

# Chapter 2

## Silencing Genes in the Heart

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### Abstract

Silencing of cardiac genes by RNA interference (RNAi) has developed into a powerful new method to treat cardiac diseases. Small interfering (si)RNAs are the inducers of RNAi, but cultured primary cardiomyocytes and heart are highly resistant to siRNA transfection. This can be overcome by delivery of small hairpin (sh)RNAs or artificial microRNA (amiRNAs) by cardiotropic adeno-associated virus (AAV) vectors. Here we describe as example of the silencing of a cardiac gene, the generation and cloning of shRNA, and amiRNAs directed against the cardiac protein phospholamban. We further describe the generation of AAV shuttle plasmids with self complementary vector genomes, the production of AAV vectors in roller bottles, and their purification via iodixanol gradient centrifugation and concentration with filter systems. Finally we describe the preparation of primary neonatal rat cardiomyocytes (PNRC), the transduction of PNRC with AAV vectors, and the maintenance of the transduced cell culture.

**Key words** siRNA, shRNA, amiRNA, AAV vectors, Iodixanol gradient centrifugation, Primary neonatal rat cardiomyocytes

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### 1 Introduction

Gene silencing induced by RNA interference (RNAi) is widely used in experimental research and has also shown its suitability for the investigation of cardiac gene function and for the treatment of cardiac diseases [1]. Small interfering (si) RNAs induce RNAi. After transfection into cells, one strand of this roughly 19 bp long double-stranded RNA binds to the mRNA of a target gene in a sequence-specific manner and induces the degradation of its target mRNA, leading to suppression of gene expression [2]. An as yet unsolved problem of the employment of siRNA in the cardiac system is the low transfection efficiency of isolated cardiomyocytes in vitro and of the heart in vivo. Moreover, cardiac specific in vivo delivery of siRNA is challenging with regard to target organ specificity and long-term efficacy. These bottlenecks can be overcome by delivery of small hairpin (shRNA) and artificial microRNAs (amiRNAs), which are processed intracellularly to mature siRNAs, by cardiotropic adeno-associated virus (AAV) vectors [3–5].

Free available computer programs can be used to select siRNA, shRNAs, and amiRNAs, directed against a specific target gene. They can then easily be inserted into expression plasmids and sub-cloned into AAV shuttle plasmids containing essential AAV genome sequences necessary for packaging of the AAV vector genome into AAV capsids [3, 6]. Over the last decade, huge efforts were made in the development of AAV vectors. Especially pseudotyped AAV vectors, comprising a vector genome derived from AAV2 packaged into the capsid of the AAV serotypes 6 and 9, showed improved cardiotropism [7–9]. The generation of AAV vectors with a self complementary (sc) vector genome represents a further milestone in AAV vector development [10]. Compared to traditional AAV vectors with single-stranded (ss) vector genomes, scAAV vectors express transgenes earlier and reach faster maximal levels in the heart [11]. This is of particular importance for investigation in cultured primary cardiomyocytes, which only survive in culture for a short time [12, 13], as well as for in vivo investigations requiring rapid gene silencing [3]. AAV vectors can be generated in culture plates, flasks, or roller bottles using helper virus-free plasmid-based packaging systems and purified by iodixanol gradient ultracentrifugation and concentrated using specific filter systems. The vectors can then be used directly for in vitro and in vivo applications [14].

Here we describe, as an example of cardiac gene silencing, the development of shRNAs and amiRNAs directed against the cardiac  $\text{Ca}^{2+}$  regulatory protein phospholamban (PLB), the generation of AAV vector shuttle plasmids with sc vector genomes, the production of pseudotyped AAV2/6 and AAV2/9 vectors in roller bottles, their purification by iodixanol gradient centrifugation and concentration with an Amicon® filter system. We further describe the preparation of primary neonatal rat cardiomyocytes (PNRC), its transduction with AAV vectors and maintenance of the transduced cell culture.

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## 2 Materials

Use double-distilled water ( $\text{H}_2\text{O}$ ) for all methods.

### 2.1 Cloning of shRNAs and amiRNAs

1. siRNA Designer.
2. pSilencer™ neo Kit (Thermo Fisher Scientific).
3. BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) containing 10× oligo annealing buffer, T4 DNA ligase (1 U/μl), and 5× T4 DNA ligase buffer.
4. *Hind*III (20,000 U/ml), *Bam*HI (20,000 U/ml), and 10× restriction buffer.
5. Agarose.
6. Ethidium bromide.

7. Gel extraction kit.
8. Calf intestine alkaline phosphatase (10,000 U/ml) and 10× CIAP buffer.
9. Oligonucleotides (200 μM).
10. Annealing buffer: 100 mM K-Acetate, 30 mM HEPES, pH 7.4, 2 mM Mg-acetate. Weigh 0.9815 g K-acetate, 0.715 g HEPES and transfer to a glass beaker add 90 ml H<sub>2</sub>O and adjust the pH to 7.4 with KOH. Add 0.0285 g Mg-acetate and fill up to 100 ml with H<sub>2</sub>O. Stir the solution with a magnet stirrer. Store the solution at 4 °C.
11. T4 Polynucleotide kinase (10,000 U/ml), 10× T4 PNK buffer, ATP (100 mM).
12. T4 DNA ligase (400,000 U/ml) and 10× T4 DNA ligation buffer.
13. Competent *E. coli* cells.
14. Plasmid mini preparation kit.
15. Plasmid maxi preparation kit.

## **2.2 Generation of AAV Shuttle Plasmids with Self Complementary AAV Vector Genome**

1. pAAV-MCS (Stratagene).
2. *MscI* (5000 U/ml), *HincII* (10,000 U/ml), and 10× restriction buffer.
3. Agarose.
4. Ethidium bromide.
5. Gel extraction kit.
6. T4 ligase (400,000 U/ml) and 10× T4 DNA ligation buffer.
7. Competent *E. coli*.
8. Plasmid mini preparation kit.
9. Plasmid maxi preparation kit.

## **2.3 Production of AAV Vectors in Roller Bottles**

### **2.3.1 Plasmids**

1. AAV shuttle plasmids.
2. pDP6rs (PlasmidFactory).
3. pHelper (Agilent Technologies, Inc).
4. p5E18-VD2/9 [3].

### **2.3.2 Cell Culture Media, Buffers and Solutions**

1. HEK293T cells.
2. DMEM-complete medium: DMEM, 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate. Add 50 ml FCS, 5 ml penicillin/streptomycin (10,000 U penicillin/10 mg/ml streptomycin), 5 ml L-glutamine (200 mM), and 5 ml sodium pyruvate (100 mM) to 500 ml DMEM (4.5 g/l glucose, w/o sodium pyruvate, w/o L-glutamine). Store the solution at 4 °C.
3. 1× PBS. Store the solution at 4 °C.

