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AAV Vectors for Efficient Gene Delivery to Rodent Hearts

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Abstract

Currently, gene therapy is one of the most promising fields in biomedicine, with great therapeutic potential for an array of inherited and acquired diseases. Adeno-associated viral (AAV) vectors have emerged as promising tools to deliver selectively a therapeutic payload to target organs, including the heart. In this chapter, we describe the production and quality control of recombinant AAV (rAAV) vectors of the serotype 9, the most cardiotropic AAV serotype when delivered systemically in rodents. We also describe the systemic administration of rAAV vectors and the local delivery of rAAV vectors by direct intramyocardial injection. Taken together, the methods described in this chapter will allow the reader to deliver efficiently therapeutic genes to the rodent heart, both globally and regionally.

Keywords

Adeno-associated virus; AAV production; AAV characterization; AAV9; AAV delivery; Rat; Mouse

1 Introduction

AAV is a small, nonenveloped virus of the family *parvoviridae* and has an approximately 4.7 kb-long, single-stranded DNA genome, which is flanked by two inverted terminal repeats (ITRs). The AAV genome encodes only two genes: the *rep* gene encoding the REP proteins, which are responsible for DNA replication and packaging, and the *cap* gene, which encodes three capsid proteins (VP1, VP2, and VP3) with overlapping reading frames. On an alternative reading frame, *cap* also encodes the assembly-activating protein (AAP), which aids in capsid assembly [1].

AAV can infect dividing and nondividing cells, is nonpathogenic, and displays limited immunogenicity. Importantly for cardiac gene delivery, AAV vectors mediate long-term transgene expression in postmitotic cells such as cardiomyocytes, even in the absence of genome integration. Because of their beneficial characteristics, AAV-based vectors are arguably the top choice of vectors for in vivo gene therapy and have been used in a large number of clinical trials. Importantly, the treatment of Leber's congenital amaurosis type 2 by an AAV2 vector delivering the defective gene, RPE65, has recently been approved by the FDA for clinical use [2]. This represents the first AAV gene therapeutic treatment approved in the United States.

In addition to the most commonly used serotypes AAV1–6, AAV8, and AAV9, more than a hundred AAV variants have been described and, together, they display a diverse and broad tissue and cell tropism. However, for the efficient delivery of genes to the entire rodent

myocardium via systemic injection, AAV9 has clearly emerged as the most promising serotype ([3, 4] and reviewed in [5]).

In the first part of this chapter, we describe the production and characterization of rAAV serotype 9 vectors and the importance of quality control, especially for studies in animal models. The generation of rAAV vectors begins with the co-transfection of HEK293T (or HEK293) cells with a plasmid that contains the experimental or therapeutic expression cassette flanked by two AAV2 ITRs, along with one or two plasmids that together express the AAV2 *rep* gene, the *cap* gene of the desired serotype, and the helper functions from adenovirus, including the virus-associated (VA) RNA, *E2A* and *E4* genes [6]. HEK293T cells provide adenovirus *E1A* and *E1B* genes, which are also necessary for AAV production [6]. Triple co-transfection, where the helper functions are distributed within two plasmids (a *rep/cap* plasmid and an adenoviral helper plasmid), is currently the most widely used methodology in research laboratories and allows AAV production of up to 10^5 viral genomes (vg) per cell ([7, 8] and reviewed in [9]). In our laboratory, we use a two-plasmid approach to produce rAAV vectors [10]. This method combines the AAV2 *rep* gene, the AAV *cap* gene (for the purpose of this chapter AAV9 *cap*), and all the adenoviral helper functions (except E1A and E1B) in a single plasmid. We use poly-ethylenimine (PEI) transfection because of its simplicity and its high level of reproducibility. Nevertheless, transfection can also be performed with calcium phosphate or other transfection approaches (reviewed in [11]). Seventy-two hours following transfection, rAAV virions are harvested from the cells or from both the cells and the cell culture supernatant. While we routinely harvest AAV9 virions from both the cells and the cell culture supernatant, this is not necessary for certain serotypes such as AAV2 ([12] and unpublished results). We then purify the rAAV virions via iodixanol gradient centrifugation [13], a method that can be used for any AAV serotype or variant. Next, purified rAAV virions undergo a series of rigorous quality control tests such as qPCR titration of viral genomes, Coomassie staining of virions run on an SDS-PAGE gel, and alkaline agarose gel electrophoresis, to quantify rAAV vector genome titers, to determine rAAV vector capsid titers and vector purity, and to ensure that the majority of packaged viral genomes are full-length (and to confirm qPCR titers), respectively. In rodents, AAV is usually delivered either systemically by tail vein injection or retro-orbital injection (for global transduction of the myocardium), or locally by open chest, intramyocardial injection (for transduction of specific areas of the heart). These approaches are described below. A third delivery method that can be used instead of tail vein injection for global transduction of the myocardium is intraventricular injection with cross-clamping of the pulmonary artery and the aorta. Because of its invasiveness, this method is rarely used; *see* [14] for a detailed description. For reviews describing delivery methods that are suitable for large animal models and humans, *see* [15-19].

2 Materials

2.1 *Plasmid Production* (See *Note 1*)

1. Cis-plasmid containing the rAAV genome to be packaged.
2. pDG9 helper plasmid: AAV9 capsid sequence cloned into the *SwaI/ClaI* digested pDG backbone (PlasmidFactory).

3. SURE2 (Agilent Technologies) or Stbl3 (Life Technologies) competent bacteria for transformation with cis-plasmid.
4. Plasmid Maxiprep Kit.
5. Lysogeny broth (LB) medium (also commonly, but incorrectly, referred to as Luria-Bertani medium).
6. *SmaI* (or the isoschizomer XmaI) restriction enzyme.

