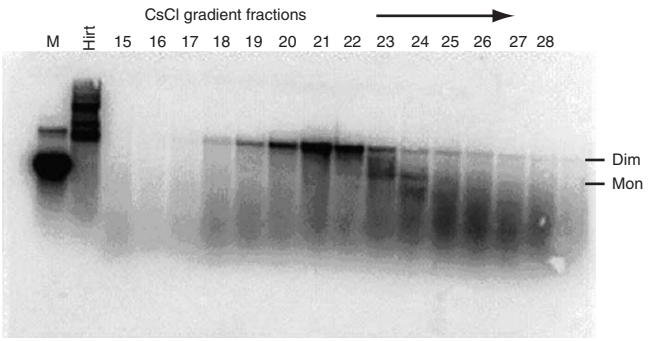


Figure 9 | Southern blot of CsCl gradient fractions of a scAAV preparation. Viral genomic DNA was extracted from each fraction and loaded onto an alkaline agarose gel to determine the fraction(s) containing scAAV (double-stranded (ds) DNA genome) based on the size of the genome. Fractions 20–22 contained the scAAV genomes (dimers ('Dim')), whereas fractions 23 and higher contained mainly incomplete genomes (monomers ('Mon')). M is the marker lane that consists of scAAV genomes (Dim) and ssAAV genomes (Mon).

75| Collect the aqueous phase and precipitate the low molecular-weight DNA by adding an equal volume of 100% isopropanol followed by centrifugation in a microcentrifuge tube at maximum speed for 15 min at 4°C.

76| Resuspend the DNA pellet in TE supplemented with 50 μg ml^{-1} RNase A.

77| Digest samples with *Dpn*I according to New England Biolabs specifications. *Dpn*I will digest the input pHelper and pXX680 plasmids that were carried over throughout the Hirt protocol.

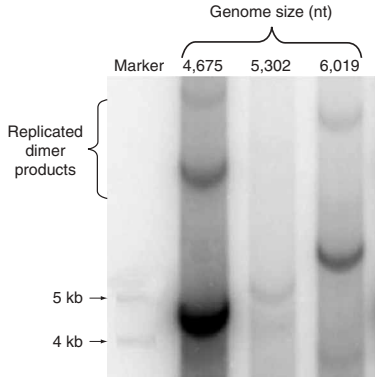


Figure 10 | Southern blot of vector DNA isolated from HEK-293 cells infected with rAAV containing genomes of sizes 4,675 nucleotides (nt), 5,302 nt and 6,019 nt in the presence of transfected adenovirus helper (XX680) and AAV2 helper (pHelper). The genome sizes are depicted at the top of the Southern blot. The correctly sized genomes and replication products are present within the cells.

Analysis of replicated DNA with electrophoresis

78| Load the samples into a 1% agarose gel for electrophoresis and transfer the DNA to a Hybond-XL nylon membrane using the infectious center assay Southern blot transfer method described in Steps 52–56.

79| Probe the membrane overnight with a radiolabeled transgene-specific probe as described in Steps 32–33.

80| Wash the membrane as described in Step 34 with low- and high-stringency buffers (**Fig. 10**).

Infectious center assay

81| Seed 5×10^4 C12 cells per well in a 48-well plate in 500 μl of medium. Gently shake the plate to disperse the cells evenly on the surface of the well.
▲ CRITICAL STEP This is critical to infect cells evenly across the well surface.

82| Incubate the cells at 37 °C for 3 h.

83| Meanwhile, perform serial dilution of each virus sample in culture medium. It is crucial to record the dilution scheme because the dilution factor is needed for calculating the number of infectious units per milliliter in the end of the experiment.

84| Dilute adenovirus to provide a multiplicity of infection of 20 per cell.

85| Coinfect cells with serially diluted recombinant AAV and adenovirus. It is important to include two negative control samples: adenovirus only (without rAAV) and uninfected.
▲ CRITICAL STEP When analyzing a set of samples for the first time, we recommend a serially diluted sample ranging 2–4 logs. After defining the dilution that can give the best readable result, retest virus samples at the specific dilution in triplicate to obtain a higher statistical confidence level.

86| Incubate infected cells at 37 °C for 42 h.

87| Soak DNA membrane in 0.4 M Tris-HCl (pH 7.5) for 10 min. For every 12 samples, prepare one piece of 3MM Whatman paper of the same size as the membrane. Also prepare five pieces of 3MM Whatman paper, with a size slightly bigger than the membrane, for each piece of membrane to be used.

