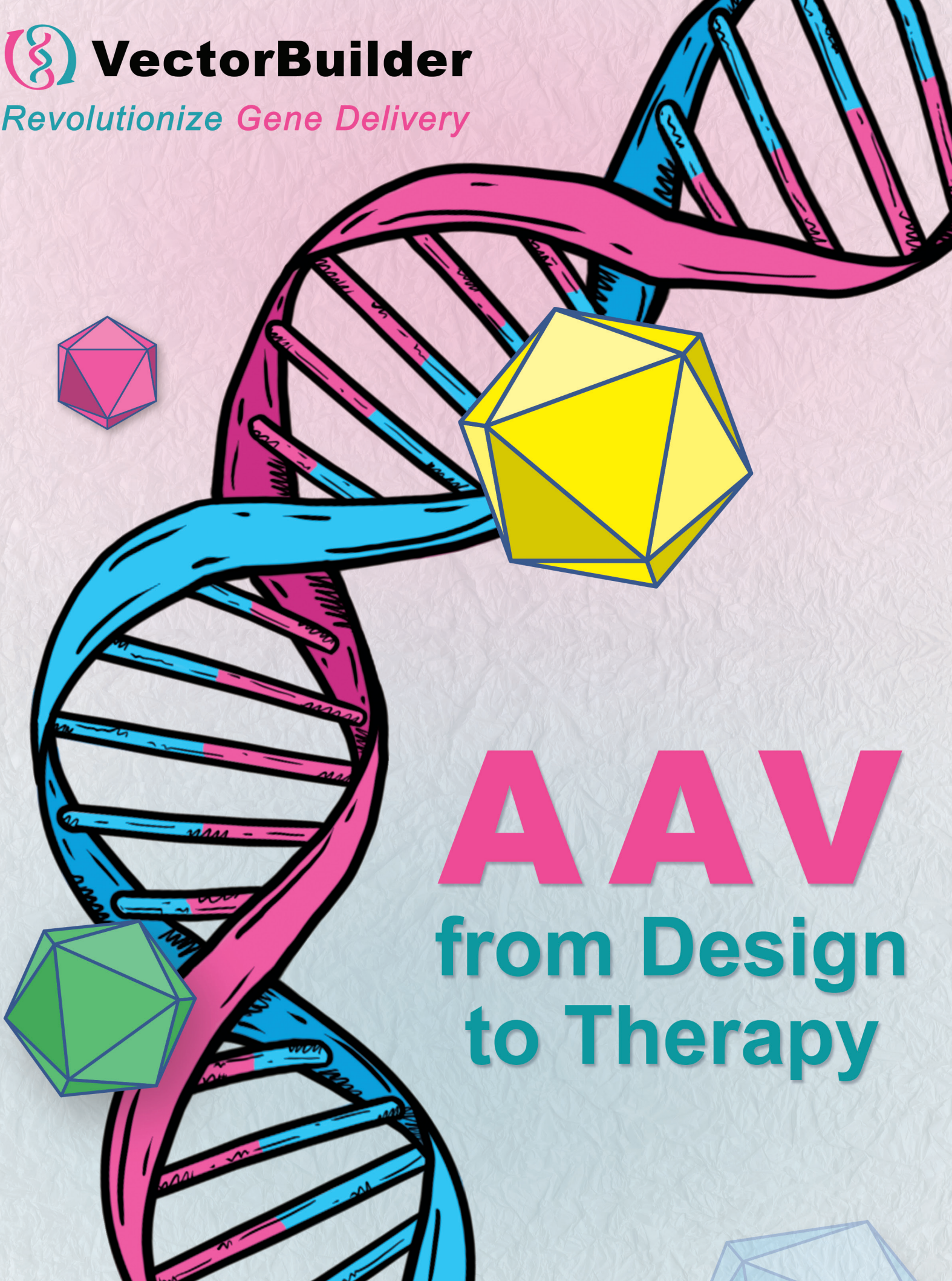
VectorBuilder

Revolutionize Gene Delivery

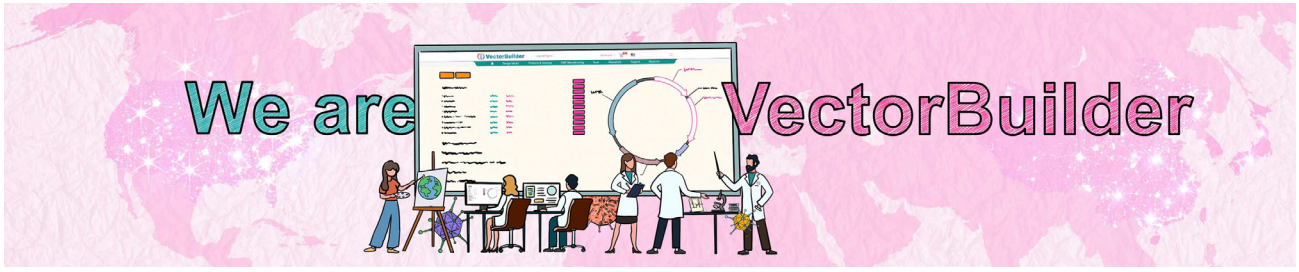


AAV

from Design to Therapy

Overview

VectorBuilder is a global leader in gene delivery technologies. As a trusted partner for thousands of labs and biotech/pharma companies across the globe, VectorBuilder offers a full spectrum of gene delivery solutions covering virtually all research and clinical needs from bench to bedside.



Offerings

Custom vectors & viruses

VectorBuilder is the world's largest provider of custom vectors for both viral and non-viral gene delivery. We currently tailor-build over 80,000 vectors a year for tens of thousands of researchers around the world. Our vector services boast several important advantages:

- **Online vector design and ordering platform:** This platform is a transformative innovation by VectorBuilder that allows researchers to design and order custom vectors online, freeing them from the tedious DIY work of cloning vectors and packaging viruses in the lab. This award-winning platform has a free web-based Vector Design Studio that employs a highly user-friendly, modular design principle. It enables even novice researchers to easily design custom vectors with just a few mouse clicks and then order their vectors online along with related services such as virus packaging and plasmid preparation.

- **Comprehensive vector systems:** VectorBuilder offers a broad range of vector systems for both viral and non-viral gene delivery, such as AAV, adenovirus (including gutless), lentivirus, MMLV, baculovirus, VSV, HSV, PiggyBac, Sleeping Beauty, etc. These vectors cover virtually all model organisms and include wide-ranging applications, such as overexpression, shRNA knockdown, CRISPR, recombinant protein expression, and in vitro transcription. Many vectors are specifically designed for clinical applications such as antibody production, CAR-T, gene therapy, mRNA vaccines, and oncolytic viral treatment.

- **Vast vector inventory:** VectorBuilder has an extensive collection of >1,100 vector backbones corresponding to a wide range of viral and non-viral vector systems. We have also amassed >400,000 in-stock vector components including ORFs, shRNAs, and gRNAs corresponding to most known genes in popular model organisms, as well as commonly used promoters, reporters, linkers, and epitope tags. Over 80% of the custom vectors we build nowadays are readily assembled from existing backbones and components, significantly reducing cost and turnaround time.

CRO services

Besides custom vectors and viruses, VectorBuilder offers a wide range of CRO services covering many more gene delivery applications in basic research and drug discovery:

- **Diverse gene delivery solutions:** We offer a comprehensive collection of premade shRNA and gRNA libraries for multiple species, as well as custom library construction and in vitro and in vivo library screening services. We also provide stable cell line generation, BAC recombineering, mRNA synthesis/encapsulation, and reverse genetics rescue of novel viruses. Additionally, we have several COVID-related offerings such as spike (S) protein pseudotyping of lentivirus and VSV.

- **Preclinical vector testing:** VectorBuilder offers preclinical non-GLP and GLP testing of vector efficacy and safety profiles, including biodistribution, ADME/PK/PD, and toxicology. These services can be performed in a variety of in vitro and in vivo systems including non-human primate (NHP) models.

- **High value-added R&D:** We recently launched several high value-added R&D services aimed at developing new delivery strategies and optimizing vector performance for tailored clinical applications, such as AAV capsid evolution, viral G protein evolution, promoter engineering and screening, and experimental codon optimization for mRNA and viral vectors. These studies can be carried out in NHPs to maximize their translatability to human use.

CDMO services

VectorBuilder is a full-service CDMO with extensive experience in cGMP vector manufacturing. Operating several state-of-the-art facilities, we have supported many customers along their entire drug-discovery pipelines, going from research-grade vectors for early discovery, to GMP-like vectors for preclinical testing, to full GMP-grade vectors for clinical trials. We have provided IND-enabling vectors to a worldwide client base in the US, Europe, Japan, China, and South Korea. Our CDMO services include process development, analytical development, cell banking, fill/finish, and regulatory support. Our GMP manufacturing covers the following systems:

- **Plasmid DNA:** VectorBuilder offers GMP-grade plasmid DNA with up to 10 g scale.

- **Viral vectors:** We offer GMP manufacturing for multiple viral vector systems including AAV, lentivirus, MMLV, adenovirus, HSV, etc. Either adherent or suspension packaging systems can be used with up to 1,500 L scale.

- **mRNA:** VectorBuilder's mRNA CDMO services include sequence engineering and optimization, DNA template construction, mRNA in vitro transcription (using modified nucleotides when appropriate), and LNP encapsulation.

IP out-licensing

With strong R&D capabilities, VectorBuilder has built an extensive IP portfolio of gene delivery technologies, some of which can be out-licensed. These include new AAV serotypes, novel viral G proteins, engineered tissue-specific promoters, high-yield recombinant protein producer cell lines, etc.

Mission

VectorBuilder is the future of gene delivery. Our mission is to provide end-to-end solutions for all gene delivery needs spanning both research and therapy.

We strive to offer innovative and high-quality products and services while maintaining rapid turnaround and exceptional affordability. Our “white-glove” customer care is supported by a PhD-level team with decades of collective experience to devise the best gene delivery solutions for our customers. As a result, we have received glowing customer testimonials and numerous citations in high-impact publications.

Operating as a multinational company with branches in North America, Europe, China, Japan, South Korea, Australia, and Israel, VectorBuilder has served over 50,000 customers from several thousand organizations worldwide, including universities, research institutions, biotech/pharma companies, and governmental agencies.

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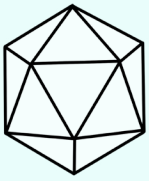
AAV Basic Biology

Adeno-associated virus (AAV) is a member of Dependoparvovirus genus and belongs to the viral family Parvoviridae. The AAV virion is a nonenveloped and relatively small (20 um) capsid, which has an icosahedral structure and contains a single stranded DNA genome. Due to its capability to infect dividing and non-dividing cells, AAV infection is common in humans and other primates but has not been reported to be pathogenic. When AAV enters host cells, it can lead to long term expression of a transgene with very low immunogenicity (unlike wildtype AAV, in host cells the viral genome of recombinant AAV exists in the form of episomal DNA). Due to all these characteristics, AAV is an increasingly popular viral vector for applications in gene therapy.



Adeno-associated virus

The AAV Genome

The genome of AAV is a positive or negative-sense single stranded DNA around 4.7 kb in length. Each AAV viral genome contains two Inverted Terminal Repeats (ITRs) at its ends that are essential to virus replication, capsid assembly, and the integration of viral DNA into the host cell's chromosome. AAV viral DNA contains two open reading frames: Rep and Cap. The Rep ORF is composed of four overlapping genes: Rep78, Rep68, Rep52, and Rep40. The Cap ORF encodes three overlapping genes that are required for capsid assembly: VP1, VP2, and VP3. The replication of the AAV genome requires a DNA polymerase provided by the host cell or a helper virus, since AAV itself lacks a viral DNA polymerase gene.

