Chapter 20

AAV Gene Transfer to the Heart

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Abstract

Recombinant adeno-associated virus (rAAV) has been widely used for gene therapy. AAV-mediated gene transfer leads to durable protein expression in non-proliferating targeted tissues, which enables long-term modulation of gene expression. Here we describe a rAAV production protocol based on PEI-mediated triple transfection of HEK293T cells, followed by purification by iodixanol density gradient ultracentrifugation. Viral yield varies, depending on the size of the viral genome, but, typically, a yield of 3E11 viral genome (vg) can be achieved using the described protocol. Our results showed that injection of rAAV9 significantly transduces cardiac cells, which supports rAAV9 being an effective tool for gene delivery in the heart in vivo.

Key words Adeno-associated virus, Gene therapy, Transfection, Ultracentrifugation, Density gradient separation

1 Introduction

With greater knowledge of the contribution of genetic mutations and human disease, approaches that modulate gene expression in vivo have been intensively investigated. Gene therapy, in which a therapeutic gene product is delivered to target cells, could enable treatment for many disorders by replacing genes disabled by mutation or by altering activity of signaling pathways that contribute to disease pathogenesis [1]. In addition to the therapeutic opportunity, gene therapy is also an exciting tool to dissect genetic disease mechanisms in vivo. For example, we used cardiac-targeted AAV to study Hippo-YAP regulation of cardiac regeneration [2–4]. Many delivery methods, including viral and nonviral routes, have been proposed to deliver nucleic acids to target cells. Whereas nonviral vehicles, such as nanoparticles, provide advantages of transient expression and the ability to fine-tune expression level and duration, viruses, particularly recombinant adeno-associated virus (rAAV), have some notable advantages compared to other methods, including cell specificity, durable expression, outstanding

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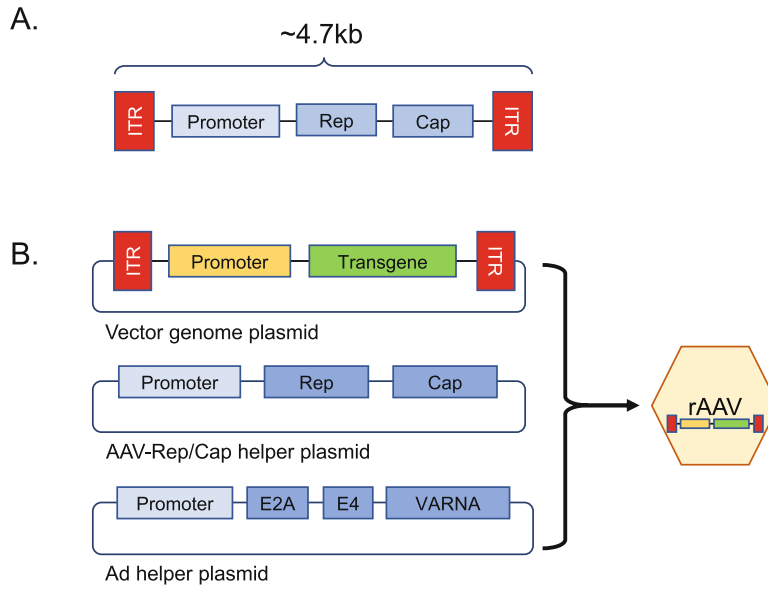


Fig. 1 System for producing helper-free, non-integrating, and replication-deficient AAV. (**a**) Schematic representation of AAV genome. (**b**) Triple-transfection system to produce recombinant AAV that is replication deficient, non-integrating, and helper free

safety profile in several clinical trials [5–7], and rare genome integration [8, 9].

