User Manual

Adeno-associated virus (AAV)

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Safe Use of AAV

1. AAV related experiments should be conducted in biosafety level 2 facilities (BL-2 level).

2. Please equip with lab coat, mask, gloves completely, and try your best to avoid exposing hand and arm.

3. Be careful of splashing virus suspension. If biosafety cabinet is contaminated with virus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting virus must be soaked in chlorine-containing disinfectant before disposal.

4. If centrifuging is required, a centrifuge tube should be tightly sealed. Seal the tube with parafilm before centrifuging if condition allowed.

5. AAV related animal experiments should also be conducted in BL-2 level.

6. AAV associated waste materials need to be specially collected and autoclaved before disposal.

7. Wash hands with sanitizer after experiment.

Storage and Dilution of AAV

Storage of AAV

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since AAV viruses are sensitive to freeze-thawing and the titer drops with repeated freeze-thawing, aliquot viral stock should be stored at -80°C freezer immediately upon arrival for long-term usage. While virus titer redetection is suggested before using if the AAV viruses have been stored for more than 12 months.

Dilution of AAV

Dissolve virus in ice water if virus dilution is required. After dissolving, mix the virus with medium, sterile PBS or normal saline solution, keeping at 4°C (using within a week).

Precautions

- Avoid AAV exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)

- Avoid introducing air into the AAV samples during vortexing, blowing bubbles or similar operations, which may result in protein denaturation.

- Avoid repeated freezing and thawing.

- Avoid exposing to “regular” plastics (especially polystyrene or hydrophobic plastics) for prolonged periods in liquid phase. Most AAV viruses are very sticky and loss can occur if exposed to regular plastics, including tubes, cell culture plates, pipette tips, if not frozen. It is best to store AAV in siliconized or low protein binding tubes. Pluronic F-68 used at 0.01%-0.1% in the formulation buffer will minimize sticking if regular plastics are used.

• Avoid diluting AAV into low salt solution. Some AAV serotypes, such as AAV2, aggregates in low salt solution, which will be non-infectious.

Introduction of AAV

In Genemedi Biosciences, recombinant adeno-associated Virus (rAAV) Expression Systems are utilized in delivering and expressing shRNA, human ORF, CRISPR *in vitro* and *in vivo*.

Adeno-associated virus (AAV) is a small single strand DNA virus infecting human and some other primate species. Currently, AAV has not known to cause disease and only induces very mild immune responses. As a member of the family Parvoviridae, wild type AAV requires the assistance of adenovirus or herpesvirus to complete the duplication, which is the reason why it's called adeno-associated virus [1,2]. The wild-type AAV2 genome consists of the viral rep and cap genes (encoding replication and capsid genes, respectively), flanked by inverted terminal repeats (ITRs) that contain all the cis-acting elements necessary for replication and packaging. The genome of typical AAV2 is about 4800bp, consisting of two upstream and downstream open read frames (ORFs) which are between two inverted terminal repeats (ITR) comprising Rep and Cap (Figure 1). ITR is required for synthesis of complementary DNA strand, while Rep and Cap can be translated into various proteins, including AAV virus cycle essential protein Rep78, Rep68, Rep52, Rep40 and enveloped protein VP1, VP2, VP3, etc. [3].

The present recombinant AAV (rAAV) vectors are generated by replacing all of the viral genome between the ITRs with a transcriptional cassette of less than 5 kilobases in length. The resulting construct is then co-expressed with two other plasmids: 1) an AAV-RC plasmid that provides the Rep and Cap genes in trans (separate from the ITR/Transgene cassette) and 2) an AAV helper plasmid that harbors the adenoviral helper genes. AAV-293 cells are used as the packaging cell line since they provide the E1a protein in trans as well. By modifying the Rep and Cap genes, scientists can control the serotypes to guide the recombinant AAV infection towards certain tissues. This 3-plasmid co-transfection system liberates the need for adenovirus during AAV production, which greatly simplifies the purification process.

To date, a total of 12 serotypes of AAV have been described with their own unique traits and tropisms [4]. Concerning high safety, low immunogenicity, long-term expression of exogenous genes, AAV is thought to be the best gene delivery tool for gene function research *in vivo*.

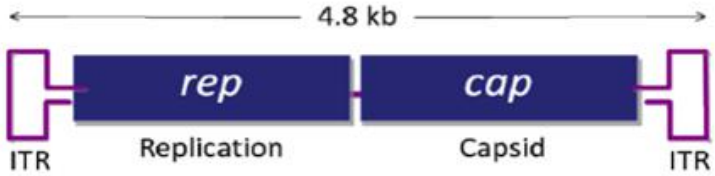

Figure 1. Schematic diagram of AAV2 genome structure.

Advantages of AAV for Gene Delivery and Expression

1. Superior biosafety rating

AAV is a naturally defective virus, requiring provision of several factors in trans for productive infection and has not been associated with any human disease. In our AAV production system, the AAV2 ITR sequences and rep/cap genes are present on separate plasmids that lack homology, preventing production of recombinant wild-type virus. These features give AAV a superior biosafety rating among gene delivery and expression vectors of viral origin.

2. Broad range of infectivity

AAV viruses infect a broad range of mammalian cells and have been used successfully to express human and non-human proteins. In contrast with other vectors of viral origin, AAV vectors have proven to be competent for gene expression in immunocompetent hosts.

3. High titer

Recombinant AAV can be produced at high titers of $\geq 10^7$ viral particles/ml with this protocol. Titers up to 10^{13} viral particles/ml after concentration have been published.

4. Infection does not require an actively dividing host cell

AAV can infect both dividing and non-dividing cells.

5. Long-term gene transfer potential

Recombinant AAV (rAAV) can be maintained in human cells, creating the potential for long-term gene transfer. In most cell populations, the viral genome typically remains epichromosomal, often forming concatemers, which are stable in slowly dividing or non-dividing cells, leading to long-term gene transfer and expression. Whereas in rapidly dividing cell populations, the AAV viruses can integrate into the host genome but not form concatemers, resulting in long-term gene expression in dividing cells, but this is a rare event. The integration occurs more frequently if an extremely high multiplicity of infection (MOI) of AAV is used or if infection occurs in the presence of adenoviral replicase, potentially supplied by the use of wild-type adenovirus. However, it will reduce the biosafety of the AAV system to increase integration events by using wild-type adenovirus.

AAV Serotypes and Native Tropism- AAV Selection Guide

Over the past decades, numerous AAV serotypes have been identified with variable tropism. To date, 12 AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or from human/nonhuman primate tissues. Different AAV serotypes exhibit different tropisms, infecting different cell types and tissue types. So, selecting the suitable AAV serotype is critical for gene delivery to target cells or tissues.