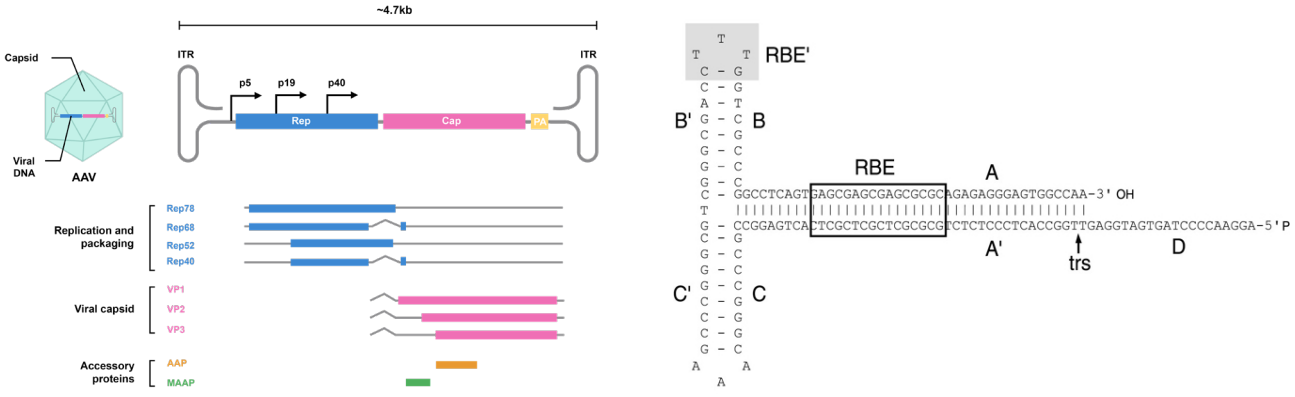


Figure 1. Wildtype AAV genome (Left) and the structure of an ITR (Right) (adapted from BioRender, 2022 & Gonçalves MA, 2005).

AAV Life Cycle

Wild-type adeno-associated virus requires a helper virus to complete its life cycle. After adeno-associated virus infects cells, it requires a helper virus, such as adenovirus or HSV, to enter the lysogenic cycle for virus replication. In the absence of a helper virus, the expression of adeno-associated virus genes is limited. Following entry into the lysogenic cycle, a part of the adeno-associated virus genome can be integrated into the AAVS1 site of human chromosome 19 through a mechanism involving the ITRs and Rep protein (Rep78). Recombinant AAV commonly used in research does not contain Rep gene thus, the transduced AAV genome exists in cells in the form of a DNA free body, which is conducive to long-term expression of the transgene in cells.

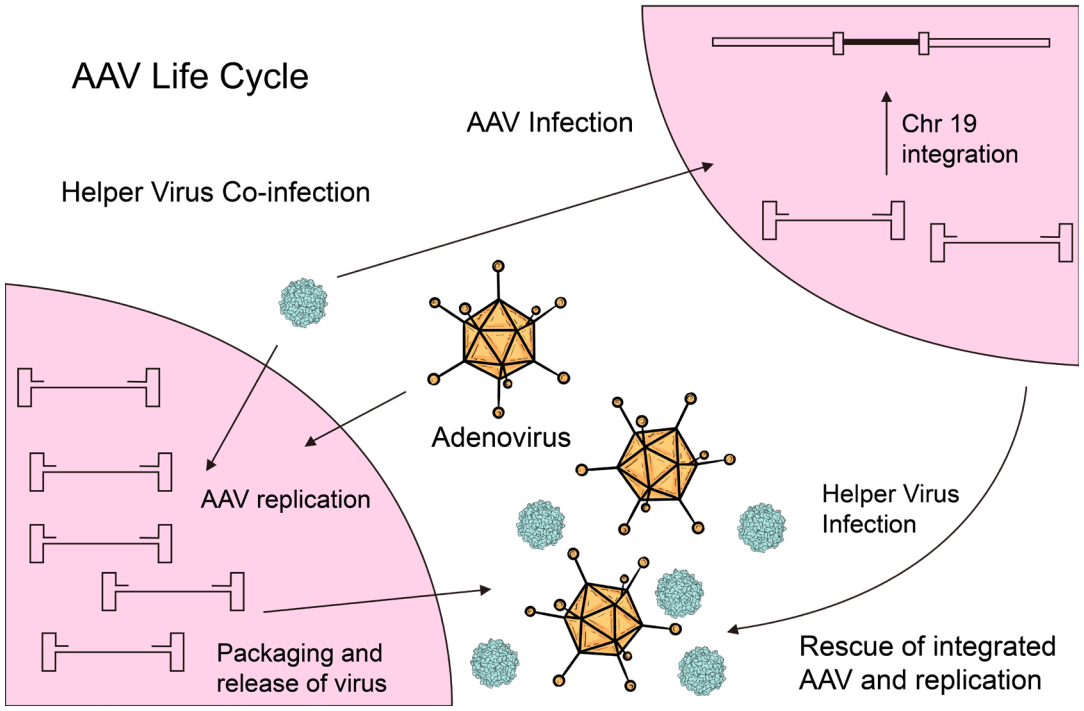
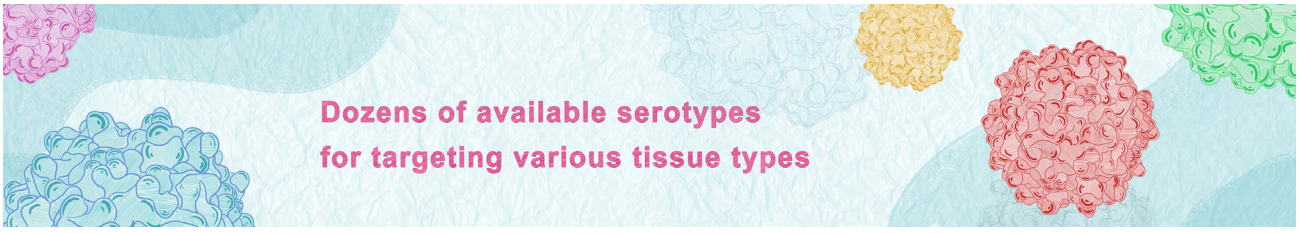


Figure 2. The life cycle of wildtype AAV virus (adapted from Daya S & Berns KI, 2008)

Serotype and Tissue Tropism

Adeno-associated virus is common in humans and other primates. At present, hundreds of AAV variants and serotypes are known in nature. Among them, AAV2, AAV3, AAV5, and AAV6 are found in human cells, AAV1, AAV4, and AAV7 through 12 are found in other primate cells, with AAV2 being the most studied. Different serotypes of AAV exhibit different cell or tissue tropism (ability to infect), which is useful in targeting AAV gene therapy to specific cells or tissues. In order to improve the gene delivery efficiency of AAV, researchers have developed many artificial serotypes of adeno-associated virus to improve gene expression, alter target tissue tropism, and increase immune evasion. For example, AAV6.2 is produced by targeted mutation of AAV6 capsid protein to increase transgene expression and AAV-DJ composed of fusion of a variety of AAV capsid sequences that exhibits broad tropism and increased immune evasion.

Go to page 22 to see more details about serotype and tissue tropism.



AAV Vectors

AAV Vector Systems

VectorBuilder offers a variety of AAV vector systems:

- **Mammalian Gene Expression**
  - ssAAV and scAAV Vector
  - Chimeric Baculovirus-AAV Vector
- **Mammalian Non-Coding RNA Expression**
  - ssAAV Vector
- **Mammalian Inducible Gene Expression (Tet-Based)**
  - Tet-On (All-In-One) AAV Vector
  - TRE Driven GOI Expression AAV Vector
  - Tet Regulatory Protein Expression AAV Vector
- **Mammalian Conditional Gene Expression (Cre-Lox Based)**
  - Cre-On Gene Expression AAV Vector
  - Cre-Off Gene Expression AAV Vector
  - Cre-Switch Gene Expression AAV Vector
- **Mammalian CRISPR Gene Editing**
  - gRNA and Cas9 Coexpression AAV Vector (single SagRNA)
  - Single gRNA AAV vector
  - Dual gRNA AAV vector
  - Cas9 Expression AAV Vector
- **Mammalian shRNA Knockdown**
  - U6-Based shRNA ssAAV Vector
  - U6-Based shRNA scAAV Vector
  - Single miR30-shRNA AAV vector
  - Dual miR30-shRNA AAV vector
  - Triple miR30-shRNA AAV vector
  - Quad miR30-shRNA AAV vector
- **Mammalian CRISPR Gene Regulation**
  - CRISPR-Based Gene Activation AAV Vector (dSaCas9-SAM system)

AAV Vector Design

ssAAV or scAAV

The AAV genome is a linear single-stranded DNA (ssDNA). When target cells are transduced with ssAAV, the single-stranded viral DNA is not transcribed until its complementary strand is synthesized. Due to this, the second-strand synthesis tends to be the rate-limiting step for the expression of a given transgene. Our scAAV transfer vector is engineered from ssAAV with two important differences. First, the trs (terminal resolution site) located in the 3' ITR is deleted in scAAV. As a result, scAAV has a tendency of forming a double-stranded DNA molecule during replication that is the concatenation of two full single-stranded genomes, one plus strand and the other minus strand. This molecule can form a self-complementary intramolecular dsDNA genome. When scAAV viral particles enter host cells, this self-complementary intramolecular dsDNA genome can skip second-strand synthesis, to quickly express genes carried on the scAAV vector. Therefore, scAAV has faster and increased transgene expression relative to ssAAV. Second, due to fact that wildtype AAV can carry up to about 4.7 kb of single-stranded DNA genome yet each scAAV DNA molecule packaged into a viral particle is the concatemer of two single-stranded genomes of opposite strands, the cargo capacity of scAAV in terms of the length of the 5' ITR to 3' ITR transgene that can be properly packaged into mature virus is only about half that of ssAAV.

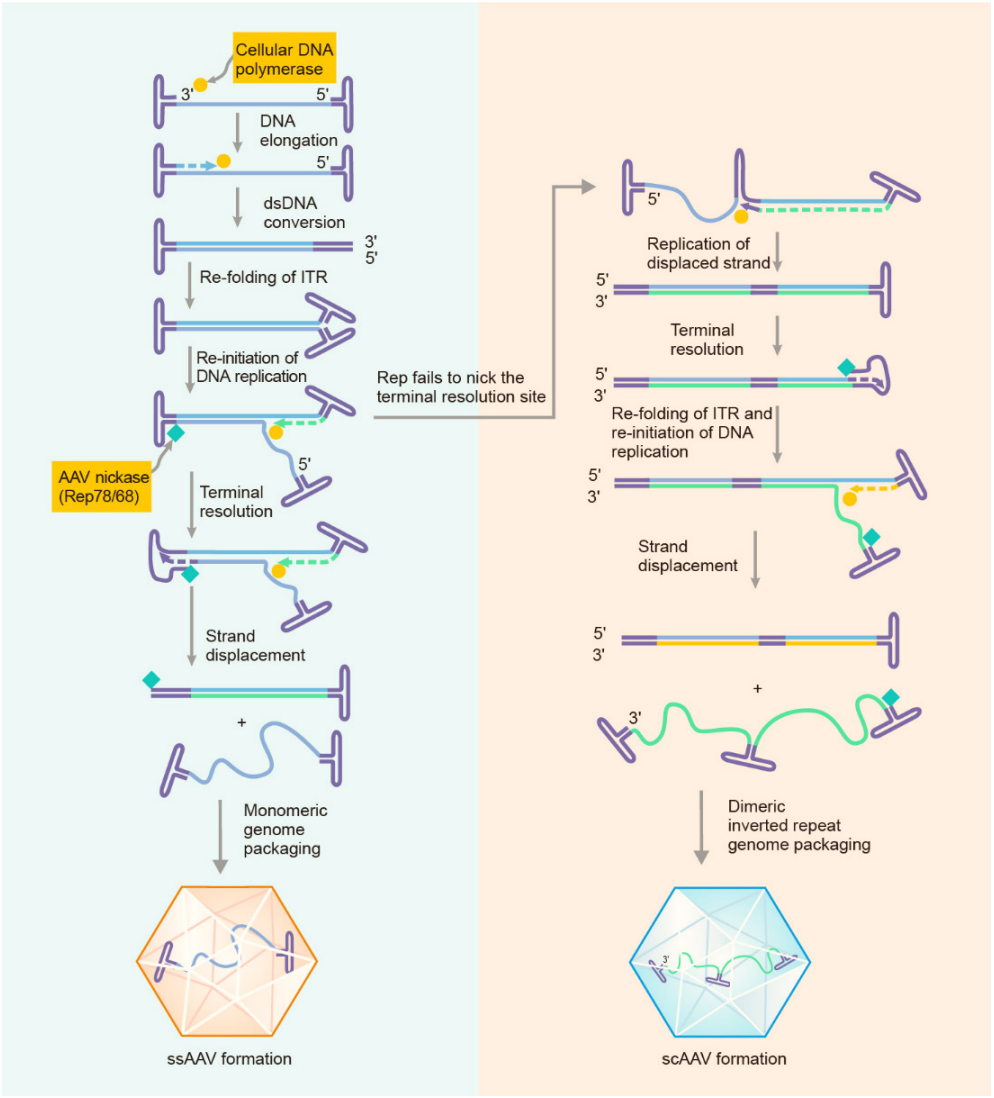
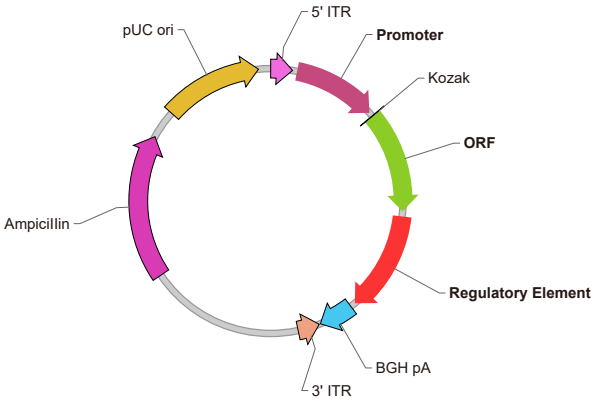


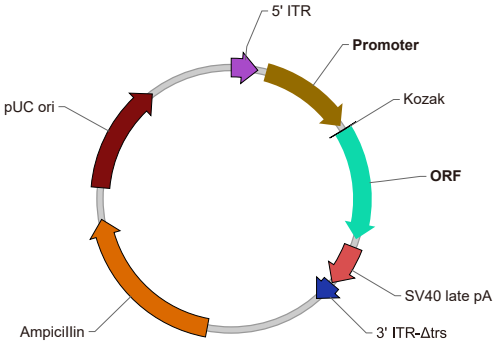
Figure 3. Virus genome formation of ssAAV and scAAV (adapted from McCarty DM, 2008).