Since its discovery as a contaminant in adenovirus preparations, AAV has been intensively studied to gain further understanding of its biology and characteristics. AAV is one of the smallest non-enveloped viruses, with a total size of approximately 25 nm and single-stranded DNA viral genome sized 4.7 kb in length [10]. As suggested by its name, AAV requires adenovirus or other viruses for its assembly and genome replication. In wild-type AAV, the viral genome contains two genes, *rep* and *cap*, which are flanked by two AAV serotype-specific inverted terminal repeats (ITRs) (Fig. 1a). Through the use of two promoters and alternative splicing, the *rep* gene encodes four proteins involved in AAV genome replication, and the *cap* gene encodes three capsid proteins and, in an alternate reading frame, the assembly-activating protein (AAP) [9]. The assembled virus attaches to and enters target cells. In the absence of a helper virus, the AAV genome establishes latency by integrating into the AAVS1 locus, a 4 kb region on chromosome 19. Integration requires a subset of the *rep* gene products, the ITRs, and a third cis-acting sequence, the integration efficiency element (IEE). In the presence of a helper virus, the AAV undergoes genome replication, viral gene expression, and virion production.

To enable the use of AAV as a vector for potential gene therapy, scientists have developed recombinant AAV (rAAV). The genome of rAAV is composed of two ITRs that flank the therapeutic cargo; all other native sequences have been removed. To produce rAAV, *rep* and *cap* gene products are provided in *trans*, e.g., by transfection of producer cells with plasmids that encode these proteins. When target cells are transduced by rAAV, absence of *rep* and *cap* genes and IEE prevents either genome integration or virion production. The rAAV genome becomes double stranded and is maintained as an episome, which can stably support expression of the transgene cargo for at least several years [11]. However, the episome does not support replication, so that rAAV will eventually be lost in proliferating cells. This unique characteristic of rAAV makes cells that do not undergo rapid division, such as cardiomyocytes and neurons, ideal targets for rAAV-mediated gene therapy.

At least 12 different naturally occurring AAV serotypes have been isolated, each with the same general organization but with specific ITR, *rep*, and *cap* sequences. Each AAV has distinct tissue tropisms, with AAV9 being among those that preferentially transduce the heart. Additional engineered capsids have been developed to modify target cell specificity. It is possible to "pseudotype" an AAV genome with the capsid of a different serotype, so that one AAV genome can be packaged with different capsids. Typically, the AAV2 ITRs are packaged with other AAV capsids by using AAV2 *rep* with the *cap* gene from other serotypes. For cardiac rAAV, AAV2 ITR and *rep* are most commonly combined with AAV9 *cap* (denoted AAV2/9).

In addition to capsid-directed cell type-specific transduction, tissue specificity can be achieved using tissue-specific promoters to drive the expression in selected cells, and miRNA target sites to suppress expression in off-target cells. For example, to drive the expression in cardiomyocytes but not in other cells, we have used the cardiac troponin T promoter [12]. Incorporating target sites of miRNAs expressed in off-target but not in on-target cells is another mechanism to selectively direct the expression of cargo to specific cell types.

Once packaged, rAAV can be delivered to the heart by various routes of injection, depending on the stage. Generally speaking, intravenous injection typically gives higher transduction in the heart with a cardiac-targeting virus. However, intravenous injection can be technically challenging in neonatal mice, for instance, via injection into the retro-orbital sinus or superficial facial vein, and consistent and efficient neonatal delivery can be achieved through subcutaneous delivery [12–15]. In adult mice, delivery options include (1) intravenous administration through the lateral-tail vein, which is efficient but can be technically challenging, particularly in darker colored mouse strains; (2) intraperitoneal injection, which can result in inconsistent and less efficient cardiac

transduction [16, 17]; and (3) injection into the retro-orbital sinus, which is an alternative intravenous route to tail-vein injection and results in consistent and efficient cardiac rAAV transduction [12]. In large animal models, intracoronary infusion of rAAV has been used to enhance cardiac gene delivery while reducing extracardiac delivery [18–20].