4. Trypsin–EDTA. Store the solution at 4 °C.
5. Collagen Type I from calf skin (Sigma-Aldrich, Cat. No. C8919). Store the solution at 4 °C.
6. 150 mM NaCl: Weigh 0.8766 g NaCl and dissolve it in 100 ml H<sub>2</sub>O. Store the solution at room temperature.
7. 2.5 M CaCl<sub>2</sub>: Weigh 27.75 g CaCl<sub>2</sub> and dissolve it in 100 ml H<sub>2</sub>O. Store the solution at room temperature.
8. Polyethylenimine (PEI). To have a final concentration of 2.58 µg/µl dissolve 129 mg PEI in 50 ml H<sub>2</sub>O. Store PEI at –20 °C.
9. Benzonase nuclease, 25 U/µl. Store the enzyme at –20 °C.
10. 1.86 M NaCl/24 % PEG 8000: Weigh 54.35 g NaCl and transfer it into a glass beaker containing 500 ml H<sub>2</sub>O. Add stepwise 120 g PEG and stir the solution strongly with a magnet stirrer (*see* **Note 1**). Store the solution at room temperature.
11. NaCl–Hepes solution: 50 mM Hepes, 150 mM NaCl, 25 mM EDTA. Weigh 1.192 g Hepes and transfer it into a glass beaker. Add 0.3 ml of 5 M NaCl and 0.5 ml of 500 mM EDTA. Fill up to 100 ml with H<sub>2</sub>O and mix the solution under continuous stirring using a magnetic stirrer. Store the solution at 4 °C.
12. Iodixanol solution (60 %, with density of 1.32 g/ml (Progen)). Store the solution at room temperature in the dark.
13. PBS-MK: 1× PBS, 1 mM MgCl<sub>2</sub>, 2.5 mM KCl. Weigh 0.95 g MgCl<sub>2</sub> and 0.1864 g KCl and transfer it into a glass beaker containing 1 l 1× PBS. Mix the solution under continuous stirring using a magnetic stirrer and autoclave it. Store the solution at 4 °C.
14. Phenol red solution. Store the solution at 4 °C.

### 2.3.3 Materials and Equipment

1. Corning® roller bottles (850 cm<sup>2</sup> surface) (Sarstedt).
2. Cannula short (18 G × 1½, Braun).
3. Cannula long (21 G × 4¾, Braun).
4. Amicon® Ultra 15 ml centrifugal filters (100 K) (Millipore).
5. Polypropylene centrifuge tubes (capacity 29.9 ml, for ultracentrifugation, Optiseal).
6. Conic centrifuge tubes (15 and 50 ml).
7. Microtubes.
8. Bottle turning device 2 levels HC 240 (VWR International GmbH).
9. Heracell™240i CO<sub>2</sub> incubator.

## 2.4 Preparation of Primary Neonatal Rat Cardiomyocytes

### 2.4.1 Solutions and Media

1. 10× CIM, 1 l (Ca-free, phosphate-buffered cell isolation medium): 1.2 M NaCl, 45.6 mM KCl, 4.4 mM  $\text{KH}_2\text{PO}_4$ , 8.4 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 250 mM  $\text{NaHCO}_3$ , 55.5 mM glucose, pH 7.5. Weigh 58.44 g NaCl, 74.55 g KCl, 136.09 g  $\text{KH}_2\text{PO}_4$ , 177.95 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 84.01 g  $\text{NaHCO}_3$ , and 180.16 g glucose and transfer it into a glass beaker filled with 900 ml  $\text{H}_2\text{O}$ . Mix the solution under continuous stirring using a magnetic stirrer. Adjust pH at room temperature to 7.5. Decant the solution into a 1 l volumetric flask and fill up to exactly 1 l using  $\text{H}_2\text{O}$ . Perform sterile filtration using a vacuum filtration system with a pore size of 0.22  $\mu\text{m}$ . Distribute 50 ml aliquots of 10× CIM into 50 ml sterile tubes under a laminar flow hood. Mark each tube with “10× CIM” and the current date. Store these aliquots at  $-20^\circ\text{C}$  not longer than 3 month until use.
2. 20× P/S: Use either lyophilized penicillin/streptomycin (10,000 U/ml/10 mg/ml) or a ready to use solution of penicillin/streptomycin (10,000 U/ml/10 mg/ml). The latter solution can be portioned into 5 ml tubes under sterile conditions and stored at  $-20^\circ\text{C}$  until use.
3. 1× CIM with P/S: Mix 10 ml 10× CIM with 5 ml 20× P/S and 85 ml  $\text{H}_2\text{O}$  under sterile conditions to obtain 100 ml 1× CIM with P/S. Store the final solution at  $4^\circ\text{C}$  (*see Note 2*).
4. 1× CIM (Ca free, phosphate buffered cell isolation medium): Mix 10 ml 10× CIM with 90 ml  $\text{H}_2\text{O}$  under sterile conditions to obtain 100 ml Ca-free 1× CIM and store this solution at  $4^\circ\text{C}$  (*see Note 2*).
5. 10× trypsin (1.25% trypsin stock solution): Dissolve 0.5 g trypsin (*see Note 3*) in a 50 ml beaker glass filled with 50 ml  $\text{H}_2\text{O}$  under continuous stirring using a magnetic stirrer. Perform sterile filtration of the resulting solution (*see Note 4*) using a vacuum filtration system with a pore size of 0.22  $\mu\text{m}$ .
6. 1000×  $\text{CaCl}_2$ : 1.26 M  $\text{CaCl}_2$ . Dissolve in a 100 ml beaker glass filled with 40 ml  $\text{H}_2\text{O}$  9.261 g supra pure  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  under continuous stirring using a magnetic stirrer. Transfer the solution in a narrow-necked volumetric flask and fill up to exactly 50 ml with  $\text{H}_2\text{O}$ . Perform sterile filtration of the resulting solution using a 50 or 10 ml syringe equipped with a syringe filter with a pore size of 0.22  $\mu\text{m}$ . Distribute 1 ml portions of the solution into sterile 1.5 ml micro tubes and store them at  $-20^\circ\text{C}$  until use.
7. 1000× FUDR (5-fluoro-2'-deoxyuridine), 2 mM FUDR: Dissolve 2.924 mg FUDR in 10 ml  $\text{H}_2\text{O}$ , filtrate the solution using a syringe with a syringe filter with a pore size of 0.22  $\mu\text{m}$ . Distribute 1 ml portions of the solution into sterile 1.5 ml micro tubes and store them at  $-20^\circ\text{C}$  until use.

8. 500× gentamycin, 10 mg/ml: Dissolve 10 mg lyophilized sterile gentamycin in 1 ml H<sub>2</sub>O or use ready to use 10 mg/ml gentamycin solution.
9. FCS: Thaw the 500 ml bottle with frozen FCS at room temperature. Inactivate the thawed FCS (if needed) for 30 min at 56 °C using a water bath. Distribute the volume into portions of either 100 ml or 50 ml under sterile conditions and store at −20 °C.
10. Horse serum (HS): Thaw the 500 ml bottle with frozen HS at room temperature. Inactivate the thawed HS (if needed) for 30 min at 56 °C using a water bath. Distribute the volume into portions of either 100 ml or 50 ml under sterile conditions and store at −20 °C.
11. CMRL1415-ATM cell culture medium (from Biochrom KG, Berlin) (*see* **Note 5**).
12. Complete CMRL1415-ATM cell culture medium: Mix 498 ml CMRL1415-ATM with 0.5 ml 1000× CaCl<sub>2</sub>, 0.5 ml 1000× FUDR, and 1 ml 500× gentamycin under sterile conditions (*see* **Note 6**).
13. Disinfectant, 1 l each. We recommend using ready-to-use disinfectant Barrycidal (Manfred Sauer GmbH). It consists of a synergistic blend of selected organic nitrogen compounds and provides a broad spectrum of activity against bacteria, yeasts, fungi, and viruses. Alternatively, 70% ethanol can be used as a disinfectant. However, the activity spectrum of the latter is not nearly as broad as Barrycidal.
14. 1× CIM with trypsin. Mix 10 ml 10× CIM, 80 ml H<sub>2</sub>O, and 10 ml 10× trypsin (*see* **Note 7**).

