Journal of Visualized Experiments

Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts

--Manuscript Draft--

Manuscript Number:	JoVE54787R3
Full Title:	Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	gene delivery, cardiomyocyte, adeno-associated virus, gene overexpression, gene knockdown, subcutaneous injection
Manuscript Classifications:	1.7: Cardiovascular System; 1.7.541: Heart; 95.51.10: biology (general); 95.51.19: genetics (animal and plant); 95.51.24: life sciences; 95.51.4: animal models; 95.51.6: biochemistry
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Abstract:	Controlling expression or activity of specific genes by myocardial delivery of genetic materials in murine models permits investigation of gene functions as well as their therapeutic potential in the heart. There are limited approaches for in vivo molecular intervention in the mouse heart. Recombinant adeno-associated virus (rAAV)-based genome engineering has been utilized as an essential tool for in vivo cardiac gene manipulation. The specific advantages of this technology include high efficiency, high specificity, low genomic integration rate, minimal immunogenicity, and minimal pathogenicity. Here, a detailed procedure to construct, package, and purify the rAAV9 vectors is described. Subcutaneous injection of rAAV9 into neonatal pups results in robust expression or efficient knockdown of the gene(s) of interest in the mouse heart, but not in the liver and other tissues. Using the cardiac specific TnnT2 promoter, high expression of the GFP gene in the heart was obtained. Additionally, target mRNA was inhibited in the heart when a rAAV9-U6-shRNA was utilized. Working knowledge of rAAV9 technology may serve the scientist in cardiovascular investigations.
Author Comments:	Here we submit the revision3
Additional Information:	

Question	Response
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

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July 5, 2016

Dear Dr. Singh and editors:

Thank you for inviting us to contribute our study to JoVE. We are now submitting a revised manuscript entitled "**Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts**" for consideration for publication.

In this study, we describe the detailed procedure to construct, package and purify the rAAV9 vectors. Subcutaneous injection of rAAV9 into neonatal pups resulted in robust expression or efficient knockdown of the genes of interest in mouse hearts.

We have carefully addressed the comments of reviewers and editors and have revised the manuscript thoroughly.

Thank you in advance for your consideration.

Sincerely yours,

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Associate Professor

TITLE:

Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts

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KEY WORDS:
Gene delivery, cardiomyocyte, adeno-associated virus, gene overexpression, gene knockdown, subcutaneous injection.

SHORT ABSTRACT:
In this manuscript, a method to prepare recombinant adeno-associated virus 9 (rAAV9) vectors to manipulate gene expression in the mouse heart is described.

LONG ABSTRACT:
Controlling the expression or activity of specific genes through the myocardial delivery of genetic materials in murine models permits the investigation of gene functions. Their therapeutic potential in the heart can also be determined. There are limited approaches for *in vivo* molecular intervention in the mouse heart. Recombinant adeno-associated virus (rAAV)-based genome engineering has been utilized as an essential tool for *in vivo* cardiac gene manipulation. The specific advantages of this technology include high efficiency, high specificity, low genomic integration rate, minimal immunogenicity, and minimal pathogenicity. Here, a detailed procedure to construct, package, and purify the rAAV9 vectors is described. Subcutaneous injection of rAAV9 into neonatal pups results in robust expression or efficient knockdown of the gene(s) of interest in the mouse heart, but not in the liver and other tissues. Using the cardiac-specific TnnT2 promoter, high expression of GFP gene in the heart was obtained. Additionally, target mRNA was inhibited in the heart when a rAAV9-U6-shRNA was utilized. Working knowledge of rAAV9 technology may by useful for cardiovascular investigations.

INTRODUCTION:
Controlling expression or activity of specific genes in various biological systems has become a valuable strategy in the study of gene function[1]. A direct means of accomplishing this goal is to manipulate nucleotide sequences and generate mutant alleles. Although making precise, targeted changes to the genome of living cells is still a time-consuming and labor-intensive practice, the development of the powerful TALEN and Crispr/Cas9 tools has opened a new era of genome editing[2-5]. A more routine laboratory method for gene manipulation has focused on the introduction of genetic materials (DNAs and RNAs containing coding sequences or siRNAs/shRNAs) into the cells to express or knockdown the gene(s) of interest[1,6].

In many cases, the major bottleneck for gene manipulation is the delivery of DNA, RNA, or protein into the cells. With regard to *in vitro* studies, efficient transfection systems have been established in many cultured cell lines. However, in the mouse model in particular, *in vivo* gene delivery is more challenging. There are a series of extra- and intracellular barriers that need to be bypassed in order to achieve efficient cellular uptake of the exogenous reagents. Additional obstacles include the rapid clearance and the short duration of the delivered materials[7,8]. One strategy to circumvent these issues is to use viral vectors as “carriers” or “vehicles” for *in vivo* gene delivery. The naturally-evolved transduction properties of viruses allow the efficient delivery of a gene of interest into cells[7,9,10]. Numerous types of viral vectors have been developed and enable flexible *in vivo* gene manipulation in different cell types and organs in mice.

The most commonly-used viral systems include Retrovirus, Lentivirus, Adenovirus, and Adeno-associated virus (AAV)[11]. Retroviruses are single-stranded RNA viruses and can introduce their genetic material to the host cell genome in a stable manner during mitotic division, providing the potential for lifelong expression of the transduced genes in the target cells and organs[12-14]. However, many types of retroviruses only infect dividing cells, and their efficacy in non-dividing cells is very low[15]. This limits their utility for gene delivery. Lentivirus is a genus of the *Retroviridae* family. Different from other retroviruses, Lentivirus can infect both dividing and non-dividing cells and has been widely used for gene transfer into post-mitotic and highly-differentiated cells[16]. The life cycle of Lentivirus also involves the integration of vector DNA into the host genome. Thus, Lentivirus-mediated gene delivery enables stable and long-duration expression of the transduced genetic elements[16-18]. However, this feature may represent a double-edged sword in the use of these viruses to manipulate gene expression, as integration of vector DNA may lead to insertional mutagenesis in the host cells and can cause artefactual effects. Adenovirus is another widely-used gene delivery system. Unlike retroviruses and lentiviruses, Adenoviruses are non-integrated and do not interfere with the genomic integrity of host cells[8,10,11,19]. In addition, Adenoviruses can transfect DNA into many cell types, and infection is not dependent upon active cell division[19]. Another important characteristic of Adenoviruses is the ease of vector purification, as the viral vectors have the ability to be replicated[19,20]. However, the major caveat of this system is that Adenovirus infection can trigger strong immune responses in target cells and organs[19], restricting its use in many investigations, particularly in gene therapy studies.

Compared with these different types of viral vectors, recombinant Adeno-associated virus (rAAV) appears to be the ideal gene delivery system[21,22]. It exhibits minimal immunogenicity and pathogenicity[23,24]. In addition, rAAV infects a broad range of cell types, including both dividing and non-dividing cells. In most cases, rAAV does not integrate into host genomes; thus, the risk of undesired genetic or genomic changes in the target cells is low[22].

Recently, rAAV systems have been successfully used for the *in vivo* delivery of DNA-encoding proteins, miRNAs, shRNAs, and Crispr-gRNAs into mouse cardiac muscle[23,25-29]. This methodology has facilitated fundamental investigations and gene therapy studies in the field of cardiovascular research. Here, the detailed procedure to generate rAAV9 vectors that efficiently overexpress or knockdown the genes of interest in mouse hearts was described. The protocol provides a simple and effective method of manipulating cardiac gene expression in murine experimental models.

PROTOCOL:

All described steps were performed under protocols approved by the Biosafety Committee and the Institutional Animal Care and Use Committee of Boston Children’s Hospital. Boston Children’s Hospital has pathogen-free mouse facilities with regulated light/dark cycles and climate control. Veterinary and animal care staff change cages and ensure the health of the mice. The facilities are AAALAC certified and have active Animal Welfare Assurance certification (AAALAC Accreditation Granted on 2/24/1992.