2.2 Cell Culture

1. HEK 293T/17 cells (ATCC).
2. Cell culture medium: DMEM with 4.5 g/L glucose, L-glutamate, and sodium pyruvate, supplemented with 10% fetal bovine serum and 1× penicillin/streptomycin.
3. 0.025% Trypsin.
4. Calcium and magnesium free PBS.
5. 175 cm^2 tissue culture flask.
6. BSL2 laminar flow tissue culture hood.

2.3 Transfection

1. Vented tissue culture TripleFlask(s) (Thermo Fisher Scientific).
2. 50 μg of cis-plasmid and 150 μg of helper plasmid (pDG9) per triple flask to be transfected.
3. Transfection media: DMEM with 4.5 g/L glucose, L-glutamate, and sodium pyruvate; and DMEM with 4.5 g/ L glucose, L-glutamate, and sodium pyruvate, supplemented with 2% fetal bovine serum (no penicillin/streptomycin).
4. Post-transfection medium: DMEM with 4.5 g/L glucose, L-glutamate, and sodium pyruvate, supplemented with 2% fetal bovine serum and 1× penicillin/streptomycin.
5. Linear 40 kDa polyethyleneimine (PEI)-Max (Polysciences Inc.).
6. 12 N hydrochloric acid.
7. 250 mL sterile filter (0.22 μm) bottles.

2.4 Harvesting and Processing

1. 200 mL polypropylene conical centrifuge tubes.
2. 50 mL polypropylene conical tubes.
3. Lysis buffer: 2 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris–HCl, pH 8.5.
4. Sorvall RC−6+ centrifuge.
5. SH-3000BK, F14-6x-250y, and F13-14×50cy rotors.

6. Pierce Universal Nuclease for Cell Lysis (Thermo Fisher Scientific) (*see* **Note 2**).
7. Ammonium sulfate.
8. 18G needle.

2.5 Preparation of Ultracentrifugation Gradients

1. Optiprep 60% iodixanol (Thermo Fisher Scientific).
2. 5× Optiprep dilution buffer (ODB): 5× PBS, 5 mM magnesium chloride, and 12.5 mM potassium chloride.
3. Iodixanol Gradient Layer Solutions (*see* Table 1).
4. Ti70 rotor (Beckman Coulter).
5. OptiSeal polypropylene 37.4 mL (26 × 77 mm) ultracentrifuge tubes (Beckman Coulter).
6. 5 and 10 mL syringes.
7. 18G needles.
8. 18G blunt-end Hamilton needles.

2.6 Dialysis

1. 10 mm flat width, 12–14 kDa MWCO, regenerated cellulose dialysis tubing (Spectrum Labs).
2. Dialysis tubing closures.
3. Lactated Ringer's solution.
4. 0.22 μm sterile syringe filter.
5. Large sterile bottle or container.
6. Orbital shaker.

2.7 Quantitative Real-Time PCR

1. Real-time PCR machine.
2. Real-time PCR plate or tubes and caps compatible with the specific machine.
3. iTaq Universal SYBR Green Supermix (Bio-Rad).
4. Forward and reverse primers that anneal perfectly to both the reference standard and the AAV sample to be quantified (*see also* **Note 3**).
5. AAV2 Reference Standard Material (ATCC VR1616) or calibrated in-house AAV reference standard (*see* **Note 4**).

2.8 Alkaline Gel

1. Horizontal ("submarine style") electrophoresis apparatus and power supply.

2. Electrophoresis grade agarose.
3. 50× Alkaline Gel Buffer: 2.5 M sodium hydroxide, 50 mM EDTA.
4. Alkaline Gel loading dye: 4× Alkaline Gel Buffer, 1.2% SDS, 20% Glycerol, 0.01% Xylene Cyanol.
5. 1 kb DNA Ladder (New England Biolabs).
6. 0.1 M Tris–HCl pH 8.5.
7. Gel Red (Biotium), SYBR Gold (Thermo Fisher Scientific), or equivalent dye that allows for the sensitive detection of single-stranded DNA.
8. UV transilluminator (Image capturing system, e.g. ChemiDoc Touch (Bio-Rad)).

2.9 Anesthesia

1. Research anesthesia machine with veterinary anesthesia vaporizer, nose cone device, and small animals' inhalational isoflurane anesthetic chamber.
2. Inhaled anesthetic: isoflurane.
3. Injectable anesthetic: 100 mg/mL ketamine HCl and 20 mg/ mL xylazine.
4. Animal scale.
5. 26G needles (for mice) and 21G needles (for rats).
6. 1 mL syringes.

2.10 Intravascular Injection Via the Tail Vein

1. 27-30G 1 mL insulin syringes, 5/8″ needle (for mice).
2. 28-30G catheter (for rats).
3. 1 mL syringes (for rats).
4. 25G needles (for rats).
5. Restraining device, such as Tailveiner restrainer (Braintree Scientific) or anesthetic.

2.11 Intravascular Injection Via the Venous Sinus (Retro-orbital Injection)

1. Isoflurane and research anesthesia machine.
2. Isothermal pad, such as PhysioSuite with RightTemp module (Kent Scientific).
3. 27.5G 0.5 mL insulin syringes, 1/2″ needle (for mice).
4. 1 mL syringes (for rats).
5. 23-28G needles (for rats).