#### 2.4.2 Materials and Equipment

1. A stack of autoclaved paper towels.
2. A small beaker glass filled with 70% ethanol for soaking instruments.
3. A large beaker glass filled with 70% ethanol for dipping neonate rats.
4. A spray bottle containing Barrycidal or 70% ethanol.
5. An aerosol can filled with disinfectant Barrycidal or 70% ethanol.
6. Test tube rack.
7. A bag for the unneeded parts of the animal attached to the hood for easy accessibility.
8. Disposable plastic tissue culture pipettes.
9. Pasteur pipettes.
10. Sterile micropipette tips.

11. Three large weigh boats.
12. Two sterile petri dishes with 20 ml 1× CIM with P/S each.
13. Large forceps for animal transfer.
14. Container with sterile surgery tools (large scissors for animal decapitation, large curved forceps for animal fixation, small scissors for thorax section, small scissors for heart removal, small forceps for transfer of removed hearts).
15. FCS.
16. HS.
17. Various sterile 0.22 µm filtration units.
18. Conic centrifuge tubes (50 ml).
19. 50 mm glass funnel with sterile gauze.
20. Culture flasks (75 cm<sup>2</sup> filter cap), culture plates (round 6-well, 12-well, 24-well on demand); culture flasks (25 cm<sup>2</sup> with filter cap instead of culture plates, if needed).

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### 3 Methods

#### 3.1 Cloning of shRNAs and amiRNAs

##### 3.1.1 Cloning of shRNAs Against Phospholamban

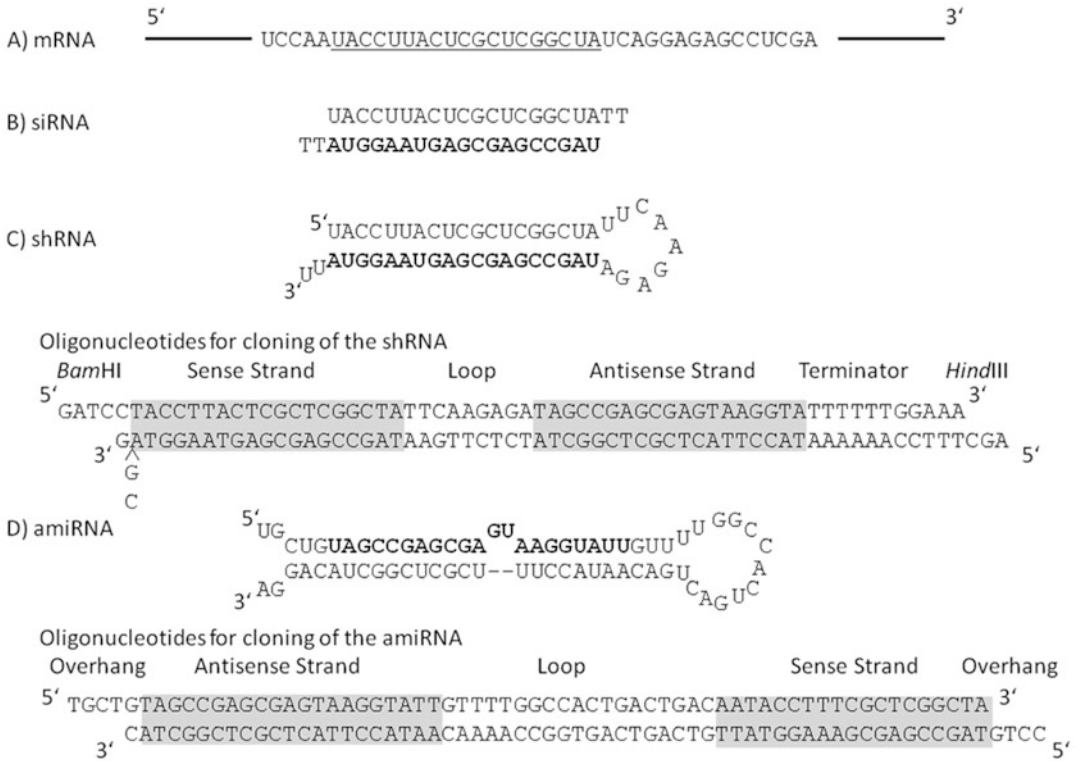
Use autoclaved double-distilled water (H<sub>2</sub>O) for all methods.

To enable vector delivery, an siRNA sequence (*see Note 8*) can be converted into the corresponding shRNA (Fig. 1). The two strands of the double-stranded RNA molecule are connected by a loop and the shRNA is usually expressed under control of an RNA polymerase III promoter, e.g., the U6 or H1 promoter. The intracellular RNAi machinery will then process the shRNA to give the mature siRNA. Various vectors are commercially available for the expression of shRNAs. An example is the pSilencer 2.1-U6 neo vector from Thermo Fisher Scientific (originally from Ambion). The oligonucleotides encoding the shRNA are cloned between a *Bam*HI and a *Hind*III site (*see Note 9*). How to clone shRNA is described in the following steps.

1. Mix 3 µg of pSilencer 2.1-U6 neo vector with 0.25 µl *Bam*HI, 0.25 µl *Hind*III, 2 µl 10× restriction buffer and H<sub>2</sub>O ad 20 µl. Incubate for 2 h at 37 °C.
2. Add 0.5 µl Calf Intestine alkaline phosphatase, 2.5 µl 10× CIAP buffer to the linearized vector and fill up with H<sub>2</sub>O to 25 µl.
3. Separate the linearized plasmid by agarose gel electrophoresis using a 1 % agarose gel containing ethidium bromide (0.5 µg/ml). Cut out the gel slice containing vector band (*see Note 10*) and isolate the DNA fragments using a gel extraction kit. Estimate the vector concentration on a separate agarose gel with a standardized marker (*see Note 11*).



