Chapter 2

Design and Construction of Functional AAV Vectors

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Abstract

Using the basic principles of molecular biology and laboratory techniques presented in this chapter, researchers should be able to create a wide variety of AAV vectors for both clinical and basic research applications. Basic vector design concepts are covered for both protein coding gene expression and small non-coding RNA gene expression cassettes. AAV plasmid vector backbones (available via AddGene) are described, along with critical sequence details for a variety of modular expression components that can be inserted as needed for specific applications. Protocols are provided for assembling the various DNA components into AAV vector plasmids in *Escherichia coli*, as well as for transferring these vector sequences into baculovirus genomes for large-scale production of AAV in the insect cell production system.

Key words: AAV, Vector plasmid, Vector performance, Transfection, Molecular cloning, Baculovirus, Vector design

1. Introduction

1.1. Basics of Vector Design

The construction of viral vectors is an exercise in compromise, as there is always some limitation as to the length and type of sequence that can be delivered, and any number of functional elements that could in theory improve the behavior of the vector. It is the goal of this chapter to provide tools and instruction to begin this process with a limited set of such elements, but it remains to the investigator to complete the effort by carefully evaluating the actual performance of the vector in therapeutically or experimentally relevant target cells and, if necessary, revisiting the construction process to correct deficiencies observed.

The basic design of an AAV vector is relatively simple, in that it consists of an appropriately sized expression cassette flanked by inverted terminal repeats (ITRs), which mediate the replication

Richard O. Snyder and Philippe Moullier (eds.), *Adeno-Associated Virus: Methods and Protocols*,
Methods in Molecular Biology, vol. 807, DOI 10.1007/978-1-61779-370-7_2,

and packaging of the vector genome by the AAV replication protein Rep and associated factors in vector producer cells. This chapter attempts to provide specific methodological details to enable an investigator to utilize standard molecular cloning techniques to assemble a functional AAV vector plasmid for use in the transient transfection system in HEK293 cells, which will suffice for many preliminary evaluations of vector performance. For those vectors slated for larger scale applications (as in large animal models or human clinical trials), we additionally provide instructions for moving existing AAV cassettes into a baculovirus genome for production in the insect cell system. A key aspect of our approach to this chapter is that we attempt to provide specific sequence details about many commonly used components of vector cassettes, to enable more specific design using either fully synthetic methods or PCR amplification from commonly available sources.

Beyond the requirements for optimal genome size and the presence of AAV ITRs, AAV vector designs are only limited by whatever functions can be conferred on a 4–5-kbp segment of DNA. We will provide below a basic introduction for two of the most common types of vectors, first for standard expression of a protein coding gene and second for the expression of non-coding RNAs to generate siRNA for targeted gene knockdown. There are other powerful vector designs beyond the scope of this chapter, but which may also be useful to the reader. In this book, Chapter 13 describes the use of AAV vectors for targeted homologous recombination, which requires the insertion of homology arms in the vector flanking the segment to be inserted into a cell genome. Another powerful technology utilizes the expression of non-coding RNA designed to interact with the cellular splicing machinery and mutant mRNA to induce "exon-skipping," which in the case of certain muscular dystrophy mutations can restore functionality to the resulting translated protein (1). Likewise, expression of ribozymes within non-coding RNA transcripts can be used to target mRNA for degradation (2).

Finally, should a vector prove to be clinically efficacious, commercialization may be affected by relevant intellectual property, which can be found by thorough review of patent databases and/or consultation with intellectual property professionals.

1.2. Protein-Coding Expression Design Concepts

A diagram of a basic expression cassette is presented in Fig. 1. Expression of protein coding mRNA begins with the initiation of transcription by RNA Polymerase II at promoter sequences, which dictate the location and orientation of transcription. Enhancer sequences, which function in a more position- and orientation-independent manner, can be located either upstream or downstream of the promoter, and modulate both the strength and tissue specificity of the promoter. Although in many cases a single contiguous fragment from a naturally derived sequence will provide

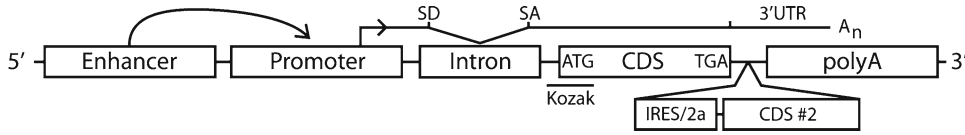

Fig. 1. Schematic diagram of a basic protein coding expression cassette. Basic components of a vector expression cassette are indicated. *SD* splice donor, *SA* splice acceptor, *CDS* coding sequence. See text for additional explanations.

both functions, in some cases chimeric promoters are constructed using separate enhancers and promoters positioned in a variety of arrangements. The selection of the promoter you wish to use in your vector depends upon the spatial and temporal control necessary for your application, the level of expression desired and the amount of space available. Although we provide information in the Subheading 3 for a few more commonly used promoters, the choices available are very broad should an investigator wish to explore more deeply. Chapters 5–9 of this book discuss tissue-specific vectors in detail, and a recent review (3) describes several regulatable promoters that have been engineered for inducible gene expression using small molecules.

Introns are frequently inserted downstream of promoters to capitalize on the fact that the splicing process facilitates mRNA export and increases steady state levels of mRNA in the cytoplasm. As the presence of a translational termination codon upstream of an intron splice junction is a trigger for initiation of the nonsense mediated mRNA degradation (NMD) pathway (4), introns should be located upstream of the end of any protein coding sequence. When transmitting very large coding sequences, one should also consider that the increase in gene expression provided by an intron may very well be negated by significant reductions in vector yield caused by exceeding the packaging capacity of the AAV system, and in those cases an intronless construct may be the best option.