The Cargo Capacity of AAV Vectors

AAV has the smallest cargo capacity of any of our viral vector systems. AAV can accommodate a maximum of 4.7 kb of sequence between the ITRs, which leaves ~4.2 kb of cargo space for a user's DNA of interest for ssAAV and ~2.2 kb for scAAV.



ssAAV



scAAV

The Advantages of AAV Gene Delivery

- **Safety:** AAV is the safest viral vector system available. AAV is inherently replication-deficient, and is not known to cause any human diseases.
- **Low risk of host genome disruption:** Upon transduction into host cells, AAV vectors remain as episomal DNA in the nucleus. The lack of integration into the host genome can be a desirable feature for in vivo human applications, as it reduces the risk of host genome disruption that might lead to cancer.
- **High viral titer:** Our AAV vector can be packaged into high titer virus. When AAV virus is obtained through our virus packaging service, titer can reach $>10^{13}$ genome copy per ml (GC/ml).
- **Broad tropism:** A wide range of cell and tissue types from commonly used mammalian species such as human, mouse, and rat can be readily transduced with our AAV vector when it is packaged into the appropriate serotype. Some cell types may be difficult to transduce, depending on the serotype used.
- **Effectiveness in vitro and in vivo:** Our vector is often used to transduce cells in live animals and it can also be used effectively in vitro.

Choose Promoter

For AAV vectors that use Pol II RNA polymerase for transcription, either a ubiquitous or tissue specific promoter can be applied to drive the expression of a GOI. However, it has been observed that the transcriptional efficiency among these promoters is significantly varied in different cell lines. VectorBuilder offers many popular promoters that users can choose from when designing their AAV vectors.

Mammalian Ubiquitous Promoter

Ubiquitous promoters are widely used to construct viral vectors due to their generally high transcriptional activity when driving the expression of a target gene. Details of these ubiquitous promotes are listed in the table below:

Name	Annotations / Notes	Size (bp)
CMV	Strong promoter; may have variable strength in some cell types.	589
CMV+intron	Strong promoter; may have variable strength in some cell types; presence of the beta-globin intron facilitates the nuclear export of mRNA by splicing and is predicted to enhance gene expression in eukaryotes.	1242
EF1A	Strong promoter	1179
EFS	Medium-strength promoter	232
CAG	Strong promoter	1733
CBh	Strong promoter	789
CBA	Strong promoter	850
SFFV	Strong promoter; drives high levels of gene expression, particularly in cell types of the myeloid lineage.	500
MSCV	Medium-strength promoter; drives gene expression in most murine or human ES cell lines and other mammalian cell lines.	515
SV40	Medium-strength promoter	344
mPGK	Medium-strength promoter	511
hPGK	Medium-strength promoter	505
UBC	Weak promoter	1178

Mammalian Tissue-Specific Promoter

In addition to selecting a specific AAV serotype, the use of tissue specific promoters is key to achieving efficient expression of transgenes in target tissues. Unlike constitutive promoters, tissue-specific promoters only drive the expression of target genes in specific organs and tissues, which can greatly reduce the non-specific expression of the AAV transgene. The tissue-specific promoters provided by VectorBuilder cover more than ten tissues and organs including nervous system, cardiovascular system, muscle, liver, lung, bone tissue, and retina. Our tissue-specific promoters are listed in the table below:

Cardiovascular system

Name	Tissue	Cells	Size (bp)
cTnT	Heart	Cardiomyocytes	413
αMHC(short)	Heart	Cardiomyocytes	2833
αMHC(long)	Heart	Cardiomyocytes	5464
Hcn4	Embryonic heart	Cardiomyocytes	2780
CD68(short)	Blood	Monocytes and macrophages	659
CD68(long)	Blood	Monocytes and macrophages	3079
Tie1	Blood vessel	Vascular endothelial cells	1142
CD144	Lung, heart, ovary, spleen, and kidney glomeruli	Vascular endothelial cells	2510

Neural system

Name	Tissue	Cells	Size (bp)
Hb9	Spinal cord	Motor neurons	4003
Camk2a(short)	Brain	Pyramidal neurons	1337
Th	Brain	CA1 pyramidal cells of the hippocampus	3015
GFAP(short)	Brain	Astrocytes	681
GFAP(long)	Brain	Astrocytes	2178
Iba1	Brain	Microglia	1889
SYN1	Brain	Mature neurons	469
NSE	Nervous system	Various neurons	1807
Nes	Nervous system at embryonic stage	Neural stem/progenitor cells	1566
Cnp	Nervous systems, testis, and thymus	Oligodendrocytes and Schwann cells	3046
Tuba1a	Nervous systems at embryonic and early postnatal stages	Developing neurons but not astrocytes or oligodendrocytes	1121

Muscle

Name	Tissue	Cells	Size (bp)
MHCK7	Skeletal and heart muscles	Differentiated postmitotic striated muscle cells	771
Myog	Muscle	Myoblasts	1139
ACTA1	Muscle	Myocytes	2162
SM22a	Adult smooth muscle	Vascular smooth muscle cells	2960
EnSM22a	Adult smooth muscle	Vascular smooth muscle cells	594

Liver

Name	Tissue	Cells	Size (bp)
Afp	Liver	Hepatocytes	2810
Alb	Liver	Mature hepatocytes	2336
TBG	Liver	Hepatocytes	460

Retina

Name	Tissue	Cells	Size (bp)
ProA1	Retina	Cone photoreceptor	2000
hRHO	Retina	Rod photoreceptor	839
hBEST1	Retina	RPE	623

Bone Tissue

Name	Tissue	Cells	Size (bp)
Col2a1	Cartilage	Chondrocytes	1033
Runx2	Bone	Early osteoblasts	2514
CD11b	Bone marrow	Mature myeloid cell lines (neutrophils, monocytes and macrophages)	1795
Col1a1	Bone, tooth and tendon	Osteoblasts and fibroblasts	3632
OC	Bone and cartilage	Osteoblasts, osteocytes and hypertrophic chondrocytes	2141

Lung

Name	Tissue	Cells	Size (bp)
SPB	Lung	AT II cells (alveolar type II epithelial cells) and Clara cells (bronchiolar epithelial cells)	635

Kidney

Name	Tissue	Cells	Size (bp)
NPHS2	Kidney	Podocytes	2589

Pancreas

Name	Tissue	Cells	Size (bp)
HIP	Pancreas	β cells	1867
Ins2	Pancreas	β cells	705
Pdx1	Embryonic developing pancreas and adult pancreatic islets	Pancreatic progenitor cells and pancreatic β cells	2930

Adipose

Name	Tissue	Cells	Size (bp)
aP2	Fat	Adipocytes	3708
Adipoq	Fat	Adipocytes	5411

Mammary gland

Name	Tissue	Cells	Size (bp)
MMTV	Mammary gland	Ductal cells of the salivary gland, mammary epithelial cells	1315
Wap	Mammary gland	Alveolar epithelial cells of mammary tissue	2444

Epidermal tissue

Name	Tissue	Cells	Size (bp)
K14	Epidermis	Keratinocytes	2268
mTyr	Epidermis	Melanocytes and melanoma cells	560
BK5	Epidermis	Keratinocytes	5200

AAV Offerings for Research

AAV Virus Packaging

Recombinant adeno-associated virus (AAV) is a versatile and popular viral vector used for in vitro and in vivo gene delivery. VectorBuilder offers superior quality AAV packaging services to support your AAV-based gene therapy experiments. We have developed a series of proprietary technologies and reagents that have greatly improved recombinant AAV production protocols in terms of titer, purity, potency, and consistency, especially for the AAV vector systems used in our vector cloning services.

Types of AAV Offered

- Single-stranded AAV (ssAAV) and self-complementary AAV (scAAV)
- 18 serotypes: 1, 2, 3, 4, 5, 6, 6.2, 7, 8, 9, rh10, DJ, DJ/8, PHP.eB, PHP.S, AAV2-retro, AAV2-QuadYF and AAV2.7m8
- AAV empty capsids or virus-like particles (VLPs)

AAV Production and QC

Triple Transfection-Based Approach

For our recombinant AAV manufacturing, the transfer plasmid carrying the gene of interest (GOI) is co-transfected with our proprietary Rep-Cap plasmid and helper plasmid encoding adenovirus genes (E4, E2A, and VA) that mediate AAV replication in HEK293T packaging cells. After a short incubation period, viral particles are harvested from cell lysates or supernatants dependent on serotype and concentrated by PEG precipitation. For ultra-purified AAV (in vivo grade), viral particles are further purified and concentrated by cesium chloride (CsCl) gradient ultracentrifugation. We use a qPCR-based approach to measure AAV titer.

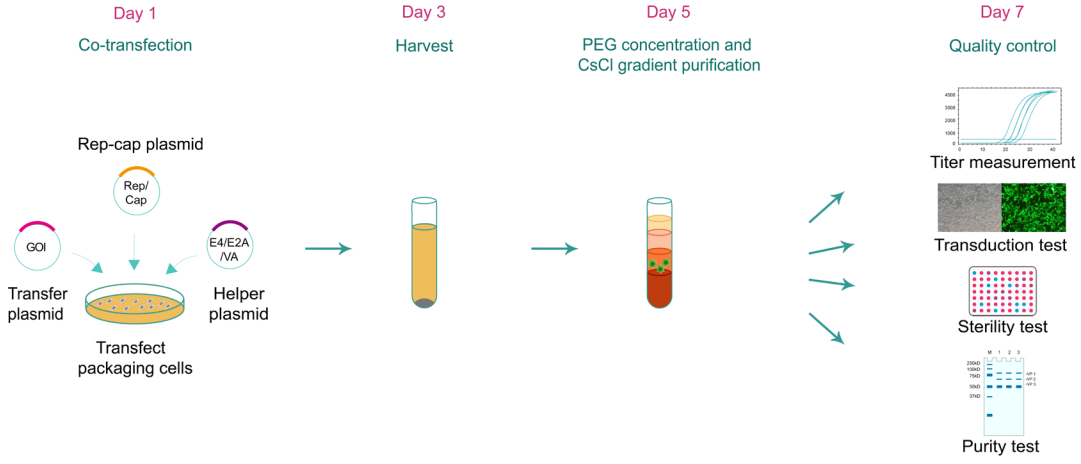


Figure 4. Typical workflow of triple transfection-based AAV packaging.

For each AAV produced by VectorBuilder, quality control includes titer measurement, sterility testing for bacteria and fungi, and mycoplasma detection. If the transfer vector encodes a fluorescent protein, we also perform a transduction test to detect corresponding fluorescence. Additionally, for ultra-purified AAV, we routinely sample virus quality by SDS-PAGE analysis and endotoxin assay.