# RNAi-mediated Silencing of Rat Phospholamban



**Fig. 1** Schematic of siRNA, shRNA and amiRNA directed against phospholamban [6]. (a) The target site in the mRNA is underlined. (b) The standard design of an siRNA comprises a 19mer duplex and two deoxythymidine overhangs at both 3' ends. (c) For the shRNA approach, the two strands of the siRNA are connected by a loop. The oligonucleotides encoding the shRNA are shown below the shRNA. Please note that the second oligonucleotide is shown in 3'–5' direction for clarity in this illustration, but must be in the 5'–3' direction when ordered. For cloning, a *Bam*HI and a *Hind*III site are added to the 5' and 3' end, respectively. A GC pair needs to be introduced upstream of the sequence encoding the sense strand in case the siRNA starts with a U or C (as in the case of the shown shRNA targeting phospholamban). A series of six Ts terminates polymerase III transcription. (d) For the amiRNA approach, the siRNA sequence is inserted into the sequence environment of a natural microRNA, e.g., the murine miR-155 encoded for example by the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen). The miR-155 flanking sequences were optimized for higher knockdown efficiency and facilitated sequence analysis. The oligonucleotides encoding the amiRNA were designed for cloning into the pcDNA 6.2-GW vector (Invitrogen). Again, the second oligonucleotide is shown in 3'–5' direction for clarity, but needs to be ordered in the 5'–3' direction. The active RNA antisense strand is always written in *bold*

- Mix 100 pmol of each of the oligonucleotides with annealing buffer ad 50 µl. Heat to 95 °C for 4 min; slowly cool to room temperature.
- Mix 2 µl of oligonucleotides annealing mixture with 1 µl ATP, 1 µl T4 polynucleotide kinase, 1 µl 10× T4 PNK buffer and H<sub>2</sub>O ad 10 µl. Incubate for 30 min at 37 °C. Inactivate T4 polynucleotide kinase by heating to 70 °C for 10 min.



6. Mix 50 ng of linearized, dephosphorylated plasmid with 5  $\mu$ l of mixture with phosphorylated oligonucleotides, 1  $\mu$ l T4 DNA ligase, 1  $\mu$ l 10 $\times$  T4 DNA ligation buffer and H<sub>2</sub>O ad 10  $\mu$ l. Incubate at 16 °C overnight.
7. Transform the ligation mixture into competent *E. coli* cells according to standard procedures.
8. Pick bacteria colonies next day and carry out plasmid preparation using a plasmid miniprep kit. Control the correctness of the plasmid by restriction analysis and sequencing. Carry out plasmid maxi preparation using a plasmid maxiprep kit (*see* **Note 12**).

### 3.1.2 Cloning of amiRNAs Against Phospholamban

For this procedure the siRNA sequence is inserted into the sequence environment of a naturally occurring microRNA, commonly miR-30 or miR-155 (Fig. 1). This approach has four major advantages: (1) amiRNA systems are highly efficient, as the endogenous miRNA pathway is used. (2) Some RNA polymerase II promoters are—in contrast to RNA polymerase III promoters—tissue-specific. (3) The amiRNA system may be used to co-express a protein-encoding cDNA, e.g., GFP as a reporter. (4) amiRNAs can be inserted into an expression cassette as repetitive copies and together with a transgene. Cloning of an amiRNA directed against PLB with the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) is described here as an example.

1. The vector pcDNA™6.2-GW/miR is supplied as a linearized plasmid.
2. Mix 5  $\mu$ l of each of the oligonucleotides (200  $\mu$ M) with 2  $\mu$ l of 10 $\times$  oligo annealing buffer and 8  $\mu$ l H<sub>2</sub>O. Heat the mixture at 95 °C for 4 min. Slowly cool down to room temperature. Dilute the mixture to obtain a final concentration of the double-stranded oligonucleotide of 10 nM.
3. Mix 2  $\mu$ l of linearized pcDNA™6.2-GW/miR with 4  $\mu$ l of the double-stranded oligonucleotides, 4  $\mu$ l of 5 $\times$  T4 DNA ligase buffer, 1  $\mu$ l T4 DNA Ligase and 9  $\mu$ l H<sub>2</sub>O. Incubate for 5 min at room temperature.
4. Transform the ligation mixture into competent *E. coli* cells according to standard procedures.
5. Pick bacteria colonies next day and carry out further analysis as described under Subheading 3.1.1, **step 8**.

### 3.2 Generation of AAV Shuttle Plasmids with Self Complementary (sc) AAV Vector Genome

To express a transgene, AAV vectors with single-stranded (ss) vector genomes need to intracellularly convert their single-stranded DNA genome into double-stranded DNA genomes [15]. This takes days to weeks and is the main reason for delayed onset of transgene expression after transduction. In contrast, transgene expression rapidly increases when scAAV vector are used [11].

B) scAAV2 vector genome after deletion of the right trs

Diagram illustrating the deletion of the right trs from the scAAV2 vector genome. The initial pAAV-MCS construct contains the Left ITR, trs, MCS, and Right ITR. The right trs is flanked by MscI and HincII sites. Digestion with MscI/HincII separates the 2800 bp and 1230 bp bands. The 1230 bp band is separated and cleaned, and the 2800 bp band is religated and transformed into bacteria. The resulting scAAV2 vector genome contains the Left ITR, trs, MCS, and Right ITR, with the right trs deleted.

**Fig. 2** Generation of AAV shuttle plasmid by deletion of terminal resolution site in pAAV-MCS **(a)** AAV vector genome sequence of left and right AAV2 ITR with *trs* and *MscI* and *HindIII* restriction sites. **(b)** Partial sequence of left and right AAV2 ITRs after insertion of a 1230 bp long *MscI/HindIII* fragment (red) into the 2800 bp long plasmid backbone of *MscI/HindIII* digested pAAV-MCS. *MCS* multi cloning site, *trs* terminal resolution site; *Dots*, stretches of unwritten nucleotides

In these vectors both DNA strands are packaged as a single molecule, forming a dimeric vector genome, which can directly be used for transcription. scAAV vectors can be generated by deleting the terminal resolution site (trs) sequence in one AAV ITR. How to delete the trs is described in the following steps (*see* also Fig. 2).

1. Mix 5  $\mu\text{g}$  pAAV-MCS (*see* **Note 13**) with 1  $\mu\text{l}$  (5 U) *MscI*, 0.5  $\mu\text{l}$  *HincII*, 5  $\mu\text{l}$  10 $\times$  restriction buffer and  $\text{H}_2\text{O}$  ad 50  $\mu\text{l}$  and incubate for 2 h at 37  $^\circ\text{C}$ .
2. Separate the digested plasmid fragments by agarose gel electrophoresis using a 1 % agarose gel containing ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ). Cut out gel slices containing a 2800 and 1230 bp band (*see* **Note 10**) and isolate the DNA fragments using a gel extraction kit. Estimate the concentration of each DNA fragment on a separate agarose gel by comparison with a standardized marker.
3. Mix 100 ng of the 2800 bp fragment with 250 ng of the 1230 bp fragment (total volume  $\leq 17 \mu\text{l}$ ), add 1  $\mu\text{l}$  T4 DNA ligase, 2  $\mu\text{l}$  10 $\times$  T4 DNA ligation buffer and  $\text{H}_2\text{O}$  ad 20  $\mu\text{l}$  and incubate for 1 h at room temperature.
4. Transform competent *E. coli* with the mixture (*see* **Notes 14** and **15**) and transfer the bacteria solution to agar plates according to standard procedures.
5. Plasmid isolation: Pick positive colonies next day and carry out plasmid miniprep using commercial plasmid miniprep

kits and control correctness of the plasmid by restriction analysis and sequencing. Use plasmid maxiprep kits for maxi-preparations of large amounts of the scAAV shuttle plasmid (*see Note 16*).

6. To generate shRNA and amiRNA expressing scAAV shuttle plasmids, the respective expression cassettes can be cut out from respective plasmids (*see Subheadings 3.1.1 and 3.1.2*) and subcloned into the scAAV shuttle plasmid (*see Note 17*) using standard cloning procedures.