2.12 Local Injection into the Ventricular Wall of the Heart

1. Rodent ventilator, such as VentElite small animal ventilator (Harvard Apparatus) for rats or VP3 veterinary anesthesia vaporizer (DRE Veterinary) for mice.
2. Isothermal pad, such as PhysioSuite with RightTemp module (Kent Scientific).
3. Rectal temperature probe.
4. High intensity fiber optic gooseneck light (Cole-Parmer).
5. Soft catheter for endotracheal intubation: 16G for rats above 200 g, 18G for rats below 200 g, and 20G for mice.
6. Soft catheter for the thoracic cavity: 18G catheter (for rats) and 22G catheter (for mice).
7. Electric hair shaver.
8. 70% ethanol.
9. Povidone-iodine 10%.
10. 1 mL syringes (for rats) and 0.3-0.5 mL syringes (for mice).
11. 25 and 30G needles (for rats) and 26 and 31G needles (for mice).
12. 0.3 mg/mL Buprenex HCl.
13. Standard small animal surgical instruments, including rib spreader, scalpel handle and blades, surgical scissors, and forceps.
14. 5-0 (for rats) and 6-0 (for mice) Vicryl sutures with cutting needle.
15. 4-0 (for rats) and 5-0 (for mice) Prolene sutures with non-cutting needle.
16. Battery-operated electrocautery device.
17. Stereo microscope.

3 Methods

3.1 Plasmid Cloning and Production

1. To prevent the potential loss of intact ITRs, all cis-plasmids are maintained in either SURE2 or Stbl3 cells and grown at 30–32 °C (*see* **Note 5**). pDG9 helper plasmid can be maintained in a regular *E. coli* strain such as DH5α and grown at 37 °C. Purify plasmids using a Maxiprep kit. For the production of the cis-plasmid, one 2 L Erlenmeyer flask with 600 mL of LB medium should produce approximately 500 μg of plasmid. Since the amount of pDG9 plasmid needed for the same number of triple flasks is three times the amount of the cis-plasmid, grow pDG9 bacterial cultures in three 2 L flasks with 600 mL of LB medium each.
2. To confirm that the majority of the purified cis-plasmids contain two intact ITRs, digest the cis-plasmid with *SmaI* (or the SmaI isoschizomer XmaI) restriction

enzyme and analyze the digested DNA on an agarose gel. Each ITR contains two SmaI/XmaI sites.

3.2 Cell Maintenance (See Note 6)

1. Maintain HEK293T cells in DMEM supplemented with 10% FBS and 1× Penicillin/Streptomycin for all growth and passaging steps unless otherwise stated. For continuous culture, grow the cells to approximately 70–80% confluency and then split 1:10, so that cells will be ready to be split again 3 or 4 days later, respectively.
2. To split cells from a 175 cm^2 flask, gently remove the culture medium, add 20 mL of PBS without Mg/Ca to cover the surface, and gently rock the flask to completely cover the cells. Remove the PBS and add 5 mL of 0.025% Trypsin to the side of the flask, not directly onto the cells. Spread over the cells by gently "rocking" the flask several times. Incubate at 37 °C for 1–2 min. Then rock the flask to completely dislodge the cells. Pipet 10 mL of medium onto the cells and pipet up and down to prepare a homogenous cell suspension. Add 25 mL of a 1:10 dilution of cell suspension in fresh medium to a new flask (*see* **Note 7**).

3.3 Transfection

1. To prepare the PEI solution for transfection, add 200 mg of PEI-Max powder to 200 mL of sterile, deionized water and stir with magnetic stir bar. Adjust pH to 4.5 by adding 12 N hydrochloric acid. Stir overnight to assure complete dissolution of the PEI-Max. The next day, sterile-filter the solution with a 0.22 μm filter bottle, and store aliquots at −80 °C. Once thawed, the solutions can be stored at 4 °C for up to 2 months.
2. The day before transfection, detach cells with Trypsin and use the cells to seed a triple flask (*see* **Note 8**). Prior to seeding the triple flask, remove a sufficient number of cells to seed a 175 cm^2 flask for the continuation of cell passaging.
3. The morning after seeding the triple flasks, check the flasks for cell confluency. If ~70% confluent, proceed to transfection immediately. If the confluency is less than ~70%, perform the transfection later in the day.
4. For each triple flask to be transfected, heat 90 mL of DMEM (2% FBS, no Pen/Strep) to 37 °C.
5. Prepare the transfection mix by adding in the following order to 20 mL of room temperature DMEM (no FBS, no Pen/Strep): 50 μg of cis-plasmid, 150 μg of pDG9 plasmid, and 250 μL of PEI-Max solution (pH 4.5). Vortex for 10–20 s and incubate at room temperature for 15 min.
6. Add the transfection mix to the prewarmed 2% FBS medium and mix by swirling.
7. Gently remove the medium from the cells in the triple flasks and replace with transfection mix medium.

8. Following incubation of cells overnight at 37 °C, replace medium with DMEM (2% FBS, 1× Pen/Strep) and further incubate the transfected cells at 37 °C.

9. Three days from the day of transfection, harvest rAAV virions from cells.

3.4 Harvesting and Processing

3.4.1 Preparation of Cell Lysate

1. Detach the cells by tapping the flask vigorously and transfer the solution to a sterile 200 mL conical tube. Pellet the cells by centrifugation at 1000 × *g* for 15 min in a Sorvall RC−6+ centrifuge and SH-3000BK swinging bucket rotor.

2. Collect the cell culture supernatant in a sterile bottle for later processing.

3. Gently add 10–15 mL of PBS to the side of the conical tube. Swirl gently to dislodge, but not break apart, the pellet. Transfer the pellet to a 50 mL conical tube. Multiple pellets can be combined into one 50 mL tube for further processing. If cells remain in the large conical tube, use the PBS to rinse the cells off the large tube and transfer to the small tube.

4. Pellet the cells by centrifugation in a Sorvall RC−6+ centrifuge and SH-3000BK rotor at 1000 × *g* for 10 min. Pour the supernatant into the bottle containing the cell culture supernatant (*see* **Note 9**). This cell culture supernatant will be used for Subheading 3.4.2, and can be frozen at −80 °C to be processed later.

