AAVanced™ AAV Cloning and Expression Vectors

Catalog # AAV5XXA-1

User Manual

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Contents

I. Introduction and Background

A. Purpose of this Manual

This manual provides detailed information necessary to generate expression constructs of your gene of interest into the AAVanced™ Adeno-Associated Virus (AAV) Cloning and Expression vectors. Specifically, instructions on amplification and cloning of cDNA into the vector backbone and verification of the final expression constructs are provided in this user manual. This manual does not include information on packaging the constructs into AAV viral particles, however, this information can be readily found in the user manual associated with the AAV packaging plasmids (not available through SBI) from various vendors. The protocol for transduction of target cells is available in the AAVanced™ AAV concentration reagent user manual (https://www.systembio.com/downloads/AAVanced-concentration-user-manual.pdf). We would encourage you to read the entire contents of the user manual before starting on your experiments.

B. Advantages of the AAVanced™ AAV Expression System

Recombinant AAV vectors (rAAV) have been widely used for gene therapy and genome editing, mainly because of their broad tropism, the lack of disease associated with wild-type virus, ability to transduce both dividing and non-dividing cells, and long term transgene expression (Vasileva A, 2005; Petrs-Silva H, 2013). Packaging rAAV with modified capsid plasmid and adenovirus gene expression plasmid provided *in trans* makes AAV production more convenient and mitigates any biosafety risks.

SBI offers AAVanced™ rAAV expression vectors based on the commonly used AAV2 serotype. The expression vectors contain inverted terminal repeat (ITR) sequences at both ends of the DNA strand and room for an open reading frame encoding a transgene driven by an exogenous promoter. To produce a high titer of viral particles, expression and packaging vectors are transiently co-transfected into suitable mammalian virus producer cells (*e.g.* HEK 293T cells) for subsequent isolation of rAAV virus particles in culture media. For a detailed description of SBI's rAAV isolation

process, please refer to the AAVanced™ AAV Concentration Reagent user manual.

C. AAVanced Cloning and Expression vectors

SBI provides a collection of cDNA cloning and expression vectors for various applications (Tables 1 and 2).

For all AAVanced vectors, there is a limit for the size of the insert(s) that can be cloned into the vectors for efficient packaging into viral particles. The entire size of the cassette between the ITRs (including insert) should be less than 5kb, otherwise, packaging efficiency may be materially impacted.

1. AAVanced™ Single Promoter Vectors

These vectors are characterized by the presence of a single mammalian promoter driving the gene on interest cloned into the MCS, either expressed by itself or co-expressed with a marker gene of interest (e.g. EGFP or Puro) in a T2A format. Vector maps and additional information can be found here: https://www.systembio.com/aav-technology-vectors/maps

Table 1: List of Single Promoter AAV Cloning and Expression Vectors

	AAV vector	Catalog #	Promoter	Application
Single Promoter	pAAVK-EF1α -MCS	AAV502A-1	EF1α	robust in most cell types, including primary, differentiated, stem cells
	pAAVK-EF1α -MCS-T2A-EGFP	AAV526A-1	EF1α	
	pAAVK-EF1α -MCS-T2A-Puro	AAV527A-1	EF1α	
	pAAVK-EF1α –MCS-T2A-mRFP	AAV528A-1	EF1α	

2. AAVanced™ Dual Promoter Vectors

These vectors are characterized by the presence of two independent mammalian promoters, one driving the gene of interest cloned into the MCS and the other driving the expression of a marker (e.g. eGFP, Puro, or mRNA). Since the gene of interest and the marker(s) are driven independently, their expression may differ depending on promoter activity. These vectors are typically used to assess transduction efficiency or generate stably selected cell lines independent of the gene of interest. Vector maps and additional information can be found here: https://www.systembio.com/aav-technology-vectors/maps

Table 2: List of Dual Promoter AAV Cloning and Expression Vectors

	AAV vector	Catalog #	Promoter	Application
Dual Promoter	pAAVK-EF1α-MCS1-CMV-MCS2	AAV503A-1	EF1α	robust in most cell types, including primary, differentiated, stem cells
	pAAVK-EF1α-MCS1-CMV-EGFP	AAV536A-1	EF1α	
	pAAVK-EF1α-MCS1-CMV-Puro	AAV537A-1	EF1α	
	pAAVK-EF1α-MCS1-CMV-mRFP	AAV538A-1	EF1α	

D. List of Components

All plasmids are shipped at a concentration of 0.5 μg/μl and total amount of 10 μg. All plasmids are shipped in dry or blue ice and should be stored at -20°C upon receipt. Properly stored plasmids are stable for 12 months from the date received.