Animal Welfare Assurance number: A3303-01). Mice were euthanized by CO_2 delivered from a compressed gas source. Tissue samples were collected after confirming that heart rate, movement, and breathing of animals had ceased. Neonatal rodents are resistant to CO_2 euthanasia and were euthanized by decapitation using sharp scissors. These methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

1. Generation of rAAV9 constructs by cloning a cDNA or shRNA expression cassette into the plasmid backbone.

Note: The rAAV9 plasmid, containing the inverted terminal repeats (ITRs) of AAV2, used for gene overexpression has been modified to harbor the chicken TNNT2 promoter (rAAV9.cTNT), which enables cardiomyocyte-specific expression of transduced genes[25,26,29]. Unique NheI and KpnI sites have been introduced into the plasmid, downstream of the promoter. The cDNA fragments encoding the genes of interest can be cloned into the rAAV9 backbone using these two restriction sites[25,26,29]. Here, as an example, the rAAV9 vector for overexpression of the GFP gene in mouse hearts was generated. The resulting plasmid contains the cTNT::GFP cassette flanked by two ITR sites (**Figure 1**). rAAV9.U6::shRNA constructs were used for gene knockdown[25]. Design shRNAs using online shRNA design servers. rAAV9.U6::shRNA can be generated either by annealing and ligating DNA oligos-containing shRNA sequences into the restriction enzyme-digested rAAV9 vectors harboring the U6 promoter, or by long-range PCR and intra-molecular Gibson assembly-based "seamless" construction[30]. The resulting plasmid should contain the U6-shRNA cassette flanked by two ITR sites (**Figure 2**). Here, as an example, the rAAV9.U6::shRNA vector was constructed to knockdown Trbp mRNA (Trbp shRNA sequence: GCAGTGATGGATATGCATCTTCTCGAGAAGATGCATATCCATCACTCG). A scramble shRNA was used as a negative control (CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG).

1.1) Clone the cDNA or shRNA expression cassette into the rAAV9 plasmid backbone. Transform the DNA into competent *E. Coli* cells [25].

Note: Use stbl2 or stbl3 *E. coli* cells for rAAV9 DNA transformation to minimize undesired ITR recombination.

1.2) Pick up the positive clone from the transformed *E. Coli* cells. Amplify the culture in 500 mL of Lilly-Barnett medium and extract the rAAV9 plasmid from the bacterial cells [25-30].

Note: Midi/Maxi prep the rAAV9 plasmid to obtain a high amount of DNA (>100 µg). Before generating the virus, always analyze the sequence integrity of the AAV plasmids by restriction digestion and agarose gel electrophoresis, as previously described (http://www.vvf.uzh.ch/cloningservice/11bpdeletion/itrintegrity.html).

2. Transfection of HEK293 cells with rAAV9 plasmids

2.1) Prepare 1 µg/µL of linear polyethylenimine (PEI) solution. Dissolve PEI powder in endotoxin-free dH_2O that has been heated to 70-80 °C. After cooling down to room temperature, neutralize the solution to pH 7.0 with 1 M HCl. Filter sterilize (0.22 µm) the solution. Aliquot the 1 µg/µL PEI stock solution (1400 µL/tube) and store the solution at -20 °C.

2.2) Culture HEK293 cells in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Culture the cells in a 37 °C incubator with 5 ± 0.5% carbon dioxide (CO_2).

2.3) At day 0, plate HEK293 cells in ten 150-mm dishes 18-20 h before transfection by splitting >90% confluent cells in a 1:2 dilution.
Note: At day 1, the cells should reach 90% confluence.

2.4) At day 1, transfect HEK293 cells with the rAAV9 plasmid (*e.g.,* rAAV9.cTNT::GFP or rAAV6.U6::shRNA constructs), Ad-Helper plasmid, and AAV-Rep/Cap plasmid using PEI[25,26,29].

2.4.1) For 10 dishes of cells at 90% confluence, mix 70 µg of AAV-Rep/Cap plasmid, 70 µg pf rAAV9 plasmid, and 200 µg of Ad-Helper plasmid in a 50-mL centrifuge tube.

2.4.2) If the cells are less confluent, adjust the DNA amount proportionally. For instance, if the cells are at 75% confluent, reduce the DNA amount proportionally (75/90 of the amount shown in step 2.4.1): mix 70 x 75/90 = 58.3 µg of AAV-Rep/Cap plasmid, 70 x 75/90 = 58.3 µg of rAAV9 plasmid, and 200 x 75/90 = 166.7 µg of Ad-Helper plasmid in a 50-mL centrifuge tube.

2.4.3) Add 49 mL of room-temperature DMEM (without FBS) to the 50-mL tube and mix well.

2.4.4) Add 1,360 µL of PEI solution to make the PEI:DNA ratio (v/w) be 4:1. Mix well. Incubate at room temperature for 15-30 min.

2.4.5) Add 5 mL of the mixture prepared in step 2.4.4 to each 150-mm dish (50 mL of the mixture for ten 150-mm dishes).

2.5) Culture the cells in a 37 °C incubator with 5 ± 0.5% CO_2 for 60-72 h.

3. Harvest of transfected HEK293 cells and purification of rAAV9 vectors
3.1) Harvest the cells 60-72 h after transfection. Dislodge and suspend the cells in the dishes by pipetting up and down with the culture medium. Transfer all the cell suspensions to sterile 50-mL tubes.

3.2) Centrifuge the cells at 500 x g for 5 min. Resuspend the cell pellet with 5 mL of PBS in each tube and combine all the cell suspensions into one 50-mL tube.

3.3) Centrifuge the cells at 500 x g for 5 min. Discard the supernatant. At this step, store the cell pellet at -80 °C or immediately purify the AAV from the pellet, as described in steps 3.4-3.15.

3.4) Prepare the lysis buffer: 150 mM NaCl and 20 mM Tris-HCl, pH 8.0. Filter sterilize (0.22 µM). Store the buffer at 4 °C.

3.5) Resuspend the pellet with 10 mL of lysis buffer.

3.6) Freeze the lysate at -80 °C or in the dry ice/ethanol bath, then thaw it at 37 °C. Vortex for 10 min. Freeze and thaw the lysate 3 times.

3.7) Add $MgCl_2$ solution to the thawed lysate (make the final concentration of $MgCl_2$ in the lysate be 1 mM). Add the nuclease to a final concentration of 250 U/mL. Incubate at 37 °C for 15 min to dissolve the DNA/protein aggregation.
Note: If the DNA/protein aggregation does not get dissolved after nuclease or endonuclease treatment, dounce homogenize the lysates 20 times.

3.8) Centrifuge the sample at 4,800 x g for 20 min at 4 °C. Collect the supernatant.

3.9) Meanwhile, prepare the Iodixanol gradient solution:

3.9.1) Prepare the 17% of the gradient solution by mixing 5 mL of 10X PBS, 0.05 mL of 1 M $MgCl_2$, 0.125 mL of 1 M KCl, 10 mL of 5 M NaCl, and 12.5 mL of density gradient medium. Adjust the total volume to 50 mL using H_2O.

3.9.2) Prepare the 25% solution by mixing 5 mL of 10X PBS, 0.05 mL of 1 M $MgCl_2$, 0.125 mL of 1 M KCl, 20 mL of density gradient medium, and 0.2 mL of 0.5% (w/v) Phenol Red. Adjust the total volume to 50 mL using H_2O.

3.9.3) Prepare the 40% solution by mixing 5 mL of 10X PBS, 0.05 mL of 1 M $MgCl_2$, 0.125 mL of 1 M KCl, and 33.3 mL of density gradient medium. Adjust the total volume to 50 mL using H_2O.

3.9.4) Prepare the 60% solution by mixing 0.05 mL of 1 M $MgCl_2$, 0.125 mL of 1 M KCl, 50 mL of density gradient medium, and 0.1 mL of 0.5% (w/v) Phenol Red.

3.10) With a needle and syringe, load the Iodixanol gradient solution into the polypropylene tube in the order of 5 mL of 17%, 5 mL of 25%, 5 mL of 40% and 5 mL of 60%, starting from the bottom. Load all the lysate obtained from step 3.8 (14-16 mL) on top of the gradient. The gradient, listed from the bottom to top, is 60%, 40%, 25%, 17%, and the lysate layer. Fill the tube with lysis buffer and cover it with the cork.

3.11) Centrifuge at 185,000 x g for 90 min at 16 °C.

3.12) Harvest the viral fraction (40% layer) with a syringe. Insert the needle (21 gauge) into the intersection between the 40% and 60% fractions, only aspirating the 40% layer.
Note: Avoid aspirating ANY of the 25% layer.