### **3.3 Production of AAV Vectors in Roller Bottles (*see Note 18*)**

Recombinant AAV vectors can be produced by co-transfection of the 293T host cell line with an AAV shuttle vector plasmid, a helper plasmid containing genetic information of adenoviral helper gene products (E2A, E4, VA RNA) and another plasmid expressing AAV replicase and capsid gene products (e.g., for generation of scAAV2/9 vectors). Alternatively the adenoviral helper function and the AAV replicase and capsid genes may be expressed from a single plasmid. Here only two plasmids, the helper and the AAV shuttle plasmid, are necessary to generate AAV vectors (e.g., for generation of scAAV2/6 vectors). The following protocol can be used for the production of ssAAV vectors and scAAV vectors. Generated vectors can be used directly for in vitro and in vivo transduction.

1. Coat roller bottle with 35 ml collagen solution (*see Note 19*) and incubate it in a Heracell 240i CO<sub>2</sub> incubator on the bottle turning device for 30 min at 37 °C. Roller bottles should spin with 0.2 rpm during this and all further incubation steps (*see Note 20*).
2. Prior to cell seeding, wash the coated surface of the roller bottles with 25 ml 1× PBS. For wash steps the bottle can also be spun by hand. To seed the cells, carefully add  $6 \times 10^7$  HEK293T cells to the bottom of the bottle (*see Note 21*). Add 200 ml DMEM complete medium to the cells and put the bottle onto the bottle turning device in the Heracell 240i CO<sub>2</sub> incubator. Let cells grow at 37 °C, 5 % CO<sub>2</sub> for 48 h. Check the confluence of the cells. If cell density reaches about 80 %, the cells can be transfected.
3. According to the desired vector, prepare the transfection solutions Mix A and Mix B as described in Table 1 using PEI (*see Note 22*). Consolidate Mix B and Mix A, vortex and incubate the transfection mix at room temperature for 15 min.
4. Remove 60 ml media from the roller bottle and store it as conditioned media.
5. Add 14 ml of the transfection mix to the roller bottle and incubate at 37 °C, 5 % CO<sub>2</sub> for 72 h.
6. Decant the supernatants (about 154 ml) into 200 ml Erlenmeyer flask (*see Note 24*) and wash the cells with 25 ml 1× PBS.

**Table 1**  
**Preparation of transfection solution for 1 roller bottle (see Note 23)**

Vector	Mix A	Mix B
scAAV2/6	7 ml 150 mM NaCl 30 µg pscAAV-shRNA or 30 µg pscAAV-amiRNA 90 µg pDP6rs	7 ml 150 mM NaCl 120 µl PEI
scAAV2/9	7 ml 150 mM NaCl 50 µg pscAAV-shRNA or 50 µg pscAAV-amiRNA 90 µg pHelper 90 µg p5E18VD2/9	7 ml 150 mM NaCl 230 µl PEI

7. Add 10 ml trypsin–EDTA into the roller bottle and incubate for 10 min at 37 °C and 5 % CO<sub>2</sub> on the bottle turning device.
8. Stop cell detachment with 25 ml conditioned media from **step 4** (see **Note 25**).
9. Transfer the suspension to 50 ml centrifuge tubes or larger centrifugation vessels if applicable.
10. Centrifuge for 20 min, 1200×*g* at room temperature (see **Note 26**).
11. Resuspend the cell pellet in 14 ml 1× PBS and transfer it into a 50 ml centrifuge tube.
12. Centrifuge at 1200×*g* for 20 min at room temperature and discard the supernatant.
13. Resuspend the pellets in 3.5 ml 1× PBS (see **Note 27**).
14. Disrupt the cells by 4 cycles of repeated freezing (–80 °C) and thawing (37 °C in a water bath).
15. Centrifuge for 30 min at 3900×*g* and transfer the supernatant to a 15 ml centrifuge tube.
16. Centrifuge for 30 min at 3900×*g* and transfer the supernatant to a new 15 ml centrifuge tube.
17. Add 35 µl benzonase nuclease (final concentration 250 U/ml) and incubate for 1 h at 37 °C. Mix the solution from time to time.
18. Centrifuge for 20 min at 3900×*g* and transfer the supernatant to a new 15 ml centrifuge tube. Mix the supernatant 1:1 with 1× PBS. The final volume is about 7 ml (see **Note 28**).

### **3.4 Precipitation of AAV Vectors from Supernatant (see Note 29)**

1. Add 2 ml 2.5 M CaCl<sub>2</sub> per 100 ml supernatant, mix and cool on ice for 1 h.
2. Distribute the solution into 50 ml centrifuge tubes or larger centrifugation vessels.

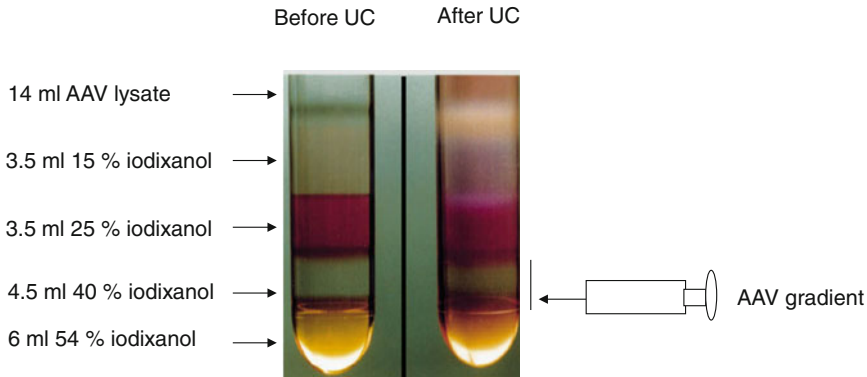
3. Centrifuge for 30 min at  $3900\times g$  and transfer the supernatant into a 400 ml Erlenmeyer flask.
4. Add 60 ml 1.86 M NaCl/24% PEG per 100 ml supernatant, mix and incubate for 72 h at 4 °C.
5. Centrifuge for 30 min at  $3000\times g$  and discard the supernatant.
6. Resuspend the pellet in 5 ml NaCl–Hepes solution.
7. Centrifuge 15 min at  $10,000\times g$  and transfer the supernatant into a 15 ml centrifuge tube.
8. Add 50  $\mu$ l Benzonase Nuclease to the supernatant (final concentration 250 U/ml) and incubate for 1 h at 37 °C. Mix from time to time.
9. Centrifuge for 20 min at  $3900\times g$  and transfer the supernatant to a new 15 ml centrifuge tube. Mix the supernatant 1:1 with  $1\times$  PBS. The final volume is about 10 ml (*see* **Note 30**).

### 3.5 Filtration of AAV Vectors with Iodixanol Gradient System

1. Prepare iodixanol dilutions as shown in Table 2.
2. Add 3.5 ml 15% iodixanol to a 22 ml polypropylene centrifuge tube.
3. Underlay 15% iodixanol with 3.5 ml 25% iodixanol, then with 4.5 ml 40% iodixanol and finally with 6 ml 54% iodixanol using a long cannula.
4. Overlay the gradient with the AAV solution produced under Subheadings 3.3 and 3.4 (*see* **Note 31**).
5. Fill-up the tube with PBS-MK to the mark on the tube (*see* **Note 32**).
6. Centrifuge at  $300,000\times g$  at 18 °C under vacuum. Use a slow deceleration protocol to avoid swirling of the layers.
7. Take the tube out of the centrifuge, dispose its cap and remove the AAV-containing layer by pricking the tube with a short cannula laterally into the tube immediately above the 54% iodixanol layer and extract the layer between 25 and 54% iodixanol layers (approximately 3 ml) (*see* **Note 33**, Fig. 3).