As the field of AAV biology and gene therapy moves forward, different viral packaging protocols have merged and been optimized in different labs and institutions. A comparison of rAAV manufacturing technologies between several gene therapy centers is well summarized in ref. [21]. Here, we describe an AAV production and titration protocol that our lab uses to produce rAAV2/9 with high yield and without helper virus contamination (Fig. 1). This protocol is based on previously described AAV production protocols [22]. In brief, viral HEK293T cells are transiently transfected with the rAAV genome plus two plasmids that supply essential *trans*-acting factors. Packaged virions are collected from the media and the cells and are purified by density gradient ultracentrifugation. The typical yield of one preparation is 3E11 viral genomes (vg).

2 Materials

1. Reagents are prepared with deionized distilled water and passed through 0.22 μm filters to remove insoluble substances and bacteria.
2. HEK293T cells: HEK293T cells are cultured with DMEM and supplemented with 10% FBS and 1% Pen-Strep, in humidified 37 °C tissue culture incubators supplied with 5% CO_2 (*see* **Note 1**).
3. Lysis buffer: Prepare the lysis buffer containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, and 1 mM $MgCl_2$. Store at 4 °C.
4. AAV precipitation solution: 40% PEG 8000 (w/v), 2.5 M NaCl. As dissolved PEG 8000 takes up significant volume, it is recommended to first dissolve PEG 8000 and NaCl in 70% of the total volume and stir until fully dissolved. Add water to the final volume. Keep at room temperature.
5. Iodixanol gradient: Prepare a series of iodixanol-based solutions of different density following Table 1 (indicated for 50 mL each). Once prepared, solutions can be stored in 4 °C for up to a month.
6. AAV plasmids: Obtain the adeno helper plasmid pHGTI/pAd-DeltaF6 (referred to as pHGTI in the following content) from Penn Vector Core (also available through Addgene). This is a low-copy plasmid, as it uses the pBR322 origin of replication.

Table 1
Iodixanol gradient preparation

% of iodixanol	10× PBS (mL)	1 M $MgCl_2$ (mL)	5 M NaCl (mL)	1 M KCl (mL)	Optiprep (mL)	Phenol red (0.5%, w/v) (mL)	ddH_2O (mL)
17	5	0.05	10	0.125	12.5	0	22.3
25	5	0.05	0	0.125	20	0.5	24.325
40	5	0.05	0	0.125	33.3	0	11.525
60	0	0.05	0	0.125	50	0.5	0

Obtain the appropriate plasmid containing rep-cap genes. For AAV2/9 pseudotyping, we use pAAV2/9 from Penn Vector Core (also available through Addgene). Prepare these two helper plasmids using Maxiprep. For a standard AAV preparation (ten plates of 150 mm dishes), one will need 320 μg of pHGTI and 140 μg of pAAV2/9 plasmid.

3 Methods

3.1 Preparation of DNA for AAV Production

1. Clone the gene of interest into the rAAV genome plasmid between the ITRs. For example, enzyme-mediated restriction digestion and ligation can be used to build a recombinant plasmid that harbors a cDNA driven by the cardiac troponin T promoter, using AAV-TNT-GFP (Addgene #105543) as a starting point (*see* **Notes 2** and **3**).
2. Once the AAV genome plasmid has been successfully constructed, transform it into competent cells, and amplify the plasmid DNA. We recommend using competent cells, such as *Stbl2*, which have been designed to maintain unstable inserts to amplify the AAV genome plasmid, pAAV2/9 plasmid, and pHGTI (adeno-miniplasmid or "adeno-helper plasmid"), respectively (*see* **Note 3**). Isolate the plasmid DNA from overnight *E. coli* cultures using a standard plasmid maxiprep kit.

3.2 Transfection of HEK293 Cells

1. For each AAV preparation, ten plates of HEK293 cells in 150 mm culture dishes are needed. Cells should be 70–80% confluent on the day of transfection. 2–5 h before transfection, change to fresh medium (10% FBS, 1% Pen-Strep in DMEM).
2. For each set of ten plates, warm 20 mL of Opti-MEM to room temperature and add 140 μg of plasmid containing transgene, 140 μg of serotype-specific *rep/cap* plasmid, and 320 μg of the helper plasmid, pHGTI. Mix well by shaking.
3. Add 2.4 mL of PEI (1 mg/mL; 1:4 μg DNA-to-μg PEI ratio). Mix immediately by shaking and leave at room temperature for 15 min.