3.13) Mix the viral fraction with sterilized polyoxyethylene-polyoxypropylene block copolymer PBS solution (10% polyoxyethylene-polyoxypropylene block copolymer stock 1:10,000 diluted in PBS) up to a total volume of 15 mL. Load the mixture into the filter tube (cut-off MW = 100 kD). Centrifuge at 2,000 x g for 30 min at 4 °C.

3.14) Discard the solution at the bottom. Refill the filter tube with polyoxyethylene-polyoxypropylene block copolymer PBS solution to a total volume of 15 mL. Centrifuge at 2,000 x g for 20 min at 4 °C. Repeat this step two more times. Collect the purified rAAV9 virus (the fraction above the filter).

3.15) Transfer the purified rAAV9 in the filter tube to 1.7-mL tubes. Aliquot the purified rAAV9 (100-400 µL/tube, depending on the volume and titer of the AAV) and store the virus at -80 °C.
Note: Avoid repeated freeze-thaws.

4. Measurement of the titer of rAAV9
4.1) Prepare standard DNA samples.

4.1.1) Design specific and efficient PCR primers for rAAV9 vectors and optimize the PCR condition.
Note: The primers used in this study are "Forward: TCGGGATAAAAGCAGTCTGG; Reverse: TCGGACGGAGATACGTGAGT". The PCR reaction was performed with the following conditions: initial denaturing at 95°C for 3 min; 35 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 10 s; and the final extension at 72 °C for 10 min. However, the optimized primers and PCR conditions are plasmid-specific, as the inset sequence in the rAAV9 vector may affect the specificity and efficiency of PCR[31].

4.1.2) Perform the PCR reaction with the conditions shown in step 4.1.1. Purify the PCR product with a gel extraction kit.

4.1.3) Measure the concentration of purified DNA using a spectrophotometer. Calculate the concentration in DNA molecular numbers based on the molecular weight/length of the PCR product.

4.1.3.1) Calculate the molecular concentration using the following equation: molecular concentration (DNA molecules or fragments/mL) = 6.23×10^{23} mol^{-1} x Con. x 10^{-6}/MW. Note: (6.23×10^{23} mol^{-1} is Avagadro's Number; Con.: DNA concentration in µg/mL; MW.: molecular weight in g/mol). For instance, if the obtained concentration of the PCR product is 100 µg/mL and its length is 200 bp, the molecular weight of the double-stranded DNA is 2 x 200 x 310 = 124,000 (the average molecular weight of each nucleotide in the single-stranded DNA is about 310 g/mol). The molecular concentration (DNA molecules/mL) = 6.23×10^{23} mol^{-1} x 100 µg/mL x 10^{-6} /124,000 g/mol = 5.18×10^{14} DNA molecules/mL.

4.1.4) Perform a dilution series of the DNA fragment and prepare the standard samples, with concentrations of 10^{13} molecules/mL, 10^{12} molecules/mL, 10^{11} molecules/mL, 10^{10}

molecules/mL, 10^9 molecules/mL, 10^8 molecules/mL, and 10^7 molecules/mL. Use 1 µL of solution for each standard sample for the quantitative PCR (qPCR, in step 4.6).

4.2) Mix 5 µL of purified rAAV9 solution with 5 µL of 10X DNAse buffer, 1 µL of DNAse (10,000 U/mL), and 39 µL of ddH_2O. The total volume should be 50 µL.

4.3) Incubate the vial at 37 °C for 30 min to remove residual unpackaged plasmid DNA.

4.4) Inactivate the DNAse at 95 °C for 10 min. Cool down the solution, add 44 µL of H_2O, 5 µL of 10X DNAse buffer, and 1 µL of Proteinase K stock (10 mg/mL).

4.5) Incubate the solution at 50 °C for 2 h. Stop the reaction and inactivate the Proteinase K at 95 °C for 10 min.

4.6) Use 1 µL of the sample for the quantitative PCR (qPCR) assay. Calculate the titer.

4.6.1) Run quantitative PCR (qPCR) with the primers designed in step 4.1.1 using the samples from step 4.1.4 (standard samples) and from step 4.5 (samples to be measured).

4.6.1.1) For each reaction, mix 10 µL of 2X Green master mix (containing Taq polymerase, dNTP mix, buffer, $MgCl_2$, and Green dye), 0.5 µL of forward primer (5 µM), 0.5 µL of reverse primer (5 µM), 8 µL of H_2O, and 1 µL of the sample to be measured. Perform qPCR with the following conditions: hold the samples at 50 °C for 2 min and 95 °C for 10 min; perform 40 cycles at 95 °C for 15 s and at 60 °C for 1 min; for the melt stage, incubate the samples at 95 °C for 30 s and 60 °C for 15 s. Generate the standard curve based on the C_T numbers of the standard samples (**Figure 3**).

4.6.2) Calculate the molecular concentration/titer of the AAV sample against the standard curve. The rAAV9 has a single-strand DNA genome, so the molecular concentration will be 2-fold higher than the calculated value (2X power (10, y), **Figure 3B**). In addition, the titer of the purified rAAV9 will be 20-fold higher than what is obtained from the calculation due to the 1:20 dilution of the virus in DNAse and Proteinase K reactions (5 µL in 100 µL total).

5. rAAV9 injection in neonatal mice and gene expression assays in the heart

5.1) Prepare rAAV9 working solutions in polyoxyethylene-polyoxypropylene block copolymer PBS solution. Make the virus stock with the titers of 1-7 x 10^{12} particles/mL.

Note: Deliver 50-70 µL of rAAV9 solution into each postnatal day 0.5-1.5 mouse by subcutaneous injection. To achieve efficient gene overexpression or knockdown, it is recommended to perform a pilot test for each study to optimize the amount of injected AAV. Use the same amount of rAAV9.cTNT::Luc or rAAV9.U6::scramble controls for each study to minimize the bias.

Note: We used 1-1.5 x 10^{11} particles/pup for overexpression and 2.5-5 x 10^{11} particles/pup for knockdown in postnatal day 0.5-1.5 mice).

5.2) Treat neonatal mice with rAAV9 at P0.5-P2.5 by subcutaneous injection.

5.2.1) Pre-fill a 29G1/2, 0.33 x 12.7 mm insulin syringe with the rAAV9 solution. Be careful to remove air bubbles.

5.2.2) Hold the pup in one hand with thumb and forefinger. Prior to injection, swipe the back skin of the pup with a swab stick saturated with 70% isopropyl alcohol to maintain the sterile condition. Insert the syringe needle into the anterior-dorsal subcutis of the animal at an angle of 5 to 10 degrees. Inject 50-70 µL of the rAAV9 solution using the insulin syringe.
Note: rAAV9 can also be delivered to the mouse via intraperitoneal or intravenous injection[26,27]. Efficient expression of the delivered genes in the heart can be obtained. However, intraperitoneal injection sometimes may result in leaky expression in the liver. After injection, the condition of the pups was monitored every day.

5.3) The level of gene expression in the heart can be monitored with qPCR, immunofluorescence, or western blot (representative results are shown in **Figures 4** and **5**)[25,26].
Note: Mice were euthanized by CO_2 delivered from a compressed gas source. Tissue samples were collected after confirming that heart rate, movement, and breathing of the animals had ceased. Neonatal rodents are resistant to CO_2 euthanasia and were euthanized by decapitation using sharp scissors. The method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

REPRESENTATIVE RESULTS:
The strategies for rAAV9 construction of rAAV9.cTNT::GFP or rAAV9.U6::shRNA plasmids are shown in **Figures 1** and **2**, respectively. As the examples, the rAAV9 vector was generated to overexpress the GFP gene in mouse hearts. The resulting plasmid contains the cTNT::GFP cassette flanked by two ITR sites (**Figure 1**). The rAAV9.U6::shRNA vector was constructed to knockdown Trbp mRNA (**Figure 2**)

The standard curve for rAAV9 titration was generated with the qPCR data by linear regression. The manipulated variable y represents the Log_{10} value of DNA molecular concentration of each standard sample, and the corresponding variable x represents the C_T value. The Log_{10} (concentration) values (y) and C_T numbers (x) exhibit a nice linear correlation ($R^2 = 0.9971$) and fit with the equation $y = -0.2832x + 14.616$ (**Figure 3A**). Titers of rAAV9 samples were calculated based on the linear equation (**Figure 3B**). With the method described in the protocol (step 4.6.2 and **Figure 3B**), a high titer of rAAV9 vectors (50-200 µL, >6 x 10^{13} particles/mL) was obtained in the representative study.

