Cat. # 6230, 6234,
6650 - 6665,
6668 - 6673,
6680 - 6685,
6690 - 6695

For Research Use

TaKaRa

AAVpro® Helper Free System

Product Manual

Serotype 9 (AAV9) series (Cat. #6690/6691/6696/6692/6693/6694/6695) are not available for sale in the United States.

v202504Da

Table of Contents

Safety & Handling of Adeno-Associated Virus Vectors

The protocols in this user manual require the handling of adeno-associated virus (AAV) vectors. It is imperative to fully understand the potential hazards of and necessary precautions for laboratory use of these vectors.

Viruses produced with AAV-based vectors could, depending on your gene insert, be potentially hazardous. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo.* For these reasons, due caution must be exercised in the production and handling of any recombinant viruses.

Follow all applicable guidelines for research involving recombinant DNA. Take appropriate safety measures when producing or handling recombinant AAVs, including working in a biological safety cabinet and wearing protective laboratory coats, face protection, and gloves.

I. Introduction

I-1. Adeno-associated viral vectors

Adeno-associated Virus (AAV) is a non-enveloped virus that belongs to the *Parvovirus* family of the *Dependovirus* genus. AAV is not thought to be pathogenic to humans and only replicates in the presence of a helper virus, such as adenovirus or herpesvirus. The AAV genome is a single-stranded DNA of approximately 4.7 kb, with T-shaped hairpin inverted terminal repeats (ITRs) at both ends (Fig. 1). The ITRs serve as the origin of replication, function as primers, and contribute to packaging of viral particles. The AAV genome encodes three ORFs: Rep, which is involved in replication and transcription; Cap, which encodes the outer coat protein of the viral particle; and AAP, a nonstructural protein essential for viral particle formation. The Rep region encodes four proteins (Rep78, Rep68, Rep52, and Rep40), and the Cap region encodes three proteins (VP1, VP2, and VP3).

There are more than 100 serotypes of AAV, and the host specificity and characteristics of the virus differ among serotypes. Takara Bio provides kits for preparation of AAV serotype 1 (AAV1), serotype 2 (AAV2), serotype 5 (AAV5), serotype 6 (AAV6), serotype 8 (AAV8), and serotype 9 (AAV9). AAV serotype 2 (AAV2) is the most commonly used serotype in AAV-based research, including gene therapy, and is characterized by a broad host range. The tissue host range of AAV1, AAV5, AAV6, AAV8, and AAV9 are more selective. AAV1 has high transduction efficiency for muscle, liver, respiratory tract, and central nervous system tissue; AAV5 has high transduction efficiency for central nervous system, liver, and retinal tissues; AAV6 has high transduction efficiency for cardiomyocyte, muscle, and liver tiisues; AAV8 has high transduction efficiency for liver, muscle, and central nervous system tissues; AAV9 has high tranduction efficiency for central nervous system, heart, live and muscle tissues.

Adeno-associated virus vectors (AAV vectors) exploit the properties of AAV for transduction of genes into cells and organisms. AAV vectors are used as research tools and also as vectors for gene therapy. In addition, AAV vectors are generally considered safer than adenoviral and retroviral vectors. AAV vectors can be used to transduce genes into both proliferating and non-proliferating cells and can impart long-term expression in non-dividing cells. In addition, AAV has little immunogenicity and is suitable for the transduction of genes into animals (as an *in vivo* transduction tool).

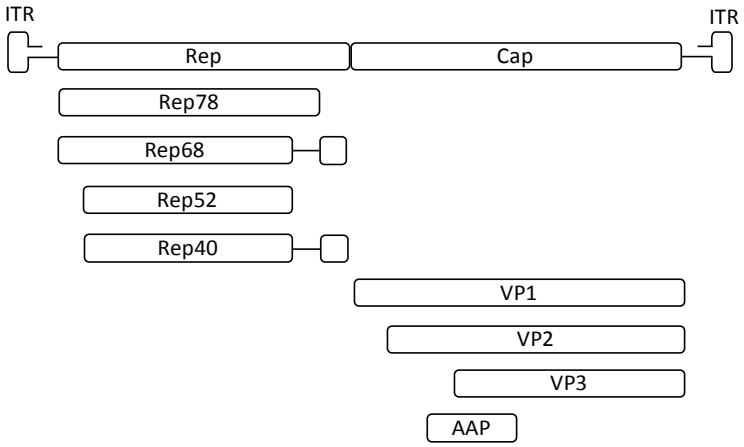


Fig. 1. Wild-type AAV genome structure and encoded proteins

I-2. Product descriptions

[AAVpro Helper Free System series]

The AAVpro Helper Free System series enables the preparation of AAV particles of serotype 1, 2, 5, 6, 8, or 9 without the use of a helper virus (Fig. 2). The AAV particles produced can be used to obtain transient expression of the target gene in mammalian cells or individual animals. For *in vivo* applications, we recommended purification of your AAV vectors prior to transduction.*1

[AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series]

RNA interference (RNAi) suppresses gene expression by degrading the mRNA of a target gene through the introduction of double-stranded RNA. RNAi is achieved in mammalian cells by using short double-stranded RNA fragments (short interfering RNA; siRNA) of 21 to 23 bases. For RNAi experiments, synthetically-produced siRNAs can be introducted directly into cells or tissue, or siRNAs can be transcribed within the target cell or tissue using expression vectors. Introduction of synthetic siRNAs results in transient RNAi, while use of expression vectors can lead to more stable RNAi.

The AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series enables preparation of AAV particles of serotype 1, 2, 5, 6, 8, or 9 that transiently express siRNA to supress expression of target gene(s) within mammalian cells or individual animals, without the use of a helper virus. Expression of short hairpin RNA (shRNA), a type of siRNA, are driven by an RNA polymerase III promoter. For *in vivo* applications, we recommended purification of AAV particles prior to transduction.*1

*1 We recommended the AAVpro Purification Kit Maxi (All Serotypes) (Cat. #6666) or the AAVpro Cell & Sup. Purification Kit Maxi (All Serotypes) (Cat. #6676) to purify AAV particles. These products provide a simple and rapid method for obtaining highly pure AAV particles.

For serotype 2, highly pure AAV particles can also be obtained using the AAVpro Purification Kit (AAV2) (Cat. #6232), which utilizes affinity purification.

