ONLINE SUPPLEMENTAL MATERIAL

Atrial-specific gene delivery using an adeno-associated viral vector

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DETAILED METHODS

Plasmid production. The AAV plasmid ubiquitously expressing GFP driven by the chicken beta actin promoter was obtained from the University of Pennsylvania vector core (pENN.AAV.CB7.CI.eGFP.WPRE.rBG; PL-C-PV1963). The sequences and location of functional elements for the TNT[1] and ANF (*Nppa*)[2] promoters have been described previously. The new AAV vectors reported in this study were constructed using standard molecular biology approaches with gBlocks synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Expression cassettes were cloned into plasmid 1179_pAAV-U6-BbsI-gRNA-CB-EmGFP (Addgene #89060)[3], which contains the Inverted Terminal Repeats (ITRs) from AAV2, and an Ampicillin resistance gene. Plasmid AAV9-TNT4-GFP (011LP-pAAV-TNT4-EmGFP-pA) expresses green fluorescent protein driven by the cardiomyocyte-specific TNT promoter. Plasmid AAV9-ANF-GFP (013LP-pAAV-ANF-657-EmGFP-pA) expresses the same transgene driven by the atrial-specific ANF promoter. Plasmid AAV9-ANF-CRE (014LP-pAAV-ANF-657-Cre-pA) expresses Cre recombinase driven by the ANF promoter. AAV vectors based on serotype 9 were packaged in HEK293T cells by the triple transfection method[4], and purified by CsCl gradient ultracentrifugation[5]. The complete vector sequences listed at the end of this document. These plasmids will be made publicly available through Addgene upon publication of the manuscript.

Production of adeno-associated virus type 9 (AAV9). The AAV9 packaging vector pAAV2/9 (PL-T-P0008-R2) and the adenoviral helper plasmid pAdDeltaF6 (PL-F-PVADF6) were obtained from the University of Pennsylvania Vector Core and generated by Puresyn, Inc. HEK293T cells (ATCC, Manassas, VA) were used for AAV9 packaging using the triple transfection method of Xiao *et al*.[4] Cells were seeded in 150 mm plates (30-50% confluent) and fed with DMEM media (Lonza, Allendale, NJ) containing 10% fetal bovine serum (Thermo Fisher, Waltham, MA), L-glutamine, and penicillin/streptomycin (Thermo Fisher, Waltham, MA) 2 hours before transfection.

After 48-72 hours, cells were harvested using TrypLE™ express enzyme medium (Thermo Fisher, Waltham, MA) and collected in PBS (Corning, New York, NY). Cells were subjected to 3 freeze-thaw cycles (-80°C for 10 minutes, 37°C for 20 minutes) in resuspension buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$). To digest cellular genomic DNA, cells were incubated with 3,000 U Benzonase (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 hour. 1/39th volume of 1M $CaCl_2$ solution and 2/3 volume of 20% PEG 8000/1.25N NaCl were used to remove the cell debris and precipitate AAV. After 2 rounds of $CsCl_2$ gradient in 45,000 rpm and 60,000 rpm, AAV was collected from the relevant fractions: refractive index = 1.3680-1.3750 for the first gradient and refractive index = 1.3680-1.3750 for the second gradient.[4]

Dialysis was conducted for AAV9 against PBS with pre-wet 10,000 MWCO Slide-A-Lyzer Cassettes (0.5–3.0 mL), and concentrated using Amicon 100 kDa MWCO centrifugal filtration device (UFC910008, EMD Millipore). AAV9 was stored at -80°C. For titer quantification, purified virus was digested with DNAse I (Sigma-Aldrich, St. Louis, MO) to remove unencapsidated DNA from the prep, followed by proteinase K digestion to free the DNA template from the AAV capsid. AAV titers were determined by qPCR relative to standard curves generated from the respective serially diluted plasmids.[4]

Immunohistochemistry. To prepare tissues for immunohistochemical analysis, standard embedding and sectioning procedures were utilized as described.[6] Mouse hearts and select organs (e.g. liver, lung, muscle, brain and kidney) were flushed or rinsed with saline (Thermo Fisher, Waltham, MA) to remove excess blood, and fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Science, Hatfield, PA) at 4°C for 1-2 hours. Tissues were then placed in 3mM glycine (Sigma-Aldrich, St. Louis, MO) at 4°C overnight to neutralize PFA. The following day, the glycine solution was discarded and the tissues were placed in 30% sucrose solution for 24-48 hours at 4°C to preserve tissue morphology. The fixed tissues were then embedded in optical cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) and stored at -80°C. A Leica cryostat was used for tissue block sectioning, at a cutting temperature from -23 to -27°C and a thickness of 10µm. Sample slides were stored immediately at -80°C until immunohistochemical analysis.