E. Additional Required Materials

For Cloning

- Restriction enzymes for digestion of the vectors and/or inserts
- High Fidelity Long-distance PCR enzymes
  - T4 DNA Ligase and ligation reaction buffer (Recommended: New England BioLabs T4 DNA Ligase (400 U/μl), Cat. # M0202S. Dilute to 40 U/μl in 1X

ligation buffer with the provided 10X buffer just before use)
- High efficiency competent *E. coli* cells (RecA$^-$) (Recommended: One Shot OmniMAX 2 T1R competent cells, Cat. # C854003)
- Petri plates containing LB Agar media with 50 μg/ml Ampicillin or Carbenicillin

For Screening Inserts and Sequencing
- Taq DNA polymerase, reaction buffer, and dNTP mix (Recommended: Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- PCR machine
- 2-3% 1X TAE Agarose gel

For Purifying cDNA Constructs after Cloning
- Plasmid purification kit (Recommended: QIAGEN Endofree Plasmid Maxi Kit, Cat. # 12362. The following kit combination can be used for Midi scale (up to 200 μg of plasmid DNA) preparation of endotoxin-free DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048

  Please visit the QIAGEN website to download the specialized protocol that is not contained in the current user manual:
  - http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

For Transfection of pAAVK Constructs into Target Cells
- Transfection Reagent (Recommended: PureFection, System Biosciences Cat. # LV750A-1)

For Packaging of pAAVK Constructs into Viral Particles
- In order to package your constructs into viral particles, you will need to obtain AAV Packaging Plasmids (typically consisting of plasmids containing adenovirus structural/regulatory genes and Rep-Cap protein specific for different serotypes of AAV). These packaging plasmids can be obtained from various vendors.

For Isolation of pAAVK Viral Particles
- We would suggest SBI's AAVanced Concentration Reagent (Cat #AAV100A-1) for simple, one-step isolation of functional rAAV particles from producer media

II. Validation Data

1. AAVanced™ Single Promoter Vectors

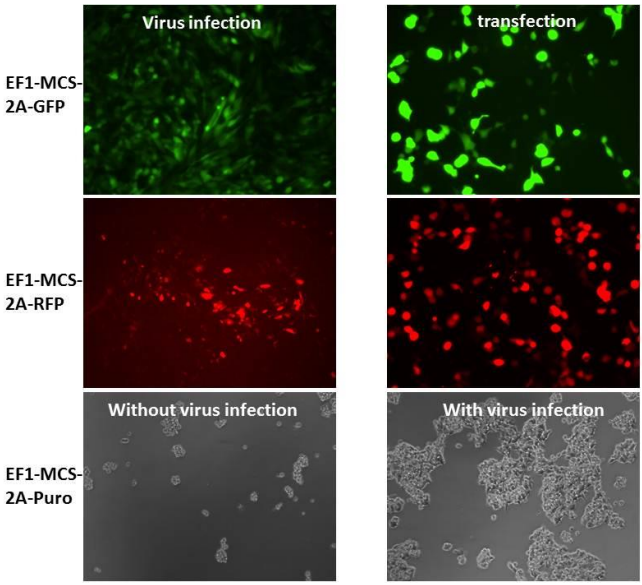


Figure 1: Representative images for GFP/RFP/Puro marker expression in HT1080 cells transduced with pAAVK vector under the control of EF1α promoter. Bottom rows show the number of cells after 3 days of puromycin selection in the well without or with pAAVK-EF1-MCS-2A-Puro virus infection.