88| For each well, transfer the medium from the well and place it into a microcentrifuge tube.

89| Wash cells with 0.5 ml 1× PBS. Place PBS into the same tube.

90| Spin cells down at maximum speed in a microcentrifuge for 10 s.

91| Remove supernatant and resuspend cells in 0.5 ml PBS.

92| Add 120 μl trypsin-EDTA into the well. Incubate at 37 °C for 6–8 min.

93| Pipette up and down ten times and transfer cells to the 0.5 ml ice-cold PBS in the microcentrifuge tube. Samples must be on ice at all times. Alternatively, add 150 μl of 10% serum-containing medium to the trypsinized cells before transferring the cells to the PBS.

94| Wash the well twice with 100 μl of PBS and transfer the PBS into the same collection tube.

95| Assemble the infectious center assay apparatus (**Fig. 11**) by soaking the piece of 3MM Whatman paper of the same size as the membrane in 0.4 M Tris-HCl (pH 7.5) just before assembly. Place the membrane on top of the Whatman paper. Assemble the apparatus by using six pieces of rubber bands to tighten the apparatus. Put suction on the apparatus briefly through a vacuum line and remove the vacuum when excess liquid has gone through the apparatus. Use eye protection.

96| Apply 12 samples dropwise to the apparatus. Apply vacuum.

97| Disassemble the apparatus. Place membrane, cell side up, on a piece of Whatman paper; let dry.

98| Place the membrane on a piece of Whatman paper saturated with denaturing solution for 8 min. Transfer to a dry Whatman paper and let dry.

Figure 11 | Picture of the infectious center assay apparatus. Twelve wells are used to vacuum samples onto a HyBond XL nylon membrane (Amersham Biosciences) placed between the top plate and middle plate.

99| Place the membrane on a piece of Whatman paper saturated with neutralization solution for 8 min. Transfer to a dry Whatman paper and let dry.

100| Cross-link DNA on membrane using UV cross-linker. Membrane can then be probed as described above.
■ **PAUSE POINT** Membrane can also be stored at room temperature indefinitely.

101| Hybridize DNA with ^{32}P-labeled probe, following the hybridization and wash protocol described in Steps 32–34.
▲ **CRITICAL STEP** Perform overnight radioactive probe hybridization using Church buffer. Using QuikHyb for 1 h will not work because the signal will be too weak to be detected by film.

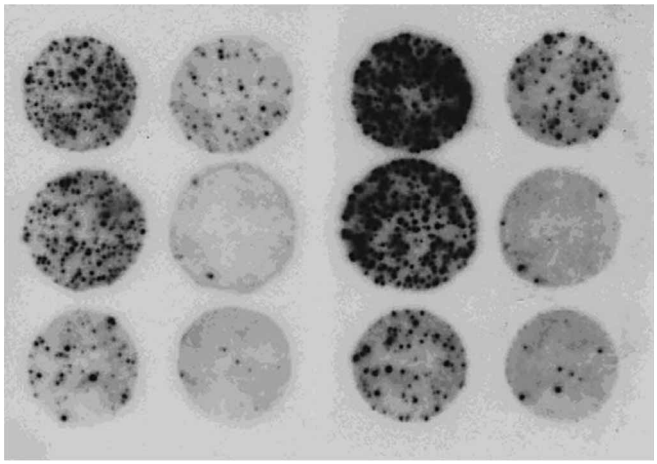
102| Expose the X-ray film to the membrane overnight (12 h).
▲ **CRITICAL STEP** Do not place cassette into –80 °C, because it will decrease resolution.
? TROUBLESHOOTING

103| Develop the film and count the number of black dots per sample. Use Kodak MR film for better resolution instead of using MS film. If signal is weak, increase exposure time (for example, 3 d to 1 week, or longer).
? TROUBLESHOOTING

104| Data analysis. Each dot on the film corresponds to one infectious viral particle that can successfully infect and deliver its genome into the nucleus for replication (**Fig. 12**). The result should represent the serial dilution scheme used at the time of infection, as demonstrated in **Figure 12**. Back-calculate the infectious titer of the virus with the known volume of virus used, as recorded in the infection scheme. The viral sample titer is denoted as infectious units per milliliter.

AAV transduction assay (determining transducing units)

105| In a six-well plate, seed 2×10^5 HeLa cells with 1.5 ml of cell culture medium.