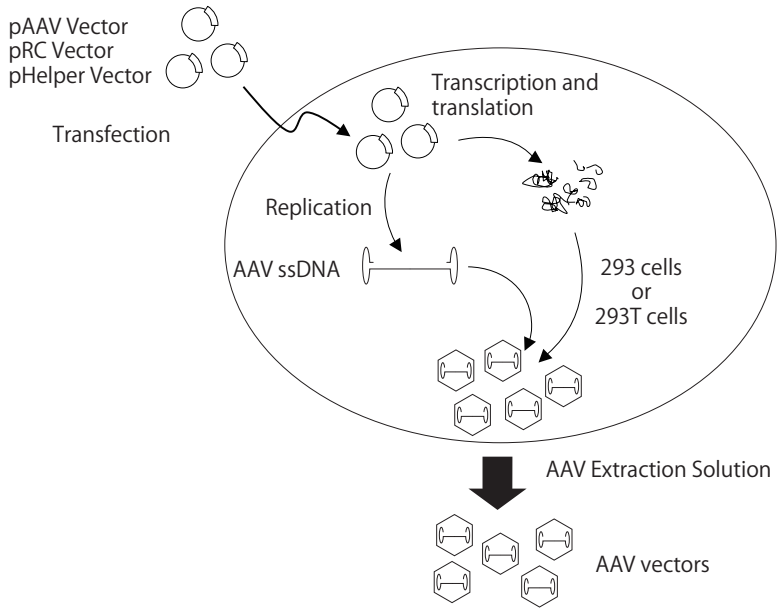


Fig. 2. Producing AAV vectors using this system

I-3. Features

1. Efficient production of AAV vectors without the use of helper viruses

The AAVpro Helper Free System series enables production of AAV vectors enabling transfection of the multiple plasmids required for AAV particle production into HEK293 or HEK293T cells.

[AAVpro Helper Free System series]

The AAVpro Helper Free System series requires the following three types of plasmids:

- pAAV Vectors: vectors containing expression cassettes for genes of interest and two ITRs

  pAAV-CMV Vectors (Cat. #6673/6230/6650/6651/6680/6690)

  These vectors contain a site for cloning a gene of interest (GOI), which is expressed from a CMV promoter. The size of the GOI cloned into the pAAV-CMV vector should be <2.5 kb as there is a limit to the size of DNA that can be encapsulated in AAV particles.

  pAAV-CRE Recombinase Vectors (Cat. #6668/6652/6653/6654/6682/6693)

  These vectors contain a *Cre* gene, a loxP-dependent recombinase. Cre recombinase is widely used in the production of transgenic mice and in various screening systems.

  pAAV-LacZ Vectors (Cat. #6669/6655/6656/6657/6683/6692)

  These vectors contain a LacZ gene. Prepared AAV-LacZ vectors can be used as control vectors for *in vitro* and *in vivo* gene transfer.

- pRC Vectors: vectors containing the AAV2 Rep gene and the Cap gene of a given serotype

pRC1 Vector	: Serotype 1 (Cat. #6673/6668/6669)
pRC2-mi342 Vector*2	: Serotype 2 (Cat. #6230/6652/6655)
pRC5 Vector	: Serotype 5 (Cat. #6650/6653/6656)
pRC6 Vector	: Serotype 6 (Cat. #6651/6654/6657)
pRC8 Vector	: Serotype 8 (Cat. #6680/6682/6683)
pRC9 Vector	: Serotype 9 (Cat. #6690/6693/6692)

  *2 pRC2-mi342 expresses hsa-miR-342, a microRNA that increases AAV2 titer in vector preparation systems. It increases titer by approximately 2-fold as compared to ordinary pRC2 vectors that express only Rep and Cap.

- pHelper Vector: vector that contains the E2A, E4, and VA genes, derived from adenovirus

[AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series]

The AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series requires the following three types of plasmids:

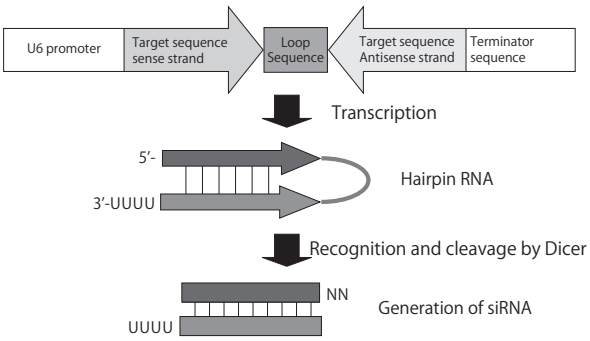
- pAAV Vectors: vector containing expression cassettes for shRNAs of interest and two ITRs.

  pAAV-U6-ZsGreen1 Vectors (Cat. #6670/6658/6659/6660/6685/6695)

  These vectors contain one site for cloning an shRNA. mU6 promoters drive shRNA expression, and a CMV promoter drives expression of ZsGreen1. ZsGreen1 expression allows for confirmation of transfection and further evaluation of transfected cells only.

  pAAV-2xU6 Vectors (Cat. #6671/6661/6662/6663/6684/6694)

  These vectors contain two separate cloning sites for cloning of two shRNAs, which are expressed from the hU6 and mU6 promoters, respectively. These vectors can be utilized for simultaneous knockdown two genes or to enhance the knockdown effect on a single gene by applying two types of shRNAs.



- pRC Vectors: vectors containing the AAV2 Rep gene and the Cap gene of a given serotype

  pRC1 Vector : Serotype 1 (Cat. #6670/6671)
  pRC2-mi342 Vector*3 : Serotype 2 (Cat. #6658/6661)
  pRC5 Vector : Serotype 5 (Cat. #6659/6662)
  pRC6 Vector : Serotype 6 (Cat. #6660/6663)
  pRC8 Vector : Serotype 8 (Cat. #6685/6684)
  pRC9 Vector : Serotype 9 (Cat. #6695/6694)

  *3 pRC2-mi342 expresses hsa-miR-342, a microRNA that increases AAV2 titer in vector preparation systems. It increases titer by approximately 2-fold as compared to ordinary pRC2 vectors that express only Rep and Cap.

- pHelper Vector: vector that contains the E2A, E4, and VA genes, derived from adenovirus

2. Simple process for AAV particle extraction

Extraction of AAV particles from AAV-producing cells is conventionally performed by freeze-thaw or sonication methods; however, these methods are time consuming and require special equipment. This kit includes AAV Extraction Solutions that allow simple and efficient AAV particle isolation while minimizing protein and nucleic acid contamination.

II. Components

II-1. Products

[AAVpro Helper Free System series]

- AAVpro Helper Free System (AAV1/AAV2/AAV5/AAV6/AAV8/AAV9)
  (Cat. #6673/6230/6650/6651/6680/6690)
- AAVpro Helper Free System (AAV-CRE Recombinase) (AAV1/AAV2/AAV5/AAV6/AAV8/AAV9)
  (Cat. #6668/6652/6653/6654/6682/6693)
- AAVpro Helper Free System (AAV-LacZ) (AAV1/AAV2/AAV5/AAV6/AAV8/AAV9)
  (Cat. #6669/6655/6656/6657/6683/6692)

[AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series]

- AAVpro Helper Free System (AAV-U6-ZsGreen1) (AAV1/AAV2/AAV5/AAV6/AAV8/AAV9)*
  (Cat. #6670/6658/6659/6660/6685/6695)
- AAVpro Helper Free System (AAV-2xU6) (AAV1/AAV2/AAV5/AAV6/AAV8/AAV9)*
  (Cat. #6671/6661/6662/6663/6684/6694)

* Not available in all geographic locations. Check for availability in your area.