Due to the exhibition of natural tropism towards certain cell or tissue types, rAAV has garnered considerable attention. Highly prevalent in humans and other primates, several AAV serotypes have been isolated. AAV2, AAV3, AAV5, AAV6 were discovered in human cells, while AAV1, AAV4, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 in nonhuman primate samples [5,6]. Genome divergence among different serotypes is most concentrated on hypervariable regions (HVRs) of virus capsid, which might determine their tissue tropisms. In addition to virus capsid, tissue tropisms of AAV vectors are also influenced by cell surface receptors, cellular uptake, intracellular processing, nuclear delivery of the vector genome, uncoating, and second strand DNA conversion [7].

In order to better improve the infection efficiency and specificity of AAV to target tissues, scientists have genetically modified the viral capsid, and generated mosaic vectors to create chimeric AAV by swapping domain's or amino-acids between serotypes [8,9], which may allow researchers to specifically target cells with certain serotypes to effectively transduce and express genes in a localized area [10].

Meanwhile, the ability of AAV to penetrate the blood-brain barrier in animals is greatly limited or improved. Traditionally, AAV could only be injected into the brain tissue by surgery for scientific research in the central nervous system, which greatly increased the difficulty of the experiment and affected the experimental results. Now the modified AAV serotype of PHP.B and PHP.eB can infect the whole brain through the blood-brain barrier by

peripheral blood injection [11]. Most popular rAAV serotypes and their tropisms are listed in the following table1.

Table 1. rAAV serotypes and their tropisms.

AAV Serotype	Tissue tropism							
	CNS	Retina	Lung	Liver	Pancreas	Kidney	Heart	Muscle
AAV1	√	√			√		√	√
AAV2		√		√		√		
AAV3		√	√	√			√	
AAV4	√	√					√	
AAV5	√	√	√		√			
AAV6	√		√	√			√	√
AAV7				√				√
AAV8		√		√	√			√
AAV9	√		√	√			√	√
AAV-DJ		√	√	√		√		
AAV-DJ/8		√		√				√
AAV-Rh10	√		√	√			√	√
AAV-retro	√	√						√
AAV-PHP.B	√						√	√
AAV-PHP.eB	√							√
AAV-PHP.S	√						√	√

AAV Product, Service and Information of Vector, List of Goods in Stock of Genemedi

Product and Service Item of Genemedi AAV

- Adeno-associated virus (AAV) customized production service (table 2).
- CRISPR/Cas9 adeno-associated virus (AAV) production service.
- Optogenetics adeno-associated virus (AAV) production service (table 3 and table 4).
- AAV-LC3 production service for autophagy flux detection (table 5).
- Pre-made adeno-associated virus (AAV) production service.
- Adeno-associated virus (AAV) control virus production service.

Product Character of Genemedi AAV

- High efficiency. The immunogenicity of AAV is extremely low. The virus titer is up to 10^{14} vg/ml, giving it high infection efficiency in various tissues.
- Long-term expression. The expression of target gene can sustain more than 2 years.
- Large serotype library. With 11 serotypes and a range of mutant subtypes, AAV could infect almost all the animal tissues and organs efficiently.
- Tissue Tropism. A lot of tissue-specific promoters are available for different cell/tissue, including the heart, liver, muscle and various nervous tissue specific promoter.
- High quality. All virus products are subjected to rigorous testing, including sterile, non-mycoplasma, non-endotoxin.

List of Main Genemedi AAV Vector and Goods in Stock

Table 2. List of AAV vector

(Some tissue-specific promoters such as heart, liver, muscle, etc. are not listed in the table, welcome to consult)

Type	AAV plasmids	Promoter	Expression characteristics	Fluorescent Label	Note
Overexpression	pAAV-CMV-MCS-T2A-ZsGreen	CMV	General and strong	ZsGreen	
	pAAV-CMV-MCS-EF1-ZsGreen	CMV	General and strong	ZsGreen	
	pAAV-CAG-MCS-T2A-ZsGreen	CAG	General and strong	ZsGreen	
	pAAV-CAG-MCS-T2A-mCherry	CAG	General and strong	mCherry	
	pAAV-CAG-DIO-MCS-T2A-ZsGreen	CAG	General and strong	ZsGreen	Cre dependent expression
	pAAV-CAG-DIO-MCS-T2A-mCherry	CAG	General and strong	mCherry	Cre dependent expression
	pAAV-hsyn-MCS-T2A-ZsGreen	hSyn	Neuron specific	ZsGreen	
	pAAV-hsyn-MCS-T2A-mCherry	hSyn	Neuron specific	mCherry	
	pAAV-CamkII-MCS-T2A-ZsGreen	CamkII	Neuron specific	ZsGreen	
	pAAV-CamkII-MCS-T2A-mCherry	CamkII	Neuron specific	mCherry	
	pAAV-GFAP-T2A-EGFP	GFAP	Astrocyte specific	EGFP	
Downregulation	pAAV-U6-MCS-CMV-ZsGreen	U6	General and strong	ZsGreen	
	pAAV-U6-CMV-mCherry	U6	General and strong	mCherry	
CircRNA Overexpression	pAAV-CMV-crRNA-EF1-ZsGreen	CMV	General and strong	ZsGreen	
Knockout	pAAV-gRNA-saCas9	U6	General and strong	None	

Table 3. List of optogenetics tools in AAV.

AAV Plasmids	Promoter and Expression Characteristics	Fluorescent Label	Cre Dependent	Optogenetics Effect
pAAV-CAG-DIO-eNpHR3.0-EYFP	CAG (general and strong expression)	EYFP	yes	Inhibition
pAAV-CAG -DIO-hChR2(H134R)-mCherry		mCherry	yes	activation
pAAV-CAG-DIO-ArchT-EYFP		EYFP	yes	inhibition
pAAV-CAG -DIO-C1V1 (t/t)-TS-mCherry		mCherry	yes	activation
pAAV-CAG-DIO-Arch3.0-EYFP		EYFP	yes	inhibition
pAAV- CAG -DIO-hCHETA-EYFP		EYFP	yes	activation
pAAV-CMV-DIO-eNpHR3.0-EYFP	CMV (general and strong expression)	EYFP	yes	inhibition
pAAV-CMV-DIO-hChR2(H134R)-mCherry		mCherry	yes	activation
pAAV-CMV-DIO-ArchT-EYFP		EYFP	yes	inhibition
pAAV-CMV-DIO-C1V1 (t/t)-TS-mCherry		mCherry	yes	activation
pAAV-CMV-DIO-Arch3.0-EYFP		EYFP	yes	inhibition
pAAV-CMV-DIO-hCHETA-EYFP		EYFP	yes	activation
pAAV-hSyn-eNpHR3.0-EYFP	hSyn (neuron specific pression)	EYFP	No	inhibition
pAAV-hSyn-hChR2(H134R)-mCherry		mCherry	No	activation
pAAV-hSyn-ArchT-EYFP		EYFP	No	inhibition
pAAV-hSyn-C1V1-(t/t)-TS-mCherry		mCherry	No	activation
pAAV-hSyn-Arch3.0-EYFP		EYFP	No	inhibition
pAAV-hSyn-DIO-hCHETA-EYFP		EYFP	yes	activation
pAAV-GFAP-eNpHR3.0-EYFP	GFAP (astrocyte specific expression)	EYFP	No	inhibition
pAAV-GFAP-hChR2(H134R)-mCherry		mCherry	No	activation
pAAV-GFAP-ArchT-EYFP		EYFP	No	inhibition
pAAV-GFAP-C1V1 (t/t)-TS-mCherry		mCherry	No	activation
pAAV-GFAP-Arch3.0-EYFP		EYFP	No	inhibition
pAAV-GFAP-hCHETA-EYFP		EYFP	No	activation
pAAV-CaMKII-eNpHR3.0-EYFP	CaMKII (neuron specific expression)	EYFP	No	inhibition
pAAV-CaMKII-hChR2(H134R)-mCherry		mCherry	No	activation
pAAV-CaMKII-ArchT-EYFP		EYFP	No	inhibition
pAAV-CaMKII-C1V1 (t/t)-TS-mCherry		mCherry	No	activation
pAAV-CaMKII-Arch3.0-EYFP		EYFP	No	inhibition
pAAV-CaMKII-hCHETA-EYFP		EYFP	No	activation