5. Resuspend the cell pellet in 5 mL of lysis buffer (*see* **Note 10**).

6. To lyse the cells, repeatedly freeze and thaw the cell suspension. The cell suspension can either be frozen in a −80 °C freezer or on dry ice. Thaw the cell suspension in a 37 °C water bath followed by brief vortexing. Repeat this freeze/thaw cycle twice. Aggregation of cellular debris during the freeze/thaw cycles is normal. Any of the freezing steps can be used as a stopping point.

7. To digest genomic and plasmid DNA, non-encapsidated viral DNA, and cellular RNA, add 1 μL (250 U) of Pierce Universal Nuclease to the thawed, crude lysate and incubate for 30 min at 37 °C (*see* **Note 2**).

8. Centrifuge the crude lysate in a Sorvall RC−6+ centrifuge and F13-14x50cy fixed angle rotor at 5000 × *g* for 20 min and transfer the supernatant to a new tube (discard the pellet). The new tube is ready for iodixanol gradient ultracentrifugation. It can be stored at −80 °C at this point.

3.4.2 Processing of the Cell Culture Supernatant

1. If the cell culture supernatant was frozen (**step 4** of Subheading 3.4.1), thaw it at 37 °C, but do not incubate longer than necessary. Add 31.3 g ammonium sulfate per 100 mL of supernatant, and shake for 1–2 min to dissolve the ammonium sulfate completely. Incubate the mixture on ice for at least 30 min (*see* **Note 11**).

2. To pellet the virus precipitate, centrifuge in a Sorvall RC-6+ centrifuge and an F14-6x250y fixed angle rotor at 8300 × *g* for 30 min. Because the pellet may be

loose, pour off the supernatant as soon as possible after centrifugation. DO NOT STERILIZE THE SUPERNATANT WITH BLEACH. INSTEAD, THE SUPERNATANT MUST BE AUTOCLAVED AND THEN DISCARDED. Ammonium Sulfate is incompatible with bleach, and treatment of the supernatant with bleach would result in the production of TOXIC GASES AND EXPLOSIVE COMPOUNDS.

3. The pellet will accumulate on the side of the centrifuge tube. Resuspend the pellet with a pipette in 4 mL of lysis buffer (*see* **Note 10**). The resuspension is complete when there is no remaining pellet on the wall of the tube and the solution is homogeneous.

4. Add 1 μL (250 U) of Pierce Universal Nuclease for digestion of possible cellular and plasmid DNA or RNA (*see* **Note 2**) and incubate 30 min at 37 °C (or according to manufacturer's instructions if using an alternative nuclease). The virus suspension is now ready for iodixanol gradient purification of the virus.

3.5 Preparation of Ultracentrifugation Gradients

3.5.1 First Gradient

1. To form the gradient, slowly underlay the following solutions: (a) 7.3 mL of 15% iodixanol, (b) 4.9 mL of 25% iodixanol, (c) 4 mL of 40% iodixanol, and (d) 4 mL of 60% iodixanol. To add each layer, use a 10 mL syringe fitted with an 18G blunt-end Hamilton needle, and lower it into the tube until the tip of the needle reaches the bottom and center of the tube (*see* **Note 12**).

2. Once the gradient is ready, add the viral solution on top of the gradient by placing the Hamilton needle on the side of the tube and dispensing the viral solution dropwise onto the top layer of the gradient.

3. If at this point the tube is not completely filled, add lysis buffer to the top of the tube to fill the tube up to the neck. Any bubbles must be removed prior to centrifugation to prevent the collapse of the tubes during centrifugation. To remove potential bubbles, slightly squeeze the tube until the bubbles rise above the neck of the tube and remove them with a pipet. Seal the tube by firmly inserting the plastic stopper.

4. Use a permanent marker to draw a line at the 40–60% interface. The interfaces are easy to see before centrifugation but more difficult to visualize after centrifugation.

5. Centrifuge at 350,333 × *g* in a Ti70 rotor for 1 h (*see* **Note 13**).

6. The majority of AAV particles will be located at the 60–40% iodixanol interface. To collect the virus, remove the stopper of the tube, insert an 18G needle connected to a 5 mL syringe just below the interface and slowly withdraw 3 mL (these 3 mL will consist of approximately the top 1 mL of the 60% layer and the bottom 2 mL of the 40% layer). Alternatively, the virus can be collected in fractions from the bottom of the gradient as described in **steps 5** and **6** of

Subheading 3.5.2. If a second gradient is not performed, collecting the virus in fractions will yield virus preparations with fewer empty particles.

7. Determine the AAV titer by qPCR (Subheading 3.7). If the viral titer is too low (*see* **Note 14**), combine the virus from up to four tubes and perform a second gradient (Subheading 3.5.2). If the viral titer is sufficiently high, proceed to Subheading 3.6.

3.5.2 Second Gradient

1. For a second round of iodixanol gradient purification, dilute the harvested AAV/iodixanol solution in 1 × ODB such that the percentage of iodixanol is lower than 25%. For this calculation, the AAV/iodixanol harvested from the first gradient is assumed to be 47% iodixanol (1 mL of 60% + 2 mL of 40%). If the virus was collected in fractions, calculate the approximate percentage of iodixanol for the corresponding fractions to be purified.
2. Add 22 mL of the AAV/iodixanol dilution into an OptiSeal ultracentrifuge tube.
3. Underlay this solution sequentially with 4 mL of 40% iodixanol and 4 mL of 60% iodixanol as described in **step 1** of Subheading 3.5.1.
4. Centrifuge the tubes at 350,333 × *g* in a Ti70 rotor for 2 h.
5. Collect 1.25 mL fractions from the bottom of the tube. To do this, remove the stopper and cover the opening with a finger (this will form a vacuum that prevents flow once a hole is punched in the bottom of the tube). With the finger still placed on the opening, make hole with an 18G needle at the bottom of the tube. Now, place a microcentrifuge tube (pre-marked at the 1.25 ml level) under the hole and release the finger to let the content drip into the microcentrifuge tube to collect the first 1.25 ml fraction. Next, place the finger back on the top opening to stop the flow, place the next microcentrifuge tube under the hole, release the finger and collect the second 1.25 ml fraction. Repeat this process until you have collected 8 fractions. The remaining content can be collected in a large conical tube to be discarded.
6. Run an alkaline gel of the fractions to identify the fractions containing the majority of full-length viral genomes and then combine those fractions.