Immunohistochemical examinations for GFP were performed on 80°C frozen materials. Briefly, heart sections were air dried for 10 min, then rinsed in PBS. Next, 10% goat serum in PBS followed by overnight incubation at 4°C the sections were incubated with overnight at 4°C with a mouse GFP monoclonal antibody (1:500, MA5-15256, Thermo Fischer, Waltham, MA). After three washes in PBS, sections were incubated with mouse IgG (H+L) cross-adsorbed secondary antibody (A-11004, Thermo Fischer, Waltham, MA) antibody at a dilution of 1:500 in 10% goat serum-PBS for 30 min at room temperature. We employed the secondary antibody alone as controls to ensure antibody specificity. All incubations with fluorescent antibodies were performed in the dark. After washing, the sections were fixed in a drop of mountain solution (Vector Laboratories, Inc, Berlingame, CA), analyzed using a confocal microscopy LSM 880 (Zeiss, Oberkochen, Germany). No nonspecific staining was observed with the secondary antibody alone.

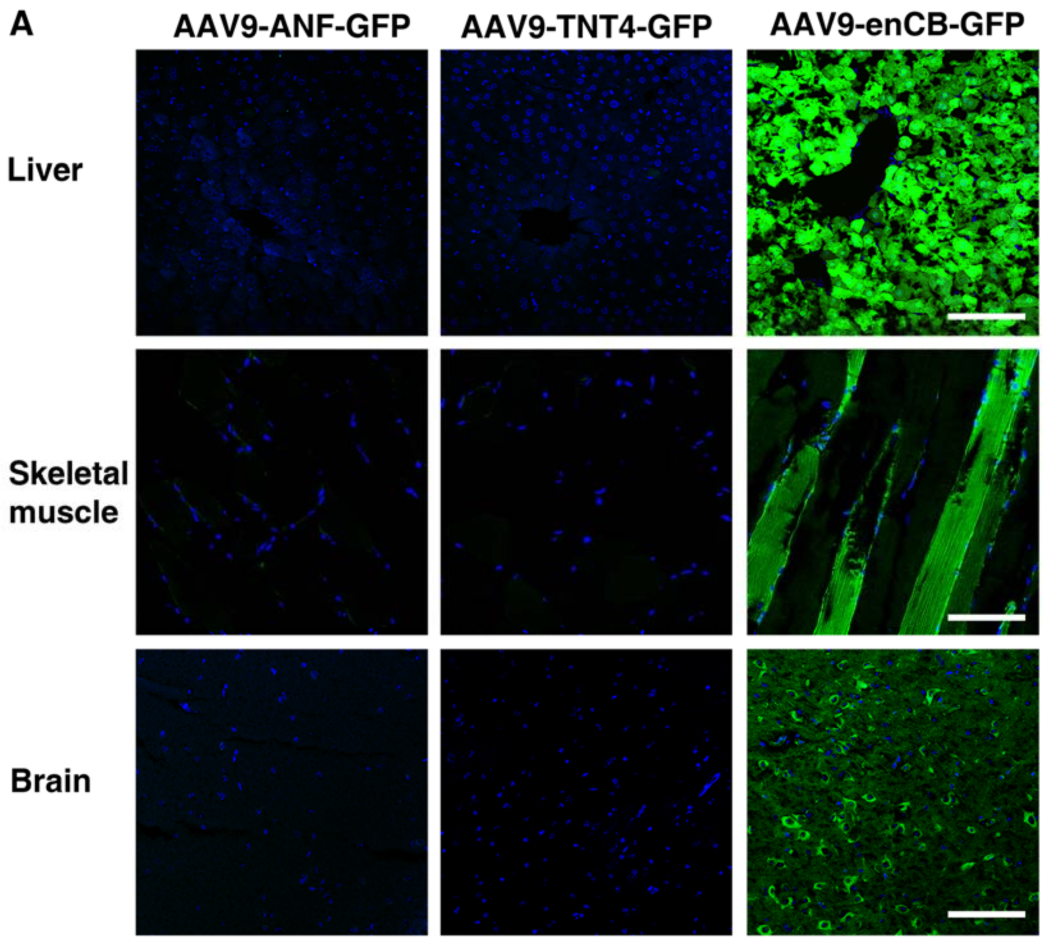
Mouse TAC model. Animals subjected to TAC/Sham ranged in age from 2.8 to 4.8 months at the time of surgery. Mice were anesthetized with 2% isoflurane mixed with 100% O_2 (0.8 L/min) and anesthesia was maintained at 1.5–2% isoflurane by endotracheal intubation and ventilation throughout the procedure. An anterior thoracotomy was performed to expose the aortic arch to the level of the third intercostal space. Constriction was performed by tying a 6-0 silk suture against a 28-gauge needle between the first and second trunk of the aortic arch. For consistency, constriction levels were quantified by measuring alterations in Doppler velocities of the right and left carotid arteries 7 days post-surgery. Right-to-left carotid peak velocity ratios ranged from 5.0 to 6.5 and 6-week post TAC ejection fractions ranged from 40%–55% in TAC groups used for experiments. The initial animal number subjected to surgery was 32 mice (22 males, 10 females). Seven mice found dead in the first 7 days after TAC surgery, before Doppler was performed. We excluded 3 animals from total of 25, because they exhibited only modest decrements in cardiac function (EF>55% by 6 weeks post TAC).