Table 4. List of chemical genetics tools in AAV.

AAV Plasmids	Promoter and Expression Characteristics	Fluorescent Label	Cre Dependent	Optogenetics Effect
pAAV-CAG-DIO-DTR-2A-GFP	CAG (general and strong expression)	EGFP	Yes	
pAAV-hSyn-DTR-2A-GFP	hSyn (neuron specific expression)	GFP	No	
pAAV-CaMKII-DTR-2A-GFP	CaMKII (neuron specific expression	EGFP	No	
pAAV-CAG-DIO-hM3D(Gq)-mCherry	CAG (general and strong expression)	mCherry	Yes	Gq-DREADD
pAAV-CAG-DIO-hM4D(Gi)-mCherry	CAG (general and strong expression)	mCherry	Yes	Gi- DREADD
pAAV-hSyn-DIO-hM3D(Gq)-mCherry	hSyn (neuron specific expression)	mCherry	Yes	Gq-DREADD
pAAV-hSyn-DIO-hM4D(Gi)-mCherry	hSyn (neuron specific expression)	mCherry	Yes	Gi- DREADD
pAAV-hSyn-hM3D(Gq)-mCherry	hSyn (neuron specific expression)	mCherry	No	Gq-DREADD
pAAV-hSyn-hM4D(Gi)-mCherry	hSyn(neuron specific expression)	mCherry	No	Gi- DREADD
pAAV-CaMKII-hM3D(Gq)-mCherry	CaMKII (neuron specific expression)	mCherry	No	Gq-DREADD
pAAV-CaMKII-hM4D(Gi)-mCherry	CaMKII (neuron specific expression)	mCherry	No	Gi- DREADD

Table 5. List of autophagy flux monitor tools with AAV

Type	AAV Plasmids	Promoter and Expression Characteristics	Fluorescent Label
Double label	pAAV-mRFP-GFP-LC3	CMV general and strong expression	mRFP+GFP
Single label	pAAV-GFP-LC3	CMV general and strong expression	GFP

Overall Experiment Procedure of AAV Production

A schematic overview of recombinant AAV production is shown in Figure 2. The first step is to clone the gene of interest (GOI) into an appropriate plasmid vector. For most applications, the cDNA of interest is cloned into one of the ITR/MCS containing vectors (table 2). The inverted terminal repeat (ITR) sequences present in these vectors provide all of the cis-acting elements necessary for AAV replication and packaging.

The recombinant expression plasmid is co-transfected into the AAV-293 cells with pHelper (carrying adenovirus-derived genes) and pAAV-RC (carrying AAV2 replication and capsid genes), which together supply all of the trans-acting factors required for AAV replication and packaging in the AAV-293 cells. Recombinant AAV viral particles are prepared from infected AAV-293 cells and may then be used to infect a variety of mammalian cells.

Upon infection of the host cell, the single-stranded virus must be converted into double-stranded in order for gene expression. The AAV virus relies on cellular replication factors for synthesis of the complementary strand. This conversion is a limiting step in recombinant gene expression and can be accelerated using adenovirus superinfection or etoposides, such as camptothecin or sodium butyrate. Whereas these agents are toxic to the target cells and can kill target cells if left on the cells, so the use of etoposides is only recommended for short-term use or in obtaining viral titers. Typically, the AAV genome will form high molecular weight concatemers which are responsible for stable gene expression in cells.

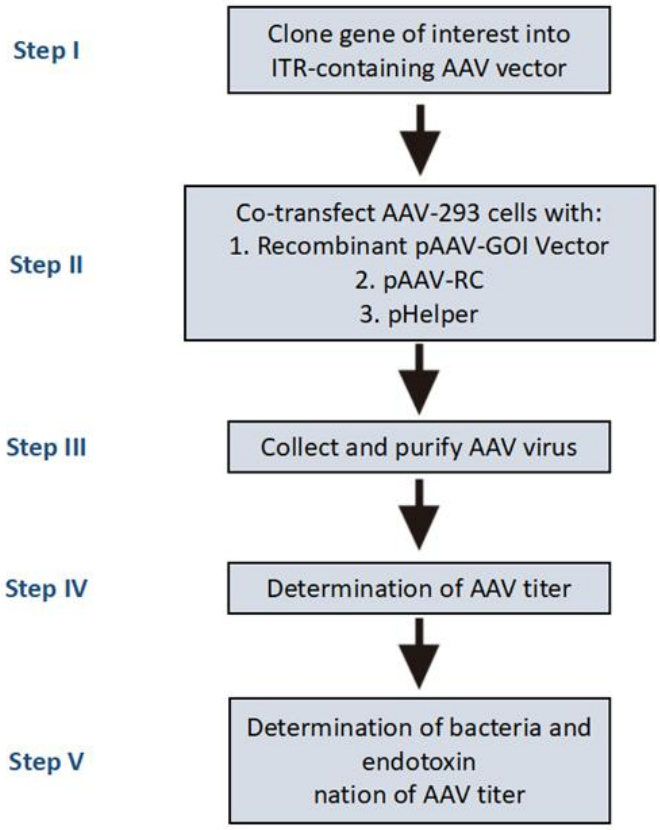

Figure 2. AAV packaging experiment flow chart.

Experimental Materials

AAV system contains plasmid with gene of interest (GOI), i.e. pAAV-GOI, packaging plasmid pAAV-RC and pHelper.

• The information of pAAV-GOI can be referred in table 2-5, while the plasmid map profiles can be consulted from www.genemedi.net.

• Bacterium strain: Escherichia coli strain Stbl3 is used to amplify AAV vectors and packaging plasmids.