II-2. Components

Each kit includes three plasmids encoding the factors necessary to prepare recombinant AAV particles, and two reagents for extracting AAV particles from producer cells.

<Serotype 1>

1. pAAV Vector (1 μg/μl)*1 — 20 μl
2. pRC1 Vector (1 μg/μl)*2 — 20 μl
3. pHelper Vector (1 μg/μl)*2 — 20 μl
4. AAV Extraction Solution A — 1.5 ml x 3
5. AAV Extraction Solution B — 150 μl x 3

*1 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6673
pAAV-CRE Recombinase Vector	: Cat. #6668
pAAV-LacZ Vector	: Cat. #6669
pAAV-U6-ZsGreen1 Vector	: Cat. #6670
pAAV-2xU6 Vector	: Cat. #6671

*2 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV1) (Cat. #6672)

pRC1 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

<Serotype 2>

1. pAAV Vector (1 μg/μl)*3 — 20 μl
2. pRC2-mi342 Vector (1 μg/μl)*4 — 20 μl
3. pHelper Vector (1 μg/μl)*4 — 20 μl
4. AAV Extraction Solution A — 1.5 ml x 3
5. AAV Extraction Solution B — 150 μl x 3

*3 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6230
pAAV-CRE Recombinase Vector	: Cat. #6652
pAAV-LacZ Vector	: Cat. #6655
pAAV-U6-ZsGreen1 Vector	: Cat. #6658
pAAV-2xU6 Vector	: Cat. #6661

*4 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV2) (Cat. #6234)

pRC2-mi342 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

Note: The following plasmid intended for clinical use can be purchased separately: AAVpro Packaging Plasmid (AAV2) (KmR) (Cat. #6266). See the product datasheet for more information.

Caution: The pRC2-Km vectors of AAVpro Packaging Plasmid (AAV2) (KmR) (Cat. #6266) do not contain the hsa-miR-342 (miRNA) expression cassette, that increased titer production 2-fold.

<Serotype 5>

1. pAAV Vector (1 μg/μl)*5 20 μl
2. pRC5 Vector (1 μg/μl)*6 20 μl
3. pHelper Vector (1 μg/μl)*6 20 μl
4. AAV Extraction Solution A 1.5 ml x 3
5. AAV Extraction Solution B 150 μl x 3

*5 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6650
pAAV-CRE Recombinase Vector	: Cat. #6653
pAAV-LacZ Vector	: Cat. #6656
pAAV-U6-ZsGreen1 Vector	: Cat. #6659
pAAV-2xU6 Vector	: Cat. #6662

*6 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV5) (Cat. #6664)

pRC5 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

<Serotype 6>

1. pAAV Vector (1 μg/μl)*7 20 μl
2. pRC6 Vector (1 μg/μl)*8 20 μl
3. pHelper Vector (1 μg/μl)*8 20 μl
4. AAV Extraction Solution A 1.5 ml x 3
5. AAV Extraction Solution B 150 μl x 3

*7 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6651
pAAV-CRE Recombinase Vector	: Cat. #6654
pAAV-LacZ Vector	: Cat. #6657
pAAV-U6-ZsGreen1 Vector	: Cat. #6660
pAAV-2xU6 Vector	: Cat. #6663

*8 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV6) (Cat. #6665)

pRC6 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

Note: The following plasmid intended for clinical use can be purchased separately: AAVpro Packaging Plasmid (AAV6) (KmR) (Cat. #6616). See the product datasheet for more information.

<Serotype 8>

1. pAAV Vector (1 μg/μl)*9 20 μl
2. pRC8 Vector (1 μg/μl)*10 20 μl
3. pHelper Vector (1 μg/μl)*10 20 μl
4. AAV Extraction Solution A 1.5 ml x 3
5. AAV Extraction Solution B 150 μl x 3

*9 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6680
pAAV-CRE Recombinase Vector	: Cat. #6682
pAAV-LacZ Vector	: Cat. #6683
pAAV-U6-ZsGreen1 Vector	: Cat. #6685
pAAV-2xU6 Vector	: Cat. #6684

*10 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV8) (Cat. #6681)

pRC8 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

Note: The following plasmid intended for clinical use can be purchased separately: AAVpro Packaging Plasmid (AAV8) (KmR) (Cat. #6866). See the product datasheet for more information.

<Serotype 9>

1. pAAV Vector (1 μg/μl)*11 20 μl
2. pRC9 Vector (1 μg/μl)*12 20 μl
3. pHelper Vector (1 μg/μl)*12 20 μl
4. AAV Extraction Solution A 1.5 ml x 3
5. AAV Extraction Solution B 150 μl x 3

*11 The following pAAV Vectors can be used for component 1:

pAAV-CMV Vector	: Cat. #6690
pAAV-CRE Recombinase Vector	: Cat. #6693
pAAV-LacZ Vector	: Cat. #6692
pAAV-U6-ZsGreen1 Vector	: Cat. #6695
pAAV-2xU6 Vector	: Cat. #6694

*12 A high volume set consisting of the virus-production plasmids (components 2 and 3) can be purchased separately:

AAVpro Packaging Plasmid (AAV9) (Cat. #6691)

pRC9 Vector (1 μg/μl)	500 μl x 2
pHelper Vector (1 μg/μl)	500 μl x 2

Note: The following plasmid intended for clinical use can be purchased separately: AAVpro Packaging Plasmid (AAV9) (KmR) (Cat. #6696). See the product datasheet for more information.

III. Vector Maps

Sequence information for each plasmid can be downloaded at takarabio.com.

[AAVpro Helper Free System series]