4. Add 2 mL of PEI:DNA mixture to each plate and rock to mix thoroughly.
5. 12–18 h later, change the medium to serum-free (SF) medium (DMEM supplemented with 1% Pen-Strep). Culture the transfected cells for 60–72 h before harvesting AAV (*see* **Notes 4–6**).

3.3 Harvesting of Crude AAV Prep

AAV virions are produced in cells and are also detectable in SF medium after transfection.

1. Harvest the cells 60–72 h after transfection. Use cell scrapers to detach the cells. There is no need to homogenize or disrupt cells during scraping, as the cells will be lysed in a later step.
2. Transfer the cells with the medium into a 250 mL sterile conical tube and centrifuge at 2000 rpm (1100 g) for 5 min at 4 °C.
3. Transfer the supernatant (200 mL) into a new 250 mL conical tube and add 50 mL of 40% PEG-containing precipitation solution. Mix well by inverting the tubes. Put the solution on ice and keep in a 4 °C cold room overnight.
4. Resuspend the cell pellet from **step 2** in 7 mL of lysis buffer. The final volume should reach approximately 10 mL.
5. Add 2 μL of benzonase (≥250 units/μL) to the cell suspension and transfer it to a 50 mL Falcon tube for easier handling.
6. Use the following freeze-thaw cycles to lyse cells (*see* **Note 7**): 30 min at −80 °C, 30 min at 37 °C, 30 min at −80 °C, and 30 min at 37 °C. Freeze the cell lysate at −80 °C after the final thawing step.
7. Next day: Thaw the cell lysate from **step 6** at 37 °C and add 3 μL of benzonase (≥250 units/μL) to remove free DNA from plasmid transfection and HEK293 cells.
8. Centrifuge the media supernatant plus PEG solution from **step 3** at 3500 rpm (3300 g) at 4 °C for 30 min. Aspirate and dispose of the supernatant. Resuspend the pellet in 5 mL of lysis buffer.
9. Combine with the cell lysate and continue to incubate at 37 °C for an additional 45 min. The total cell lysate contains crude AAV, which could be frozen at −80 °C until ready for AAV purification.

3.4 Purification of AAV

1. Thaw the AAV lysates at 37 °C. Centrifuge at 4000 rpm (4500 g) at 4 °C for 30 min.
2. While waiting for the centrifugation, prepare the iodixanol-containing Optiprep gradient in Optiseal tubes (refer to material for detailed recipes). Start from the lowest density layer and add increasingly more concentrated solution to the bottom of

the former layer. Add each layer slowly to the bottom of the tube using a blunt-ended 16G needle and syringe. Volumes for each layer are shown below:

(a) 6 mL of 17% iodixanol.

(b) 5 mL of 25% iodixanol.

(c) 4 mL of 40% iodixanol.

(d) 5 mL of 60% iodixanol.

3. Layer the supernatant from **step 1** on top of the density gradient. Add slowly using a pipet pressed against the tube above the top of the density gradient. Try not to disturb the density gradient.
4. Balance the Optiseal tubes with the AAV lysis buffer.
5. Place the centrifuge tubes in a VTi50 or equivalent ultracentrifuge rotor. Centrifuge at 170,000 × *g* for 110 min at 16 °C.
6. After centrifugation, extract 40% of the layer with an 18G needle and syringes. The 40% layer will be yellow and can be identified easily in a well-lit environment. Remove the cap of the tube. Puncture the tube on the side of the wall with a needle and syringe. Insert the needle at approximately 2 mm lower than the 40%/60% interface with the bevel facing up. AAV was reported to have density at 1.266 g/mL, which is approximately similar to a 50% iodixanol solution [23]. Therefore, AAV largely accumulates at the bottom of the 40% layer and the interface with the 60% layer. Avoid getting the 25% layer or the interface of 40% with 25% where cell debris and protein accumulate.
7. Mix the extracted AAV-containing layer (approximately 4 mL) with 10 mL of PBS + 0.001% nonionic surfactant Pluronic F-68 in spin concentrators with a 100 kDa molecular weight cutoff (for example, Fisher Scientific Cat#UFC910024).
8. Centrifuge at 5000 × *g* for 30 min at 4 °C.
9. Remove the flow-through and add 15 mL of new F68-containing PBS (0.001% F68 in PBS). Repeat **steps 8** and **9** for a total of three washes.
10. Store the purified AAV in −80 °C until if needed. To avoid repeated freeze-thaw cycles, aliquot the purified AAV.