2. AAVanced™ Dual Promoter Vectors

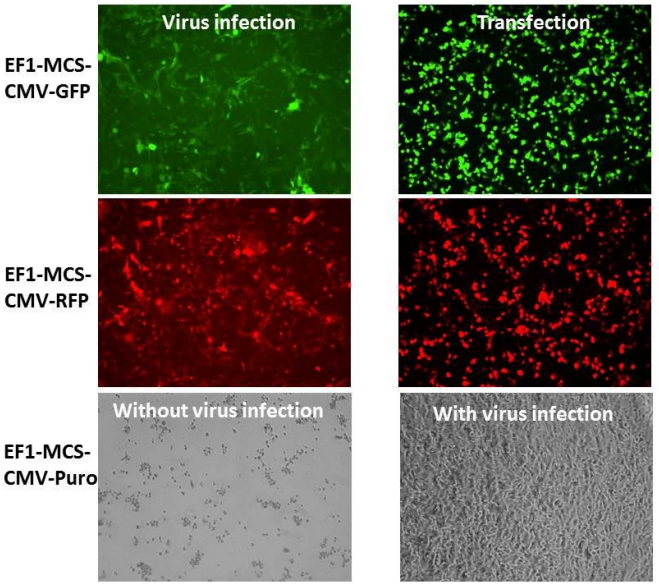


Figure 2: Representative images for GFP/RFP/Puro marker expression in HT1080 cells transduced with pAAVK dual promoter vector. GFP/RFP/Puro marker is umder the control of CMV promoter. Bottom rows show the number of cells after 3 days of puromycin selection without or with pAAVK-EF1-MCS-CMV-Puro virus infection.

III. Protocol

The following section provides general guidelines for the cloning of cDNA, amplified by PCR, into pAAVK vectors.

A. cDNA Amplification

Full length cDNA fragments can be recloned from another plasmid or amplified by PCR. PCR-based cloning is the most convenient way for full-length cDNA cloning in pAAVK vectors. The pAAVK does not contain an ATG initiation codon. A translation initiation sequence must be incorporated in the insert cDNA if the cDNA

cDNA fragment to be cloned does not already have an ATG codon. We also recommend including a Kozak sequence (*i.e.* GCCACC) before the ATG for optimal translation. For amplification of the target cDNA fragment, design a 5'-primer (containing a Kozak sequence and ATG codon) and 3'-primer with unique restriction sites present in the MCS of the pCDH vector but not present in the cDNA sequence. Amplify the cDNA fragment by high fidelity long-distance PCR using about 200 ng of plasmid template DNA and a minimum number of cycles (usually 12-15 cycles), purify, digest the amplified product with end-specific restriction enzyme(s) and purify the digested PCR product in a 1.2% agarose gel to prevent contamination with the original plasmid used for amplification.

B. Primer Design for Cloning into Vectors with T2A Sequence

Since the gene of interest and the reporter gene in cDNA expression vectors containing a T2A peptide sequence will form one open-reading frame, extra attention should be paid when designing the 3' primer for amplifying the target sequence. First of all, do not include a stop codon at the 3' end of target sequence—this would prevent the expression of the reporter gene; secondly, place the target sequence in-frame with the 2A peptide. The ATG start site of the insert that cloned into the MCS must be in-frame with the GAG of the T2A sequence. Please verify the cloning strategy to ensure that this will occur prior to cloning.

C. Preparation of Digested Vector

Digest the vector with the corresponding restriction enzymes used in the preparation of the cDNA fragments, and then verify complete digestion of the vector by agarose gel electrophoresis. We suggest that you perform only preparative gel purification of the digested vector if more than one restriction enzyme is used. If you use a single restriction enzyme, dephosphorylation as well as gel purification of the vector is necessary to reduce the background in the vector ligation step.

D. Cloning of cDNA into Vector

The optimal insert-to-vector molar ratio may be different for different inserts. Always try at least two different ratios (*e.g.*, 10:1 and 30:1) for each experiment. Also make sure to include one negative control reaction, which contains only the digested vector.