• Cell line: AAV packaging cell AAV-293; Medium: DMEM with penicillin-streptomycin and 10% FBS. To ensure stability and continuity of experiment, it is best to freeze enough AAV-293 cells at logarithmic phase as backup cell bank.

Notices:

If the cell line is contaminated by mycoplasma, to reach a better cultured cell state, we recommend the use of Genemedi anti-mycoplasma reagent CurePlasma™.

Vector Construction of AAV

Before AAV packaging, gene of interest should be constructed into the vector of ITR-containing plasmids as listed in table 2. Genemedi also provides various AAV vectors with alternative promoters and fluorescent labels. Customization of the vector with tissue specific promoter or conditional dependent regulatory element (e.g cre-dependent FLEX/DIO, tet-on) is also feasible. At the same time, Genemedi has plenty of premade AAV vector goods carrying some genetic tools in stock, such as optogenetics, DRADDs and LC3-indicated autophagy flux detection, etc. (for more details, refer in table 2-5).

Note:

In order to construct vectors quickly and efficiently, it is strongly recommended to use Genemedi - ClonEasy™ One Step Cloning Kit（Cat. GM-GC-01/02/03.

Packaging of AAV

Propagate AAV-293 cells in DMEM with 10% FBS and 1% pen/strep. The day before transfection, plate the cells in a 10cm dish such that the cells reach 70-80% confluency the next day. On the day of transfection, set up the 3-plasmid co-transfection as table 6.

Table 6. Plasmid and transfection reagent required for transfection of a standard 10cm Dish in AAV Production.

Component	Amount
pAAV-RC	10 μg
pHelper	20 μg
pAAV-GOI	10 μg
LipoGene™	100 μl

DMEM needs to be preheated to 37℃ with water bath. LipoGene™ transfection reagent needs to be warmed up to room temperature before use, and mix gently before use. Replace the transfection medium of 10cm dish with fresh medium 6 hours post transfection.

Note:

1. LipoGene™ transfection reagent is from Genmedi, please refer to LipoGene™ manual during transfection.

2. Before transfection, the cells should be in a good state.

3. Please equip with disposable gloves and conduct in BL-2 level.

Collection and Purification of AAV

Around 72 hours after transfection, harvest the cells from the 10cm plate with a cell scraper.

Spin the cells at 1,500g for 5 minutes to collect the cell pellet. Resuspend the cell pellet in 0.5ml lysis buffer (10 mM Tris-HCl (pH8.5), 150 mM NaCl). Freeze/thaw the cell pellet 3 times through a dry ice/ethanol bath and a 37°C water bath to obtain the crude lysate.

Spin down the crude lysate at 3,000g for 10 minutes. Collect the supernatant fraction, which contains harvested rAAV. Keep the virus in -80℃.

Unless otherwise specified, all AAV viruses are purified with iodixanol gradient ultracentrifugation at 350,000g for 90 min in a T70i rotor at 10℃ to separate out contaminants from the impure AAV preparations. The 15% iodixanol step help destabilize ionic interactions between macromolecules with addition of 1M NaCl. The 40% and 25% steps are used to remove contaminants with lower densities, including empty capsids. The 60% step acts as a cushion for genome-containing virions. The prep with full (genome containing) particles will be enriched after several steps of gradient ultracentrifugation.

AAV capsid contains VR1 82kDa, VR2 72kDa and VR3 62kDa, which can be detected using the method of polyacrylamide gel electrophoresis (PAGE) followed by silver staining or Coomassie blue staining. Pure AAV should display only three major protein bands, such as the following virus purified in Genemedi shown in Fig 3. We guarantee the purity of the AAV virus based on the specification that we give for different services.

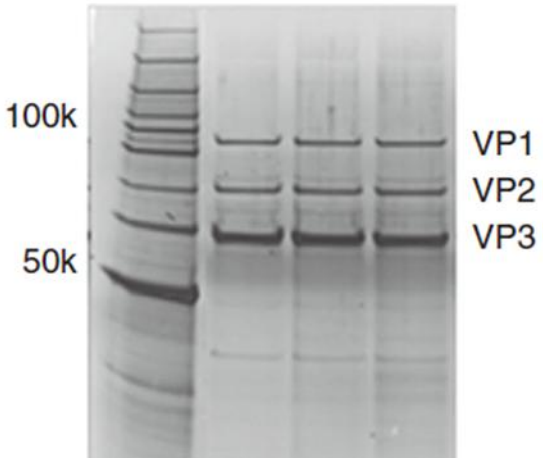

Figure 3. Purity of purified AAV virus.

Titer Detection of AAV

AAV viruses are titered by real-time quantitative PCR using primers targeted the ITR. The amplicons are detected using SYBR green technology. Titer values are then determined by comparison to a standard curve of a plasmid sample of known concentration. An example of the Ct value of standard sample and sample to be tested is shown in table 7, while the standard curve is displayed in figure 4.

Table 7. Ct value of standard and sample in a typical absolute quantification process.

Standard	Copy Number (vg/ml)	Ct value 1	Ct value 2	Ct value 3
Standard_1	10^{10}	4.51	4.32	4.42
Standard_2	10^{9}	7.21	7.61	7.25
Standard_3	10^{8}	10.97	10.55	10.67
Standard_4	10^{7}	14.19	14.38	14.35
Standard_5	10^{6}	17.75	17.58	17.68
AAV Sample		12.78	12.65	12.81

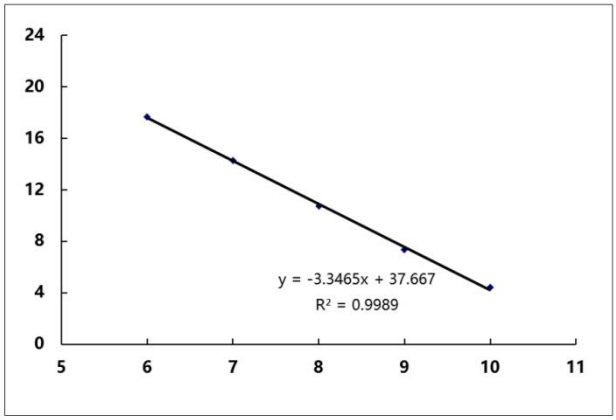

Figure 4. Standard curve in absolute quantification.

Set the average Ct value of each group as Y-axis, the logarithm of corresponding group as X-axis. Substitute the average Ct value of samples to be tested into formula, obtaining X = 7.45. Substitute the AAV virus titer calculation formula: $10^{x} \times 40000$ vg/ml= $10^{7.45} \times 40000 = 1.1 \times 10^{12}$ vg/ml.

Cell Infection Test of AAV

After AAV titer detection, the infection activity needs to be evaluated before animal experiments. Test the expression of target gene by infecting cells, such as 293T, CHO. MOI will be controlled ranging from 10^{4} to 10^{5} (MOI is multiplicity of infection, namely the number of virus particle needed for infecting a cell).