3.6 Dialysis

1. Soak dialysis tubing in lactated Ringer's solution overnight.
2. The next morning, squeeze out any lactated Ringer's solution left in the tubing, clamp one side of the tubing with a dialysis clip, pipet the AAV/iodixanol solution into the tubing, and clamp the other end with a dialysis clip. To allow for easy recovery of the virus, leave at least 2 in. of tubing hanging outside one of the clamped ends.
3. Place the closed dialysis tubing in a large sterile bottle with at least 100 dialyzate volumes of lactated Ringer's solution.

4. Agitate the dialysis solution gently for 1–2 h at 4 °C on an orbital shaker platform and replace the lactated Ringer's solution with an equal volume of fresh solution.

5. Agitate the dialysis solution for an additional 4–5 h at 4 °C and replace with fresh lactated Ringer's solution with an equal volume of fresh solution.

6. Agitate overnight at 4 °C. Remove the clamp on the side with additional tubing and collect the contents of the tubing in a 50 mL tube. Filter through a 0.22 μm sterile syringe filter. Aliquot and store at −80 °C (*see* **Note 15**).

3.7 Viral Titer Determination by Quantitative Real-Time PCR (qPCR)

1. Prepare a master mix of reagents (5 μL of 2X iTaq Universal SYBR Green Supermix, 0.5 μL of 10 μM forward and reverse primers, and 2 μL of water) that will be sufficient for duplicates of the following samples: four dilutions of standards, two dilutions of AAV sample, and one no-template control.

2. Thaw an aliquot of the ATCC AAV reference virus (or a well-characterized in-house AAV standard) (*see* **Notes 3, 4** and **16**), as well as an aliquot of the AAV to be quantified.

3. Prepare a standard curve of the reference virus by diluting the virus stock: 1:1,000; 1:10,000; 1:100,000 and 1:1,000,000.

4. Dilute the AAV sample 1:10,000 and 1:100,000.

5. Add 8 μL of master mix and 2 μL of virus dilutions or water to each tube/plate well for a final reaction volume of 10 μL, and perform the qPCR according to the PCR reagents and instrument manufacturer's instructions.

6. Calculate the viral titer based on the standard curve and dilution of virus sample using the qPCR machine software (*see* **Note 4**).

3.8 Alkaline Gel Electrophoresis

1. Perform alkaline gel electrophoresis to determine vector genome integrity and titer. Prepare the alkaline agarose gel by adding 1 g of agarose to 98 mL of water and microwave until all the agarose is dissolved. Let the solution cool until it can be handled without gloves, then add 2 mL of 50× Alkaline Electrophoresis Buffer, swirl to mix, and pour the solution into the tray to cast the gel.

2. Place the gel into an electrophoresis apparatus, fill with cold 1× Alkaline Running Buffer, and place everything in a cold room.

3. Dilute 500 ng of DNA ladder in 25 μL of water.

4. Mix 25 μL of AAV sample or diluted DNA ladder with 8.5 μL of freshly prepared 4× alkaline sample loading buffer, heat to 95 °C for 3 min, and cool on ice prior to loading on the gel.

5. Load the gel and run overnight at 20 V in the cold room using a dedicated power supply (*see* **Note 17**).

6. Remove the gel from the electrophoresis apparatus, place the gel in a container, and cover it with 0.1 M Tris–HCl pH 8.5 and rock gently for 1 h.
7. Discard the buffer and replace it with 4× GelRed or SYBR Gold in 0.1 M NaCl and rock protected from light for 2 h.
8. Rinse the gel briefly in tap water.
9. Visualize the gel with a UV transilluminator and capture a digital picture (*see* Fig. 1 and **Note 18**).

3.9 Vector Delivery Approaches for Cardiac Transduction

In the following sections, we describe the intravascular injection of rAAV vectors to rodents via the tail vein or the venous sinus, as well as local injection into the ventricular wall of the heart. Vector delivery by intraventricular injection combined with crossclamping of the aorta and pulmonary artery and local injection in the pericardium are described elsewhere [20].

1. Approval of the study by the local Institutional Animal Care and Use Committee is required before the initiation of the study.
2. The animals should be free of all endoparasites, ectoparasites, mycoplasma species, and common rodent viruses.
3. House rodents in a room at 22 ± 2 °C with a relative humidity of 50 ± 10%, and feed rodents a commercial laboratory diet and water *ad libitum* [21]. Ventilation in the room should be between 12 and 15 air changes per hour of 100% prefiltered outside air. The light cycle period should be controlled on a 12/12 h light/dark cycle with no twilight transition.
4. Males and females must be housed separately, as well as males from different litters, and overcrowding of cages must be avoided (*see* ref. 22 for further information on housing recommendations).

3.10 Anesthesia

Different methods of anesthesia can be employed, depending on the AAV vector administration procedure to be performed, the animal model used, and the available methods in the laboratory facilities. Here, animals are anesthetized with inhaled anesthetic for retro-orbital injections and with injectable anesthetic for local intramyocardial injection into the ventricular wall of the heart.