Price and turnaround

Scale	Application	Typical Titer	Minimum Titer	Volume	Price (USD)	Turnaround
Pilot	Cell culture	>10^{12} GC/ml	>2x10^{11} GC/ml	250 ul (10x25 ul)	$449	6-12 days
Medium				1 ml (10x100 ul)	$649	
Large		>5x10^{12} GC/ml	>2x10^{12} GC/ml	1 ml (10x100 ul)	$1,099	
Ultra-purified pilot	Cell culture & in vivo	>2x10^{13} GC/ml	>10^{13} GC/ml	100 ul (4x25 ul)	$1,399	7-14 days
Ultra-purified medium				500 ul (10x50 ul)	$1,999	
Ultra-purified large				1 ml (10x100 ul)	$3,099	

Baculovirus-Based Approach

Our baculovirus-based AAV packaging workflow consists of two main steps as shown in Figure 5 below. Step 1 involves the generation of two recombinant baculoviruses, one expressing the gene of interest (GOI) flanked by the AAV inverted terminal repeats (ITRs) and a second helper baculovirus expressing the AAV Rep and Cap genes. In step 2 recombinant AAV particles are generated by co-infecting insect cells with the two recombinant baculoviruses produced in step 1.

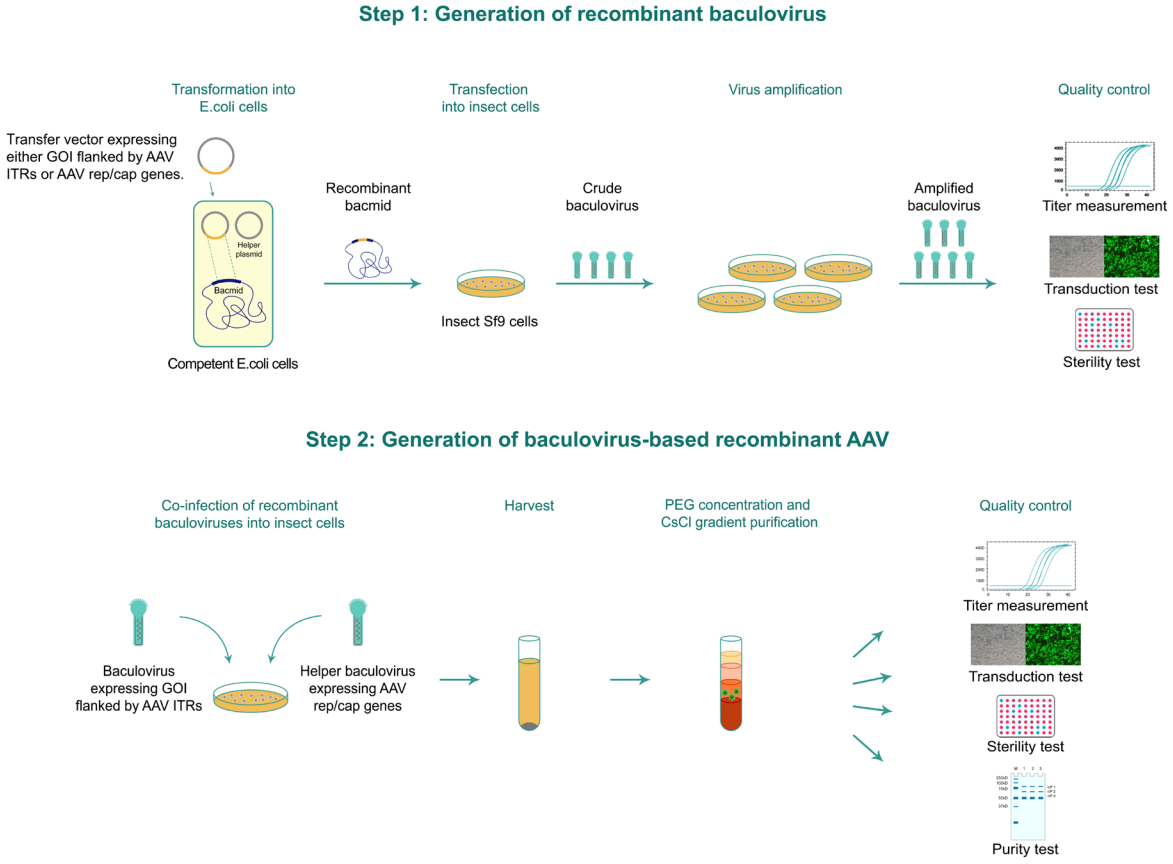


Figure 5. Typical workflow of baculovirus-based AAV packaging.

For generating the two recombinant baculoviruses (one expressing the GOI flanked by AAV ITRs and the other expressing the AAV Rep/Cap genes), the expression cassette for each baculovirus is first cloned into a baculovirus transfer vector. The baculovirus transfer plasmid is then co-transformed with a helper plasmid expressing the Tn7 transposase into the bacterial host harboring the empty baculovirus shuttle vector (a.k.a. bacmid) to generate recombinant bacmid. The recombinant bacmid is then transfected into insect Sf9 cells. After a short incubation period, viral particles are harvested from the media and further concentrated by sucrose cushion centrifugation. We use a qPCR-based approach to measure baculovirus titer. Both recombinant baculoviruses are then co-infected into insect cells. After an incubation period of 72-96 hours, AAV particles are harvested from the cell lysate as well as the supernatant and concentrated by PEG precipitation. For ultra-purified AAV (in vivo grade), viral particles are further purified and concentrated by cesium chloride (CsCl) gradient ultracentrifugation. We use a qPCR-based approach to measure AAV titer.

Price and turnaround

Scale	Application	Minimum Titer	Volume	Price (USD)	Turnaround
Ultra-purified pilot	Cell culture & in vivo	>5x10^{13} GC/ml	1 ml (10x100 ul)	$5,599	35-49 days
Ultra-purified medium			5 ml (25x200 ul)	$20,199	35-49 days
Ultra-purified large			10 ml (50x200 ul)	$38,199	35-49 days

Note: Baculovirus-based approach is available for the following serotypes: 1, 2, 5, 6, 8, 9.

AAV Serotype Testing Panel

Adeno-associated viruses (AAVs) have emerged as the most effective viral vectors for gene therapy due to their ability to transduce a wide variety of mammalian cell types and their low immunogenicity in host organisms. VectorBuilder offers the AAV serotype testing panel to enable users to select the optimal AAV serotype for specific applications by systematic comparison of a variety of serotypes in cells or in animals.

Ordering Information

Product	Catalog No.	Titer & Volume	Unit Price (USD)
In vitro grade AAV serotype testing panel (CMV-EGFP)	PANEL-AAVS01	>10^{12} GC/ml, 25 ul	$79 per aliquot
In vitro grade AAV serotype testing panel (CAG-EGFP)	PANEL-AAVS02		
In vivo grade AAV serotype testing panel (CMV-EGFP)	PANEL-AAVSP01	>10^{13} GC/ml, 25 ul	$139 per aliquot
In vivo grade AAV serotype testing panel (CAG-EGFP)	PANEL-AAVSP02		

Experimental Validation

We have developed a number of proprietary techniques to optimize our triple transfection-based AAV packaging protocol and our virus has been validated to exhibit high transduction efficiency in mammalian cells.

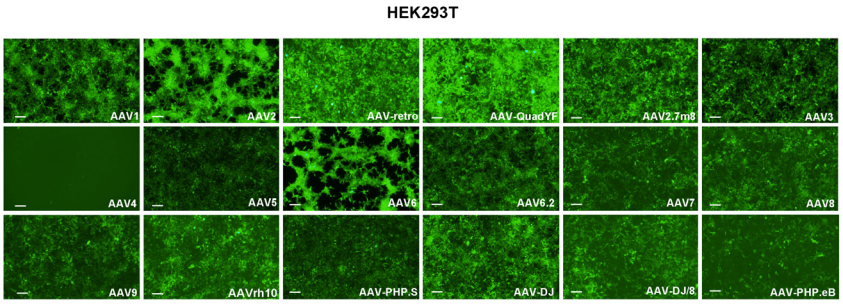


Figure 6. HEK293T cells were transduced with 18 serotypes of recombinant AAV packaged from the same CMV>EGFP vector (VB010000-9394npt). Representative EGFP expression at 48 h post transduction in different cell lines is shown as indicated. Scale bars: 100 um.

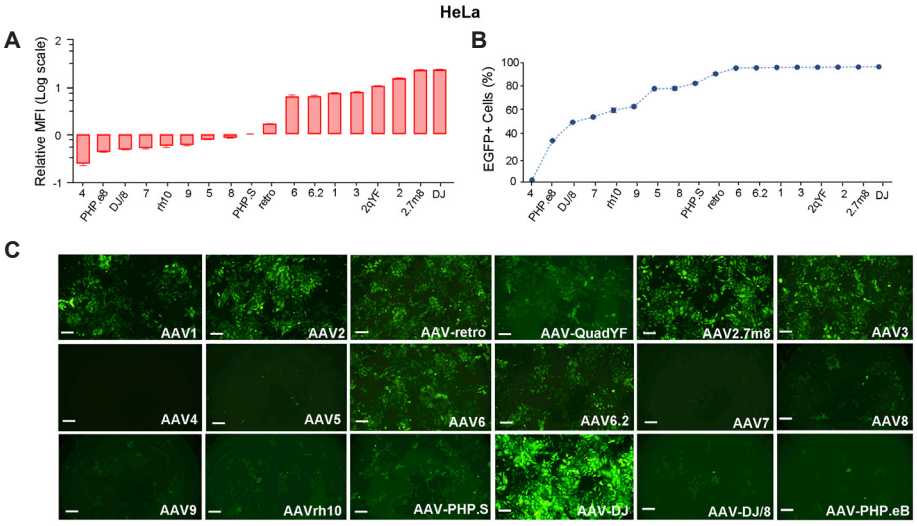


Figure 7. HeLa cells were transduced with 18 serotypes of recombinant AAV packaged from the same CMV>EGFP vector (VB010000-9394npt). EGFP (A) Mean Fluorescence Intensity (MFI) and (B) positivity were quantified using flow cytometry 48 h post transduction. (C) Representative EGFP expression in different cell lines is shown as indicated. Scale bars: 100 um.

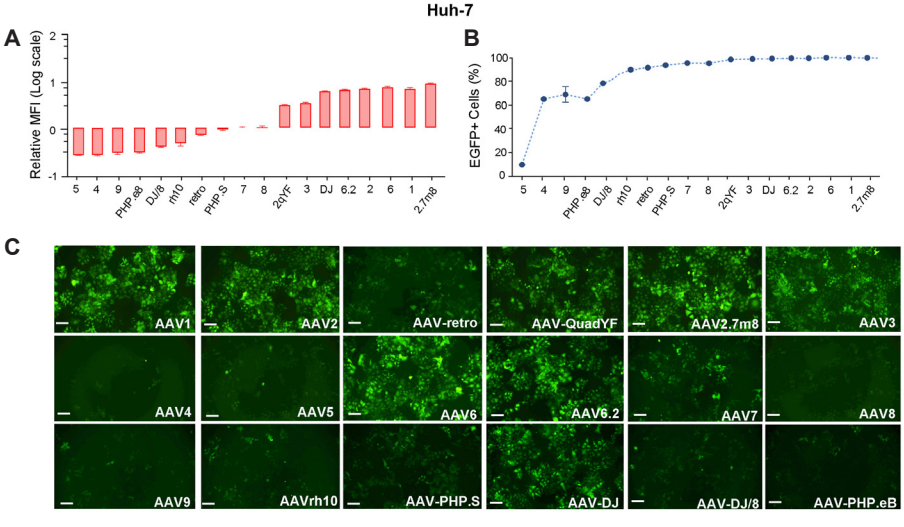


Figure 8. Huh-7 cells were transduced with 18 serotypes of recombinant AAV packaged from the same CMV>EGFP vector (VB010000-9394npt). EGFP (A) Mean Fluorescence Intensity (MFI) and (B) positivity were quantified using flow cytometry 48 h post transduction. (C) Representative EGFP expression in different cell lines is shown as indicated. Scale bars: 100 um.

shRNA (3+1) AAV Packaging

The knockdown effects of empirically designed shRNAs are often limited by variations in specificity and efficiency observed from one shRNA to another. Therefore, it is important to test multiple shRNAs to find the most potent shRNA for knocking down your GOI. VectorBuilder's shRNA (3+1) virus packaging services enable you to select optimal shRNAs for your target genes at highly affordable prices. This offering includes cloning and packaging three custom shRNA viruses targeting your GOI and one scramble control virus.

