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 CO2 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