Recommended protocol for *in vitro* cell transduction:

1. Thaw the AAV virus on ice.

2. Start cell transduction at MOI of 10^4 and 10^6 vg/cell when cells are in a good state and ready for transduction.

3. Incubate cells with the virus-containing media for at least 6-12 hours. Exchanging the virus-containing media during infection is not necessary until 12 hours later. It may take 3-7 days for expression of target genes.

4. If the packaged AAV encoding a fluorescent label protein, the efficiency of viral infection can be observed with a fluorescence microscope, 24h, 48h, 72h and 96h after transduction.

Note:

AAV infection is cell type and serotype dependent, and the transduction efficiency of some commonly used cell lines are listed in table 8. Generally, AAV has a weak ability to infect cell lines compared to lentivirus and adenovirus, so it is not recommended for *in vitro* experiments.

Table 8. Transduction efficiency comparison among different AAV serotypes.

Cell Line	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV8	AAV9	AAV-DJ	AAV-DJ/8
Huh-7	13	100	2.5	0	0.1	10	0.7	0	500	0.2
HEK293	25	100	2.5	0.1	0.1	5	0.7	0.1	500	0.3
Hela	3	100	2	0.1	6.7	1	0.2	0.1	667	0.2
HepG2	3	100	16.7	0.3	1.7	5	0.3	ND	1250	0.5
Hep1A	20	100	0.2	1	0.1	1	0.2	0	400	0.1
911	17	100	11	0.2	0.1	17	0.1	ND	500	0
CHO	100	100	14	1.4	333	50	10	1	25000	5
COS	33	100	33	3.3	5	14	2	0.5	500	0.3
MeWo	10	100	20	0.3	6.7	10	1	0.2	2857	1
NIH3T3	10	100	2.9	2.9	0.3	10	0.3	ND	500	0.1
A549	14	100	20	ND	0.5	10	0.5	0.1	1000	0.1
HT1180	20	100	10	0.1	0.3	33	0.5	0.1	333	0.2
Monocytes	1111	100	ND	ND	125	1429	ND	ND	100	ND
Immature DC	2500	100	ND	ND	222	2857	ND	ND	200	ND
Mature DC	2222	100	ND	ND	333	3333	ND	ND	100	ND

Animal Experiment with AAV

For normal tissues or organs, such as heart, liver, kidney, breast, pancreas, ovary, brain, eye, skeleton muscle, adipose tissue, etc., Genemedi systematically organizes the corresponding optimal AAV serotypes, gene delivery methods and injection volume for mouse and rat tissue infection, which are listed in table 9.

Table 9. AAV gene delivery methods in specific organs.

Infection organ	Recommended serotype	Injection route	Animal	Injection volume (μl)
Heart	AAV9	Multiple points in situ	Rat	10-15/point, 3-5 points
			Mouse	10-15/point, 3-5 points
		Tail vein	Rat	250 (200g body weight)
			Mouse	100
Liver	AAV8 or AAV9	Tail vein	Rat	200 (200g body weight)
			Mouse	100
Whole brain	AAV-PHP.eB	Tail vein	Rat	200 (200g body weight)
			Mouse	100
Lateral ventricle	AAV9	Stereotactic	Rat	1-5
			Mouse	1-5
Brain tissue	AAV9	Stereotactic	Rat	2-3
			Mouse	1-2
Fat	AAV9	Intraperitoneal - intraperitoneal fat	Rat	300
			Mouse	150-200
		in situ injection - subcutaneous fat	Rat	10-15/point, 3-5 points
			Mouse	10-15/point, 3-5 points
Skeletal muscle	AAV1 or AAV9	In situ injection	Rat	10-15/point, 3-5 points
			Mouse	10-15/point, 3-5 points
Eyes	AAV2, AAV10 or AAV-DJ	vitreous chamber injection	Rat	3-5
			Mouse	1-3
		subretinal space injection	Rat	1-2
			Mouse	1-2
Lung	AAV6	Intratracheal injection	Rat	100 (200g weight)
			Mouse	50-75
Kidney	AAV2 or AAV9	Renal pelvis injection	Rat	10-15/point, 3-5 points
			Mouse	10-15/point, 3-5 points
Intestine	AAV1 or AAV5	Enema	Rat	200 (200g weight)
			Mouse	100

As an advanced and safe tool for gene delivery *in vivo*, AAV has been proved as the most excellent gene therapy vector. How to perform virus injection in specific organs? Four kinds of for AAV gene delivery methods are fully described as follows.

Tail Vein Injection

Infection sites: Heart, liver and whole brain (AAV-PHP.eB)

1. Put mouse into the device for tail vein injection (If no such device, it can be retrofitted with 50ml centrifuge tube. The whole device should be ventilated and fixed).

2. Disinfect the tail of mouse with 70% alcohol. Be careful to keep the tail warm, the blood vessels will contract if it's too cold.

3. Hold the mouse's tail with the thumb of the left forefinger, straighten it gently, and then move the forefinger forward, and bend the tail slightly. You can insert needle in the bending place and inject AAV (27-30G insulin needle, 100μl/mouse).

4. Inject slowly, hold for 5-10 seconds to prevent virus from flowing back.

5. Pull out the needle, then press the injection site with fingers or dry aseptic cotton balls or gauze for a few seconds.

Figure 5. Diagram of tail vein injection.

Hepatic Portal Vein Injection (local delivery of AAV in liver)

Infection sites: liver

1. Inject Xylazin/Ketamine mixture (10mg/kg, 100mg/kg) (Xylazin/Ketamine, Sigma-Aldrich X1251, K2753) subcutaneous to anesthetize mouse.

2. Apply ointment on mouse's eyes (Oculentum simplex, Teva Pharmachemie) to prevent eyes from drying.

3. Adjust the mouse's abdomen to make it upward, shave the hair between the second ribs and the area between the fourth nipples. This step can be operated 1 days ahead without anesthesia.

4. Disinfect the depilation site repeatedly. Then cut 3cm from the ribs to the area of the fourth nipple with a scalpel. Be careful not to hurt other tissues such as mammary glands, intestines, liver and so on.

5. Carefully pull out the internal organs such as the large intestine and small intestine with a sterile cotton swab. And gently cover them with gauze to avoid touching, until the hepatic portal vein can be seen.

6. Suck the virus into a 32g syringe in advance, insert the needle slowly into the hepatic portal vein at an angle of less than 5 degrees and at a distance of about 1 cm below the liver. Insert 3-5 mm along the hepatic portal vein (if it is difficult to see the portal vein, it can be identified by anatomical mirror).

7. Inject the virus slowly. After the injection is completed, stay for 3-5s and then dispense the needle, and gently press it with a cotton swab for a while. Then cover the hepatic portal vein with hemostatic cotton, and gently press with a cotton swab for 5 minutes to help stop bleeding. Make sure hemostasis is done, otherwise continue to press the wound.

8. Remove the hemostatic cotton (If the tissue is tightly attached to the hemostatic cotton, it can be moistened with sterile PBS).