**Table 2**  
**Dilution of iodixanol**

	15% iodixanol	25% iodixanol	40% iodixanol	54% iodixanol
PBS-MK	37.5 ml	28 ml	16.5 ml	5 ml
Iodixanol	12.5 ml	20 ml	33 ml	45 ml
Phenol red	–	100 $\mu$ l	–	100 $\mu$ l



**Fig. 3** Iodixanol gradient before and after centrifugation. Positioning of the cannula for harvesting the AAV vector is shown on the right site. *UC* ultra centrifugation

### 3.6 Concentration of AAV Vectors

1. Dilute scAAV vector preparation 1:10 with PBS-MK.
2. Fill 15 ml of the scAAV vector preparation into an Amicon® Ultra 15 ml centrifugal filter.
3. Centrifuge for 15 min at  $4000\times g$ . Discard flow-through and repeat until the entire preparation has been centrifuged (see **Notes 34** and **35**).
4. Rinse the filter areas of the Amicon® Ultra 15 ml centrifugal filters with the remaining buffer (about 150  $\mu$ l) several times to resuspend the vector particles, if necessary dilute with PBS-MK (see **Note 36**).

### 3.7 Isolation of Primary Neonatal rat Cardiomyocytes (PNRC), Its Transduction with AAV Vectors and Maintenance of the Transduced Cell Culture

To characterize the posttranscriptional inhibition of selected cardiac targets (e.g., PLB) using small interfering RNAs such as shRNA or amiRNAs, cultured primary neonatal rat cardiomyocytes (PNRC) have been proven to be a valuable experimental model [4, 6, 13]. Therefore, the preparation of primary neonatal heart cell cultures from ventricular tissue of 1–3-day-old rat pups, described by Vetter et al. [12] earlier, will be specified here as a detailed protocol.

1. Wipe down a hood with either Barrycidal or 70 % ethanol.
2. Pour 20 ml ice-cold  $1\times$  CIM with P/S into each of two or three sterile 100 mm petri dishes.
3. Wash five neonatal rats (see **Note 37**) at a time in two sequential weigh boats with 70 % ethanol.
4. Holding a pup with large forceps, dip briefly into 70 % ethanol, decapitate with large scissors, place the decapitated neonate on its back on the stack of paper towels. Repeat this procedure for the remaining four pups. Before continuing, wash another set of five pups with 70 % ethanol and transfer into the proper weigh boats. Hold the decapitated animals down with forceps,

dab the ventral thorax area with a sterile swab using another pair of forceps and make a midline incision through the sternum using a small pair of scissors (*see Note 38*). Press downward with the large forceps to pop the heart out through this incision. Clip the heart out with the small pair of scissors and carefully transfer to the petri dish filled with 20 ml 1× CIM with P/S with the small forceps. Repeat this procedure with the other pups. Up to 25 hearts per dish can be pooled.

5. After all the hearts have been collected, trim away the atria and any other tissue such as connective tissue or fat from each heart. Transfer the remaining heart (consisting now of the right and left ventricle only) to a second petri dish with 20 ml ice-cold 1× CIM with P/C, where the remaining heart should be cut into 3–4 tissue pieces using small forceps and scissors. Alternatively, cutting into pieces can also be performed using two scalpels. Finally, pool all tissue pieces of the dissected hearts in the latter dish (*see Note 39*).
6. Carefully withdraw 1× CIM with P/C from the petri dish using a Pasteur pipette. Add 20 ml ice-cold 1× CIM to wash the tissue pieces and withdraw the solution from the petri dish again. Repeat the latter procedure once more. Thereafter, add 15 ml 1× CIM into the dish (*see Note 40*).
7. Transfer the complete content of the petri dish including all tissue pieces into a sterile spinner flask. Remove the buffer with a sterile Pasteur pipette and add depending on the number of hearts used 8–15 ml antibiotic-free 1× CIM with trypsin as indicated in Table 3.
8. Incubate the content in the spinner flask jacketed with 36–37 °C thermostatic H<sub>2</sub>O under continuous stirring with 150 rpm. Remove and discard the digestion buffer after 3–5 min (*see Note 41*).
9. Add 10 ml 1× CIM with trypsin and incubate at 36–37 °C. Remove the digestion buffer carefully after 15 min

**Table 3**  
**Used 1× CIM with trypsin per number of hearts**

Number of hearts	1× CIM with trypsin, ml
0–14	8
15–19	9
20–25	10
25–29	11
30–34	13
35–50	15



using a sterile culture pipette and transfer the content into a prepared 50 ml conic centrifuge tube filled with 5 ml ice-cold HS (In case you have added 15 ml 1× CIM with trypsin, the removed digestion buffer should be transferred into a prepared 50 ml conic centrifuge tube filled with 6.5 ml ice-cold HS). Mix the content of the centrifuge tube carefully. Add another 10 or 15 ml 1× CIM with trypsin to the Spinner flask and incubate as indicated before. While this next digestion is running, centrifuge the centrifuge tube at  $300\text{--}400\times g$  for 8 min using centrifuge equipped with a swing out rotor. Set the centrifugation conditions to slow acceleration, minimal brake, and room temperature. Use this schedule to perform another five digestion steps, with subsequent cell collection, using centrifugation in each step (*see* **Note 42**).

10. Carefully remove and discard the supernatant from the centrifuge tube after centrifugation. Add 1 ml FCS plus 1 ml CMRL1515-ATM to this tube and resuspend the cell pellet carefully. Perform this procedure for all cell pellets in the six centrifuge tubes obtained after centrifugation and pool the contents of the six tubes into a single tube.
11. Filter the pooled crude cell suspension through two layers of gauze into one sterile 50 ml conic centrifuge tube using a sterile 40 mm glass funnel. Centrifuge the tube at  $300\text{--}400\times g$  for 8 min as indicated under **step 9**. Remove and discard the supernatant carefully after centrifugation (*see* **Note 43**).
12. Resuspend the obtained cell pellet in complete gentamycin-containing CMRL1415-ATM cell culture medium supplemented with 10% of FCS and 10% HS (FCS/HS-CMRL1415-ATM) each and transfer the crude cell suspension into a 175 ml culture flask.
13. Incubate the cell suspension in an incubator for 90 min at 37 °C for selective attachment of non-muscular cells. In general, use 1 ml of this medium per one heart used for digestion, i.e., for 10 hearts use 10 ml medium, for 20 hearts use 20 ml, etc. At the end of the incubation period, carefully remove the cardiomyocyte-enriched medium from the culture flask and transfer it into a 50 ml conic centrifuge tube using a disposable plastic tissue culture pipette.
14. Pipette 50 µl of the obtained cell suspension into a micro tube together with 50 µl 1× PBS and 50 µl Trypan blue and mix the contents carefully. Immediately use a sample of this mixture to count the number of PNRC using a Neubauer improved hemocytometer and a microscope. Count the number of PNRC in each of four quadrants and calculate the mean value. For final calculation of the cell density, take into consideration the 1:3 dilution of the original cardiomyocyte-enriched medium obtained under **step 13**.