3.10.1 Injectable Anesthesia

1. Prepare a mixture of 100 mg/mL ketamine and 20 mg/mL xylazine.
2. Weigh the animal and inject intraperitoneally a dose equivalent to 50 mg/kg ketamine and 10 mg/kg xylazine with a 26G needle (for mice) or 21G needle (for rats) fitted with a 1 mL syringe.

3.10.2 Inhaled Anesthesia

1. Place the animal into an appropriately scavenged inhalational isoflurane anesthetic chamber. Introduce isoflurane into the chamber at a rate of 1.5–3 L/min at a 5% (v/v isoflurane/oxygen) concentration.
2. After 3–5 min, assess the level of anesthetic depth by observing the loss of the righting and palpebral reflexes such as pedal reflex (firm toe pinch), and by muscular tone, response to painful stimulation, and rate and depth of respiration.
3. Once the animal is completely anesthetized, switch the system to flow through a nose cone, and quickly remove the animal from the chamber and position it in the nose cone (*see* **Note 19**). Adjust the anesthetic delivery to 2.5% in 50% oxygen/50% air concentration to maintain surgical plane.
4. Proceed with AAV vector delivery, monitoring anesthetic depth throughout the procedure and adjusting the vaporizer as needed.

3.11 Intravascular Injection Via the Tail Vein

1. Prepare the AAV vector solution by diluting the dialyzed virions to the desired concentration in either PBS or lactated Ringer's solution (*see* **Note 20**).
2. Place the rodent under a heat lamp for a few minutes to promote peripheral vasodilation.
3. Immobilize the animal with a restraining device or lightly anesthetize the animal.
4. Hold the needle parallel to the posterior tail vein of the animal with the bevel side up at the lower portion of the tail (*see* **Note 21**). Use a 27–30G 1 mL insulin syringe for mice, or a 28–30G catheter or 1 mL syringe fitted with a 25G needle for rats (reviewed in [23]).
5. Puncture the vein and inject the vector solution (*see* **Note 22**).
6. Return the animal to the cage for recovery.

3.12 Intravascular Injection Via the Venous Sinus (Retro-Orbital Injection) (See Note 23)

1. Prepare the AAV vector solution by diluting the dialyzed virions in a volume of PBS or lactated Ringer's solution not exceeding 150 μL [24].
2. Anesthetize the animal with a volatile anesthetic drug such as isoflurane (*see* Subheading 3.10.2) and place it in left lateral recumbency with the head facing to the right on an isothermal heating pad (*see* **Note 24**). Apply gentle pressure to the skin dorsal and ventral to the eye to partially protrude the mouse right eyeball from the eye socket (*see* **Note 25**).
3. Using a 27.5G insulin syringe (for mice) or a 1 mL syringe fitted with a 23–28G needle (for rats), introduce vector solution carefully into the medial canthus with the bevel down at an angle of approximately 30 degrees (*see* **Note 25**). When the needle reaches the base of the eye, slowly inject the vector solution (do not

aspirate before the injection) and, when completed, withdraw the needle carefully and slowly to prevent the solution to leak out.

4. Return the animal to the cage for recovery.

3.13 Local Intramyocardial Injection into the Ventricular Wall of the Heart

1. Prepare the AAV vector solution by diluting the dialyzed virions in a volume of PBS or lactated Ringer's solution not exceeding 30 μL (for mice) or 400 μL (for rat). The temperature of the solution should be approximately body temperature.
2. Thirty minutes prior to anesthetization, inject all animals intraperitoneally with 0.1 mg/kg of the analgesic buprenorphine with a 26G needle (for mice) or 25G needle (for rats) fitted with a 1 mL syringe.
3. Anesthetize the animal with an intraperitoneal injection of the anesthetic ketamine/xylazine mixture (*see* Subheading 3.10.1). This injection will sedate the animal within 15–20 min.
4. When the animal is fully sedated, perform an endotracheal intubation with a 16–18G catheter (for rats) or a 20G catheter (for mice) using a fiber optic gooseneck light and connect the endotracheal tube to the ventilator. Test if the animal is breathing either by observing the heaving of the chest or by using a cold mirror to observe condensation of humidity from the animal's exhaled breath.
5. Set the ventilator to the correct tidal volume and respiratory rate (*see* **Note 26**). Maintain the surgical plane with inhaled anesthesia (*see* Subheading 3.10.2).
6. Place the anesthetized animal in a right lateral semi-decubitus position on an isothermal heating pad to maintain body temperature, and place a rectal temperature probe in the animal for temperature monitoring.
7. Remove the hair on the chest and disinfect the surgical area by washing with 10% povidone-iodine, followed by 70% ethanol.
8. Perform a left antero-lateral thoracotomy incision in the fourth intercostal space. Insert a small self-retaining rib spreader (*see* **Note 27**). Move the lung towards the posterior to allow access to the heart, and incise the pericardium. Once the myocardium is exposed, inject the virus directly into the left ventricle with a 1 mL syringe fitted with a 30G needle (for rats) or a 0.3–0.5 mL syringe fitted with a 31G needle (for mice) at an angle of 45 degrees in relation to the left ventricle wall. The puncture should go only a few millimeters deep as the ventricular wall is very thin (*see* **Note 28**).
9. Once hemostasis is achieved, begin closing the ribs and intercostal muscles with 4–0 Prolene suture (for rats) or 5–0 Prolene suture (for mice). Before tying the sutures, insert an 18G catheter (for rats) or a 22G catheter (for mice) into the thoracic cavity, then finish tying the sutures. Next, close the skin and subcutaneous space with running 5–0 Vicryl (absorbable) suture (for rats) or 6–0 Vicryl suture (for mice).

10. After tying the skin sutures, use a 3 mL syringe (for rats) or a 0.5 mL syringe (for mice) to evacuate air and fluid from the pleural cavity through the catheter that was inserted into the thoracic cavity, in order to regain the normal negative physiologic pressure.