9. Gently put the organ back into the belly of the mouse. Suture the wound using a 4-0 line, approximately 10-15 needles.

10. Inject 100μl of bupivacaine (5mg/ml) to the wound site to relieve pain. Hydration was performed by subcutaneous injection of 500μl of sterile PBS with a 26G insulin needle. The entire operation took about 30 minutes.

11. It is necessary to keep the body of the mouse warm enough (such as a 37-degree heating lamp, a thermostat, etc.) to facilitate recovery when the experiment is completed.

Brain Stereotactic Positioning

Infection site: brain

1. Inject Xylazin/Ketamine mixture (10mg/kg, 100mg/kg) (Xylazin/Ketamine, Sigma-Aldrich X1251, K2753) subcutaneous to anesthetize mouse.

2. Shave the hair between the mouse's head and the ear, then place it on the warm tray of the stereo positioner.

3. Zero the ruler between teeth and ears. Apply ointment on mouse's eyes (Oculentum simplex, Teva Pharmachemie) to prevent eyes from drying.

4. Fix the upper incisors of the mouse into the slots of the fixed plate to ensure that the head remains fixed. Adjust Bregma and Lambma on the same sagittal line and horizontal plane. Make sure the mouse's nose is centered and stable.

5. Use a scalpel to peel off the skin of the mouse's head and scrape off the surface with a scalpel. If blood flows out, please dry it with a cotton swab to ensure that the position of the bregma and lambda is clearly visible (plus schematic).

6. Zero the bregma coordinates (X, Y, Z = 0), ensuring that the lambda position is on the same level as the bregma.

7. Set the coordinates according to the target brain area. Once the coordinates are fixed, mark the skull, a hole of

about 0.015 mm is drilled at the mark within 10 seconds. Be careful to protect brain tissue from being damaged.

8. Suck the AAV into the syringe and insert according to the coordinates set before, then the virus is slowly injected at a rate of 0.05-0.1μl/min. Typically, each mouse is injected at a volume between 0.05μl and 2μl.
9. Keep it for 10 minutes, when the injection is completed, and then return the needle slowly.

10. If blood is flowing out during the operation, use a cotton swab to dry the blood in time.

11. The last step is to suture the scalp. It is necessary to keep the body of the mouse warm enough (such as a 37-degree heating lamp, a thermostat, etc.) to facilitate recovery after the experiment.

Intrathecal Injection

Infection site: spinal nerve

1. Inject Xylazin/Ketamine mixture (10mg/kg, 100mg/kg) (Xylazin/Ketamine, Sigma-Aldrich X1251, K2753) subcutaneous to anesthetize mouse.

2. Apply ointment on mouse's eyes (Oculentum simplex, Teva Pharmachemie) to prevent eyes from drying.

3. Remove the hair at about $2cm^2$ of the skin near the tail, in order to see where the needle is inserted.

4. Put the mouse on a special rack and place a 15ml centrifuge tube in the lower part of the body to arch the needle insertion site slightly.

5. Suck the virus into 25μl Hamilton with 30G needles.

6. Position the L6 (the most prominent one) of the spine and gently press it with your fingers to make it smooth.

7. Carefully insert the needle into the groove between the L5 vertebrae and L6 vertebra. Note that the tail of the mouse is slightly upturned, which indicates that the needle is successfully inserted into the myelin sheath.

8. Once successfully inserted, secure the needle with one hand and slowly inject 5-10μl of virus with the other hand. Note that the injection volume should be appropriate, small volume will increase the experimental error and a large volume will increase the intrathecal pressure.

9. If necessary, repeat the injection after 24h.

10. After the experiment, it is necessary to keep the body of the mouse warm enough (such as a 37-degree heating lamp, a thermostat, etc.) to facilitate recovery.

11. As previously reported, it will be better to inject 8 points with 1μl per point. Choose two cross-sections at different depths, and 4 points on a cross-sectional line. The schematic is shown in Figure 6.

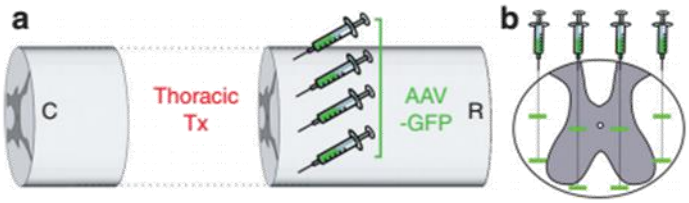

Figure 6. The schematic of multiple points in situ injection.

In addition, Genemedi also organizes the injection video of normal tissues and organs, welcome to enquiry.

Usually, it will take at least 3 weeks before the target gene can be detected *in vivo* by frozen section, paraffin section, qPCR or WB since virus injection. In general, for tissues in situ injection, if AAV is labeled with GFP or RFP, the efficiency of viral infection can be observed with a fluorescence microscope at the injection site since 3 to 4 weeks after injection.

Case sharing

Case 1. AAV Liver Transduction

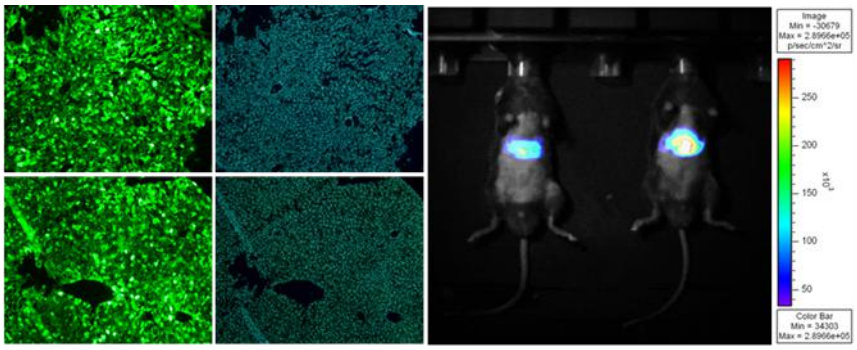

Figure 7. Selected transduction results of AAV9-GFP in mouse liver.

Virus and titer: AAV-pTBG-Luc, 1.4×10^{12} vg/ml
Animal: mouse, C57, 2 months
Gene delivery method: tail vein, 100μl
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy, *in vivo* imaging
Conclusion: intravenous injection of AAV-pTBG-Luc only infects liver cells and no non-specific infection

Case 2. AAV Heart Transduction

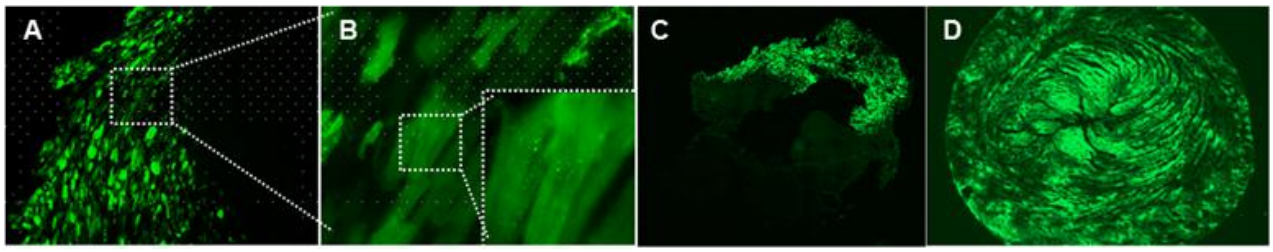

Figure 8. Selected transduction results of AAV9-GFP in rat heart (A, B, C) and mouse heart (D).