To monitor the efficiency and tissue specificity of rAAV9.cTNT vectors, P0.5 pups were treated with same amount (1 x 10^{11} particles/pup) of rAAV9.cTNT::Luciferase (AAV-Luc) or rAAV9.cTNT::GFP (AAV-GFP) by subcutaneous injection. Two weeks after injection, the GFP signal was monitored in various tissues of the mice. Robust expression of GFP was detected in the heart, but not in other organs (**Figure 4,** n > 3). Thus, efficient and heart-specific gene expression was achieved with the rAAV9.cTNT vector.

To monitor the knockdown efficiency and tissue specificity of rAAV9.U6 vectors, P0.5 mice were treated with same amount (3 x 10^{11} particles/pup) of rAAV9.U6::Scramble (AAV-Scramble) or rAAV9.U6::Trbp shRNA (AAV-shTrbp) by subcutaneous injection. Two weeks after injection, expression of Trbp in various tissues of was monitored by qPCR (**Figure 5,** n = 3). The mRNA level of Trbp in the heart was substantially reduced by rAAV9.U6::shTrbp (68% downregulation, P = 0.0004452). Down regulation of Trbp was also detected in the liver tissue from rAAV9.U6::shTrbp-treated mice. However, the change is much lower.

FIGURE LEGENDS:

Figure 1. The strategies to construct the rAAV9.cTNT::GFP plasmid. (A) The scheme of the cTNT::GFP cassette. (B) The rAAV9 plasmid has been modified to harbor the chicken *TNNT2* promoter (rAAV9.cTNT) followed by the two unique restriction sites (NheI and KpnI). The GFP open reading frame was cloned into the rAAV9.cTNT vector by restriction site-mediated ligation to generate the rAAV9.cTNT::GFP plasmid. (B) The rAAV9.cTNT::GFP plasmid can be constructed by Gibson assembly.

Figure 2. The strategies to construct the rAAV9.U6::shRNA plasmid. (A) The scheme of the U6::shRNA cassette is shown. Expression of shRNA is driven by the U6 promoter (Blue). (B) rAAV9-U6-shRNA cassettes can be generated by annealing and ligating DNA oligos containing shRNA sequences into the restriction enzyme-digested rAAV9 vectors harboring the U6 promoter. (C) rAAV9.U6::shRNA cassettes can be generated by long-range PCR and intra-molecular Gibson assembly-based “seamless” construction. The 5’ arm, loop, and 3’ arm of shRNA are shown in green, orange, and red, respectively.

Figure 3. Calculation of the rAAV9 titer. (A) The standard curve for rAAV9 titration was generated by linear aggression using the qPCR data. The manipulated variable y represents the Log_{10} value of DNA molecular concentration of each standard sample, and the corresponding variable x represents the C_T value. (B) Titers of rAAV9 samples are calculated based on the linear equation of the standard curve.

Figure 4. Expression pattern of rAAV9.cTNT::GFP in mice tissues. P0.5 pups were treated with same amount (1 x 10^{11} particles/pup) of rAAV9.cTNT::Luciferase (AAV-Luc, negative control) or rAAV9.cTNT::GFP (AAV-GFP) by subcutaneous injection. Two weeks after injection, the tissue samples were harvested. Expression of GFP was monitored under a fluorescent dissection scope. Both bright field and fluorescence images are presented. The experiments have been repeated more than 3 times (n > 3). Bar = 2.0 mm. SkM, skeletal muscle.

Figure 5. Knockdown gene expression with AAV-shRNA. P0.5 mice were treated with the same amount (3 x 10^{11} particles/pup) of rAAV9.U6::Scramble (AAV-Scramble) or rAAV9.U6::Trbp shRNA (AAV-shTrbp) by subcutaneous injection. Two weeks after injection, the mRNA levels of Trbp in various tissues were monitored by qPCR (n = 3). Data are presented as the Mean ± SEM. The cut-off P value is 0.05. NS, P > 0.05, not significant. **, P < 0.01. SkM, skeletal muscle.

DISCUSSION:

It is important to minimize undesired ITR recombination during plasmid construction. Before generating the virus, one must always monitor the ITR integrity of the AAV plasmids by using restriction digestion and agarose gel electrophoresis. It is impossible to obtain 100% intact plasmids, but the recombination ratio should be minimized as much as possible. Less than 20% is acceptable for successful rAAV9 packaging. Of note, culturing the bacteria at lower temperature (30 °C) with a lower shaking speed (180-200 rpm) can reduce the chance of ITR recombination.

It is essential to ensure that the HEK293 cells are healthy for successful transfection and rAAV9 packaging. “Healthy” cells are usually highly proliferative and grow quickly. However, rapid proliferation and growth of HEK293 cells does not necessarily guarantee the high efficiency of rAAV9 packaging. Thus, it is important to start the experiments with fresh cells. It is recommended to use low-passage HEK293 cells (<10 passages, the cells are passaged every 2-3 days) for rAAV9 packaging. Of note, other serotypes of rAAV may need to be purified using different procedures[32].

The investigator is granted flexibility in the generation of rAAV9 plasmids. Either restriction site-mediated ligation or Gibson assembly can be used[30]. For the rAAV9.U6::shRNA construction, the intra-molecular Gibson assembly-based strategy is an effective method (**Figures 1** and **2**). Multiple AAV-shRNA plasmids or pooled AAV-shRNAs can be rapidly constructed. To overexpress genes using rAAV9, there exists a size limitation for inserted cDNA sequences. Generally, the size fragment between ITRs needs to be smaller than 5 kb[33]. Intein-catalyzed protein splicing can be used to circumvent the packaging size limit of rAAV9 vectors[34].

Other viral systems, including retrovirus, lentivirus and adenovirus, have also been developed and enable flexible gene manipulation. Compared with these different types of viral vectors, rAAV has specific advantages: high efficiency, high specificity, low genomic integration rate, minimal immunogenicity, and minimal pathogenicity. Thus, rAAV-based genome engineering is emerging as an ideal tool for *in vivo* gene manipulation.

Previous studies have shown that the rAAV9 system enables the efficient expression of delivered genes *in vivo*. With the cTNT (chicken TnnT2) promoter, heart-specific gene expression was obtained (**Figure 4**)[25,26]. Although the U6 promoter is ubiquitously active in mouse tissues, a most striking inhibition of target mRNA (Trbp) by rAAV9.U6::shRNA was observed in the heart, but not in other organs (**Figure 5**). The liver is the most common organ transduced by different serotypes of rAAV[35,36]. However, the knockdown efficiency (36% reduction of mRNA level) in the liver is much lower than that in the heart (68% reduction of mRNA level). This is consistent with the previous study showing that, despite higher viral genome presence in the liver, systemic delivery of shRNA by rAAV9 provides more efficient gene knockdown in the heart[35]. It is possible that hepatocytes are more proliferative than cardiomyocytes and are more actively undergoing cell division after rAAV9 administration at the neonatal ages, which results in substantial vector genome dilution in the liver tissue. However, this also suggests that the serotype of rAAV9 may more efficiently transduce cardiomyocytes in comparison to other cell types. As demonstrated by Lovric *et al*., in differentiated myocytes, the DNA damage response MRN complex proteins are repressed. MRN complex proteins bind AAV genomes and inhibit AAV transduction through transcriptional silencing. Thus, permissivity to AAV transduction can be induced by the terminal differentiation of cardiomyocytes[36], making the rAAV9 system, comparing to other viral vectors, very suitable for gene manipulation in the heart. To further minimize the undesired knockdown effects in other organs (*e.g.,* the liver), one also can use cardiac-specific cTNT promoter-driven miR-30a-based shmiR to repress the genes of interest in the heart[29]. This manuscript provided the reader with the specific technicalities of capitalizing upon the rAAV9 technology in cardiovascular investigations.

ACKNOWLEDGEMENTS:
We thank Dr. Zaffar Haque for careful reading of the manuscript. We thank Drs. Masaharu Kataoka and Gengze Wu for discussions and help. Work in the Wang lab is supported by the American Heart Association, Muscular Dystrophy Association, and NIH (HL085635, HL116919, HL125925).