11. Administer sterile lactated Ringer's solution subcutaneously to the animals (2 mL for rats and 0.3 mL for mice) and cease administration of isoflurane.

12. When the animal starts making spontaneous breathing motions and neck movements, disconnect the ventilator from the endotracheal tube and remove the endotracheal tube from the animal.

13. Return the animal to the cage for recovery.

14. For pain control, inject the animals subcutaneously with 0.3 mg/kg of Buprenex (for mice) or 0.1 mg/kg of Buprenex (for rats) immediately after surgery and again every 12 h for 3 days.

4 Notes

1. Subheadings 2.1–2.8 and 3.1–3.8, as well as associated Notes and display items, are reproduced with permission from [25].

2. Alternative nucleases that digest double-stranded DNA, single-stranded DNA, and RNA, such as Benzonase (Sigma), can also be used to achieve the same result. If Benzonase is used, add 2 μL of Benzonase (25 unit/μL) to every 1 mL of crude lysate and incubate for 30 min at 37 °C.

3. Extensively characterized AAV2 and AAV8 reference standard material can be obtained from ATCC (#VR-1616 and #VR-1816, respectively). The broad use of these reference standards should facilitate the reproducibility of titer determinations among different laboratories. For economic reasons, it might be preferable to employ the ATCC standard viruses to prepare a thoroughly characterized in-house reference virus preparation. However, unless ITR-specific primers are used [26], the reference standards can only be used for the titration of AAVs that share other common sequence elements with the AAV standards. A detailed protocol for in-house production of reference standard can be found in [27].

4. If a single-stranded in-house reference standard is used and the virus to be titrated is self-complementary, the final titer should be divided by 2. In this context, the accurate titer determination of double-stranded AAVs by qPCR has additional pitfalls, as previously described [28].

5. For further information on designing and constructing cis-plasmids, we refer the reader to [29].

6. Cells can be grown in the presence or absence of antibiotics. HEK293T cells are a human cell line, and all work must be performed in accordance with Biosafety Level 2 (BSL2) regulations. This includes the use of a BSL2 laminar flow tissue

culture hood. Adeno-associated viruses are BSL1, but all work with AAV should be performed under sterile conditions.

7. HEK293T cells can be passaged for about 1–2 months after thawing the initial vial, as long as they are regularly split at least twice a week.

8. To achieve approximately 70% cell confluency seed the cells of 2 confluent 175 cm^2 flasks per triple flask.

9. We routinely combine this PBS pellet wash solution with the cell culture supernatant from the previous step, rather than discarding it, because it can contain AAV virions.

10. The maximum volume of crude lysate that can be loaded in a single ultracentrifugation tube is 10 mL. However, the cell pellets from up to three triple flasks can be combined and resuspended in 10 mL of lysis buffer. After freeze/thaw and clearing of the lysate, the combined lysate can be loaded into a single tube. Similarly, it is possible to resuspend the pellets of precipitated AAV from cell culture supernatant of up to three triple flasks.

11. Although incubation on ice for as little as 30 min is sufficient for virus precipitation, keeping the ammonium sulfate/cell culture supernatant mixture for several days at 4 °C will not affect the quality of the virus preparation. However, once the virus precipitate has been spun down, the pellet should be resuspended and loaded onto a gradient the same day. Resuspending the pellet and freezing for a later gradient is not recommended as aggregation appears upon thawing.

12. The creation of multiple iodixanol gradients can be facilitated by using a multichannel peristaltic pump (Watson-Marlow) to assemble several gradients simultaneously. With such a system the different iodixanol solutions can be delivered to multiple tubes at the same time, reducing the number of necessary pipetting steps. Using this method, up to four centrifugations (with Ti70 rotors, holding 8 tubes each) can be performed in one day.

13. If a different rotor is used, the speed and run times must be adjusted using the k-factors of the Ti70 rotor at 350,333 × g (k = 44.9) and the respective k-factor of the alternate rotor at a given speed. A convenient tool for this calculation can be found at: https://www.beckmancoulter.com.

14. Calculate whether the AAV titer is sufficiently high for the planned experiments. Especially for in vivo experiments, when small injection volumes are required, it might be necessary to concentrate the virus further. In addition to concentrating the virus, if the virus was collected as a single fraction during the first gradient purification, this second ultracentrifugation gradient also allows the removal of empty capsids that may have been collected together with the genome-containing AAV particles from the first gradient.

15. For long-term storage, AAV is preferably stored at −80 °C. However, AAVs are extraordinarily stable; recent reports have shown no loss of activity after one week of storage at room temperature [30]. This can have important implications

for the shipping of AAVs. In particular, the quality of the virus is unlikely to be affected if all dry ice has evaporated due to unanticipated delays in the delivery of the virus. Because of the danger of damaging the AAV genome, never expose AAV to UV light (such as that from a biosafety cabinet).

16. The quality of AAVs purchased from academic vector cores or especially commercial vendors can vary significantly. This is especially true for double-stranded AAVs or AAVs with a genome size approaching the maximum packaging capacity of the AAV capsid. Therefore, we recommend a thorough in-house characterization before performing any experiments. Capsid titers and virus purity can be assessed by Coomassie staining of virions run on an SDS-PAGE gel [25]. Although this is not routine for in vitro or small animal experiments, we recommend that researchers that are not familiar with the procedures described in this chapter use SDS-PAGE and Coomassie staining to confirm the purity of their viral preparations. Once the routine production of high-quality AAV vector preparations has been firmly established in a laboratory, SDS-PAGE followed by Coomassie Blue staining may be omitted. However, we strongly recommend the use of alkaline gel electrophoresis to confirm vector genome integrity.