Ordering Information

Scale	Application	Price (USD)*	Turnaround**
Pilot	Cell culture	$1,499	10-19 days
Medium		$1,999	
Large		$2,999	
Ultra-purified pilot	Cell culture & in vivo	$4,199	11-21 days
Ultra-purified medium		$5,699	
Ultra-purified large		$8,799	

** Price includes both vector cloning and virus packaging.*

*** Turnaround includes the production time for both vector cloning and virus packaging. It does not include transit time for shipping final deliverables to the customer.*

Control AAV Virus

AAV control virus is designed to match the biological application of the custom virus and to be used for testing AAV transduction. For example, if the custom virus overexpresses a gene, then the control virus provided will be EGFP control AAV (AAV overexpressing EGFP), and if the custom virus expresses an shRNA against a gene, then the control virus provided will express a scramble shRNA. Detailed information on the control virus is shown below:

Vector System	Control Virus Vector Name	Control Virus Vector ID
ssAAV gene expression system	pAAV[Exp]-CMV>EGFP:WPRE	VB010000-9394npt
scAAV gene expression system	pscAAV[Exp]-CMV>EGFP	VB010000-9304aud
ssAAV U6-based shRNA knockdown system	pAAV[shRNA]-EGFP-U6>Scramble_shRNA	VB010000-0023jze
scAAV U6-based shRNA knockdown system	pscAAV[shRNA]-EGFP-U6>Scramble_shRNA	VB010000-9343nhh
ssAAV miR30-based shRNA knockdown system	pAAV[miR30]-CMV>EGFP:Scramble_miR30-shRNA:WPRE	VB010000-9397wgw
ssAAV CRISPR system	pAAV[Exp]-CMV>EGFP:WPRE	VB010000-9394npt

AAV Virus-Like Particles (VLPs)

VectorBuilder offers premade as well as custom AAV virus-like particles that can be used for a variety of applications during the development of AAV gene therapy vectors, including optimization of analytical assays, biodistribution assessment, and evaluation of in vivo immune responses to specific serotypes of AAV capsids. Our AAV virus-like particles are extensively characterized and can therefore, serve as reliable standards for evaluating the quality of AAV virus-like particles generated in house. Moreover, they can be used as negative controls in studies intended to demonstrate that an observed effect is caused by the expression cassette harbored in an AAV vector rather than its capsid.

Highlights

- Ready-to-ship in vivo grade AAV virus-like particles of serotypes 1, 2, 3, 5, 6, 8, 9, 2-retro, DJ, and DJ/8
- Custom services available for AAV virus-like particles produced using any desired serotype, production system, purification method, or QC requirement
- Thorough characterization using various methods including SDS-PAGE, BCA, endotoxin testing, mycoplasma detection, and full/empty capsid ratio analysis

Ordering Information

Scale	Volume	Price (USD)
10 ug (1.6x10^{12} VPs)	25 ul	$399
100 ug (1.6x10^{13} VPs)	0.1 ml (1x100 ul)	$2,299
500 ug (8x10^{13} VPs)	0.5 ml (5x100 ul)	$8,399
1 mg (1.6x10^{14} VPs)	1 ml (10x100 ul)	$12,599

Experimental Validation

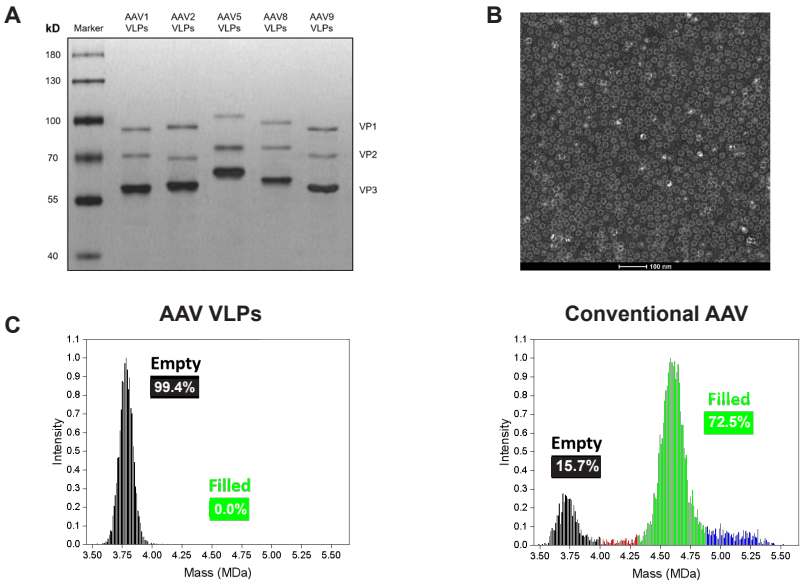


Figure 9. (A) Silver stained SDS-PAGE of ready-to-ship AAV virus-like particles of serotypes 1, 2, 5, 8, and 9. (B) Transmission electron microscopy of serotype 9 AAV virus-like particles showing empty (black-filled spheres) and filled (white-filled spheres) capsids. (C) Charge detection mass spectrometry analysis indicating percentages of empty (black) and filled (green) capsids for AAV virus-like particles and conventional AAV preparations of serotype 8.

AAV GMP Manufacturing

Virus Manufacturing

VectorBuilder has extensive expertise in producing many different types of viruses. We can provide viruses at different scales and with different quality control requirements to meet the full range of demands along the gene therapy drug development pipeline. We have established and validated a platform of technologies for large-scale GMP manufacturing of adeno-associated virus (AAV).

Different Grades of AAV Offered

AAV for Research

Research-grade AAV is suitable for basic research and drug discovery studies. It is produced in conventional cell culture facilities. Stringent QC assays are performed to ensure the quality of the virus meets the standards requested by customers.

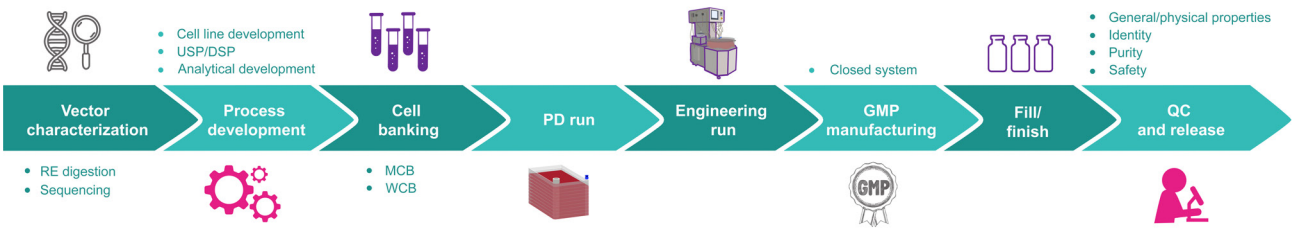
GMP-like AAV

GMP-like AAV is suitable for pre-clinical studies including drug safety and metabolism testing in animals. The manufacturing of GMP-like virus follows key features of GMP guidelines, such that the production process and quality attributes are comparable to GMP with document control and traceability. GMP-like grade is therefore intended to be a small-scale mimic of the final GMP product, but with significantly lower cost and a much faster timeline. Product release is accompanied by a certificate of analysis (COA). TSE/BSE statement can be provided upon request.

GMP AAV

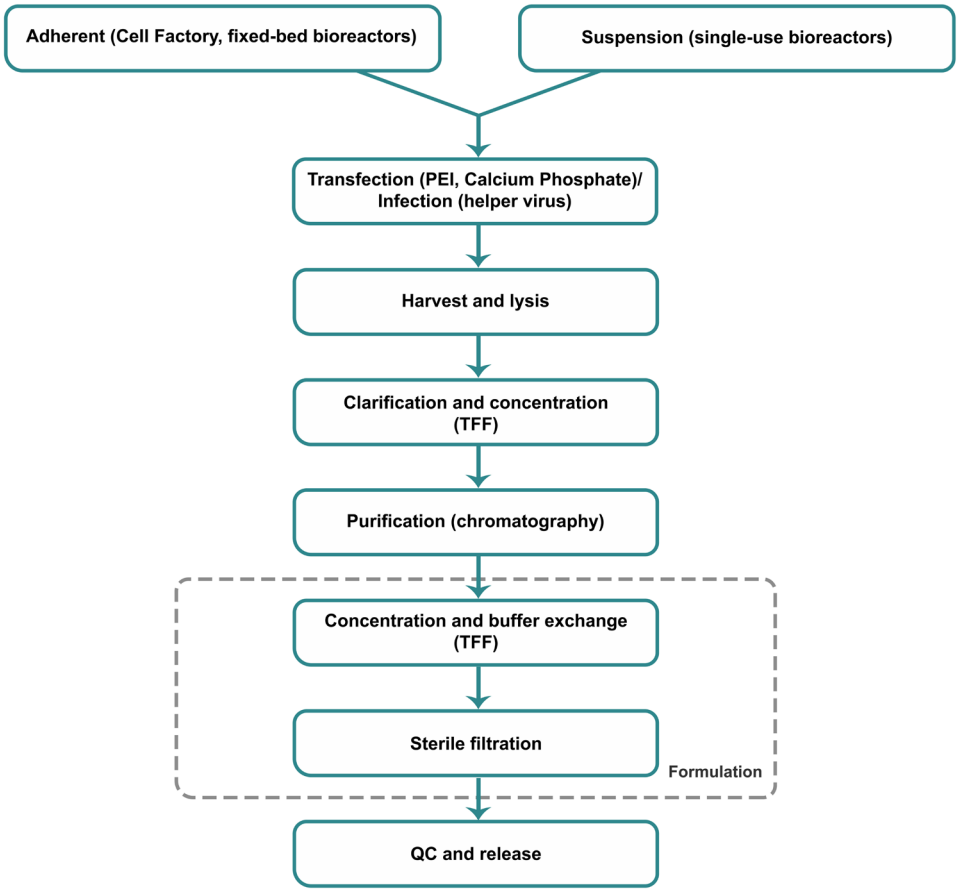
GMP-grade AAV is produced in our certified GMP cell culture suite following strict GMP guidelines. A comprehensive quality assurance system is embedded throughout the manufacturing process, including an array of in-process and release QC assays to ensure that the virus meets customer specifications and regulatory standards for quality and safety. At product release, a COA and a batch release report fully documenting the production process are provided.

Workflow for GMP AAV Production



Platform Technologies

We package AAV in HEK293T cells under either adherent conditions (Cell Factory or fixed-bed bioreactors) or serum-free suspension conditions (up to 200 L single-use bioreactors). We also package AAV in suspension Sf9 insect cells. We can achieve a scale of up to 10^{17} GC AAV per batch.



GMP Facilities

VectorBuilder currently has about 100,000 sq ft of modern GMP facilities with advanced designs and state-of-the-art equipment. All our facilities are designed to meet GMP regulations and guidelines of the US, EU, Japan, China, and PIC/S. They are suitable for Phase I/II/III clinical trials and commercial manufacturing. Our facilities currently include 10 GMP manufacturing suites, multiple fill/finish suites, QC laboratories totaling 9,500 sq ft, and multiple GMP suites totaling 8,400 sq ft for PD/pilot runs.

Additionally, our Stage 3 GMP facilities (~500,000 sq ft), capable of large-scale commercial gene and cell therapy drug manufacturing, are under construction and will be operational in early 2024.

AAV CRO Services

Library Construction

VectorBuilder can help you build custom pooled libraries to perform large-scale functional screens. We have considerable experience in constructing high-quality pooled libraries utilizing our optimized vectors and proprietary technologies. We can deliver your library as an E. coli stock, DNA, or recombinant virus, depending on your needs. Our custom libraries are fully validated by next-generation sequencing (NGS) to ensure uniform clone representation and low error rates.

Libraries Offered

- CRISPR libraries (CRISPR KO, CRISPRa/i, CROP-seq, Perturb-seq, etc.)
- shRNA libraries
- Barcode libraries
- Enhancer/promoter screening libraries
- Peptide expression libraries
- DNA shuffling libraries
- Two-hybrid libraries
- Capsid screening libraries
- And many more

AAV Capsid Evolution

Directed evolution is a high-throughput approach widely used for engineering novel AAV capsids. It mimics the process of natural selection through repeated genetic diversification and selection. Directed evolution of AAV capsids is performed by mutating the wildtype AAV capsid gene to generate highly diverse AAV capsid libraries, which are then screened to identify novel capsid variants with improved properties.