1. Ligation of cDNA to Vector

a. Dilute the gel-purified, digested vector to 10 ng/μl.

b. Set up 10 μl ligation reactions for each sample and control as follows:

1.0 μl	Digested pAAVK Vector (10 ng/μl)
7.0 μl	cDNA insert (usually 30-50 ng) or Nuclease-free water
1.0 μl	10X T4 DNA Ligase Buffer
1.0 μl	T4 DNA ligase (40 U/μl)
10.0 μl	Total volume

c. Incubate the ligation reactions at 16°C for 1-2 hrs if it is sticky-end ligation. For blunt-end ligation, use an overnight incubation

2. Transform *E. coli* with the ligation product

Transform competent cells** (with a transformation efficiency of at least $1x10^9$ colonies/μg pUC19) with the whole ligation reaction (10 μl) following the protocol provided with the competent cells. Plate the transformed bacteria on LB-Ampicillin/Carbenicillin agar plates.

3. Identify Clones with the cDNA Insert

a. Depending on the ratio of colony numbers for the cDNA sample vs. the negative control sample, randomly pick 5 or more well-isolated colonies and grow each clone in 100 μl of LB Broth with 75 μg/ml ampicillin at 30°C for 2 hours with shaking.

b. Use 1 μl of each bacterial culture for screening cDNA inserts by PCR and continue to grow the culture for another 4 hours. Store the culture at 4°C.

Prepare a PCR Master Mix with PCR primers flanking the cDNA insert:

1 rxn	10 rxn	Composition
0.5 μl	5 μl	PCR primer 1 (10 μM)
0.5 μl	5 μl	PCR primer 2 (10 μM)
0.5 μl	5 μl	50X dNTP mix (10 mM of each)
2.5 μl	25 μl	10X PCR Reaction Buffer
19.5 μl	195 μl	Nuclease-free water
0.5 μl	5 μl	Taq DNA polymerase (approx. 5 U/μl)
24.0 μl	240 μl	Total volume

c. Mix the master mix very well and aliquot 24 μl into each well of 96-well PCR plate or individual tubes.

d. Add 1 μl of each bacterial culture from step (b) into each well (or tube).

e. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min/1 kb*	25 cycles
68°C, 3 min	1 cycle

* depending on the size of final PCR product, use a shorter or longer time.

f. Take 5 μl of the PCR reaction and run it on a 1.2% agarose/EtBr gel in 1X TAE buffer to identify clones with correct insert.

Grow a positive clone with the cDNA insert in an appropriate amount of LB-Amp Broth, and purify the construct using an endotoxin-free plasmid purification kit

Confirm identity of the cDNA insert by sequence analysis of the construct using the following primer.

EF1a-F: 5'-CTCCACGCTTTGCCTGACCCTGCTT-3'

IV. References

Vasileva A, Jessberger R. Precise hit: adeno-associated virus in gene targeting. Nat Rev Microbiol. 2005 Nov;3(11):837-47.

Petrs-Silva H, Linden R. Advances in recombinant adeno-associated viral vectors for gene delivery. Curr Gene Ther. 2013 Oct;13(5):335-45.

M.J. Osborn, A.P. Mortari, R.T. McElmurry, S.K. Bell et al. A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system. Molecular Therapy. (2005) 12:569-574

M.L. Donnelly et al. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage" mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal "skip". J. Gen. Virol. (2001) 82:1013-1025

X.Yu, X. Zhan, J.D'Costa, V.M. Tanavde, Z.Ye, T. Peng, M.T. Malehorn, X. Yang, C.I. Civin, and L.Cheng. lentiviral vectors with two independent internal promoters transfer high-level expression of multiple transgenes to human hematopoietic stem-progenitor cells. Molecular Therapy, (2003) 7:827-838

Mizuguchi M, Xu Z, Ishii-Watabe A,Uchida E and Hayakawa T. "IRES-Dependent Second Gene Expression is significantly lower than Cap-Dependent First gene expression in a bicistronic vector" Molecular Therapy (2000) 1, 367-382

V. Appendix

A. Maps and Features for AAVanced Vectors

For the latest annotated vector maps and features for all of the AAVanced vectors, please visit our website at

https://www.systembio.com/aav-technology-vectors/maps

Additionally, the vector sequence files for all of the vectors can be requested by emailing tech@systembio.com. Proof of vector purchase will be required at time of inquiry.