Virus and titer: AAV9-GFP, 1×10^{12} vg/ml
Animal: Rat, SD, 2 months (Figure 8A, B, C); mouse, C57, 8 months (Figure 8D)
Infection site: heart
Gene delivery method: myocardial in situ injection, 10μl/site, 5 sites in total (Figure 8A, B, C);
intraorbital intravenous injection (Figure 8D)
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 3. AAV Lung Transduction

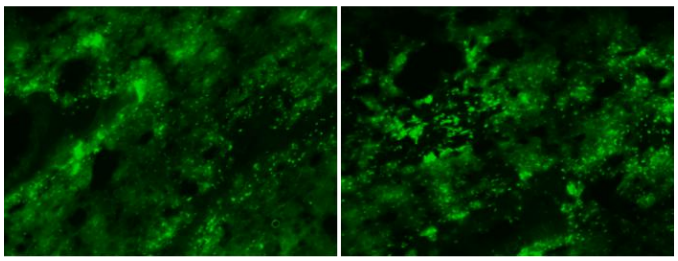

Figure 9. Selected transduction results of AAV9-GFP in mouse lung.

Virus and titer: AAV9-GFP, 1×10^{12} vg/ml
Animal: Rat, SD, 2 months
Infection site: Lung
Gene delivery method: Trachea injection, 10μl/site, 5 sites in total
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 4. AAV Brain Transduction

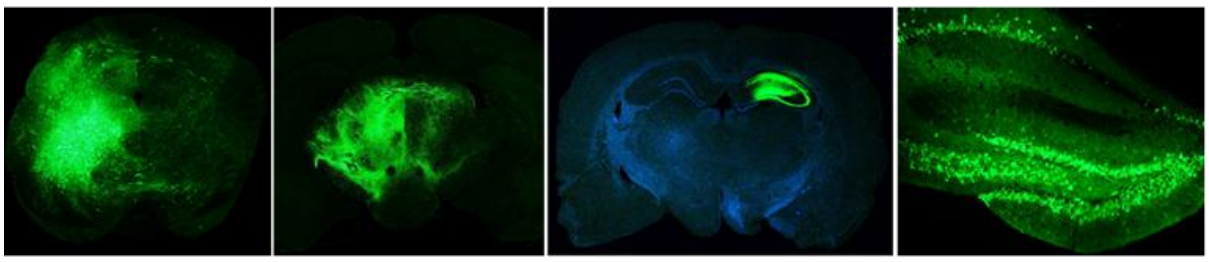

Figure 10. Selected transduction results of AAV9-GFP in mouse brain.

Virus and titer: AAV9-GFP, 1×10^{12} vg/ml

Animal: Mouse, C57, 2 months
Infection site: Brain
Gene delivery method: Brain localization injection, 1μl
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 5. AAV Hippocampus Transduction

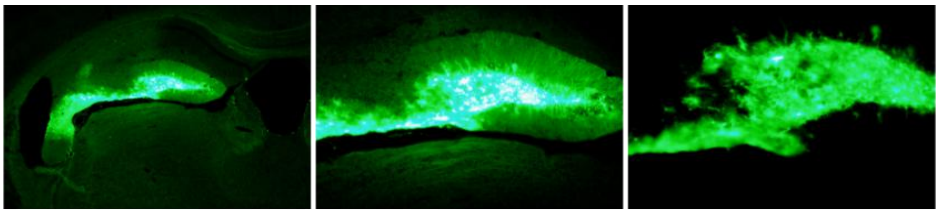

Figure 11. Selected transduction results of AAV2-GFP in mouse hippocampus.

Virus and titer: AAV9-GFAP-GFP, 1×10^{12} vg/ml
Animal: mouse, C57, 2 months
Infection site: Hippocampus
Gene delivery method: Brain localization injection, 1μl
Determine assay: 3 weeks post infection, wholemount, immunofluorescence microscopy

Case 6. AAV Retina Transduction

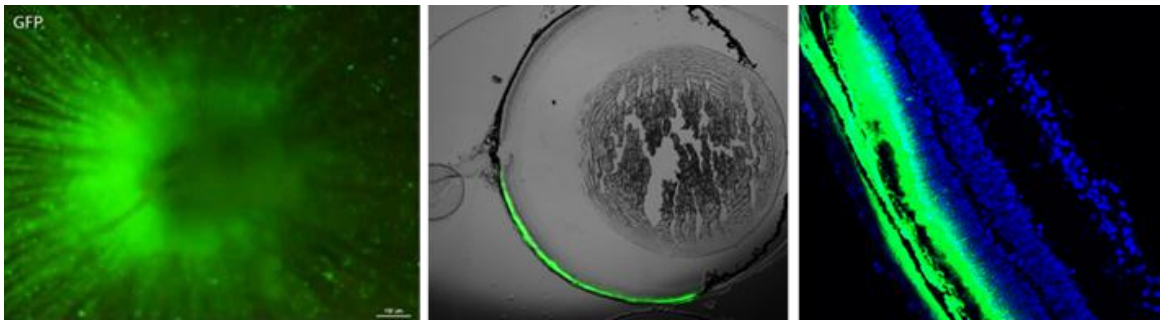

Figure 12. Selected transduction results of AAV2-GFP in mouse retina.

Virus and titer: AAV2-GFP, 1×10^{12} vg/ml
Animal: mouse, C57, 2 months
Infection site: retina
Gene delivery method: subretinal cavity injection, 3μl
Determine assay: 3 weeks post infection, wholemount, immunofluorescence microscopy

Case 7. AAV Skeletal Muscle Transduction

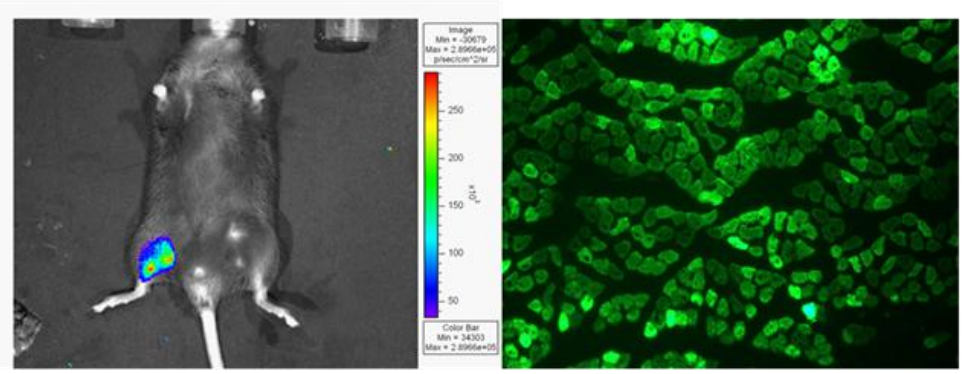

Figure 13. Selected transduction results of AAV2-GFP in mouse skeletal muscle.