Capsid Evolution Services Offered

- Full-service platform to fulfill all your needs along the workflow of generating high diversity AAV capsid libraries
- High-complexity capsid libraies construction via any mutagenesis or combinatorial approach, including error-prone PCR, random peptide display, DNA family shuffling, and in silico design
- High-titer packaging of viral capsid library by either one-step or two-step approaches
- In vivo screening in multiple species including mice, rats, and NHPs
- Full technical support covering every aspect of your AAV capsid project from library design and construction to in vivo screening and NGS analysis

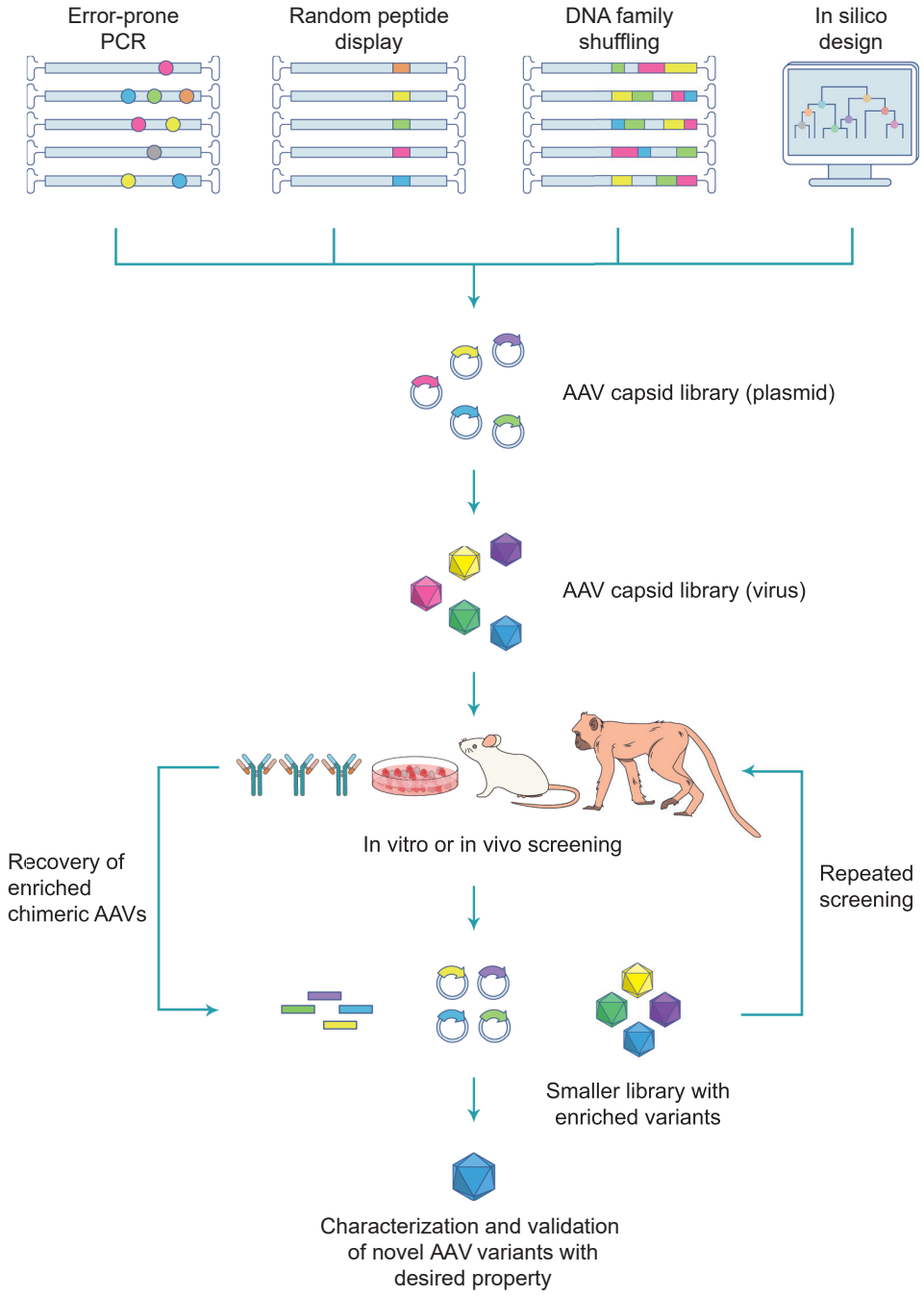


Figure 10. Above is the typical workflow for screening novel AAV capsids by directed evolution.The first and most critical step in the entire workflow of AAV capsid evolution is the generation of a highly diverse AAV capsid library in which each plasmid carries a chimeric AAV genome consisting of a Rep gene and a capsid gene variant. The capsid gene variants can be efficiently generated using various approaches, such as error-prone PCR, random peptide display, DNA family shuffling, or in silico design. The capsid library is then packaged into viral particles, and each viral particle harbors a corresponding capsid variant in its genome. The viral library is then subsequently subjected to a screening process. Viral genomes that pass screening are recovered from target cells and made into a smaller library for the second round of screening. Multiple rounds of screening are usually performed to enrich high-confidence hits. The resulting hits are then validated and characterized to identify novel AAV capsid variants with enhanced properties.

AAV Biodistribution Profiling

Assessing the distribution and persistence of AAV vectors in various body tissues and organs at the developmental and preclinical stages is critical to ensure the success of AAV-based gene therapy. AAV biodistribution studies have been highly instrumental in identifying off-target effects, thereby playing a significant role in the safety assessment of AAV vectors.

Service Highlights

- Full-service platform to fulfill all your needs along the workflow of biodistribution assessment for your AAV vectors
- Services available for multiple species, including mice, rats, and NHPs
- Multiple analytical assays, including fluorescence imaging, flow cytometry analysis, luciferase assay, qPCR, and RT-qPCR
- Multiplexing analysis using barcodes and NGS for assessing the biodistribution of different vectors within the same animal
- Multiple routes of AAV administration by highly trained experts, including tail vein injection, facial vein injection (for neonatal mice and rats), intracerebroventricular injection, intrathecal injection, subretinal injection, intravitreal injection, intratympanic injection, and intramuscular injection
- Full technical support that covers every aspect of your AAV biodistribution project

Experimental Validation

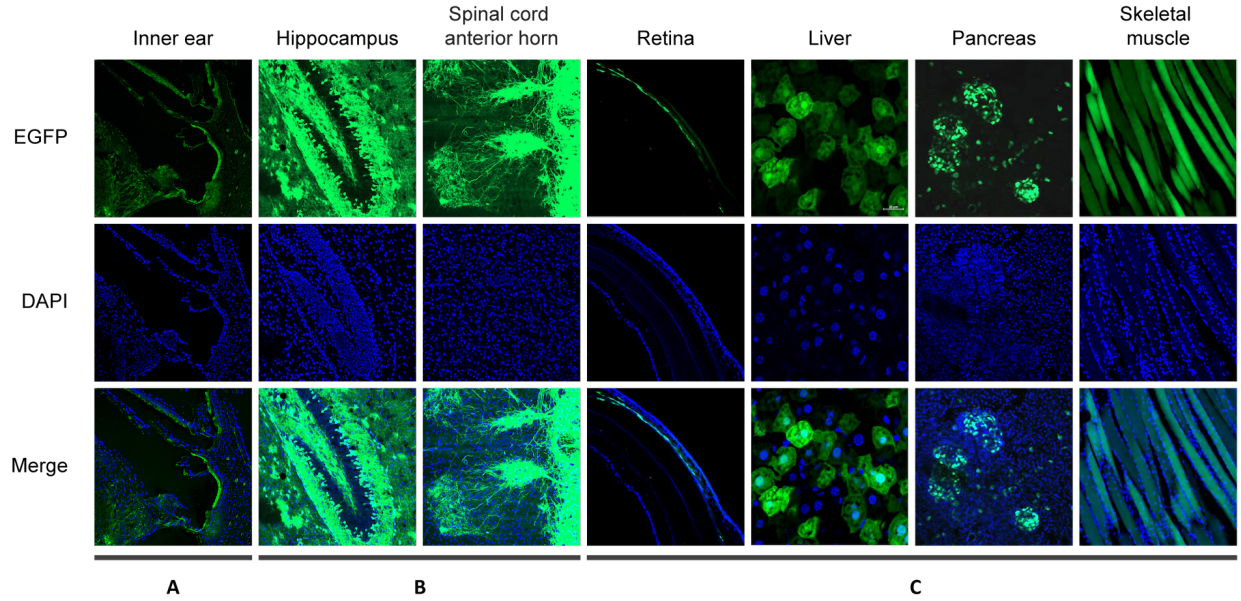


Figure 11. AAV9 carrying CAG promoter driving EGFP was administered to mice by various routes. EGFP and DAPI fluorescence was analyzed in the following organs: (A) inner ear, images were taken 13 days after vector delivery by intratympanic injection to the left ear; (B) hippocampus and spinal cord anterior horn, images were taken 10 days after vector delivery by facial vein injection; (C) retina, liver, pancreas, and skeletal muscle, images were taken 12 days after vector delivery by tail vain injection.

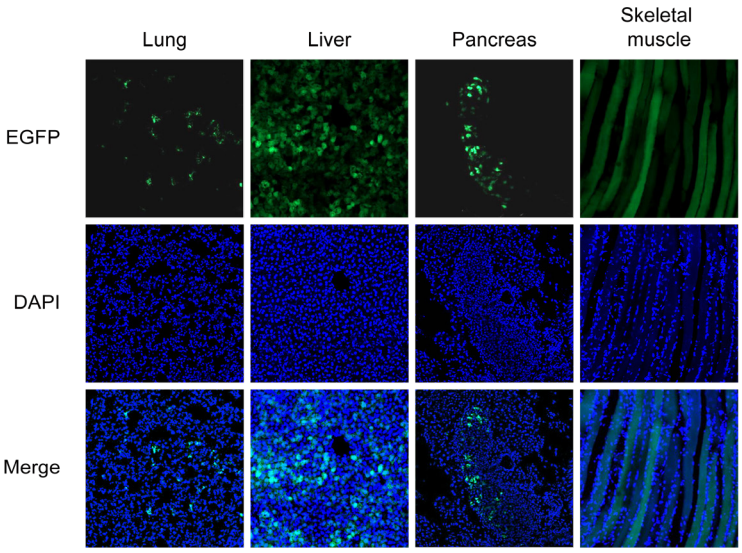


Figure 12. AAV1 carrying CMV promoter driving EGFP was administered to mice by tail vein injection. EGFP and DAPI fluorescence was analyzed in lung, liver, pancreas, and skeletal muscle. Images were taken 12 days after injection.

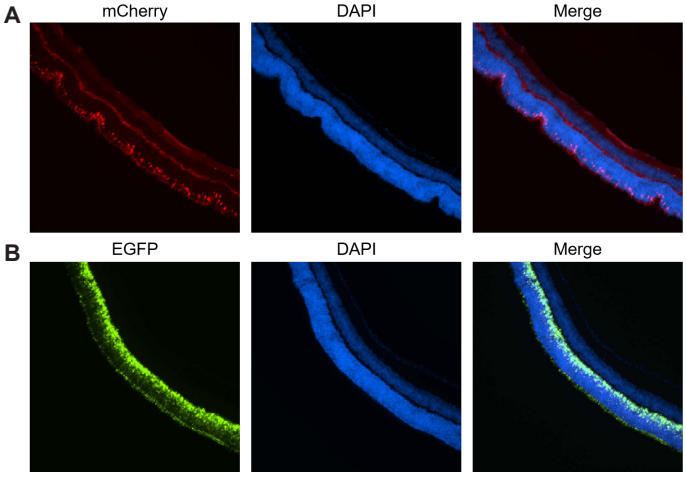


Figure 13. (A) AAV8 carrying ProA1 promoter driving mCherry or (B) AAV8 carrying hRHO promoter driving EGFP was administered to mice by subretinal injection. mCherry, EGFP, and DAPI fluorescence was analyzed in retina. Images were taken 20 days after injection.