Transthoracic echocardiography. Mice were anesthetized using 1.5% isoflurane in 100% O_2 at 1.5 L/min. Vital signs were continuously monitored to ensure similar heart and respiration rates. Body temperature was maintained between 36.5–37.5°C on a heated platform. Cardiac function was assessed using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described.[7, 8]

Western blotting. Atrial and ventricular lysate was made after flash freezing tissue in liquid nitrogen followed by homogenization in RIPA-CHAPS lysis buffer and sonication 3x for 1 second. Protein lysates were denatured for 10 minutes at 70°C in Laemmli buffer with beta-mercaptoethanol prior to electrophoresis on a 10% acrylamide gel. Proteins were transferred overnight at 4°C to a PVDF membrane. Membranes were blocked 30 minutes at RT in 5% Bovine Serum Albumin/ 5% non-fat milk-tris-buffered saline (TBS) followed by overnight incubation at 4°C with primary antibodies for GFP (1:5000, MA5-15256, Thermo Fischer, Waltham, MA), JPH2 (1:1000, a custom rabbit polyclonal Yenzym antibody raised against a synthetic peptide consisting of the amino acid sequence 458-CRPRESPQLHERETPQPEG-475), and GAPDH (1:10000, MAB374, EMD Millipore, Burlington, MA), membranes were incubated in goat anti-mouse IgG (H+L) superclonal™ secondary antibody, Alexa Fuor 680 (1:10000, A28183, Thermo Fischer, Waltham, MA) or goat anti-rabbit IgG (H&L) antibody DyLight™ 800 conjugated (1:10000, 611-145-002, Rockland, Limerick, PA) prior to imaging with Li-Cor Odyssey Blot Imager.