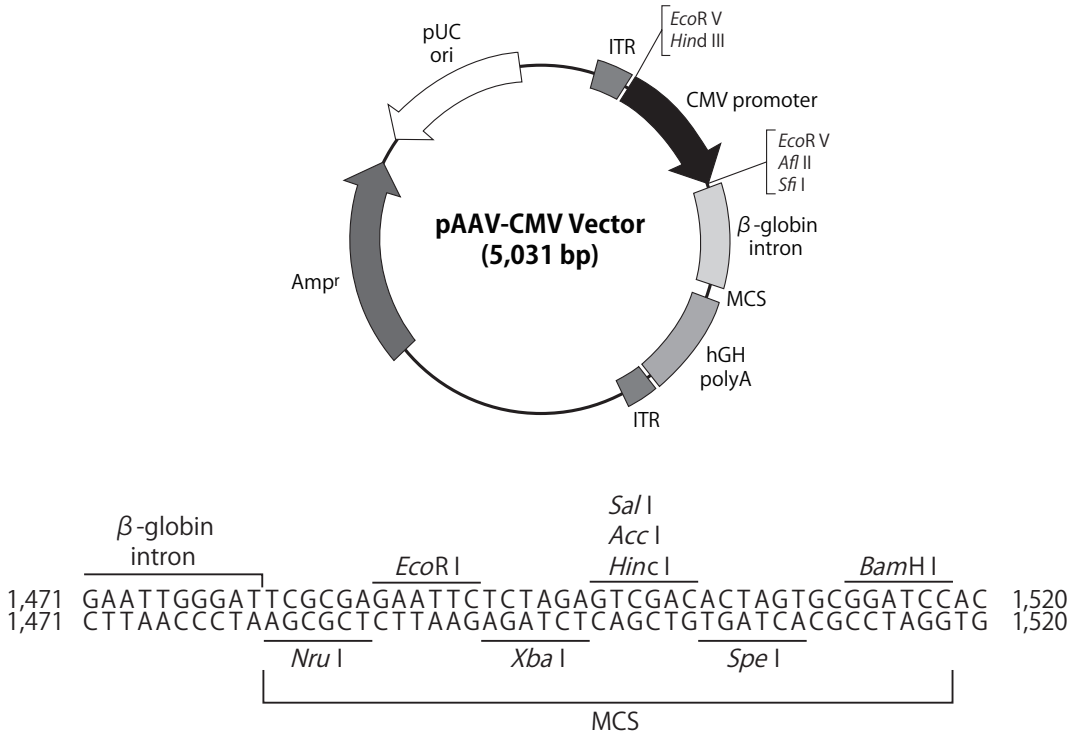


Fig. 3. pAAV-CMV Vector map and multiple cloning sites (MCS)

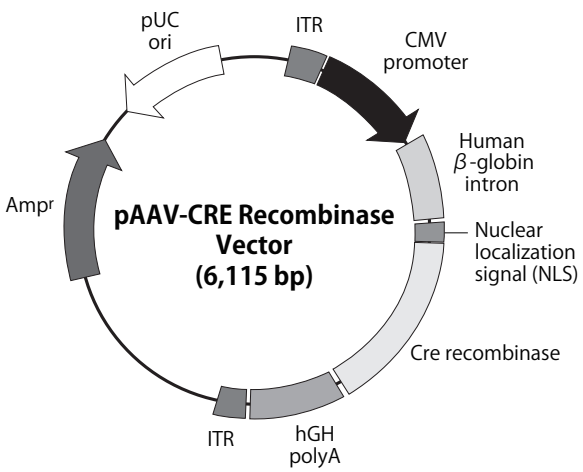


Fig. 4. pAAV-CRE Recombinase Vector map

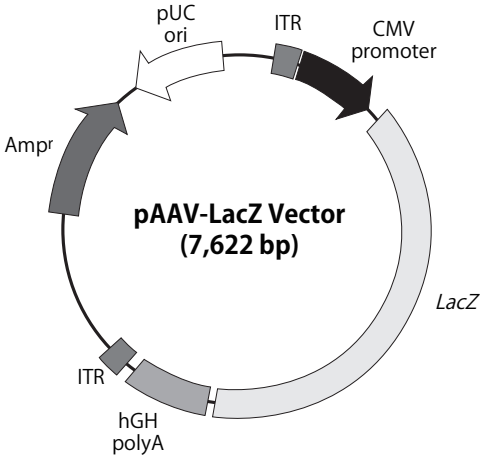


Fig. 5. pAAV-LacZ Vector map

[AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series]

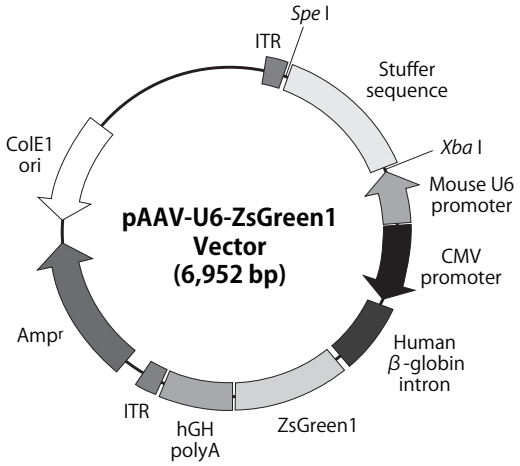


Fig. 6. pAAV-U6-ZsGreen1 Vector map

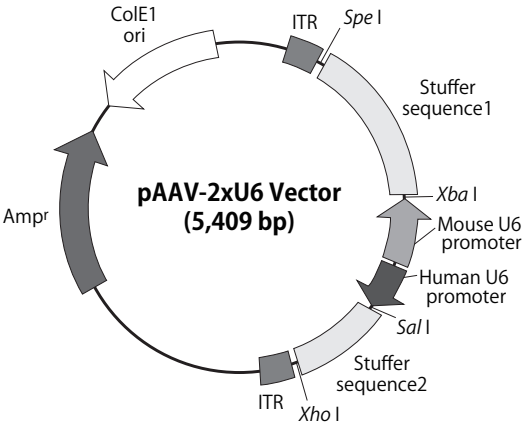


Fig. 7. pAAV-2xU6 Vector map

[Common to both series]