106| Twenty-four hours later, use serially diluted rAAV in culture medium for infection.

107| Remove culture medium 2–3 h post-infection and replace with fresh medium. Incubate cells in a 37 °C incubator with 5% CO_2 atmosphere.

Figure 12 | Infectious center assay of a purified rAAV preparation. Serial dilutions of the virus are made and used in duplicate to determine the infectious titer of the prep. One black dot represents an infectious particle, meaning that a single genome entered the nucleus and replicated producing the signal. To help ensure that one virus infects a single cell, fewer viral genome–containing particles than C12 cells are added per well.

108| Harvest cells 24 h post-infection and subject them to flow cytometry analysis of GFP expression (**Fig. 13**).
? TROUBLESHOOTING

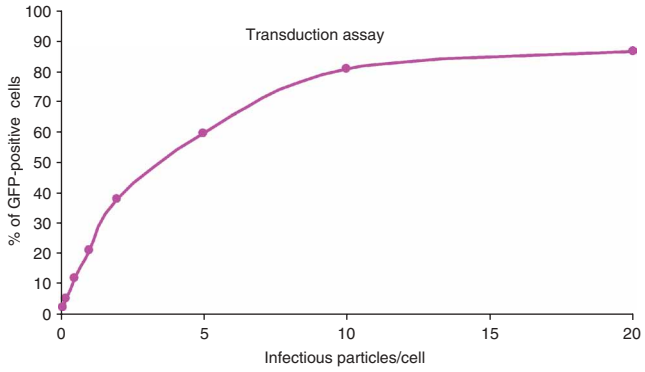


Figure 13 | HeLa cells were infected by serially diluted scAAV (0.1 IU/cell–20 IU/cell). Cells were harvested 24 h post-infection and subjected to FACS analysis.

● TIMING

Transfection (Steps 1–3): 72–96 h
Isolation of rAAV from the nuclei of producer HEK-293 cells (Steps 4–11): 1–3 h (varies depending on number of plates to be harvested)
Gradient purification of rAAV (Step 12): 8–16 h (varies depending on the number of samples to be processed)
Dot blot for titering (digestion of virus particles to release DNA; Steps 13–24): 6 h
Dot blot for titering (preparation of standards; Steps 25–26): 0.5 h
Dot blot for titering (hybridization; Steps 27–35): 16 h
Dot blot for titering (quantification of dot-blot analysis; Steps 36–37): 2 h
Electron microscopy (Steps 38–45): 3–4 h (varies depending on number of samples)
Alkaline gel electrophoresis (extraction of rAAV genomes; Step 46): 6 h
Alkaline gel electrophoresis (denaturation and electrophoresis; Steps 47–51): 12 h
Alkaline gel electrophoresis (Southern hybridization; Steps 52–57): 8 h + 12 h + 2 h
Replication assay (viral genome replication; Steps 58–60): 48 h
Replication assay (harvesting replicated DNA; Steps 61–77): 16 h
Replication assay (analysis of replicated DNA with electrophoresis; Steps 78–80): 20 h
Infectious center assay (rAAV infection; Steps 81–86): 48 h
Infectious center assay (harvesting cells and releasing genomes; Steps 87–100): 4 h
Infectious center assay (hybridizing membrane and analysis of results; Steps 101–104): 24 h
AAV transduction assay (Steps 105–108): 48–72 h

? TROUBLESHOOTING

Low titer (Step 12)

Transfection efficiency is crucial for achieving high yield of vectors. Test the transfection solutions for their efficiencies before carrying out vector purification prep. In addition, requantify plasmid concentration used for transfection. Moreover, the pH of the medium is important for efficient transfection. Therefore, remove plates from incubators immediately before the addition of the transfection complex. Also test for the integrity of the TRs (if serotype 2 TR is used) by restriction digest. There are two *Sma*I restriction sites on the T-shaped palindromic structure of TR2, and the presence of the *Sma*I sites would confirm that the TRs are intact and thus should lead to successful replication and packaging of the viral vector. If the above suggestions can be dismissed, an aliquot of the sample can be saved from each step of the purification process for analysis by dot blot.

Testing the virus quickly (Step 12A(xiii))

Viruses in iodixanol can be used to infect cells *in vitro* directly because the solute is non-toxic to cells.

High empty-to-full particle ratio (Step 12A(xiii))

Try to insert the needle into the centrifuge tube with the bevel upward, and then turn the bevel downward to avoid collecting the upper portion of the 40% layer, which is where the empty particles would be.