15. Adjust the cell density cardiomyocyte-enriched medium (obtained under **step 13**) to  $4 \times 10^5$  cells/ml using FCS/HS-CMRL1415-ATM with gentamycin. Distribute this cardiomyocyte containing medium either to cell culture plates or cell culture flasks depending on the demands of the planned experiments. For later transduction of the cultured cardiomyocytes with viral vectors a seeding cell density of  $1.25 \times 10^5$  cells/cm<sup>2</sup> is recommended. To reach this density, volumes of 3.0 ml, 1.5 ml, and 1 ml/well of the cardiomyocyte-enriched medium with  $4 \times 10^5$  cells/ml are needed if distributed to round 6-well, 12-well, or 24-well culture plates, respectively (*see Note 44*).
16. Incubate the cell culture plates for 24 h at 37 °C in an incubator (*see Note 45*).
17. After the latter incubation period remove and discard the medium and add to each well of the culture plates the indicated volumes (as described under Subheading 3.7, **step 15**) of complete CMRL1415-ATM cell culture medium with 10% FCS containing also 2 µM FUDR in addition to 20 µg/ml gentamycin (*see Note 46*).
18. After another 48 h of incubation replace the medium by fresh CMRL1415-ATM cell culture medium with 10% FCS, 2 µM FUDR, and 20 µg/ml gentamycin and transduce the cells with scAAV vectors by directly adding the vector into the cell culture medium and incubate at 37 °C as before (*see Note 47*).
19. Twenty four hours later the cell culture medium containing the viral vector can be removed and discarded. The wells of the plates are washed once with 1 ml, 1.5 ml, or 3 ml 1× PBS, depending on the type of plates used, respectively, to get rid of the non-absorbed viral vector. Thereafter, add fresh CMRL1415-ATM cell culture medium with 10% FCS, 2 µM FUDR, and 20 µg/ml gentamycin to the wells and incubate at 37 °C as indicated above. Using medium changes every 24–48 h, the spontaneously contracting PNRC can be cultured until they are harvested after 4–14 days in culture (*see Note 48*).

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## 4 Notes

1. Using this procedure the volume of the solution will be increased by 150 ml.
2. Do not store this glucose containing solution longer than 3 days at 4 °C.
3. Most commercially available trypsin preparations for cell cultures purposes will not work properly for neonatal cardiomyocyte preparation. Best results can be obtained with crude trypsin preparations, although not all crude enzyme prepara-

tions will work properly and vary from lot to lot. Therefore, it is necessary to prescreen several lots of crude trypsin before selecting a lot to purchase. We have successfully used crude porcine trypsin 1:250 from (Belger Biochemie, Kleinmachnow, Germany, Lot 0110795).

4. First use a 20 or 50 ml syringe with a disposable filter unit (0.45  $\mu$ M pore size) to filter the entire volume. Thereafter, use a 10 ml syringe with another disposable filter unit (0.22  $\mu$ M pore size) to filter 10 ml portions to sterility of the prefiltered 10 $\times$  trypsin. This procedure is recommended to prevent plugging of the 0.22  $\mu$ M filter.
5. CMRL stands for Connaught Medical Research Laboratories and ATM stands for atmosphere. The bicarbonate-free medium has a high buffering capacity and a 5 % CO<sub>2</sub> atm and thus a CO<sub>2</sub> incubator is not needed for cell cultures growing in this medium. Moreover, cell cultures can be handled outside the incubator without substantial changes in the pH of the medium.
6. Usually 500 ml CMRL1415-ATM are delivered by the supplier in 500 ml bottles. It is recommended to remove 2 ml from such a bottle. Thereafter, add the indicated volumes of ingredients of 1000 $\times$  CaCl<sub>2</sub> and 1000 $\times$  FUDR to the CMRL1415-ATM containing bottle. This will guarantee the correct final concentrations of CaCl<sub>2</sub> (1.26 mM), FUDR (2  $\mu$ M), and gentamycin (10  $\mu$ g/ml).
7. We routinely calculate approx. 25 ml of this solution for isolation of cardiomyocytes from five neonatal rat hearts. Accordingly, a higher volume is needed if higher numbers of hearts are used, e.g., prepare 250 ml of this solution for 50 hearts.
8. Various criteria for the design of efficient siRNAs have been suggested, including a GC content between 30 and 52 %, A or U in positions 15–19, lack of internal repeats, A at positions 3 and 19, U at position 10, G or C at position 19 and G at position 13 [16]. In addition, structural restrictions of the target site have to be taken into consideration [17]. Various programs are provided at no cost to support the design of siRNAs, e.g.: <http://dharmacon.gelifesciences.com/design-center/> or <http://rnaideigner.thermofisher.com/rnaiexpress/design.do>. Alternatively, use one of the known active siRNAs published for most human and rodent genes in recent years. Furthermore, various companies offer predesigned and validated siRNAs with a silencing guarantee. It is still advisable to test three to four siRNAs for a given target to find the most efficient candidate. The standard design for siRNAs comprises a 19mer duplex with two nucleotide overhangs at both 3' ends. Figure 1 shows an example of an active siRNA targeting phospholamban.

9. As the prices for DNA synthesis have dropped dramatically in recent years, one may choose the option not to clone oligonucleotides downstream of the promoter, but rather to have parts or the entire expression cassette synthesized. This strategy is usually much faster, less labor-intensive and only slightly more expensive.
10. Put the agarose gel on a UV transilluminator to visualize the bands and cut out the gel slices with a scalpel. Be careful to protect your eyes with UV protective glasses and wear protective gloves to prevent contact with the gel, as ethidium bromide, used to make the DNA bands visible, is toxic. The vector backbone (2800 bp DNA fragment) can be dephosphorylated to prevent the *MscI* sites from religating.
11. Dephosphorylation enhances the efficiency of cloning by preventing religation of vector DNA which was only cleaved by one of the restriction enzymes.
12. Plasmid maxiprep yield large amounts of plasmid DNA, making it easier to subclone an expression cassette into AAV shuttle plasmids. In principle, plasmids isolated by plasmid mini preparation can also be used for subcloning.
13. pAAV-MCS is a plasmid containing an AAV vector genome which consists of the 5' and 3' ITRs from AAV2.
14. We suggest using recombination deficient (*recA*) bacteria, e.g., XL10-Gold Ultracompetent cells (Stratagene) for cloning to prevent recombination of AAV ITRs.
15. After restriction with *MscI*/*HincII*, the 1230 bp DNA fragment has blunt ends. Therefore it is possible that after ligation the fragment is inserted in two different orientations in the plasmid, leading to destruction of either the left or right AAV ITR. Either plasmid, containing a deletion of the right or left ITR, can be used as AAV shuttle plasmid to generate scAAV vectors.
16. Plasmid mini- and maxiprep kits are supplied by several companies. The plasmid maxiprep kits are typically used to produce high amounts of plasmid DNA which are necessary for AAV preparation. To prevent possible toxic side effects during AAV production, use endotoxin free plasmid maxiprep kits.
17. The vector DNA present in the AAV shuttle plasmid (representing the nucleotide number from the 5' end of the left to the 3' end of the right ITR in the AAV shuttle plasmid) should not exceed 2.4 kb for reliable packaging. Despite reports that larger AAV vector genomes can be packaged, we were unable to consistently package scAAV genomes exceeding this critical threshold. ShRNAs and amiRNA expression cassettes are typically smaller than this size, and therefore packaging of the respective expression cassettes in AAV genomes should be unproblematic. The lower limit of scAAV vector genome size

is currently unknown. We found that scAAV vector genomes with total length of 975 bp can be successfully packaged.