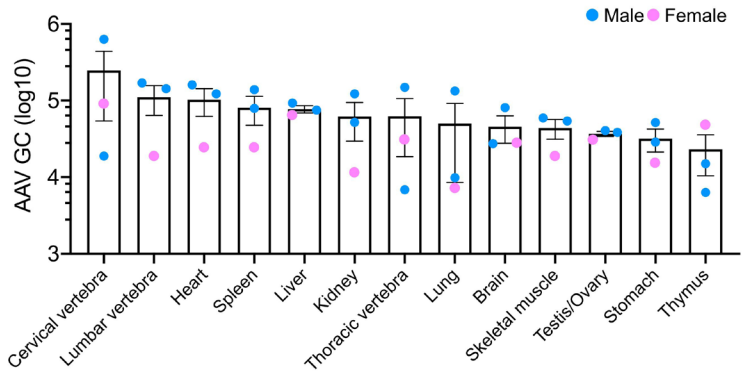


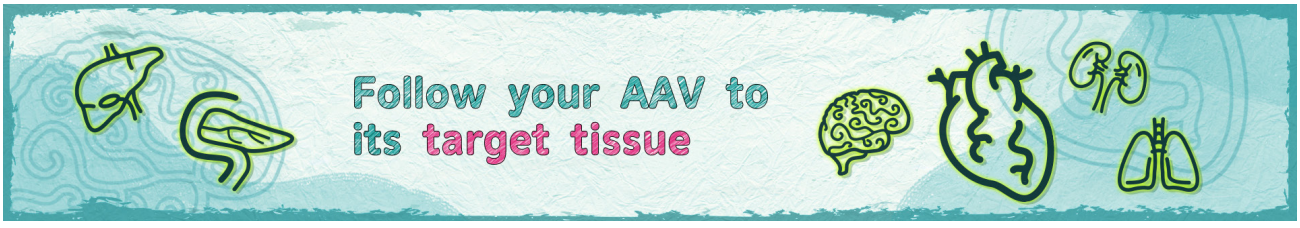
Figure 14. Recombinant AAV9 particles (~1.5X10^{10} GC/mouse) were intravenously administered to three neonatal mice (two male and one female) within 48h after birth. AAV9 biodistribution in several organs was quantified using qPCR six weeks after the injection.

AAV Knowledge Base

AAV Serotypes and Tissue Tropism

Many strains of AAV have been identified in nature. They are divided into different serotypes based on different antigenicity of the capsid protein on the viral surface. Different serotypes can render the virus with different tissue tropism (i.e. tissue specificity of infection). When our AAV vectors are packaged into the virus, different serotypes can be conferred to the virus by using different capsid proteins for the packaging. The table below lists the tissue specificity conferred by different AAV serotypes.

Tissue type	Recommended AAV serotypes
Smooth muscle	AAV1, AAV2, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh10
Skeletal muscle	AAV1, AAV9
CNS	AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh10, AAV-PHP.eB
PNS	AAV-PHP.S
Brain	AAV1, AAV2, AAV5, AAV7, AAV8, AAV-DJ/8
Retina	AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh10, AAV2-QuadYF, AAV2.7m8
Inner ear	AAV1, AAV2, AAV6.2, AAV8, AAV9, AAV2.7m8
Lung	AAV1, AAV3, AAV4, AAV5, AAV6, AAV6.2, AAV9, AAVrh10
Liver	AAV1, AAV2, AAV3, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh10, AAV-DJ, AAV-DJ/8
Pancreas	AAV1, AAV2, AAV6, AAV8, AAV9, AAVrh10
Heart	AAV1,AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10, AAV-DJ
Kidney	AAV2, AAV4, AAV8, AAV9, AAVrh10, AAV-DJ, AAV-DJ/8
Adipose	AAV6, AAV8, AAV9
Testes	AAV2, AAV9
Spleen	AAV-DJ, AAV-DJ/8
Spinal nerves	AAV2-retro
Endothelial cells	AAV2-QuadYF



Protocols for AAV In Vitro and In Vivo Applications

Storage and Handling

1. VectorBuilder's non-ultra-purified AAV is recommended for in vitro applications. Our non-ultra-purified AAV is stored in a Tris-based buffer. VectorBuilder's ultra-purified AAV is recommended for in vivo applications. Our ultra-purified AAV is stored in a PBS-based buffer.
2. After thawing, AAV can be dispensed into smaller aliquots according to the quantity used in your experiment and then refrozen. If you need to dilute the virus, you may use PBS, but do so ONLY immediately prior to use.
3. Upon receiving, AAV should be stored at -80°C for long term storage (stable for at least 1 year), or -20°C for short term storage (stable for 2~3 weeks).
4. Thaw the vial of AAV on ice prior to use and keep it on ice during the experiment. Thawed AAV can be stored at 4°C for 1-2 weeks without significant loss of biological activity.

Note: Do not freeze and thaw your AAV sample multiple times. AAV can be frozen and thawed several times with minimal loss of activity, but it is better to avoid this.

AAV In Vitro Transduction

Transduction of Targeted Cells

AAV transduction is cell type dependent. Some cell types exhibit low transduction efficiency, while others transduce very readily. When designing AAV transduction experiments, it is recommended to use different serotypes with a reporter vector such an AAV expressing EGFP (e.g. VectroBuilder's Serotype Testing Panel) to determine the optimal serotype for transduction of your tissue or cell culture. Start transducing the cells at a multiplicity of infection (MOI) between $1x10^4$ and $1x10^6$ genome copies (GC) per cell if the cells are readily transducible. With some cell lines a higher MOI might be needed, look for the highest transduction with minimal cell death. For some cell lines, high transduction levels cannot be achieved.

Protocol for Transducing Mammalian Cell Lines

1. **Day before transduction (Day 0)**

   Plate target cells in appropriate medium so that they will be 30-50% confluent at the time of transduction. Incubate 18-20 hours at 37°C in a humidified 5% CO_2 incubator. For example, when using HEK293T cells, we usually plate $3x10^5$ cells per well in a 6-well plate.

2. **Day of transduction (Day 1)**

   - Thaw virus on ice. Take the appropriate amount of virus as needed to achieve the desired MOI, place in an appropriate amount of medium, and mix gently (but do not vortex). To maximize transduction efficiency, use the minimum amount of medium necessary to cover the surface of the plate. For example, when infection is performed in 6-well plates, we use 1 ml of medium per well.
   - Aspirate old medium from target cells, then add the virus-containing medium onto the cells.
   - Swirl the plate gently to mix and cover the cells. Incubate at 37°C in a humidified 5% CO_2 incubator overnight.

Note: If you are concerned that exposure to the viral supernatant may adversely affect the target cells, limit the transduction to 6-8 hours.

3. **Day 2**

   Remove the virus-containing medium and replace with fresh complete culture medium. Incubate at 37°C in a humidified 5% CO_2 incubator overnight.

4. **Day 3 and onward**

   Analyze gene expression at desired time points following viral infection. In general, detectable levels of your gene product should be evident 24-48 hours after transduction.

Note: In actively dividing cells (i.e. doubling time of approximately 24 hours), transgene expression is generally detectable within 24 hours of transduction, with maximal expression observed at 48-96 hours (2-4 days) post transduction. Expression levels generally start to decline 5 days post-transduction. In cell lines that exhibit longer doubling times or non-dividing cell lines, high levels of transgene expression normally persists for a longer time. If you are transducing AAV into your mammalian cell line for the first time, we recommend performing a time course study to determine the optimal temporal conditions for expression of your transgene.

Anticipated Results

An example of successful transduction is shown below:

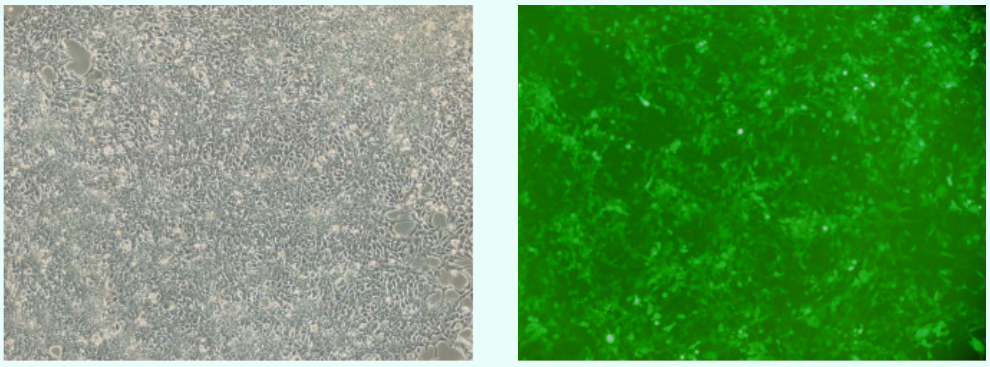


Figure 15. EGFP expressing AAV2 was used to transduce HEK293T cells at MOI 10,000 using the protocol in this document. Images were taken at 100X. Left: bright field; right: EGFP.

AAV In Vivo Transduction

Protocol for Mouse Tail Vein Injection

Intravenous (IV) injection via the lateral tail vein is an efficient means of transducing mouse tissues with AAV. Tail vein injections are preferred for their simplicity, as they do not require surgery or anesthesia. Other injection sites include the portal and jugular veins, but these require surgery. Tail vein injections are more efficient at delivering AAV to tissues compared to portal vein injections.

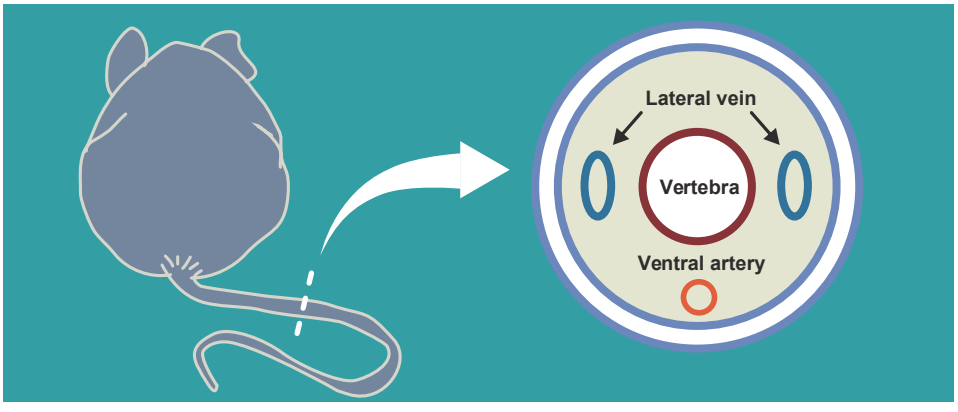


Figure 16. Diagram of a transverse sectional view of mouse tail lateral veins and ventral artery.

1. **Physical restraint**

   Mice can be physically restrained using a commercially available restraining device. Gently introduce the mouse to be injected into restrainer making sure the tail is exposed for further manipulations.

2. **Vasodilation**

   To induce peripheral vasodilation, warm the mouse by either dipping the tail in warm water (43°C) or placing the animal near a heat lamp for 5 to 10 minutes prior to injection.

Note: When using a heat lamp, place the lamp about 15 to 25 cm above the cage and observe the mice for 5 minutes. Stop heating once the mice huddle together in one corner of the cage.

3. **Injection**

   - Making sure the mouse is placed in the restraining device in a stern position, slightly rotate the tail to visualize the lateral vein. Disinfect the injection site with 70% ethanol and wipe off excess alcohol with gauze pad.
   - Using a 0.5 ml syringe with a 27 G needle, draw up at least 0.1 ml of ultra-purified AAV particles. Carefully remove air from the needle.
   - Grasp the distal part of the tail and twist it slightly to the lateral vein's side (Figure 16). Insert the needle at a shallow angle (as the tail vein is relatively close to the surface) and inject 0.1 ml of diluted AAV into the vein. The injection should proceed smoothly without any resistance if the needle is correctly positioned, and the vein will transiently shift to a clear color. Stop injecting if any local swelling is observed, as in that case the virus is being injected into the tail tissue, not the vein.

Note: Start your injection from the lower portion of the tail about 1/3 from the tip. This allows you to move up the tail if the previous injection was unsuccessful.

   - Remove the needle and apply pressure with a clean gauze until bleeding stops.
   - Return mouse to original cage.

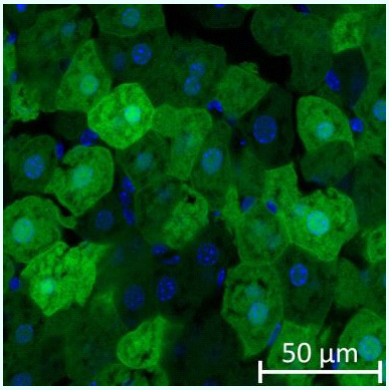
4. **Validation**

   AAV particles are predominantly sequestered by tissues following intravenous injection. To determine the efficiency of in vivo transduction, mice can be sacrificed at day 7 after virus infusion and target tissue(s) can be processed for analysis.

Anticipated Results

An example of successful in vivo transduction is shown below:

Figure 17. Mice were injected with the ultra-purified EGFP expressing AAV9 at a dose of $1x10^{13}$ GC/kg using the protocol of IV injection in this document. 10 days after the injection, the livers from injected animals were dissected for analysis. Images were taken by a confocal microscope. Green: EGFP. Blue: DAPI.