B. Common Features of AAVanced Cloning and Expression vectors:

- **Multiple Cloning Site (MCS)—**for cloning the gene of interest in the MCS located downstream of the promoter.
- **WPRE element—**enhances stability and translation of the CMV-driven transcripts.
- **SV40 polyadenylation signal—**enables efficient termination of transcription and processing of recombinant transcripts.
- **Inverted Terminal Rpeat (ITR)**—serves as the origin for viral DNA replication and essential for virus packaging, rescue, and integration.
- **SV40 origin—**for stable propagation of the pCDH plasmid in mammalian cells.
- **pUC origin—**for high copy replication and maintenance of the plasmid in *E.coli* cells.
- **Ampicillin resistance gene—**for selection in *E.coli* cells.

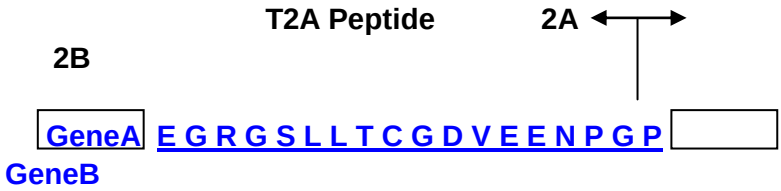
C. Additional Considerations

2A Peptide-enabled dual expression system

Coexpression of a reporter gene together with a gene of interest is a useful approach for selecting transfected or

transduced cells. This is commonly achieved by using two independent internal promoters, such as CMV and EF1 in pAAVK-EF1-MCS-CMV-EGFP, or by linking two transgenes with an internal ribosomal entry site (IRES) element in a single bicistronic transcript. Many dual promoter pairs have shown a high level of expression of both transgenes in standard cell lines—however, promoter interference often occurs in some cell lines. There are also two main problems that limit the use of IRES: the large size and the imbalanced expression between the first and second cistrons (H. Mizuguchi, 2000; X.Yu, 2003).

The "self-cleaving" 2A peptides have been used successfully to generate multiple proteins from a single promoter in many applications (P. de Felipe, 2004; M.J. Osborn, 2005; P. de Felipe, 2006). The 2A-like sequences exist in several viruses and are used to mediate protein cleavage from a single open reading frame. Through a ribosomal skip mechanism, the 2A peptide prevents normal peptide bond formation between the 2A glycine and the 2B proline without affecting the translation of 2B (M.L. Donnelly, 2001):



SBI's cDNA expression vectors incorporate the 2A-like sequence (T2A) from the insect virus *Thosea asigna* to mediate the coexpression of a reporter gene with the target cDNA. Reporter genes have been cloned at either the first or second positions, and we achieved high expression levels at both locations. It is important to note that the final cassette will constitute a single ORF and thus when cloning the stop coden from the first gene has to be removed. An effective Kozak consensus sequence (GCCACC) in front of the start codon ATG will significant increase gene expression.

D. Safety Guidelines

AAV is human infectious virus with no known disease associated. Wild type virus have the ability preferentially integrated into human choromosome 19q; recombinant vectors loss this specificity and appear to integrate randomly, thereby increasing the risk of insertional mutagenesis.

The NIH Guidelines state that "AAV types 1 through 4, and **all recombinant AAV constructs**, in which the transgene dose not encode either a potentially tumorigenic gene or a toxic molecule abd are produced in the absence of a helper virus" can in most cases be handeled at biosafety level1 (BSL-1).

E. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

System Biosciences (SBI)
265 North Whisman Rd.
Mountain View, CA 94043

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

Fax: (650) 968-2277

E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VI. Licensing and Warranty Statement

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Use of the AAVanced cDNA Cloning and Expression Vector (i.e., the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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CMV Promoter

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242.

SBI has pending patent applications on various features and components of the Product. For information concerning licenses for commercial use, contact SBI.

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