Virus and titer: AAV9-Luc, AAV9-GFP, 1×10^{12} vg/ml
Animal: Mouse, C57, 2 months
Infection site: Skeletal muscles
Gene delivery method: Muscle in situ injection, 10μl/site, 4 sites in total
Determine assay: 4 weeks post infection, *in vivo* imaging, frozen section, immunofluorescence microscopy

Case 8. AAV Kidney Transduction

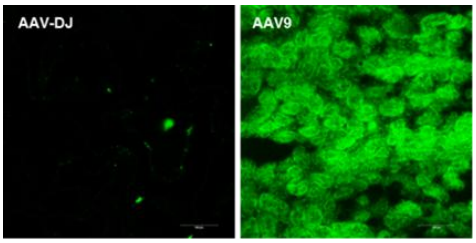

Figure 14. Selected transduction results of AAV2-GFP in mouse muscle.

Virus and titer: AAV9-GFP, DJ-GFP, 1×10^{12} vg/ml
Animal: mouse, C57, 2 months
Infection site: Kidney
Gene delivery method: Multiple sites injection in kidney 10μl/site, 6 sites in total
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 9. AAV Breast Transduction

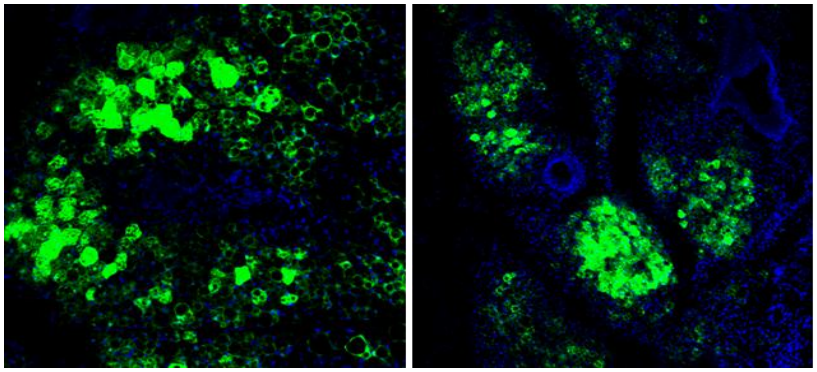

Figure 15. Selected transduction results of AAV2-GFP in mouse muscle.

Virus and titer: AAV9-GFP, 1×10^{12} vg/ml
Animal: mouse, C57, 2 months
Infection site: Mammary fat pad
Gene delivery method: Breast injection, 10μl/site, 4 sites in total
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 10. AAV Tumor Transduction

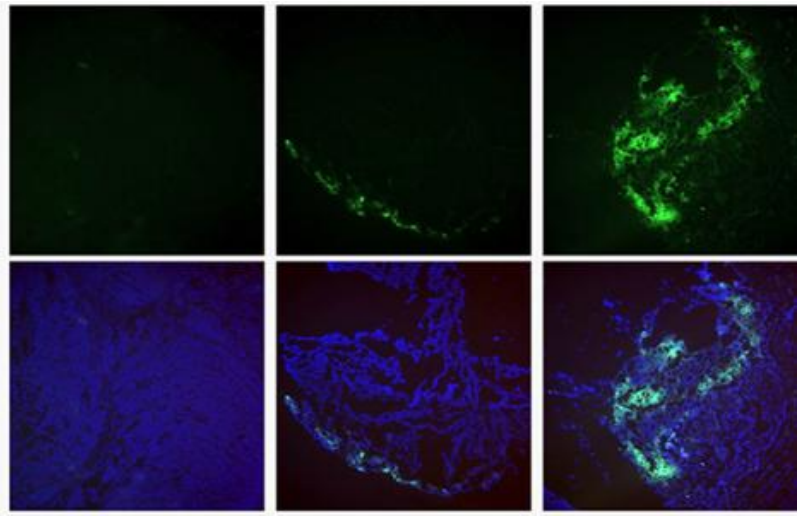

Figure 16. Selected transduction results of AAV-DJ/9-GFP in tumor.

Virus and titer: AAV-DJ/9-GFP, 1×10^{12} vg/ml
Animal: Nude mouse, 2 months
Infection site: Subcutaneous transplant of bowel cancer cells
Gene delivery method: Tumor injection, 10μl/site, 4 sites in total
Determine assay: 3 weeks post infection, frozen section, immunofluorescence microscopy

Case 11. AAV Transduction *in vitro*

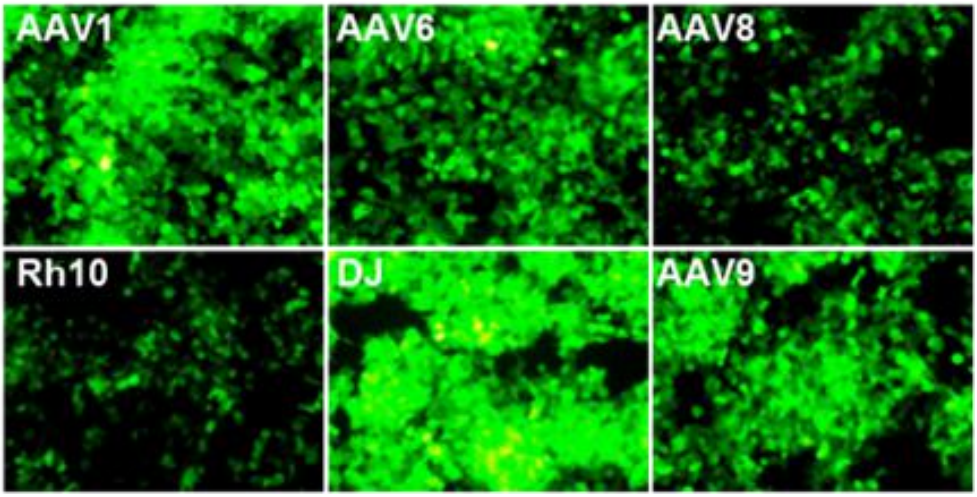

Figure 17. Selected transduction results of AAV *in vitro*

Virus and titer: AAV1/6/8/Rh10/DJ/9-GFP, 1×10^{12} vg/ml
Cells: HEK-293T
MOI: MOI=1×10^{4}
Determine assay: 36 hours post infection, immunofluorescence microscopy

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