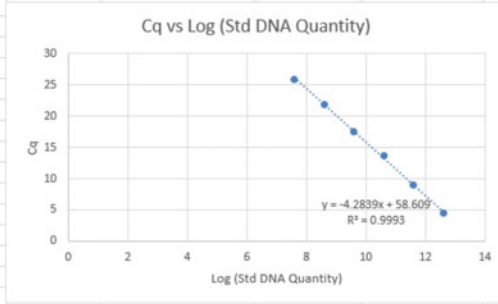
3.5 QPCR-Based Quantification of AAV Titer

1. DNA standard for viral titration: Design specific and efficient quantitative PCR (qPCR) primers for viral titer measurement. Target sequences should be between two ITRs that can be easily accessed and amplified, following regular QPCR primer design guidelines [24]. Typical choices of sequence for QPCR-based titration include promoters (for comparison between different cargos), cDNA (for comparison between various *cis*-

elements), as well as ITRs, which are commonly used in many studies [25–27] and often shared between many different rAAV genomes. However, there have been reports of artificially high titers as a result of ITR-based titration [28]. In this protocol, we use a plasmid-containing CAG promoter (Addgene #37825) and designed primers in the promoter region: Fwd: 5'GTGGCTGCGTGAAAGCCTTGAG 3′; Rev: 5' GGCATGAACATGGTTAGCAGAGGCTCTAG 3′. If cardiac-only delivery is desired, one could also use a cardiac-restricted promoter, such as a cardiac troponin T promoter. We have deposited Tnnt2-containing rAAV2 genomes into a publicly accessible repository (Addgene #86558, 87682, 69915). Generate DNA standards by PCR amplification of target sequences using optimized PCR conditions. For the troponin primers above, we used GoTaq polymerase and the following PCR program: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 5 min. Run the PCR product on a 2% agarose gel and confirm that the PCR generates a single band. Use a gel purification kit to extract the DNA from the gel. Measure the DNA concentration using a UV spectrophotometer (e.g., Nanodrop). Aliquot the DNA standard and store in 10 mM Tris pH 8, 1 mM EDTA at −20 °C. Avoid repetitive freezing and thawing cycles, as this will degrade the DNA.

2. Digest the DNA not assembled into virions by DNase I treatment: 5 μL of purified AAV + 5 μL of 10x DNase buffer (100 mM Tris–HCl; 50 mM $MgCl_2$; 5 mM $CaCl_2$) + 1 μL 2 U/μL DNase I + 39 μL ddH_2O. Incubate at 37 °C for 30 min. Inactivate the DNase by treatment at 95 °C for 10 min.
3. Remove the capsid and release the viral genome by proteinase K treatment: Dilute 20 mg/mL of proteinase K by 20-fold in H_2O. To DNase-treated sample from **step 2**, add 5 μL of 10x DNase buffer, 35 μL of ddH_2O, and 10 μL of diluted proteinase K. Incubate at 37 °C for 15 min and terminate at 95 °C for 20 min. Chill on ice.
4. QPCR (perform technical duplicates for QPCR-based AAV titration): Use a DNA standard to create 10 μL of solution with a concentration of 1 ng/μL. Make tenfold serial dilutions of the 1 ng/μL solution. Each dilution should be 10 μL. The DNA solutions should cover the range from 1 to 10^{-8} ng/μL. Dilute the pretreated AAV from **step 3** by 1:10, 1:100, and 1:1000. Set up the QPCR reaction using the previously designed primers and optimized PCR programs: 10 μL of 2X SYBR Green Mastermix, 0.5 μL of F primer (10 μM), 0.5 μL of R primer (10 μM), 8 μL of ddH_2O, and 1 μL of diluted AAV or standard DNA.