17. Moving the power supply frequently from the cold room to room temperature will damage the power supply due to water condensation.

18. Alkaline gel electrophoresis can also be used to determine AAV titers. For this, load a dilution series of a mass DNA ladder, or dilutions of a linear DNA fragment of known concentration and, preferably, of similar size to the AAV genome (e.g., the cis-plasmid digested with *SmaI* if the only *SmaI* sites are in the viral ITRs) onto the same gel as the AAV samples to be quantified. Quantification can then be performed with the freely available ImageJ (http://imagej.nih.gov/ij/) or similar software. This method is especially useful if the AAV samples to be quantified do not share sequence elements with the reference standard and for double-stranded AAVs.

19. During the system change, the animal might start to awaken. In this case, wait until it is fully anesthetized again before starting with the AAV vector administration procedure. The use of a nose cone can cause eye dryness. Use an eye lubricant compatible with the vector delivery procedure while using the nose cone, if necessary.

20. For systemic delivery, the dose of AAV vector usually ranges from 2×10^{10} vg to 5×10^{11} vg for adult mice and 5×10^{11} vg to 2×10^{12} vg for rats. The injection volume should be around 5 mL of solution per kg (reviewed in [31]). For reference, commonly used injection volumes are 100–200 μL for mice and should not exceed 500 μL for rats.

21. For administration of the vector solution with a needle into small rodents such as mice, slightly bending the needle 30 degrees up prevents having to excessively bend the rodent tail and, thus, helps with the injection procedure.

22. When injecting the vector solution intravenously, the vein loses its purple color and acquires a whiter color due to the vector solution displacing the blood contained in the vein. This is a positive sign that the vector solution is being properly injected into the vein. Swelling of the tail is indicative of the vector solution being injected subcutaneously instead of intravenously. In the latter case, remove the needle and try a second administration attempt puncturing the vein closer to the tail base than the initial attempt, as the vein might have been ruptured at the location of the initial attempt.

23. Despite the fact that this technique might seem aesthetically distasteful, it has been argued to be a more humane alternative compared to the more commonly used tail vein injection because retro-orbital injection has a lower failure rate, and it can easily be applied to adult and neonatal mice ([32] and reviewed in [24]). It is important to not perform more than one injection per eye per day, and not more than two injections in total per eye (with one day interval between injections).

24. A drop of ophthalmic anesthetic can also be applied on the eye to minimize pain to the animal upon completion of the procedure. Excess ophthalmic anesthetic can be removed carefully from the medial canthus with an absorbent gauze pad.

25. Special care must be taken while performing this technique because excessive pressure to the ventral cervical vessels (jugular veins and carotid arteries) will obstruct blood flow and impede administration of the vector solution, and pressure to the trachea may cause it to collapse and restrict air flow. Also, the needle must have minimal movement once inserted into the retro-orbital plexus to prevent vessel rupture and subsequent bleeding and loss of vector solution into the tissue behind the eye. There should be little-to-no bleeding following withdrawal of the needle, and the whole procedure should not take more than 1 min.

26. Use a convenient calculator for appropriate tidal volumes and respiratory rates at https://www.kentscientific.com/Products/Specs/SpecPopUp.asp?Mode=SpecBody&SpecTypeId=3&SpecId=216, or use a formula found in [33]. Keep the peak pressure at or below 10 cmH_2O.

27. For easier evaluation, perform the surgical procedure with a stereo microscope at ×10 magnification.

28. The viral solution should be injected in the apex and posterolateral wall of the left ventricle.

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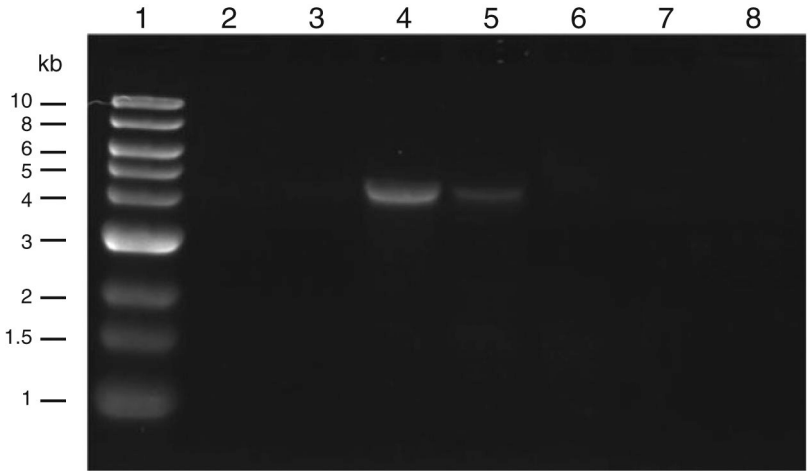

Fig. 1.

Alkaline gel of fractions collected from an iodixanol gradient. *Lane 1*:1 kb DNA Ladder (500 ng). *Lanes 2–8,* 20 μL of fractions 2–8 of a first iodixanol gradient of AAV9-LMNA. The capsid protein content in fractions 5 is higher than in fraction 4, indicating that fraction 5 contains empty capsids (SDS-PAGE, not shown)

Table 1

Components to prepare iodixanol gradient layer solutions sufficient for one complete (8 tubes) Ti70 Rotor

Iodixanol gradient layer (%)	Optiprep (mL)	1 M $MgCl_2$ (µL)	5 M NaCl (mL)	10× PBS (mL)	Phenol Red (µL)	Water (mL)	Total for 1 full rotor (mL)
15	14.6	58.4	11.68	5.84	–	26.02	58.2
25	16.33	39.2	–	3.92	78.4	18.83	39.2
40	21.33	32	–	3.2	–	7.44	32
60	31.95	32	–	–	16	–	32