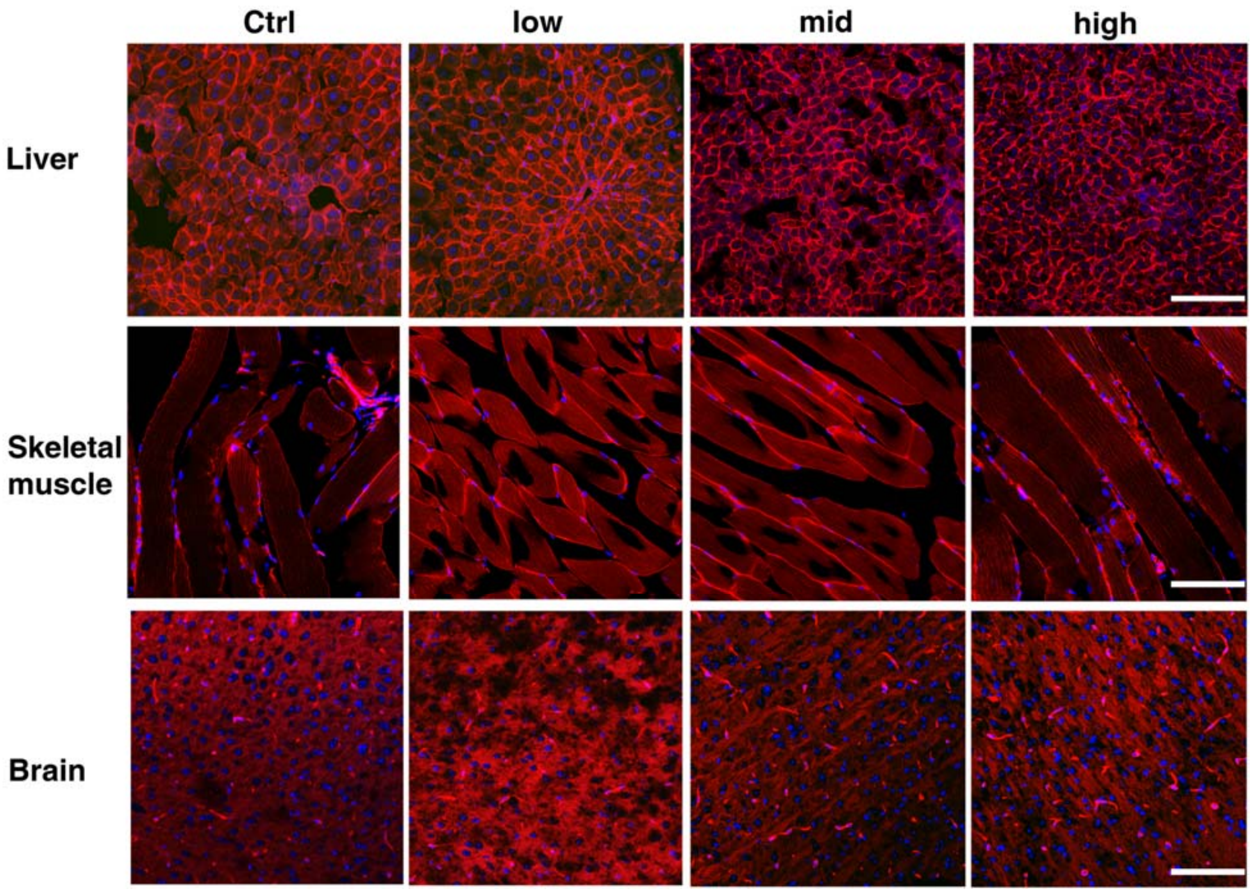
Atrial myocyte Ca^{2+} imaging. Atrial myocytes were isolated using a collagenase method as described.[9] Atrial myocytes were loaded with 2 mmol/L Fluo-4-AM (Invitrogen, Carlsbad, CA) in normal Tyrode solution containing 1.8 mmol/L Ca^{2+} for 30 minutes at room temperature. Cells were subsequently imaged by confocal microscopy. Line scans were used to obtain Ca spark data and transient amplitudes. Caffeine was used to determine the SR load. Data were analyzed using Image J and the Spark Master plug-in.