DISCLOSURES:
The authors have nothing to disclose.

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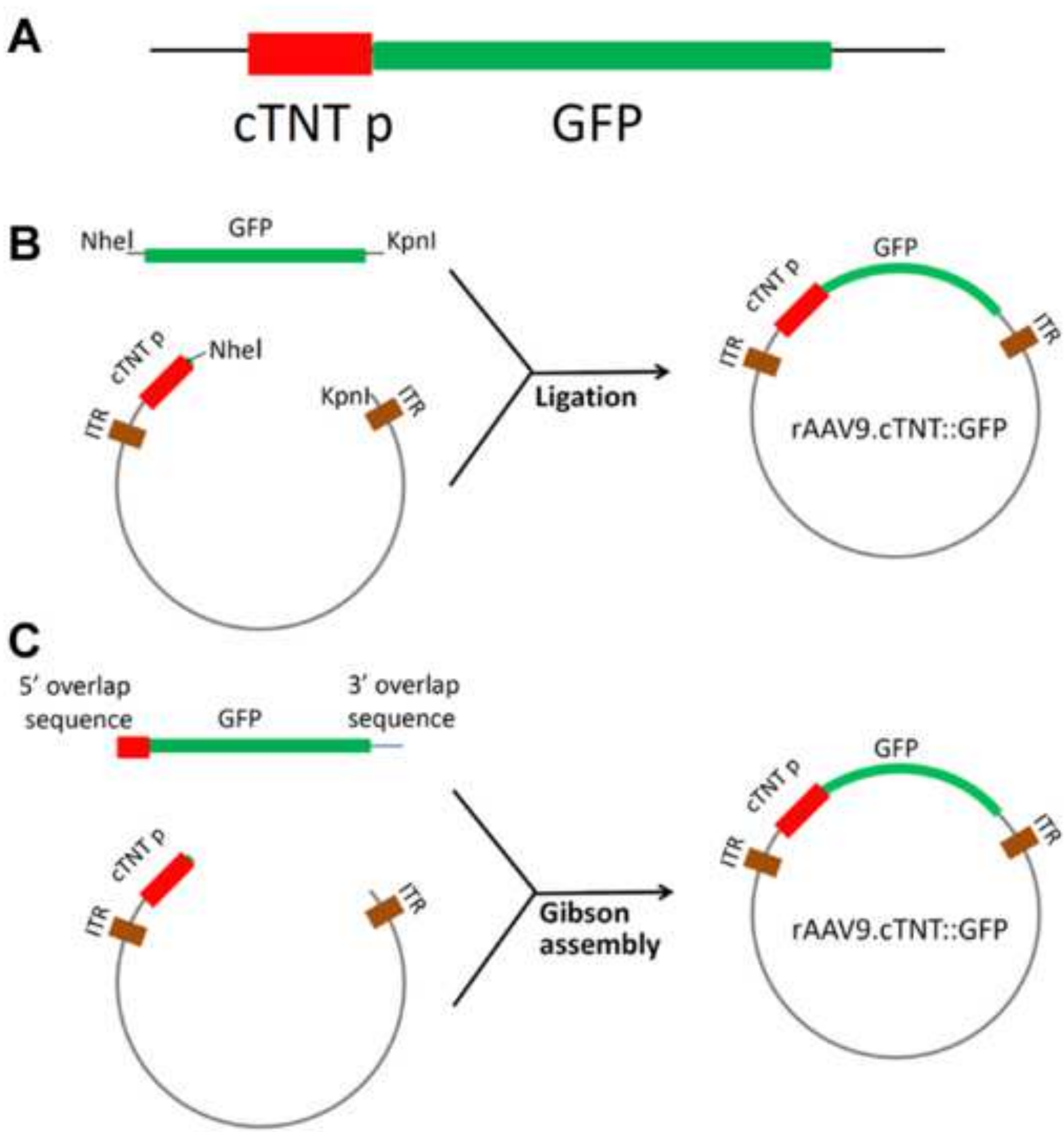
Figure 1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B
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ITR
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ITR
rAAV9.cTNT::GFP
C
5′ overlap
sequence
GFP
3′ overlap
sequence
cTNT p
ITR
ITR
Gibson
assembly
cTNT p
GFP
ITR
ITR
rAAV9.cTNT::GFP

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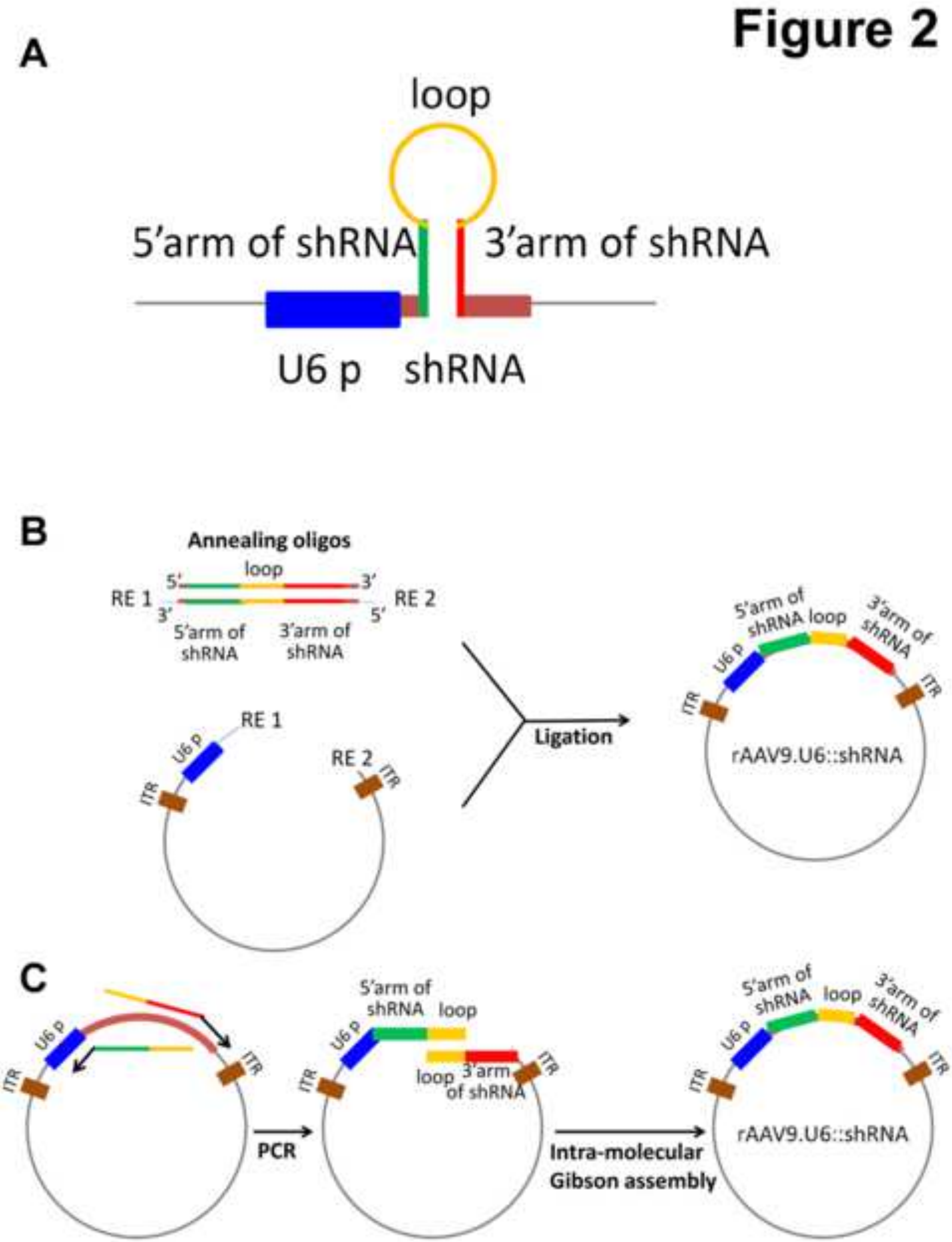
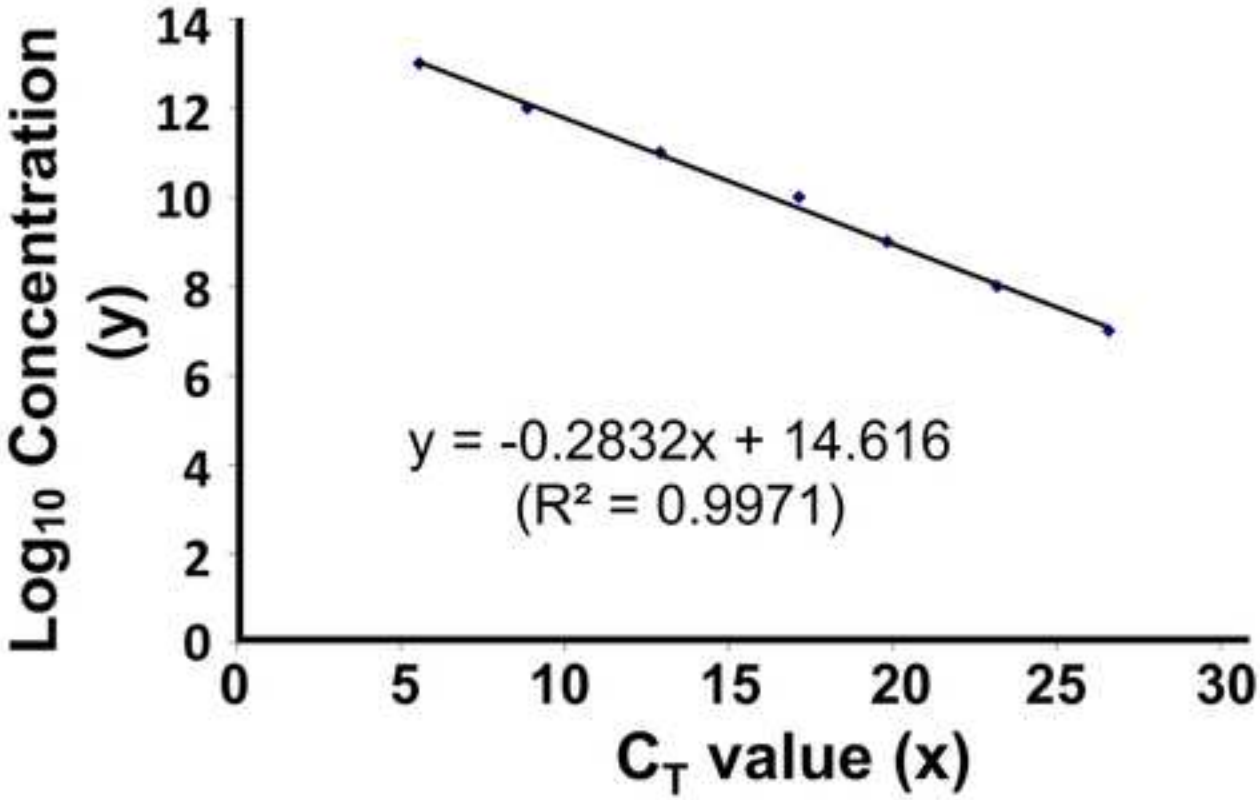