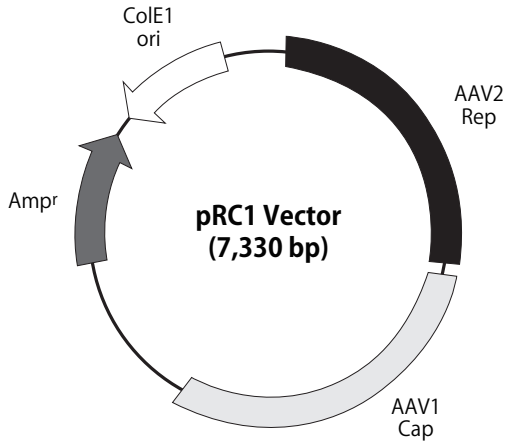


Fig. 8. pRC1 Vector map

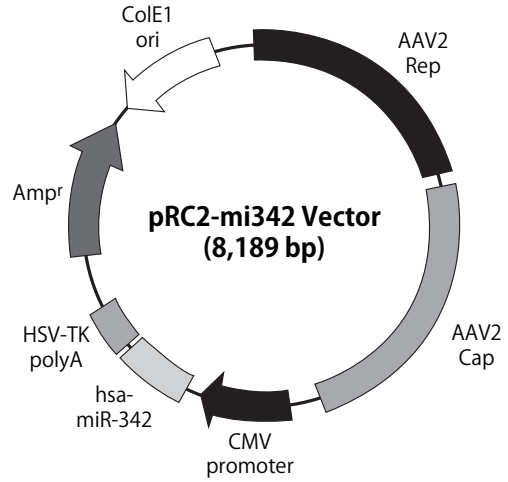


Fig. 9. pRC2-mi342 Vector map

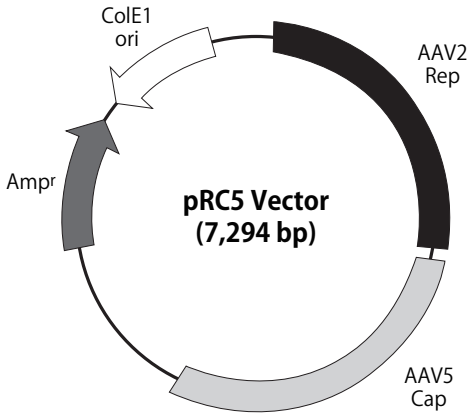


Fig. 10. pRC5 Vector map

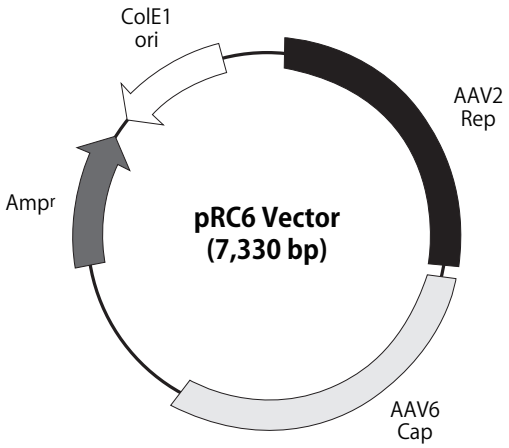


Fig. 11. pRC6 Vector map

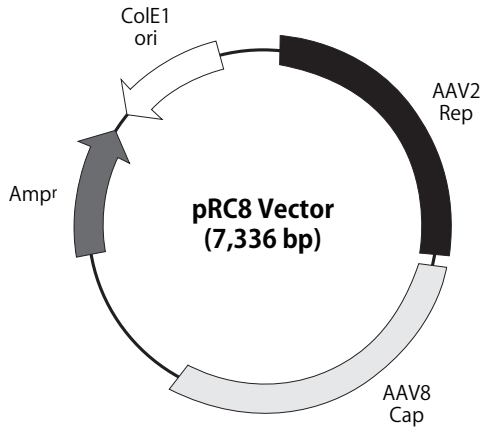


Fig. 12. pRC8 Vector map

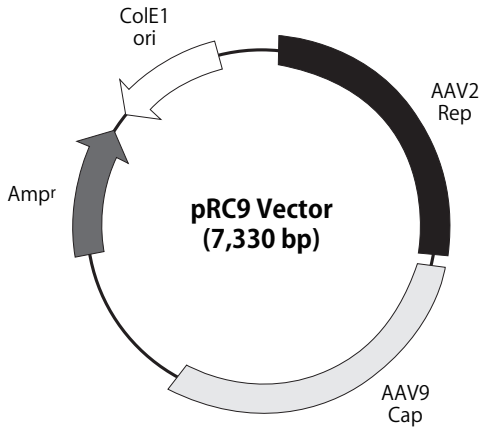


Fig. 13. pRC9 Vector map

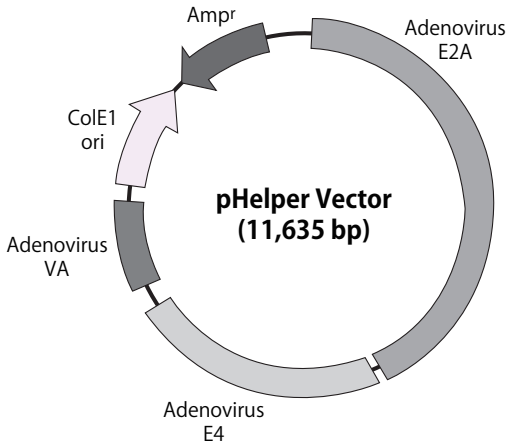


Fig. 14. pHelper Vector map

IV. Storage

Store at -20℃

Store AAV Extraction Solution A and AAV Extraction Solution B at room temperature after thawing.

Use within 2 years of receipt.

V. Materials Required but not Provided

V-1. Equipment

- 100 mm tissue culture-treated dishes
- General equipment required for cell culture

V-2. Reagents

- Transfection reagent:
  a. CalPhos™ Mammalian Transfection Kit (Cat. #631312) or
  b. *Trans*IT-293 Transfection Reagent (Cat. #MIR2704)*1 or
  c. Xfect™ Transfection Reagent (Cat. #631317)
- Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/L Glucose with L-Glutamine (584 mg/L)
- Fetal Bovine Serum (FBS)
- Trypsin EDTA
- HEK293 or HEK293T cells*2
- 0.5 M EDTA (pH8.0) (EDTA Buffer Powder, pH 8.0 (Cat. #T9191))
- Positive control vectors:
  pAAV-ZsGreen1 Vector (Cat. #6231)*3
  pAAV-ZsGreen1 Vector (KmR) (Cat. #6610)*3, 4
- In-Fusion® Snap Assembly Master Mix (Cat. #638947, etc.)*5
- Double-stranded DNA oligo preparation buffer (10 mM Tris-HCl (pH 8.0), 50 mM NaCl)*5, 6

*1 Not available in all geographic locations. Check for availablity in your area.

*2 Several HEK293 and HEK293T cell lines are commercially available, and viral production is highly variable depending on the cell line. We recommend the AAVpro 293T Cell Line (Cat. #632273), which has been optimized for AAV vector production. Please check titers of the produced AAV vectors before use.

*3 pAAV-ZsGreen1 Vector expresses the green fluorescent protein ZsGreen1, and is useful as a positive control for checking transfection efficiency and biological titers of the produced AAVs.

*4 If using the AAVpro Packaging Plasmid (KmR), use the pAAV-ZsGreen1 Vectors (KmR) for cloning your gene of interest according to your needs.

*5 This reagent is only required for the AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series.

*6 A general 1X PCR buffer can also be used for preparing double-stranded DNA oligos (e.g., 10X *Ex Taq* Buffer (20 mM Mg^{2+} plus) provided with *TaKaRa Ex Taq®* (Cat. #RR001A) diluted to 1X).

VI. Protocol

VI-1. Overview of AAV particle production

1. Clone a GOI into a pAAV-CMV Vector
   or
   Clone dsDNA oligo into a pAAV-U6-ZsGreen1 Vector or pAAV-2xU6 Vector
   ↓
2. Prepare recombinant plasmid (pAAV vector)
   ↓
3. Culture HEK293 or HEK293T cells
   ↓
4. Co-transfect HEK293 or HEK293T cells with pAAV, pRC, and pHelper vectors
   ↓
5. Change culture medium
   ↓
6. Collect AAV vector-producing cells (2 - 3 days after transfection)
   ↓
7. Extract virus particles from AAV-producing cells

VI-2. Protocol

1. Cloning into pAAV series vectors

[When using the AAVpro Helper Free System]

Using standard cloning methods, the coding sequences of genes of interest (GOI) are inserted into the multiple cloning sites (MCS) of the chosen pAAV-CMV Vector. Additionally, the *Eco*RV site of the chosen pAAV-CMV Vector can be used to replace the CMV promoter region with another promoter (see Fig. 3).

In-Fusion Snap Assembly Master Mix (e.g., Cat. #638947) can also be used for cloning. This kit allows easy cloning of PCR products into any linear vector. For transformation following the In Fusion reaction, we recommend Stellar™ Competent Cells (Cat. #636763)*.