Online Figure I



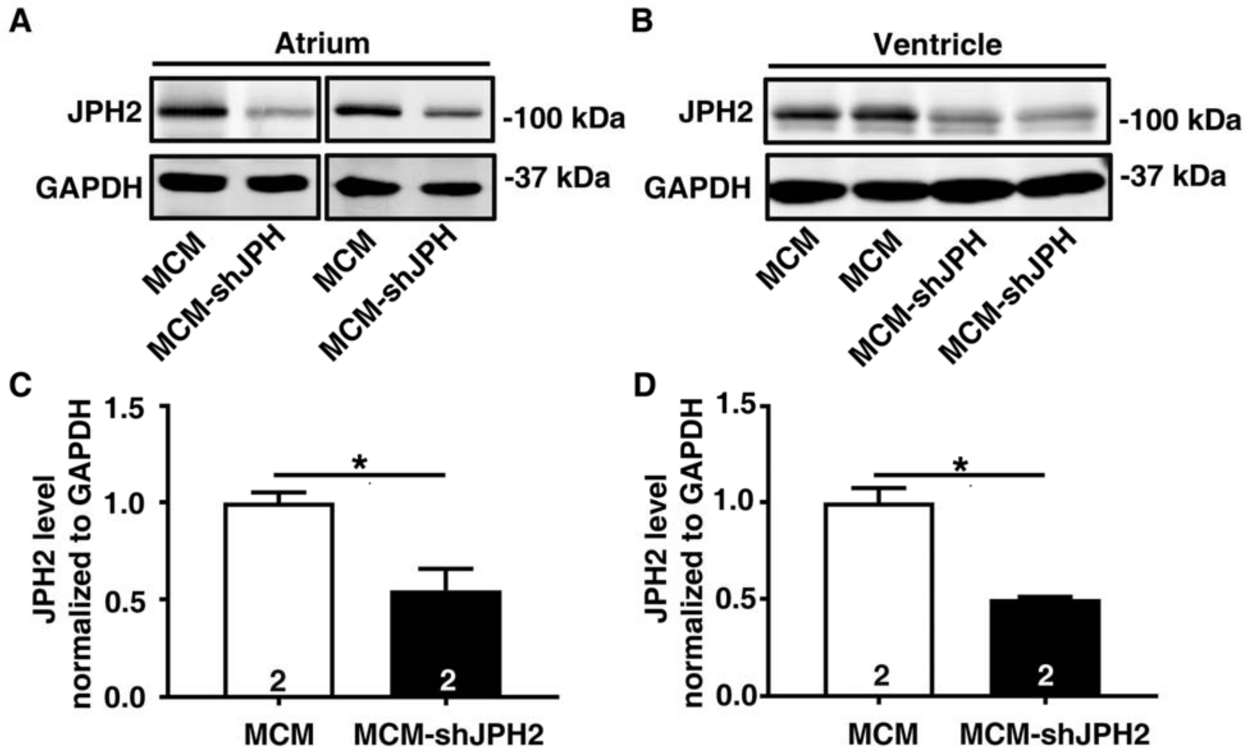
Atrial-specific expression of GFP after systemic injection of AAV9-ANF-GFP in C57Bl/6J mice. Sections from C57Bl/6J mice injected retro-orbitally with AAV9-ANF-GFP (1×10^{12} GC) exhibited no extracardiac GFP expression in representative tissues (liver, skeletal muscle, brain). Scale bars, 100 μm.

Online Figure II



Absence of extracardiac Cre-mediated recombination in *mTmG* mice injected with AAV9-ANF-GFP. Sections from *mT/mG* mice injected retro-orbitally with AAV9-ANF-GFP (5×10^{11} GC low dose; 1×10^{12} GC mid dose; 5×10^{12} GC high dose) exhibited no or minimal extracardiac Cre-mediated recombination in representative tissues (liver, skeletal muscle, brain). Scale bars, 100 μm). Ctrl, control (saline, no virus).

Online Figure III



Reduced JPH2 protein levels in atria and ventricles of MCM-shJPH2 mice injected with tamoxifen. Western blots showing reduced JPH2 protein levels normalized to GAPDH loading controls in both atrial and ventricular tissue of shJPH2 mice injected with AAV9-TNT-Cre. *$p < 0.05$.

Online Table I

Echocardiographic parameters of mTmG mice

	Sham+saline (n=5)	TAC+saline (n=6)	Sham+AAV9 (n=5)	TAC+AAV9 (n=6)
HR (bpm)	432.8±17.4	500.4±9.8**	448.1±20.6	514.8±8.4#
EF (%)	75.6±3.8	48.0±2.2***	73.5±1.9	46.5±2.0###
FS (%)	44.1±3.2	24.0±1.3***	41.8±1.7	23.0±1.1###
LVAW;d (mm)	0.92±0.04	1.04±0.02*	0.90±0.03	1.12±0.04##
LVID;d (mm)	3.62±0.03	4.25±0.22*	3.49±0.06	4.08±0.22#
LVPW;d (mm)	0.95±0.06	1.34±0.04***	1.01±0.09	1.34±0.08#
LVAW;s (mm)	1.17±0.07	1.18±0.04	1.12±0.03	1.27±0.04#
LVID;s (mm)	2.17±0.14	3.27±0.19**	2.19±0.04	3.16±0.21##
LVPW;s (mm)	1.40±0.07	1.60±0.07	1.45±0.13	1.60±0.07