No access to an electron microscope (Step 38)

An ELISA kit commercially available from American Research Products can measure the number of total particles in a viral preparation, but it is specific only to AAV2 capsids (it utilizes the A20 antibody specific to AAV2). It is a colorimetric-based assay formatted to a 96-well plate, and samples are analyzed by a plate reader. The number of empty particles can be calculated by subtracting the number of viral genome–containing particles (determined by dot-blot or quantitative PCR) from the total number of particles (determined by the ELISA).

Signal is too weak (Step 102)

Increase exposure time to 3–7 d using the Kodak MR film.

Cannot visualize dots well enough for counting (Step 103)
Place the film on a light box. Place a transparency sheet on top of the film so that marks can be made for easier counting.

Transgene expression level is low (Step 108)
For conventional single-stranded rAAV (ssAAV), adenovirus type 5 (Ad5) can be added at a MOI of 5. Cells are harvested and analyzed for transgene expression using an assay specific for the transgene (e.g., flow cytometry, immunohistochemistry, histological staining, drug resistance, etc.). The addition of Ad5 can increase transduction efficiency of conventional single-stranded rAAV due to increase in efficiency of second-strand synthesis[32]. However, adenovirus has a cytopathic effect because of its antigenicity and should not be used *in vivo*. Thus, a titer derived with use of adenovirus *in vitro* may not accurately reflect an *in vivo* competency. It is up to each investigator to establish a standard procedure for titering different rAAV preps.

ANTICIPATED RESULTS

Dot-blot analysis
Figure 4 depicts a dot blot. The titer of the viral preps can be calculated according to the methods described in Steps 36–37.

EM analysis
As depicted in **Figure 8**, empty particles have been defined as those that contain a darkly stained center, whereas full particles lack this specific staining[16].

Alkaline gel electrophoresis
The integrity of the vector genome is also important, especially in the case of the scAAV. As shown in **Figure 9**, the scAAV genome, also known as double-stranded (dimer) AAV, was separated from the incomplete (monomer) genomes in a CsCl gradient owing to their difference in weight contributed by the genomes. Using the pHpa-trs-SK cloning vector and the production method described above, $>95\%$ purity of scAAV vectors can be obtained. As described in the PROCEDURE, genome integrity of rAAV containing single-stranded genomes is determined utilizing the same method as for scAAV. The genome size is directly compared to reference standards. A sharp, distinct band reflective of the size of the genome should be present as depicted in **Figure 9**.

Replication assay
As described in the previous section, cells permissive for replication are infected with rAAV; 24 h after infection, Hirt DNA is isolated from the cells; and the integrity of the replicated viral genomes are assessed via Southern blot (**Fig. 10**).

Infectious center assay
C12 cells[27] containing AAV serotype 2 *rep* and *cap* integrated in the cell genome are used as a tool to score for rAAV2 particles that can successfully deliver their genomes into the nucleus; these are reported as infectious particles. As the intact cells are lysed on the DNA membrane, the replicated AAV genome from each cell is bound locally on the membrane surface as a dot (**Fig. 12**). A dot represents replicated viral genome products (monomer, dimer or concatamer) from a single viral genome in the nucleus of the cell. Replication of the viral genomes is essential to increase the concentration of viral genomes and yield a signal (dot) on the membrane. Therefore, each dark spot on the film indicates one infectious rAAV unit (IU) (**Fig. 12**). Because a known volume of virus was used in the dilution series, the infectious units per milliliter can be back calculated for the vector preparation. The ratio of viral genome–containing particles to infectious units can also be determined if a dot blot was performed on the sample. It is important to determine the infectious titer if the transgene expression (or transducing units) cannot be easily detected or quantified.

Determining transducing units
In order to determine the transducing titer of virus preps, a transduction assay must be carried out. An example of a transduction assay is shown in **Figure 13**. A positive transduction signal indicates that the vector has successfully infected the cell, unpackaged and expressed the transgene to a level sufficient to allow detection by the appropriate expression assay. Transducing titers can vary with the cell type used, but with a standard cell line, transducing titers can be used to compare rAAV preps of the same transgene (**Fig. 13**). This titer can be compared to the particle number to determine the transducing unit-to-particle ratio. Titers may also vary when comparing immortalized cell lines in culture to *in vivo* transduction. Because different target cells of interest are of different sizes and their doubling time differs, appropriate density of cells should be seeded into multiwell tissue culture plates.

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