Sequence information for all pAAV-CMV Vectors can be downloaded at takarabio.com.

* Not available in all geographic locations. Check for availability in your area.

[When using the AAVpro Helper Free System (AAV-U6-ZsGreen1)/(AAV-2xU6) series]

<DNA oligo design for shRNA expression>

For cloning of the double-stranded DNA (dsDNA) oligo (that express desired shRNAs) into the pAAV-U6-ZsGreen1 or pAAV-2xU6 Vector series, we recommend using In-Fusion Snap Assembly Master Mix (Cat. #638947, etc.).

Both a top and bottom strand DNA oligo need to be synthesized, and the sequence should contain the following elements as shown below: a linking sequence (15 bases), the shRNA target sequence (sense) (N), a hairpin loop sequence, the shRNA target sequence (antisense) (N), a terminator sequence , and finally, another linking sequence (15 bases). Since the transcription start point of the pol III promoter is preferably a purine base (G or A), if the target sequence does not start with G or A, a G or A should be included before the target sequence.

After synthesis of the top and bottom strand oligos and preparation of the dsDNA oligo, the dsDNA oligo is inserted downstream of the promoter using In-Fusion cloning. shRNA oligo DNA-1 is inserted into the *Xba* I-*Spe* I site, and shRNA oligo DNA-2 is inserted into the *Sal* I-*Xho* I site.

The CTGTGAAGCCACAGATGGG (Boden *et al.*[7]) hairpin loop sequence below is given as an example of a loop sequence, but GTGTGCTGTCC (Miyagishi *et al.*[8]) has also been confirmed to be functional. Other hairpin loop sequences have been reported by Lee *et al.*[9], Paddison *et al.*[10], Paul *et al.*[11], and Sui *et al.*[12]. When two shRNA expression units are carried in one vector, we recommended using separate loop sequences for each shRNA to reduce the risk of recombination within the vector.

We recommened using the TTTTTT terminator sequence, as four consecutive T's stop transcription from pol III promoters. Depending on the combination of the target sequence and the hairpin loop sequence used, there may be four or more consecutive T's within the sequence before the terminator. After designing the DNA oligo, confirm that the top strand does not have four or more consecutive T's prior to the terminator sequence.

◆ Synthetic DNA sequences for In-Fusion reactions

shRNA oligo DNA-1 (*Xba* I-*Spe* I site)
<Linking sequence - target sequence (sense) - loop sequence - target sequence (antisense) - terminator sequence - linking sequence>

Transcription start point*

	Linking sequence	target sequence (sense)	Hairpin loop	target sequence (antisense)	Terminator	Linking sequence
Top strand	5'-GAGAAAAGCCTCTAG(G/A)	NNNNNNNNNNNNNNNNNNN	CTGTGAAGCCACAGATGGG	NNNNNNNNNNNNNNNNNNN	(C/T)TTTTTT	CTAGTGATATCGATA-3'
Bottom strand	3'-CTCTTTTCGGAGATC(C/T)	NNNNNNNNNNNNNNNNNNN	GACACTTCGGTGTCTACCC	NNNNNNNNNNNNNNNNNNN	(G/A)AAAAAA	GATCACTATAGCTAT-5'

shRNA oligo DNA-2 (*Sal* I-*Xho* I site)
<Linking sequence - target sequence (sense) - loop sequence - target sequence (antisense) - terminator sequence - linking sequence>

Transcription start point*

	Linking sequence	target sequence (sense)	Hairpin loop	target sequence (antisense)	Terminator	Linking sequence
Top strand	5'-GAAAGGACGAGTCGA(G/A)	NNNNNNNNNNNNNNNNNNN	CTGTGAAGCCACAGATGGG	NNNNNNNNNNNNNNNNNNN	(C/T)TTTTTT	TCGAGAGATCTAGGA-3'
Bottom strand	3'-CTTTCCTGCTCAGCT(C/T)	NNNNNNNNNNNNNNNNNNN	GACACTTCGGTGTCTACCC	NNNNNNNNNNNNNNNNNNN	(G/A)AAAAAA	AGCTCTCTAGATCCT-5'

*If the first base of the target sequence is not G or A, insert G or A before the target sequence.

<Sequence primers>

shRNA oligo DNA-1 sequence confirmation primer	5'-AAGCTTGAATTCGATCCGACG-3'
shRNA oligo DNA-2 sequence confirmation primer	5'-GAATTCAAGCTTAAGGTCGGG-3'

Note: It is also possible to clone dsDNA oligos by ligation using the restriction enzyme sites that sandwich the stuffer sequence on the vector. For the design of DNA oligos in such cases, see "VII-2. Cloning of DNA oligos by ligation."

<Cloning DNA oligos into the pAAV-U6-ZsGreen1 Vector>

1) Prepare the dsDNA oligo.
   (1) Dilute each single-stranded DNA oligo (top strand and bottom strand) with double-stranded DNA oligo preparation buffer to a final concentration of 20 pmol/μl.
   (2) Incubate at 95℃ for 5 min using a thermal cycler or similar device, then slowly cool to 25℃ over 30 min or more.

2) Linearize the pAVV-U6-ZsGreen1 Vector.
   Digest the pAAV-U6-ZsGreen1 Vector with *Xba* I and *Spe* I. This will separate the ~900 bp stuffer sequence from the rest of the plasmid, which will be about 6 kb. Run the digest on an agarose gel, and purify the ~6 kb linearized vector fragement.

3) Perform the In-Fusion reaction.
   Prepare the following reaction mixture:

Reagent	Volume
5X In-Fusion Snap Assembly Master Mix	2 μl
Purified DNA vector	50 - 100 ng
dsDNA oligo DNA	3 pmol
dH_2O	x μl
Total	10 μl

   Incubate at 50℃ for 15 min, then transfer to ice.

   Note: For details, see the In Fusion Snap Assembly User Manual.

4) Transform competent cells with the In-Fusion reaction mixture. We recommended Stellar Competent Cells (Cat. #636736) for transformation.

<Cloning DNA oligos into the pAAV-2xU6 Vector>

The pAAV-2xU6 Vector has two cloning sites. To ulitize both sites, clone a dsDNA oligo into one site in the same way as with a pAAV-U6-ZsGreen1 Vector, then purify the plasmid and use it to clone the second dsDNA oligo into the other site. When digested with *Xba* I and *Spe* I, the linearized pAAV-2xU6 Vector is about 4.5 kb; when digested with *Sal* I and *Xho* I, the linearized pAAV-2xU6 Vector is about 4.9 kb.

2. Preparing cloned pAAV series vectors for transfection

After confirming the presence of the correct inserts, prepare plasmid DNA for transfection using a plasmid purification kit, such as NucleoBond Xtra Midi/Maxi (Cat. #740410.10/740414.10, etc.)*. Adjust the plasmid DNA concentration to 1 μg/μl.

Note: To minimize insoluble impurities in the prepared DNA solution, centrifuge the obtained plasmid DNA at 13,500*g* for 5 min. Use the supernatant as the plasmid DNA solution.