	DNA Std Size (bp)	DNA Std MW (g/mol)	DNA Std Srial Dilution Concentration (ng/μl)	Quantity/ml	Log of (Quantity/ml)	Average Cq
Pre-calculated→	170	112200	0.698	3.8757E+12	12.58835061	4.443130394
			0.0698	3.8757E+11	11.58835061	8.977782282
			0.00698	38757040998	10.58835061	13.62220717
			0.000698	3875704100	9.588350612	17.55337362
			0.0000698	387570410	8.588350612	21.80132118
			0.00000698	38757041	7.588350612	25.94995454



AAV Sample Dilution	Cq1	Cq2	Average Cq	Log Quantity	Diluted AAV Quantity/ml	Undiluted AAV Quantity after pre-treatment/ml	Undiluted AAV quantity before pre-treatment/ml	Average Undiluted AAV quantity/ml
1/10	12.47414536	12.58106804	12.5276067	10.75687885	5.71E+10	5.71319E+11	1.14264E+13	1.45484E+13
1/100	16.34544718	16.31625923	16.33085321	9.869078828	7.40E+09	7.3974E+11	1.47948E+13	
1/1000	20.44423096	20.17663009	20.31043053	8.940117526	8.71E+08	8.71199E+11	1.7424E+13	

Fig. 2 Example calculation of AAV titer calculation using a standard curve and qPCR

5. Run QPCR: Calculate the AAV titer using the Cq from the DNA standards and AAV samples. Calculate the number of molecules in the DNA standard and its diluted series. For example, a primer set designed to amplify a fragment of a CAG promoter generates a PCR product of 170 bp, which is equivalent to the molecular weight (MW) of $170 * 2 * 330 = 112{,}200$ g/mol. The number of molecules per mL is calculated using the following equation:

$$\text{DNA molecules mL}^{-1} = \frac{\text{DNA conc.}\left(\text{g mL}^{-1}\right) \times 10^{6} \times 6.23 \times 10^{23}}{\text{MW}\left(\text{g mol}^{-1}\right)}$$

Establish a linear standard curve using the Cq of DNA standards and Log [standard DNA molecules/mL] (Fig. 2). The generated equation is then utilized to calculate the viral genomes per mL using the Cq of AAV samples. The pretreatments (DNase and proteinase K) of AAV cause 20-fold dilution, which should be taken into consideration when calculating the original titer of the AAV.

3.6 Delivery of AAV to Mice

1. Obtain the appropriate biosafety and animal care and use regulatory approvals prior to delivering AAV to mice. For initial pilot experiments, use an AAV such as AAV-CAG-GFP (Addgene #37825), where validation of effective delivery to the heart is straightforward.