18. The method is described for production of AAV vectors in one roller bottle. One roller bottle is sufficient for production of a total of  $1 \times 10^{10}$ – $1 \times 10^{11}$  scAAV vectors (determined as vector genomes (vg) by quantitative PCR). For large-scale production we generally use a minimum of 6–10 roller bottles. You can also use standard disposable cell culture vessels (flasks or plates) for production. One roller bottle replaces approximately 10 cell culture plates (14.5 cm diameter).
19. The collagen is available from Sigma-Aldrich (Cat. No. C8919). For details of preparation of the collagen solution go to: <http://www.sigmaaldrich.com/technical-documents/articles/biofiles/collagen-product-protocols.html#sthash.Y5GaCDUp.dpuf>
20. Removed collagen solution can be reused. We have reused it for up to 10 times. Coated roller bottles can be stored at 4 °C for several days.
21. In each step when a solution is added/removed from the roller bottle set down the bottle gently to avoid shaking off cells. Also avoid spraying added solutions onto the walls of the bottle where cells are growing, as this may lead to unintentional detachment of the cells.
22. Before use, PEI must be warmed up at 65 °C for 10 min to ensure the PEI is completely dissolved.
23. For packaging of pseudotyped scAAV2/6 vectors (containing ITRs of AAV2 and capsids of AAV6) the pDP6 plasmid is used. It contains essential adenoviral helper functions and the replicase gene of AAV2 and the capsid gene of AAV6. For packaging of pseudotyped scAAV2/9 vectors (containing ITRs of AAV2 and capsids of AAV9) the adenoviral helper functions (E2a, E4, and VA RNA) and the replicase gene of AAV2 and the capsid gene of AAV9 are on separate plasmids, pHelper and p5E18VD2/9, respectively.
24. The supernatant also contains AAV vectors, but the yield is usually low compared to the cells collected from the roller bottle. Therefore isolation of AAV vectors from the supernatant (*see* Subheading 3.4) is often not recommended.
25. Rinse the lateral surfaces of the roller bottle with the cell solution while gently spinning the bottle by hand to detach all cells from the walls.
26. Prolonged centrifugation (30 min) leads to more compact pellets. Be careful with decantation to avoid discarding the cell pellet.
27. The suspension can directly be used in the next step or be stored at –80 °C until use.

28. The solution can be stored at  $-80^{\circ}\text{C}$  or directly filtered with the iodixanol gradient system.
29. In most cases this step will not lead to a drastic increase of total vector yield. Therefore this step is optional.
30. Store the solution at  $-80^{\circ}\text{C}$  or go to filtration with iodixanol gradient system.
31. Let the AAV solution run down on the wall of the tube very slowly to avoid swirls within the iodixanol layers. The maximum capacity of the tube for AAV solution is about 14 ml. Do not overload the gradients: A ratio of 2 roller bottles of AAV isolates per gradient should not be exceeded.
32. The centrifuge tube has an obvious mark on its neck. Be sure that the tube is filled exactly to the mark, otherwise the tube will collapse during ultracentrifugation. Tare the centrifuge tubes as exactly as possible (e.g., to the fourth digit on a milligram scale).
33. If the cannula is pricked into the tube, the open angle of the cannula must point upwards. When you remove the cannula from the tube, the contents of the tube will leak out the punctured site. Therefore, we hold the tube over a 50 ml centrifuge tube and drop it into the tube immediately after pulling out the cannula. AAV vector solution can be stored at  $-80^{\circ}\text{C}$ .
34. If solution remains in the tube and does not pass through (which is a result of remaining iodixanol in the preparation), resuspend and rinse the membrane of the Amicon® Ultra 15 ml centrifugal filters with the remaining volume or extend centrifugation time.
35. For large volumes of AAV, several Amicon® Ultra 15 ml centrifugal filters can be used in parallel. After centrifugation the concentrated AAV solutions can be combined and concentrated further using a fresh Amicon® Ultra 15 ml centrifugal filter. Be careful if an Amicon® Ultra 15 ml centrifugal filter is centrifuged several times. The more often a column is centrifuged, the higher the probability of leaks and therefore loss of AAVs.
36. Use a suitable micropipette for resuspension to avoid formation of foam. Concentrated scAAV vectors can be stored at  $-80^{\circ}\text{C}$ .
37. Newborn Sprague Dawley or Wistar rats can be used. However, use 1–3 day old pups only. Do not use neonates older than 3 days, because the hearts of these animals have a more extensive extracellular matrix, which hampers its trypsin-catalyzed digestion and needs more digestion time. Extending digestion times may lead to enhanced trypsin-related damage of PNRC. As a consequence the number of viable PNRC will decline and an excessive number of non-surviving cells will be found in culture.

38. The length of the incision should be less than half of the length of the body. It should not extend across the thorax–abdomen border.
39. Complete removal of atria is very important. Contaminating atrial pace maker cells in the culture of spontaneously beating PNRC will result in a markedly increased contraction rate.
40. These washing steps are necessary to get rid of penicillin and streptomycin which could be harmful to the PNRC during the following enzymatic digestion.
41. This first digestion allows the removal of broken cells as well as blood cells. The temperature during this digestion step as well as the following ones should be continuously checked and protocolled. It should not exceed 37 °C. Otherwise trypsin-catalyzed digestion will result in excessive cell damage leading to an increased number of dead cells in the sought culture.
42. Too rapid acceleration of the centrifuge rotor may damage the enzymatically isolated PNRC, while too rapid deceleration will result in loss of PNRC due to turbulence causing some pelleted cells to be lost to the supernatant.
43. This filtration step through sterile gauze is used to remove feasible cell debris of larger size from the cell suspension.
44. For firm attachment and growth of the isolated PNRC we strongly recommend using flasks and cell culture multidishes with Nunclon™ Delta surface coating. If you intend to use other types of plastic culture material for attachment and growth of the PNRC, they must first be tested. In some cases premature surface coating with collagen or another suitable surface coating substance may be needed.
45. Using CMRL1415-ATM medium during incubation no CO<sub>2</sub> supply is needed. Therefore, if a CO<sub>2</sub>-incubator is being used, adjust the CO<sub>2</sub> supply to zero. This also holds true for all following incubation steps.
46. The mitosis inhibitor FUDR is added to reduce division and growth of contaminating fibroblasts, which, in contrast to PNRC, will otherwise proliferate substantially in the serum-containing medium.
47. At this time point spontaneously contracting PNRC usually will have reached a confluence of about 70–80 %.
48. We have been successful keeping contracting PNRC in culture for up to 21 days. However, keep in mind that the number of fibroblasts in the culture will increase continuously, even in the presence of 2 µM FUDR. Higher concentrations of the inhibitor to achieve complete suppression of fibroblast mitosis should not be used, due to its toxicity to PNRC.



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