Figure 3

A



B

	C_T value	Log10 (Concentration)	Concentration	Titer(particles/mL)
Sample	x	y	2XPower (10, y)	20X2XPower (10, y)
1	8.514024734	12.2048282	3.20522E+12	6.41045E+13
1	8.577964783	12.18672037	3.07433E+12	6.14866E+13
2	7.933270454	12.36929781	4.68088E+12	9.36177E+13
2	8.045316696	12.33756631	4.35107E+12	8.70214E+13

Figure4

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Figure 4

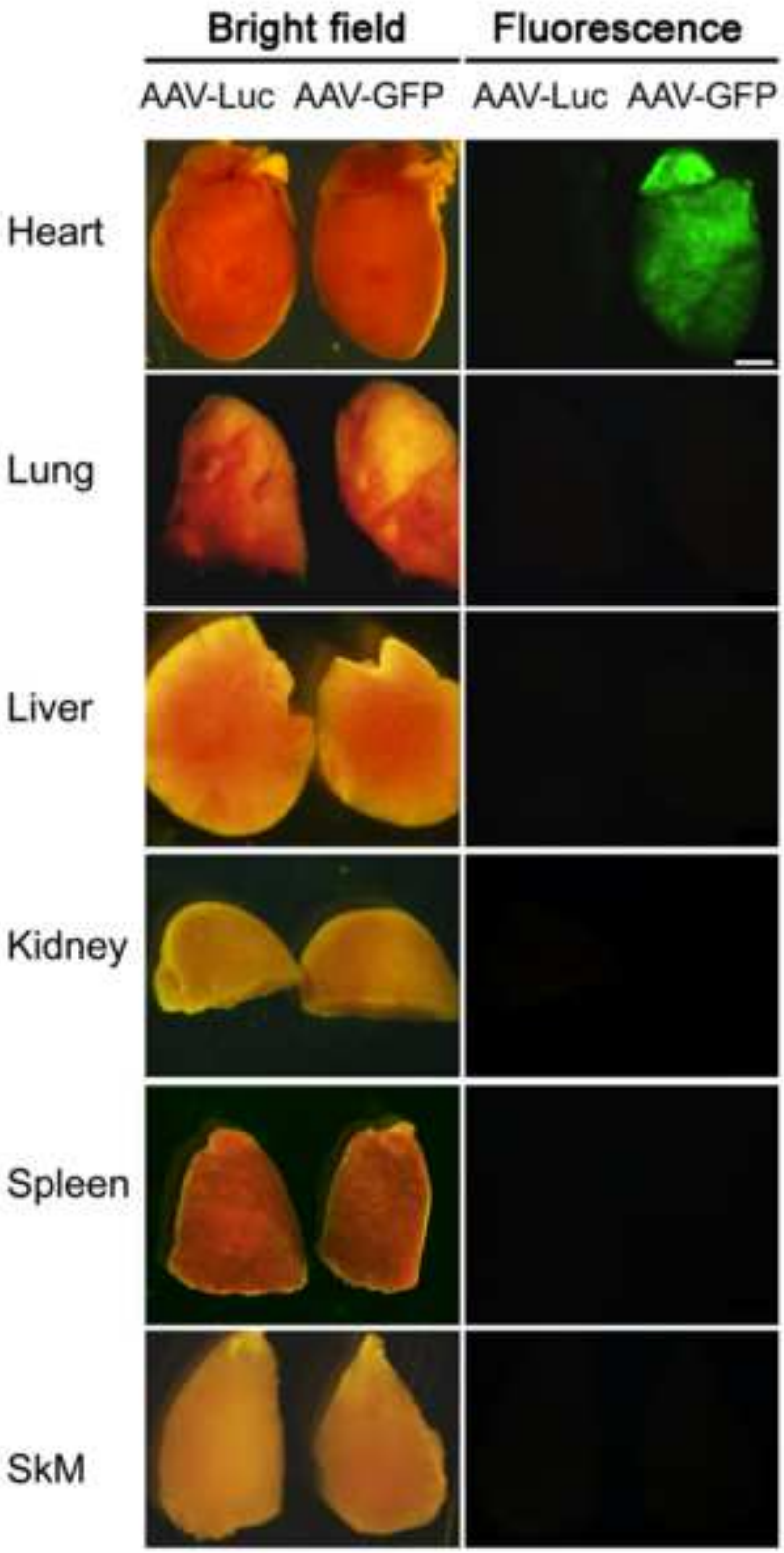
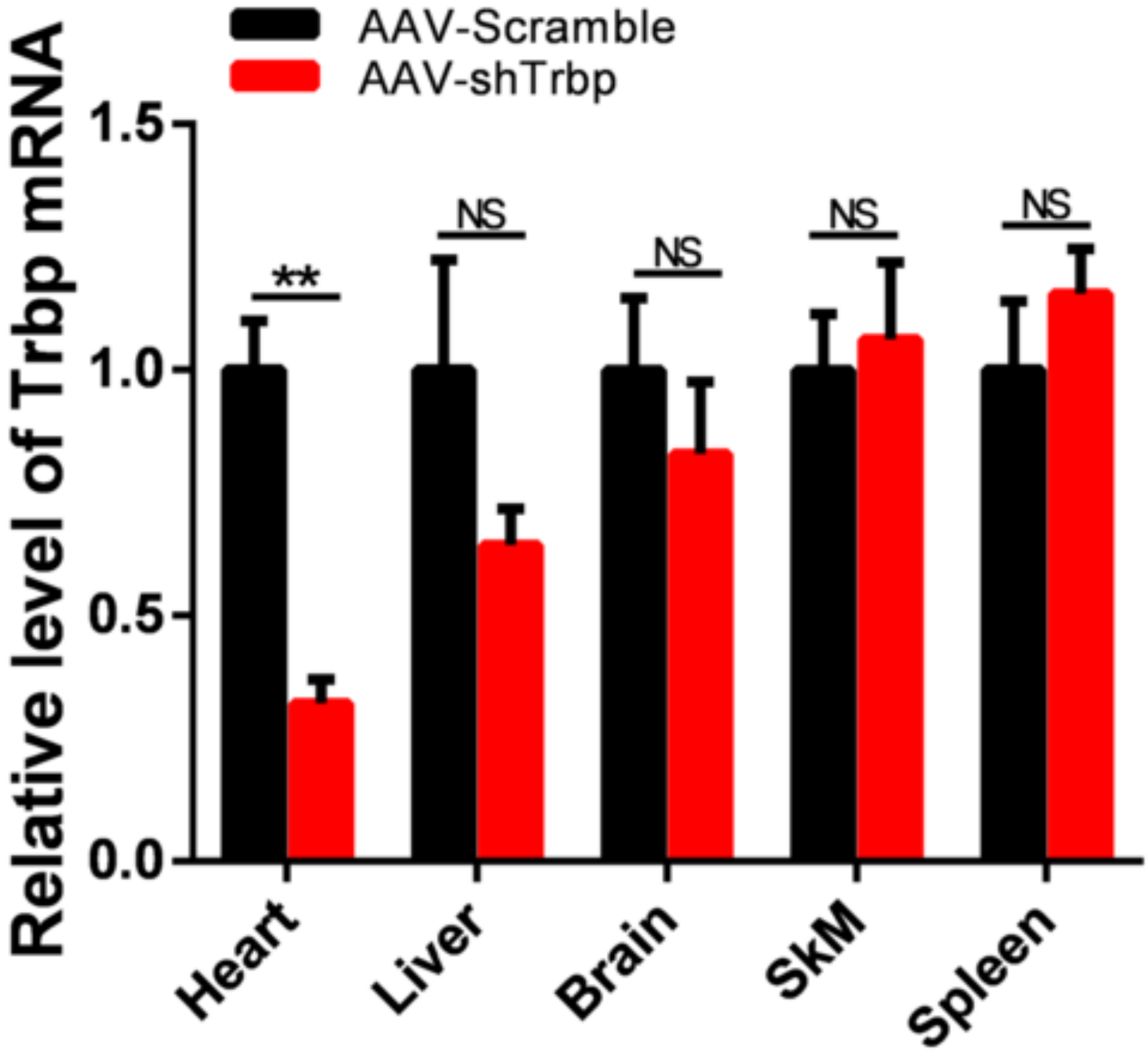