Protocol for Mouse Intramuscular Injection (IM)

1. Restrain the animal. Assistance for restraint or use of anesthesia is helpful.
2. Secure the rear foot nearest you.
3. Injections can be made into the thigh muscles caudal to (behind) the femur (semitendinosus - semimembranosus) or those cranial to (in front of) the femur (quadriceps).
4. Isolate the muscle mass to be used with your fingers.
5. Insert the needle to avoid the sciatic nerve. Direct the needle caudally (toward tail) if using the caudal thigh muscles or cranially (toward head) if using the quadriceps muscle. 25-30 G needle is needed.
6. Aspirate to ensure that you have not entered a blood vessel. If blood is aspirated, redirect the needle and aspirate again. For mouse, the injection volume generally is 25 ul. If no blood is aspirated, slowly inject the material.
7. Withdraw needle. If there is any bleeding at the injection site, apply pressure to the site for approximately 1 minute until bleeding stops.
8. Return the animal to its cage and observe the animal for any signs of pain or distress.

Protocol for Mouse Intraperitoneal Injection (IP)

Intraperitoneal injection is a simple, rapid and non-invasive AAV delivery method. Glybera, a commercial AAV gene therapy drug for lipoprotein esterase deficiency, was administered by intraperitoneal injection. The protocol of intraperitoneal injection is as follows:

1. Draw up the amount of AAV virus to be administered into the syringe and needle (the maximum volume per intraperitoneal injection is 10 ml/kg).
2. Restrain the mouse in an appropriate manner: “scruff” the mouse (gather the skin) with your non-dominant hand and hold the animal with its head slightly lower than the abdomen.
3. Identify anatomical landmarks to locate the appropriate IP injection site. Typically, the injection site is the animal’s lower right quadrant of the abdomen to reduce the chances of damage to the urinary bladder, cecum and other abdominal organs.
4. Disinfect the injection site with 70% ethanol. Insert needle at a 30-40^{o} angle to the horizontal plane of the animal with the bevel facing up.
5. Direct the needle towards the head of the animal. With a 25-27 G needle, approximately 1/2 the needle length is inserted.
6. When the needle inserts into the abdomen, the resistance from tissue dissipates. Pull back on the plunger to ensure negative pressure and that nothing is aspirated before injecting.

> **Note: If aspirate is green material, likely the bowel has been punctured. If aspirate is a yellow liquid, likely the bladder has been punctured. If aspirate contains blood, urine or gastrointestinal contents, the syringe and needle must be discarded. Restart the injection using new syringe and needle. Record any possible complications for the animal.**

7. Proceed with the injection. The injection can be completed in 1-2 seconds. Do not allow the needle to move further in or out of the abdomen.
8. When the injection is completed, remove needle from the abdomen at the same angle as it was inserted.
9. Return the animal to its cage and observe for any complications.

Protocol for Mouse Intra-Cerebroventricular Injection

Intra-cerebroventricular (ICV) injection is a procedure to deliver the ultra-purified AAV into the left and right lateral ventricles, followed by its diffusion into the central nervous system (CNS). The brain ventricles and CNS contain cerebrospinal fluid (CSF) which is produced in the two lateral ventricles. The CSF starts circulating from the lateral ventricles and eventually flows into the subarachnoid space. Since the subarachnoid space covers the entire brain, spinal cord, and sacrum, the ICV injection can be used to introduce the virus sample or other therapeutic agents to the CNS without being obstructed by the blood-brain barrier. To determine the optimal injection dose for your study, you could conduct pilot testing in your animals by using reporter AAV, such as EGFP-expressing AAV.

1. **Preparation of operating environment**
   - Wipe the operating table with 70% ethanol.
   - Cool down the brain stereotaxic device by adding 5 ml of absolute ethanol in the groove of the operating table. Consistently add dry ice to the absolute ethanol to stabilize the temperature of the operating table at about 0°C. At the same time, pre-warm a small heating pad.

Note: To avoid the death of mice, the temperature of the operating environment should not be below 0°C.

2. **Preparation of injection syringe**
   - Take a 33-34 G needle and sleeve it tightly with a PE tube, and then tighten the other side of the PE tube with a 10 ul syringe.
   - Draw up 5 ul of ultra-purified AAV into the needle, and carefully remove air from the needle.
   - Restrain the syringe and needle in the stereotaxic device.
3. **Anesthesia**

   Anesthetize the mouse to unconsciousness by gently placing the mouse on ice for 4 minutes.
4. **Physical restraint**

   After anesthesia, the head of the mouse can be physically restrained on the stereotaxic device gently.
5. **Injection**

5.1. **Left ventricle**
   - Disinfect the mouse head with 70% ethanol and wipe off excess alcohol with gauze pad.
   - Place the needle just above the lambda structure of the mouse (Figure 18). Record the indexes of x and y axis on the vernier scale.

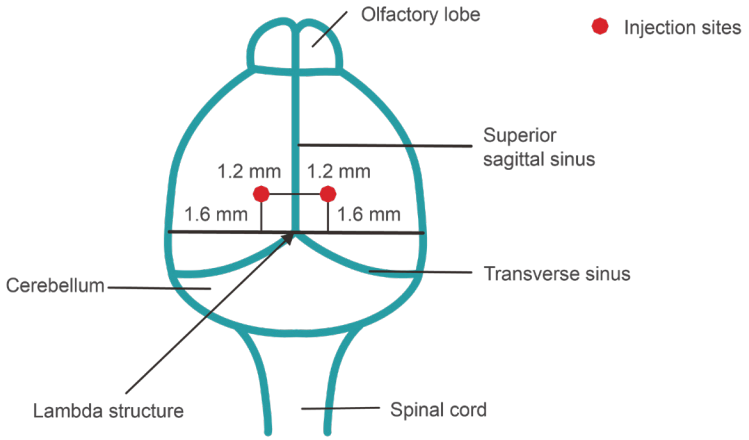


Figure 4. Diagram of the top view of mouse lambda structure and injection sites

Figure 18. Diagram of the top view of mouse lambda structure and injection sites.

- Place the needle just above the left ventricle (x + 1.2 mm, y + 1.6 mm). Then pierce the mouse skull with the needle by adjusting the z axis.
- Immediately after piercing the skull, withdraw the needle out of the mouse slowly.

Note: The z axis position of the mouse skull can be slightly shifted when being pierced due to the pressure from the needle. Withdrawing the needle immediately after piercing the skull can help the skull move back to the original position

- Carefully put the needle back into the skull and move the needle down until the needle tip just touches the brain. Record the index of z axis (z_1) on the vernier scale.
- Lower the needle slowly for 2.5~3 mm so that the needle tip penetrates the center of the left ventricle. The indexes on the vernier scale should be x + 1.2 mm, y + 1.6 mm, z - 2.5~3 mm.
- Push the syringe slowly and inject 2.5 ul virus sample into the left ventricle. To prevent the AAV leaking from the brain, wait for 1 minute or even longer after injecting the virus sample.
- Slowly adjust the z axis to withdraw the needle from the mouse. This process should take about 2 minutes. Withdrawing the needle too fast may lead to AAV leakage.

5.2. Right ventricle

- Place the needle above the right ventricle (x - 1.2 mm, y + 1.6 mm). Then pierce the mouse skull with the needle by adjusting the z-axis.
- Immediately after piercing the skull, withdraw the needle out of the mouse slowly.

Note: The z axis position of the mouse skull can be slightly shifted when being pierced due to the pressure from the needle. Withdrawing the needle immediately after piercing the skull can help the skull move back to the original position.

- Carefully put the needle back into the skull and move the needle down until the needle tip just touches the brain. Record the index of z axis (z_2) on the vernier scale.
- Lower the needle slowly for 2.5~3 mm so that the needle tip penetrates the center of the right ventricle. The indexes on the vernier scale should be x - 1.2 mm, y + 1.6 mm, z - 2.5~3 mm.
- Push the syringe slowly and inject 2.5 ul virus sample to the right ventricle. To prevent the AAV leaking from the brain, wait for 1 minute or even longer after injecting the virus sample.
- Slowly adjust the z axis to withdraw the needle from the mouse. This process should take about 2 minutes. Withdrawing the needle too fast may lead to the AAV leakage.

Note: To avoid the death of mice, mice should not be kept at low temperature for over 30 minutes.

6. Recovery

Right after the injection, place the mouse on the pre-warmed heating pad until the body temperature and movement are restored.

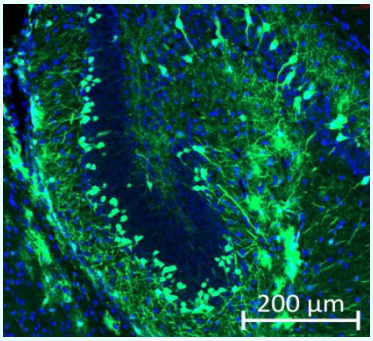
7. Observation

Return the mouse to the cage with the mother mouse. Monitor the condition of the mice until starting further experiments.

Anticipated Results

An example of successful in vivo transduction is shown below:

Figure 19. Mice were injected with the ultra-purified EGFP expressing AAV9 at a dose of $1x10^{13}$ GC/kg using the protocol of ICV in this document. 10 days after the injection, the brains from injected animals were dissected for analyses. Images of hippocampus were taken by a confocal microscope. Green: EGFP. Blue: DAPI.



Comparisons of AAV Delivery Methods In Vivo

CNS Gene Delivery

Delivery methods	Advantages	Disadvantages
Direct injection	Efficiently transduce a small area	Invasive
ntracisternal injection Intraventricular injection	Allows broad distribution of the vector across the CNS	Limited penetration into the brain parenchyma
Intrathecal injection	Distributes the vector through the CSF, but mostly targets the spinal cord and dorsal root ganglia (DRGs)	N/A
Intramuscular injection	Targets motor and sensory neurons via retrograde transport	N/A
Intravascular administration	Widespread transduction throughout the CNS	Requires high vector doses and peripheral exposure to the vector

Liver Gene Delivery

Delivery methods		Advantages	Disadvantages	Species
Direct Liver Delivery	Liver lobes injection	High Transduction efficiency	Highly invasive, require minor to major surgery, risk of operative and postoperative complications in coagulation-deficient animal models	Mouse
	Portal vein injection (PV)			Rat, Dog, NHP
	Splenic capsule injection			Mouse
	Hepatic artery injection (HA)			NHP
Peripheral Vector Delivery	Intraperitoneal injection (IP)	Less invasive, easy to perform	Less effective in large animal model due to the increased volume of the peritoneal cavity	Mouse
	Intravenous injection (IV)		Limited injection volume compared to IP	Mouse, Dog, NHP, Human

Cardiac Gene Delivery

Delivery methods	Advantages	Disadvantages
Peripheral intravenous injection	• Non-invasive • Simple	• Dilution issues • High expression in nontargeted organs • Low myocardial transduction rate
Antegrade coronary injection	• Clinically relevant • Less invasive	• Lower transduction efficiency • Limited delivery to the ischemic area
Retrograde coronary injection	Higher transduction efficiency	Requires blockage of antegrade flow
Aortic clamping	Higher transduction efficiency	Highly invasive
Direct intramyocardial injection	• No endothelial barrier • No first pass-effect of liver and spleen • Reduced T-cell triggered inflammation • Independent of neutralizing antibodies • Endomyocardial approach being less invasive	• Surgical delivery being the most invasive delivery method • Damage along the needle track • Region of interest might be not completely covered as a result of a restricted area of injection • Limitation of vector delivery because of leakage at the injection site
Pericardial injection	• Minimally invasive • High concentration and long duration of vector possible • Little systemic vector distribution	• Transduction mostly in pericardial cells and only very limited in myocardial cells • Possible leakage of injected agent into thorax cavity with transduction of nontargeted organs • In future clinical use, difficult to apply after pericarditis or previous cardiovascular surgery

Muscle Gene Delivery

Delivery methods		Advantages	Disadvantages	Diseases
Localized Delivery	Intramuscular injection	• Easy to administer High transduction in muscle • Low off-target vector delivery • Low immune response	Not able to transduce large muscle mass	A1ATD, LGMD2D, Pompe disease, etc.
	Transvenous limb perfusion			DMD
	Intracoronary infusion			Heart failure, myocardial infarction and LGMD
	Recirculating delivery			Heart failure
	Transendocardial injection			Heart failure and DMD
Systemic delivery	Intravascular injection	Widespread transduction of large muscle mass, potentially the muscles of whole body	• Off-target vector delivery Immune responses • Need of high doses of vector • Limited to certain rAAV serotypes	FSHD, myocardial infarction
	Intraperitoneal injection			DMD, LGMD

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August 2024 (EN)