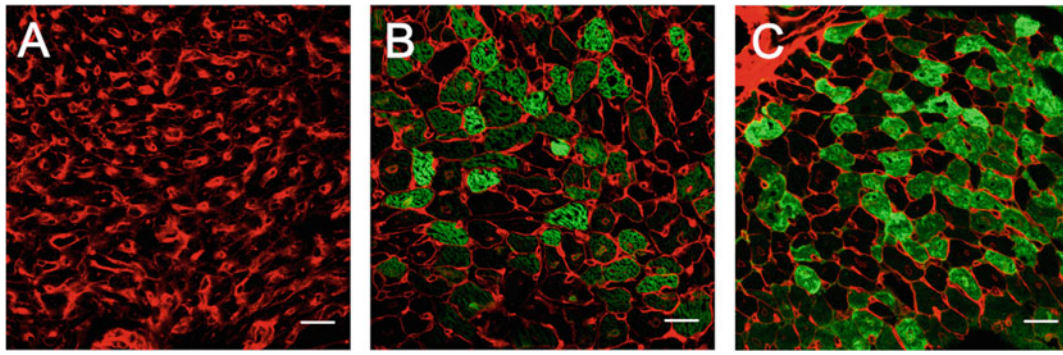


Fig. 3 Example of expression of GFP reporter gene in the heart after the injection of rAAV-CAG-GFP. GFP expression is visualized by immunofluorescent staining of hearts treated by saline (**a**) or rAAV9-CAG-GFP (**b, c**). GFP expression was examined 21 days after injection in P1 neonatal mice via subcutaneous injection (**b**) or adult mice via retro-orbital injection (**c**). Cell membrane is counterstained and shown in red using WGA. Scale bars represent 20 μm

2. For neonatal mice: Prepare the rAAV-containing injectate in sterile saline or PBS. Inject up to 50 μL to newly born mice (P0–P5). The volume can slightly increase as mice grow (*see* **Note 8**). Subcutaneous injection can be conveniently done on either the chest area or the back of the neonate by one person using an insulin needle and syringe. The efficiency of cardiac transduction can be determined by immunofluorescent staining of the reporter gene or the transgene. Figure 3b shows an example using rAAV9-CAG-GFP.
3. For adult mice: Use retro-orbital injection for virus delivery to the heart. The rAAV-containing injectate is prepared in sterile saline or PBS, up to 100 μL per eye. Insulin needles and syringes are used for the injection. Cardiac transduction can be examined by immunofluorescent staining. Figure 3c shows an example using rAAV9-CAG-GFP.

4 Notes

1. 293T cells should be passaged with a 1:5 to 1:7 ratio and split every 2–3 days. Keeping the cells at low passage helps maintain their healthy condition and allowing the HEK293T cells to reach confluency reduces their competency and may compromise transfection efficiency. When preparing the cells for transfection, switch the passage ratio to 1:3 or 1:4 to enhance cell proliferation.
2. Other cloning technologies, such as Gibson assembly, can also be applied to achieve construction of the desired plasmid. The packaging capacity of a single-stranded rAAV, namely the number of base pairs between ITRs, is generally about 5 kb, whereas self-complementary rAAV can only contain about 2.5 kb DNA.

3. ITRs are not stable in *E. coli*. Therefore, it is recommended to always check for the integrity of ITRs in plasmids carrying the rAAV viral genome when DNA is replicated by *E. coli*. Due to the complexity of the ITR's secondary structure, it is difficult to perform regular Sanger sequencing on intact ITRs. An easy approach is to digest the ITRs with the restriction enzyme, SmaI. Each ITR from AAV2 contains two SmaI sites for restriction digestion, separated by 5 nt and, therefore, allows for a brief checkup of the ITRs by restriction digestion using SmaI. As a result of enzymatic cutting, the viral genome-containing plasmid will be cut into two major fragments, namely the bacterial backbone and the transgene flanked by ITRs, unless there is another SmaI site included in the designed promoter/trangene/any other cis-elements between the ITRs. An alternative choice for a restriction enzyme is XmaI, which is also present as a pair separated by 5 nt in AAV2-derived ITRs. We also recommend using bacterial strains, such as Stbl2, that are designed for cloning and amplifying unstable plasmids. Reduced culture time and temperature (e.g., 14 h at 30 °C) can also reduce the risk of recombination.
4. PEI transfection is effective as early as 8 h after the addition of PEI-DNA complex, and thus overnight transfection time is sufficient.
5. PEI-containing medium should be replaced with fresh medium at the recommended time to avoid the cytotoxicity of PEI.
6. Serum-free medium slows down cell proliferation but does not block AAV production.
7. Cell lysis can also be achieved by nonmechanical methods, such as detergent.
8. Depending on the desired therapeutic window, limit of injection volume on different neonatal stages should be consulted with the veterinary office at each animal facility.

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