* Not available in all geographic locations. Check for availablity in your area.

3. Culturing HEK293 or HEK293T cells

Inoculate a 100-mm cell culture dish with 2.5 - 4.0 x 10^6 HEK293 or HEK293T cells in DMEM culture medium supplemented with 10% FBS.

Note: If you are planning to use the CalPhos Mammalian Transfection Kit or Xfect Transfection Reagent in Step 4 below, use 10 ml of culture medium. If you are planning to use *Trans*IT-293 Transfection Reagent, use 15 ml of culture medium.

4. Co-transfecting the pAAV Vector, pRC Vector, and pHelper Vector into HEK293 or HEK293T cells

One day after plating HEK293/HEK293T cells from Step 3, co-transfect the cells with the purified pAAV Vector purified in Step 2, and the pRC Vector and pHelper Vector included in the AAVpro Helper Free System series.

The following three types of transfection reagents are recommended.

a. CalPhos Mammalian Transfection Kit (Cat. #631312)

b. *Trans*IT-293 Transfection Reagent (Cat. #MIR2704)

c. Xfect Transfection Reagent (Cat. #631317)

The CalPhos Mammalian Transfection kit is the most cost-effective transfection option. *Trans*IT-293 Transfection Reagent and Xfect Transfection Reagent, although less cost effective, transfect cells with higher efficiency, leading to higher AAV titers. The transfection protocols for each reagent are provided below.

<a. Co-transfection with the CalPhos Mammalian Transfection Kit>

The following protocol has been partially modified from the protocol included with the CalPhos Mammalian Transfection Kit for use with the AAVpro Helper Free System series. This modified protocol results in higher AAV titers.

1) Bring 2X HEPES-Buffered Saline to room temperature.
2) Dilute 2 M Calcium Solution with sterile H_2O (included in the kit) to obtain a 333 mM Calcium Solution (6-fold dilution), and bring to room temperature.
3) Combine vector DNA and Calcium Solution as shown below.

Reagent		Volume
pAAV-GOI/dsDNA oligo Vector	1 μg/μl	6 μl
pRC Vector	1 μg/μl	6 μl
pHelper Vector	1 μg/μl	6 μl
Calcium Solution	333 mM	1,000 μl
Total		1,018 μl

4) Add an equal volume of room-temperature 2X HEPES-Buffered Saline to the mixture from 3). Close the tube and mix by shaking vigorously up and down 15 times.
5) Incubate at room temperature for 3 min.

   Caution: Adhere to a strict 3 min incubation time, then proceed quickly to the next step. With longer incubation, large calcium phosphate-DNA complexes will form and transfection efficiency will decrease.
6) Add the mixture dropwise to the HEK293/HEK293T cell cultures that were plated in Step 3. Return HEK293/HEK293T cell cultures to a cell culture incubator.

<b. Co-transfection with *Trans*IT-293 Transfection Reagent>

1) Bring *Trans*IT-293 Transfection Reagent to room temperature. Mix by vortexing before use.
2) Combine serum-free DMEM and vector DNA as shown below. Gently pipet the mixture up and down to mix.

Reagent		Volume
pAAV Vector	1 μg/μl	5 μl
pRC Vector	1 μg/μl	5 μl
pHelper Vector	1 μg/μl	5 μl
Serum-free DMEM or Opti-MEM		1,500 μl
Total		1,515 μl

3) Add 45 μl of *Trans*IT-293 Transfection Reagent to the mixture from 2) and gently pipet the mixture up and down to mix. Let stand at room temperature for 15 to 30 min.
4) Add the mixture dropwise to the HEK293/HEK293T cell cultures that were plated in Step 3. Return HEK293/HEK293T cell cultures to a cell culture incubator.

<c. Co-transfection with Xfect Transfection Reagent>

1) Vortex the Xfect Polymer.
2) Combine Xfect Reaction Buffer and vector DNA as shown below. Vortex vigorously for 5 sec.

Reagent		Volume
pAAV Vector	1 μg/μl	13 μl
pRC Vector	1 μg/μl	13 μl
pHelper Vector	1 μg/μl	13 μl
Xfect Reaction Buffer		561 μl
Total		600 μl

3) Add 11.7 μl of Xfect Polymer to the mixture from 2). Vortex for 10 sec.
4) Incubate at room temperature for 10 min.
5) Centrifuge the solution briefly. Add the mixture dropwise to the HEK293/HEK293T cell cultures that were plated in Step 3. Return HEK293/HEK293T cell cultures to a cell culture incubator.

5. Changing culture medium

At least 6 hours after transfection (up to 25 hours), completely replace the culture medium with fresh DMEM containing 2% FBS.

Note: If the CalPhos Mammalian Transfection Kit was used for transfection, calcium phosphate crystals will be visible under a microscope.

6. Collecting AAV-producing cells (2 - 3 days after transfection)

1) Add 1/80 volume of 0.5 M EDTA (pH 8.0) to a culture medium containing AAV-producing cells and mix well. Allow to stand at room temperature for 10 min.
2) Collect the detached cells in a sterile centrifuge tube. Centrifuge at 1,750*g* at 4℃ for 10 min. Completely remove the supernatant and collect the cell pellet.

   Note: Confirm that the supernatant has been completely removed before proceeding; viral particle isolation may be affected by residual supernatant.

7. Extracting AAV particles from AAV-producing cells

The use of the AAV Extraction Solution included in the kit is strongly recommended. This method yields AAV particles with higher purity and titer than standard freeze-thaw or sonication methods.

1) Loosen the cell pellet from Step 6 by tapping or vortexing the tube.
   Note: If the cell pellet has not been loosened sufficiently, the efficiency of extraction may decrease. Confirm that there are no clumps of cells before proceeding.
2) Add 0.5 ml of AAV Extraction Solution A.
3) Resuspend the cell pellet by vortexing for 15 sec.
4) Incubate at room temperature for 5 min. Resuspend cells again by vortexing for 15 sec.
5) Centrifuge at 2,000 - 14,000*g* at 4℃ for 10 min.
6) Collect the supernatant into a new tube.
7) Add 50 µl of AAV Extraction Solution B. Mix by pipetting.
   Note 1: If recovered AAV titer is low, extraction efficiency may be improved by repeating steps 3) - 5).
   Note 2: Extracted AAV particles can be stored at -80℃.
   Note 3: Some samples may turn pink when the AAV Extraction Solution B is added. This does not affect the success fo the extraction.

VII. Additional Information

VII-1. Measuring viral titer

Viral titer can be measured by real-time PCR (vector genome assay) or by infection assay (biological titer measurement). Real-time PCR analysis of vector genomes provides rapid quantification, whereas determining titer by infection into cells is generally more accurate to determine infectious virus titer. There are other titration methods for AAV vectors that involve assay of viral capsid proteins, but these methods may detect nonfunctional (empty) particles.