Figure 5


AAV-Scramble
AAV-shTrbp
Relative level of Trbp mRNA
1.5
1.0
0.5
0.0
**
NS
NS
NS
NS
Heart
Liver
Brain
SkM
Spleen

Name of Material/ Equipment	Company
Polyethylenimine, Linear (MW 25,000)	Polysciences, Inc.
Tube, Polypropylene, 36.2 mL, 25 x 87 mm, (qty. 56)	Beckman Coulter, Inc
Nuclease, ultrapure	SIGMA
Density Gradient Medium(Iodixanol)	SIGMA
Centrifugal Filter Unit with Ultracel-100 membrane	EMD Millipore Corporation
Laboratory pipetting needle with 90° blunt ends,gauge 14, L 6 in., nickel plated hub	SIGMA
Poloxamer 188 solution (Pluronic® F-68 solution)	SIGMA
Proteinase K	SIGMA
DNase I	Roche
Centrifuge machine	Thermo Scientific
Centrifuge System	Beckman Coulter
Ultracentrifuge	Beckman Coulter
DMEM medium	Fisher Scientific
Fetal Bovine Serum	Atlanta Biologicals
rAAV9 vector	Penn Vector Core

Catalog Number	Comments/Description
#23966-2	
# 362183	
#E8263-25KU	
#D1556-250ML	
#UFC910008	
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CORRESPONDING AUTHOR:

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Article Title: Prepare rAAV9 to overexpress or knckdown genes in mouse

Signature:

Date: March 30, 2016

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Editorial comments:
•*NOTE: Please download this version of the Microsoft word document (File name: 54787_R2_050916) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.*

•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.
•Formatting:
-Please define all abbreviations at first occurrence (ie ITR).
Response: Done.

-Section 1 does not contain any steps, although some parts are phrased in the imperative tense. Please reformat Section 1 to contain at least 2 steps in the imperative tense with a combination of steps and notes.
Response: Done.

-Please include all media in the materials table, or supply the medium composition in the text.
Response: Done.

-Please make units of time consistent (use "h" for "hr", etc.).
Response: Done.

•Please copyedit the manuscript for numerous grammatical/typographical errors. Such editing is required prior to acceptance. A subset of errors is indicated below.
-Please rewrite the title as follows: Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts
Response: Done.

-Short abstract – Please use present tense and correct the typo at the end of the sentence.
Response: Done.

-Long abstract – Please use present tense when describing the protocol (ie "a detailed procedure...is described."
Response: Done.

-Line 64 – "described Subcutaneous"
-Line 58 – "therapeutic potentials"
-Line 68 – "is utilized." – It should be and "was utilized."
-All sentences should begin with capital letters, including those in notes.
-3.12, 3.13 – "vial fraction"
-5.3 – "Monitor the level of gene expression in the heart can be monitored"
-Line 363 – "Of not"
-Line 367 – Please clarify "good status" and "good caliber". These phrases are not typically used

to describe cells in English.
Response: Done.

•Visualization: Steps 3.11-3.15 should be included in the highlighted material for filming, as they appear to contain critical steps in vector isolation.
Response: Done.

•Additional detail is required:
-2.4 – Please provide citations for plasmids.
-3.10 – What volume of the gradient solutions is used?
-4.1.2 – Please indicate whether a kit is used.
-4.1.3 – How is DNA concentration measured?
-4.6.1 – Please indicate the reaction composition without using brand names.
Response: Done.

•Branding:
-Please remove all trademark symbols from the materials table.
Response: Done.

-Penn Vector Core, P1967 – Please move plasmid company and catalog number to the materials table and remove from the protocol section.
Response: Done.

-3.7 – Benzonase
Response: Done.

-3.9, 3.10 – Please reduce the number of Iodixanol mentions in the protocol to no more than 2.
Response: Done.

•Discussion: Please discuss the future applications of the protocol. Please also discuss the significance with respect to alternative methods and include independent citations. Some material could be moved from the introduction to the discussion for this purpose.
Response: Done. We have now compared AAV with other alternative viral systems in the discussion section.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
Response: All the figures or tables are original, and have not been used for other publications.

•JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more

information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.
Response: We have included DOIs in the references.

•IMP: Please copyedit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
Response: Done. Dr. Zaffar Haque, who is a native English speaker, has carefully proof read the manuscript.

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is the second revision of a manuscript that describes a method for using adeno-associated virus (AAV) to overexpress and knockdown genes in the mouse heart. This manuscript is well written and will be a useful resource for researchers in the molecular cardiology field.

Response: Thanks for your positive comments.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
N/A

Major Concerns:
-Line 65: Please indicate that the delivered genes are preferentially expressed in the heart, not the liver and other tissues.
Response: AAV-GFP signal was only detected in the heart, but not in other tissues. We have revised the sentence in the abstract.

-Is there a variation in expression efficiency between P0.5 and P2.5?
We did not see significant a variation in expression efficiency between P0.5 and P2.5 injections.

-Does any part of the skin yield similar expression pattern?
Response: We have systemically delivered AAV.cTNT::Luc into neonatal pups, but did not detect any signal in the skin.