Data are expressed as mean ± SEM. * P<0.05, ** P<0.01, *** P<0.01 vs Sham+saline, and #P<0.05, ## P<0.01, ### P<0.01 vs TAC+saline. HR, heart rate; EF, ejection fraction; FS, left ventricular fractional shortening; LVAW, left ventricular anterior wall thickness; LVID, Left ventricular internal diameter; LVPW, left ventricular posterior wall thickness. Subscript letters represent during diastole or systole.

Online Table II

Characteristics of Ca^{2+} sparks in atrial myocytes.

	shJPH2+saline	**shJPH2+AAV9-AAV-Cre**
N (sparks)	89	225
N (mice)	3	3
Amplitude(F/F0)	0.84±0.02	0.83±0.01
FWHM (μm)	1.37±0.06	1.42±0.04
FDHM (ms)	24.7±1.14	23.8±0.73
TtP (ms)	18.8±1.48	17.8±0.83
dV/dt (mV/ms)	77.1±1.39	74.9±1.07
Tau (ms)	27.4±2.14	24.2±1.38

FWHM, Full width at half maximum; FDHM, Full duration at half maximum; TtP, time to peak.

Online Table III

Echocardiographic parameters of shJPH2 mice.

	shJPH2+saline n=7	**shJPH2+AAV9-AAV-Cre** n=9
HR (bpm)	537.2±22.8	570.0±8.7
EF (%)	67.8±4.3	69.2±0.7
FS (%)	37.6±3.1	38.2±0.5
LVAW;d (mm)	0.70±0.03	0.78±0.02
LVID;d (mm)	3.53±0.15	3.54±0.06
LVPW;d (mm)	0.73±0.02	0,.77±0.03
LVAW;s (mm)	1.13±0.06	1.23±0.04
LVID;s (mm)	2.31±0.21	2.32±0.05
LVPW;s (mm)	0.89±0.03	0.92±0.03

Data are expressed as mean ± SEM. HR, heart rate; EF, ejection fraction; FS, left ventricular fractional shortening; LVAW, left ventricular anterior wall thickness; LVID, Left ventricular internal diameter; LVPW, left ventricular posterior wall thickness. Subscript letters represent during diastole or systole.

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1. Werfel S, Jungmann A, Lehmann L, Ksienzyk J, Bekeredjian R, Kaya Z, Leuchs B, Nordheim A, Backs J, Engelhardt S, Katus HA, Muller OJ. Rapid and highly efficient inducible cardiac gene knockout in adult mice using aav-mediated expression of cre recombinase. *Cardiovasc Res*. 2014;104:15-23.
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VECTOR SEQUENCES

AAV9-TNT4-GFP

Features:

5'ITR	1..170
hTNNT2 -502 to +42 Promoter Werfels et al	180..723
attB1	791..815
ATG Start	824..826
3X FLAG	827..892
HA tag	896..922
GGAGG flexible linker	929..943
EmGFP	944..1663
SV40 polyA	1678..1899
3'ITR	1919..2088
pEMBL8 Plasmid Backbone	2113..4528

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AAV9-ANF-GFP

Features:

5'ITR	1..170
ANF promoter	180..836
attB1	904..928
ATG Start	937..939
3X FLAG sequence	940..1005
HA tag	1009..1035
GGAGG flexible linker	1042..1056
EmGFP	1057..1776
SV40 polyA	1791..2012
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pEMBL8 Plasmid Backbone	2226..4641

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AAV9-ANF-CRE

Features:

5'ITR	1..170
ANF promoter	180..836
ATG START	890..892
SV40 NLS	893..913
CRE Recombinase	914..1939
HA	1940..1966
SV40 polyA	1984..2205
3'ITR	2225..2394
pEMBL8 Plasmid Backbone	2419..4834

Sequence:

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