<Vector genome quantification>

The AAVpro Titration Kit (for Real Time PCR) Ver.2 (Cat. #6233) can be used to measure virus titer by real-time PCR analysis using the viral ITR domain as a target.

<Measuring biological titers of AAV particles with a ZsGreen1 gene>

Biological titer measurement involves detecting the expression of the introduced genes. The protocol below enables measuring biological titers of AAV vectors carrying the ZsGreen1 gene (prepared using a pAAV-ZsGreen1 Vector (Cat. #6231) and the AAVpro Helper Free System series).

1) Prepare target cells at 2 - 4 x 10^4 cells/ml in DMEM containing 10% FBS.
2) Seed cells at 0.5 ml/well into a cell culture surface-treated 24-well plate. Incubate the plate in a cell culture incubator overnight.
3) Prepare serial dilutions of the prepared AAV particle solution using DMEM with 10% FBS and then infect the cell with the diluted virus solution. The dilution ratio depends on the virus titer, but serial dilutions in the 1,000 - 100,000-fold range are recommended.
4) Two to three days after infection, detach cells with Trypsin/EDTA, harvest cells, and analyze ZsGreen1 expression by flow cytometry.

VII-2. Cloning of DNA oligos by ligation

Note: This section only applies to the AAVpro Helper Free System (AAV-U6-ZsGreen1)/ (AAV-2xU6) series.

Both a top and bottom strand DNA oligo need to be synthesized prior to cloning by ligation. The sequences should contain the following elements as shown below: a linking sequence, the shRNA target sequence (sense) (N), a hairpin loop sequence, the shRNA target sequence (antisense) (N), a terminator sequence , and finally, another linking sequence. Since the transcription start point of the pol III promoter is preferably a purine base (G or A), if the target sequence does not start with G or A, a G or A should be included before the target sequence.

The CTGTGAAGCCACAGATGGG (Boden *et al.*[7]) hairpin loop sequence below is given as an example of a loop sequence, but GTGTGCTGTCC (Miyagishi *et al.*[8]) has also been confirmed to be functional. Other hairpin loop sequences have been reported by Lee *et al.*[9], Paddison *et al.*[10], Paul *et al.*[11], and Sui *et al.*[12]. When two shRNA expression units are carried in one vector, we recommended using separate loop sequences for each shRNA to reduce the risk of recombination within the vector.

We recommened using the TTTTTT terminator sequence, as four consecutive T's stop transcription from pol III promoters. Depending on the combination of the target sequence and the hairpin loop sequence used, there may be four or more consecutive T's within the sequence before the terminator. After designing the DNA oligo, confirm that the top strand does not have four or more consecutive T's prior to the terminator sequence.

<Xba I-target sequence (sense) - loop sequence - target sequence (antisense) - terminator sequence - *Spe* I>

		Transcription start point*					
		Xba I ↓	target sequence (sense)	Hairpin loop	target sequence (antisense)	Terminator	*Spe* I
Top	5'-pCTAGA(G/A)	NNNNNNNNNNNNNNNNNNN	CTGTGAAGCCACAGATGGG	NNNNNNNNNNNNNNNNNNN	(C/T)TTTTTT	A-3'	
Bottom	3'-T(C/T)	NNNNNNNNNNNNNNNNNNN	GACACTTCGGTGTCTACCC	NNNNNNNNNNNNNNNNNNN	(G/A)AAAAAA	TGATCp-5'	

<Sal I - target sequence (sense) - loop sequence - target sequence (antisense) - terminator sequence - *Xho* I>

		Transcription start point*					
		Sal I ↓	target sequence (sense)	Hairpin loop	target sequence (antisense)	Terminator	*Xho* I
Top	5'-pTCGAC(G/A)	NNNNNNNNNNNNNNNNNNN	CTGTGAAGCCACAGATGGG	NNNNNNNNNNNNNNNNNNN	(C/T)TTTTTT	C-3'	
Bottom	3'-G(C/T)	NNNNNNNNNNNNNNNNNNN	GACACTTCGGTGTCTACCC	NNNNNNNNNNNNNNNNNNN	(G/A)AAAAAA	GAGCTp-5'	

*If the first base of the target sequence is not G or A, insert G or A before the target sequence.

The pAAV-U6-ZsGreen1 Vector cloning sites are *Xba* I upstream side and *Spe* I downstream. The pAAV-2xU6 Vector has two cloning sites to allow for cloning of two shRNA expression units; one with *Xba* I upstream and *Spe* I downstream, and one with Sal I upstream and Xho I downstream.

Caution: When cloning DNA oligos by ligation, be sure to phosphorylate the 5' end of DNA oligo and to dephosphorylate the digested vector. Multiple DNA olgios may be inserted with ligation-based cloning.

VIII. References

1) Miyake K *et al. J Nippon Med Sch.* (2012) **79**(6): 394-402.
2) Van Vliet K M *et al. Methods Mol Biol.* (2008) **437**: 51-91.
3) Wu Z *et al. Mol Ther.* (2006) **14**(3): 316-327.
4) Keiya Ozawa et al. Protein, Nucleic Acid and Enzyme (2007) **52**(10): 1288-1293.
5) Zincarelli C *et al. Mol Ther.* (2008) **16**(6): 1073-1080.
6) Ellis B L *et al. Virol J*. (2013) **10**: 74.
7) Boden *et al*. *Nucleic Acids Res*. (2004) **32**: 1154-1158.
8) Miyagishi *et al*. *J Gene Med*. (2004) **6**: 715-723.
9) Lee *et al*. *Nat Biotech*. (2002) **20**: 500-505.
10) Paddison *et al*. *Genes and Dev*. (2002) **16**: 948-958.
11) Paul *et al*. *Nat Biotech*. (2002) **20**: 505-508.
12) Sui *et al*. *Proc Natl Acad Sci USA*. (2002) **99**: 5515-5520.

IX. Related Products

pAAV-ZsGreen1 Vector (Cat. #6231)
pAAV-ZsGreen1 Vector (KmR) (Cat. #6610)
AAVpro® Purification Kit Maxi (All Serotypes) (Cat. #6666)
AAVpro® Cell & Sup. Purification Kit Maxi (All Serotypes) (Cat. #6676)
AAVpro® Purification Kit (AAV2) (Cat. #6232)
AAVpro® Titration Kit (for Real Time PCR) Ver.2 (Cat. #6233)
AAVpro® Extraction Solution (Cat. #6235)
AAVpro® Freeze-Thaw Extraction Buffer (All Serotypes) (Cat. #6679)
AAVpro® 293T Cell Line (Cat. #632273)
CalPhos™ Mammalian Transfection Kit (Cat. #631312)
*Trans*IT-293 Transfection Reagent (Cat. #MIR2704/MIR2700/MIR2705/MIR2706)*
Xfect™ Transfection Reagent (Cat. #631317/631318)
In-Fusion® Snap Assembly Master Mix (Cat. #638947, etc.)
Stellar™ Competent Cells (Cat. #636763)*

* Not available in all geographic locations. Check for availability in your area.