-Section 5.2.2. Please discuss: does intraperitoneal or intravenous injection also lead to higher expression in the heart than in any other tissues?
Response: We have revised it as following: "With intraperitoneal or intravenous injection, efficient expression of the delivered genes in the heart can be obtained. However, intraperitoneal injection sometimes may result in leaky expression in the liver".

Minor Concerns:
-Line 399: please indicate n=?
Response: We have included the information (n=3) in the revised manuscript.

-Figure 4. n?
Response: We have included the information (n>3) in the revised manuscript.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
In this article Ding et. al. describes a detailed procedure to construct, package, and purify the rAAV9 vectors. Overall, the methodology to produce recombinant AAV particles is clearly described.

Major Concerns:
N/A

Minor Concerns:
1. The AAV nomenclature is somewhat confusing. The serotype of AAV is determined by the type of Cap gene on the rev:cap plasmid used to prepare viral particles, while the viral genome encapsulated within AAV particles typically contain the ITRs of AAV2. The authors should standardize the nomenclature of the AAV throughout the text and clarify the origin of the ITRs in the vectors. For example, the plasmids/constructs should be named as AAV.cTnT:GFP and AAV.U6:shRNA. Also, lines 186-187 should read: "At day 1, transfect HEK293 with AAV plasmid, Ad-Helper plasmid and AAV9.Rep/Cap plasmid using PEI." Line 209 should read: "3. Harvesting of transfected HEK293 cells and purification rAAV9 viral particles", lines 63-64: "pathogenicity. Here, a detailed procedure to construct, package, and purify the rAAV9 viral particles..."

Response: The rAAV vector we are currently using contains the ITRs of AAV2. We clarified the origin of the ITRs in the vectors and included the information in the revised manuscript. The nomenclature is from Penn Vector Core. We have standardized nomenclature of AAV.cTnT:GFP and AAV.U6:shRNA throughout the text in the revised manuscript.

2. Typos should be avoided e.g. line 304 "product is 100 µg/ µmL", line 64 "vectors was described Subcutaneous..." ; line 163 "example, the rAAV9-U6-shRNA vector was construct to knockdown..."; line 166: "undesired ITR recombination, ."
Response: Done.

3. The sequences targeting Trbp and the scramble sequence should be provided.
Response: We have included the sequences in the revised manuscript.

4. In lines 445-446 the authors state that "Before generating the virus, one must always monitor the ITR integrity of the AAV plasmids by restriction digestion and agarose gel electrophoresis." However, the methodological details are not included in the text.
Response: We have included the weblink (http://www.vvf.uzh.ch/cloningservice/11bpdeletion/itrintegrity.html) describing how to monitor ITR integrity in the manuscript.

5. The authors refer to the rAAV as an 'emerging technology in cardiovascular investigations" or an "emerging as an essential tool for in vivo cardiac gene manipulation" that "has yet to reach its full potential". However, the rAAV systems have has been used in cardiovascular research in numerous studies for more than a decade. These sentences should be rephrased.
Response: We have removed the words "emerging" and revised the sentences in the manuscript.

Additional Comments to Authors:
N/A

Reviewer #4:
Manuscript Summary:
Ding et al. describe procedures for construction of AAV vector plasmids for AAV9 vector-mediated heart-specific overexpression and shRNA expression, procedures for production, purification and titration of AAV9 vectors, and procedures of vector administration into neonatal mice via subcutaneous injection for the purpose of in vivo genetic manipulation of the murine heart. Although the emergence of the CRISPR-Cas9 system has significantly accelerated the process of the creation of genetically modified mice, it would be attractive if one can attain the same goal by in vivo genetic manipulation without undergoing transgenesis. In this regard, the authors take advantage of the strong cardiotropic nature of AAV9, and show that, when combined with the heart specific TNNT2 promoter or the human U6 snRNA promoter-driven shRNA, it is possible to overexpress or down-regulate genes of interest in a heart-specific manner by a simple subcutaneous injection of AAV9 vector into neonatal mice. Although none of the concepts, approaches or techniques described in the manuscript are new and the impact of the paper may not be high in the fields relevant to cardiovascular and gene therapy research,

visual presentation of the contents described in the manuscript may help readers who are not familiar with AAV vectors and neonatal injection.

Major Concerns:
1. The major concern resides in whether or not this article will have general impact strong enough for publication. The procedure for AAV vector production and purification using iodixanol has already been published in JVE 2011 and neonatal AAV9 vector injection has also been published in JVE 2014. There are a number of publications on the methods for heart-specific manipulation of gene expression.
Response: We recognize that rAAV systems have been widely used for the investigation of gene function in vivo, including in the cardiovascular research field. In our manuscript, we have optimized the procedures of rAAV9 construction, packaging and injection. For instances, we used intramolecular Gibson assembly, which facilitate the construction of rAAV9 vectors; We minimized the usage of FBS, reducing the cost of rAAV9 preparation. We showed that efficient expression of rAAV9 in the heart could be obtained with a simple and easy S.C. injection in neonatal mice. We believe that publishing this manuscript and visual presentation of our procedure will allow for much more *widespread* applications of this promising molecular intervention tool for both basic and therapeutic studies.

2. Several descriptions in the manuscript do not appear to be correct or at least are not presented clearly. For example, it is not clear how the difference between the strandedness in double-stranded DNA standard (PCR products) and that in the viral genomes (either single-stranded or double stranded) is taken into account in the measurement of the AAV9 vector titers.
Response: We have used previously purified and titer-calculated rAAV9 virus, instead of PCR products, as standard samples. We have clarified this in the revised manuscript.

Another example is "This suggests that the serotype of rAAV9 can more efficiently transduce cardiomyocytes in comparison to other cell types." This statement may not necessarily be correct because reference 35 used P8 and the authors used P0.5-2.5 animals. At these ages, hepatocytes are still undergoing cell division, which results in vector genome dilution. It could be possible that substantial gene knock-down might have been observed in the liver if they had analyzed gene expression earlier.
Response: We have discussed these possibilities in the revised manuscript.

Minor Concerns:
1. The authors need to clarify the strandedness of AAV vectors.
Response: We have indicated that the rAAV9 contains single-strand genome in the revised manuscript.

2. "0.36 fold decrease" etc. are confusing. According to the results shown in the paper, they are most likely 36% decrease or 1.56-fold decrease.
Response: It is 36% decrease. We have corrected it accordingly the revision.

3. Were animals perfused at sacrifice?
Response: Mice were euthanized at sacrifice by CO_2 delivered from a compressed gas source.

4. Fig. 5. The difference in the liver would be statistically significant if the authors took 0.05 as the cut-off p value. Because of this reason, the authors had better show actual p values instead of NS.

Response: We use p0.05 as the cut-off P value. The p value for liver tissue is 0.0583682, which is higher than 0.05.

5. The manuscript contains many grammatical errors and typos, which should have been carefully checked before submission. Examples are:
Line 53, 58, 64,73, 190, 251, 315: extra/missing period, comma, space or parenthesis.
Response: We have corrected the typo in the revised manuscript.

Line 176: Singular/plural inconsistency.
Response: We have corrected the error in the revised manuscript.

Line 262, 266: Vial is used instead of viral.
Response: We have corrected the typo in the revised manuscript.

Line 315: DNAse instead of DNase.
Response: We have corrected the typo in the revised manuscript.

Line 362: every day instead of everyday.
Response: We have corrected the typo in the revised manuscript.

Line 364: Monitor, can be monitored, which is a grammatical error.
Response: We have corrected the typo in the revised manuscript.

Line 448: Of not is a grammatical error.
Response: We have corrected the typo in the revised manuscript.

Mixed use of present tense and past tense without any clear reasons
Response: We have checked and corrected the use of present tense and past tense in the revised manuscript.

6. It is not clear whether the final viral prep has been sterilized with a 0.22 micron filter.
Response: We did not sterilize the final viral prep with 0.22 micron filter. However, the wash buffer and rAAV9 storage buffer have been sterilized. We did not detect any pathogenic responses in rAAV9-injected mice.

7. It would be helpful if the authors provide more information about the "filter tube" (MW cut-off etc.)-Line 268.
Response: The MW cut-off is 100KD. We have included the information in the revised manuscript.

8. The authors' intention to include AAV-Luc data in Figure 4 is not clear.

Response: The AAV-Luc was used as the negative control. We have included the information in the revised manuscript.

Additional Comments to Authors:
N/A