In order to achieve optimal translation efficiency, the coding sequence must be positioned such that the ATG initiator codon is the first ATG of the spliced message. The use of optimal Kozak sequences just upstream of the ATG significantly improves translation initiation efficiency and ensures that ribosomal scanning of the vector-encoded mRNA does not lead to translation of aberrant products lacking N-terminal amino acids (5). Although the most important element of this consensus is a purine at the –3 position, the small size of the full optimal sequence (CCACCATG) enables easy incorporation into any construct coding sequence generated synthetically or by PCR.

Additional enhancement of gene expression can be provided by a process called “codon optimization,” which involves the construction of a cDNA with alternate codons chosen to facilitate robust gene expression while still encoding the same amino acid sequence. The concept behind this technique is that though there

are multiple triplet codons that can encode for a single amino acid, it is often the case that the best expression is achieved when amino acids are coded by the codons most commonly found in highly expressed genes. Codon usage is naturally biased to favor a subset of codons in different species and also in highly expressed genes. Although these biases can originate via a variety of mechanisms (6), it has been shown that "optimal" codons can enhance translational efficiency by utilizing the most abundant charged tRNAs in the cell (7). Codon optimization can be performed by commercial gene synthesis companies, which have in many cases developed algorithms for automated selection of the codons in a gene and have convenient online ordering forms. The cost can depend upon the length of the gene being synthesized, and range from $0.39 to $2 per base pair. It should be noted, however, that the potential for introduction of sequences that negatively affect gene expression is also a risk with this process, and it is difficult to predict which sequences will have such an effect. For example, it has been shown that the sequences coding for protein domain boundaries are more likely to be coded by "translationally slow" codons (8), providing support for the hypothesis that slower translation at domain boundaries enhances fitness by allowing each domain to fold co-translationally in distinct temporal phases (9). Codon-optimized genes should therefore be tested carefully to confirm that the resulting protein product is not improperly folded.

Although in most cases fully spliced cDNAs are used in vectors to preserve space, in some cases coding sequences in vectors are interrupted with introns, such that splicing allows regeneration of an intact open reading frame. One example of this application is the use of native intronic sequences from the first intron of the human Factor IX gene, which allowed incorporation of the endogenous tissue-specific enhancers present in those sequences (10). Other applications include the insertion of regulatable polyA cassettes in an intron to allow regulation of complete expression (11) and the splitting of large coding sequences between two vectors genomes, which can, after head-to-tail joining in vivo, transcribe through both genomes to generate transcripts longer than 5 kb and which are spliced to yield an intact coding sequence (12).

Although clinical vectors almost invariably exclude them, additional exogenous peptides are often fused or co-expressed with the primary gene of interest for research and pre-clinical applications. Epitope tags are small and allow the use of commonly available antibody reagents to quantitate expression in cell lysates from tissues transduced with the vector. Signal peptides, when not encoded by the natural cDNA sequences, can be inserted at the N-terminus of an open reading frame to direct proteins to be secreted from the cell. Co-transmission of a second, separate coding sequence has numerous research and clinical applications, most commonly in allowing the co-expression of fluorescent marking

proteins to track cells transduced with the vector. This can be achieved using internal ribosome entry sites (IRES), or more recently by using specialized "2A peptide" sequences, which, when translated, cause a failure in peptidyl bond formation between the two coding open reading frames, resulting in efficient co-expression of two separate proteins in an approximately 1:1 ratio (13).

Sequences downstream of the translational stop codon form the 3′ untranslated regions (3′ UTR) of a transcript. Recent discoveries of the mechanisms of action for non-coding RNAs have illuminated the importance of these sequences as mediators of micro-RNA (miRNA) binding and regulation of both RNA stability and translation (14). For some genes such as Factor IX, sequences in the 3′ UTR facilitate optimal expression of vector-derived transcripts (10), whereas in other genes omission of endogenous 3′ UTR sequences can prevent unwanted degradation (15). In another creative application of these natural interactions, insertion of multiple miRNA target site sequences rendered expression of an otherwise ubiquitously expressed transcript specific to non-hematopoietic tissues, enhancing evasion of immunological consequences of expression in those tissues (16).

1.3. Noncoding RNA Expression Design Concepts

Small 21–25-nucleotide (nt) long RNA fragments in cells have been shown to be powerful modulators of mRNA translation and stability, and are increasingly utilized for experimental and therapeutic purposes. These short, interfering RNAs (siRNAs) act via their assembly into an RNA-Induced Silencing Complex (RISC), wherein the RNA fragment provides specificity via base complementarity to allow the RISC to target mRNAs for degradation or translation inhibition, depending upon the degree of complementarity between the two RNAs. Construction of an expression vector to generate siRNAs in vivo has largely been accomplished using two strategies, short hairpin RNAs (shRNAs) (17) and microRNA mimics (18, 19). Although shRNA cassettes can be more potent than miRNA mimics (20), miRNA mimics have been shown to have reduced toxicity (21).

The structure of these two types of non-coding RNA expression cassettes are presented in Fig. 2, along with an illustration of the different processing steps leading to the loading of the RISC with the siRNA. Transcription of an shRNA cassette (Fig. 2a) is typically mediated by RNA Polymerase III to generate a hairpin free of additional nucleotides beyond the hairpin ends. This hairpin exits the nucleus where it is processed by the cytoplasmic enzyme Dicer, which cleaves the loop sequence and allows the resulting RNA fragment to be loaded onto the RISC (17). MicroRNA mimics (Fig. 2b), on the other hand, can be generated using RNA Pol II, typically by inserting an extended RNA sequence derived from a naturally occurring primary miRNA transcript into either an intron or the 3′ UTR of a pre-existing protein coding

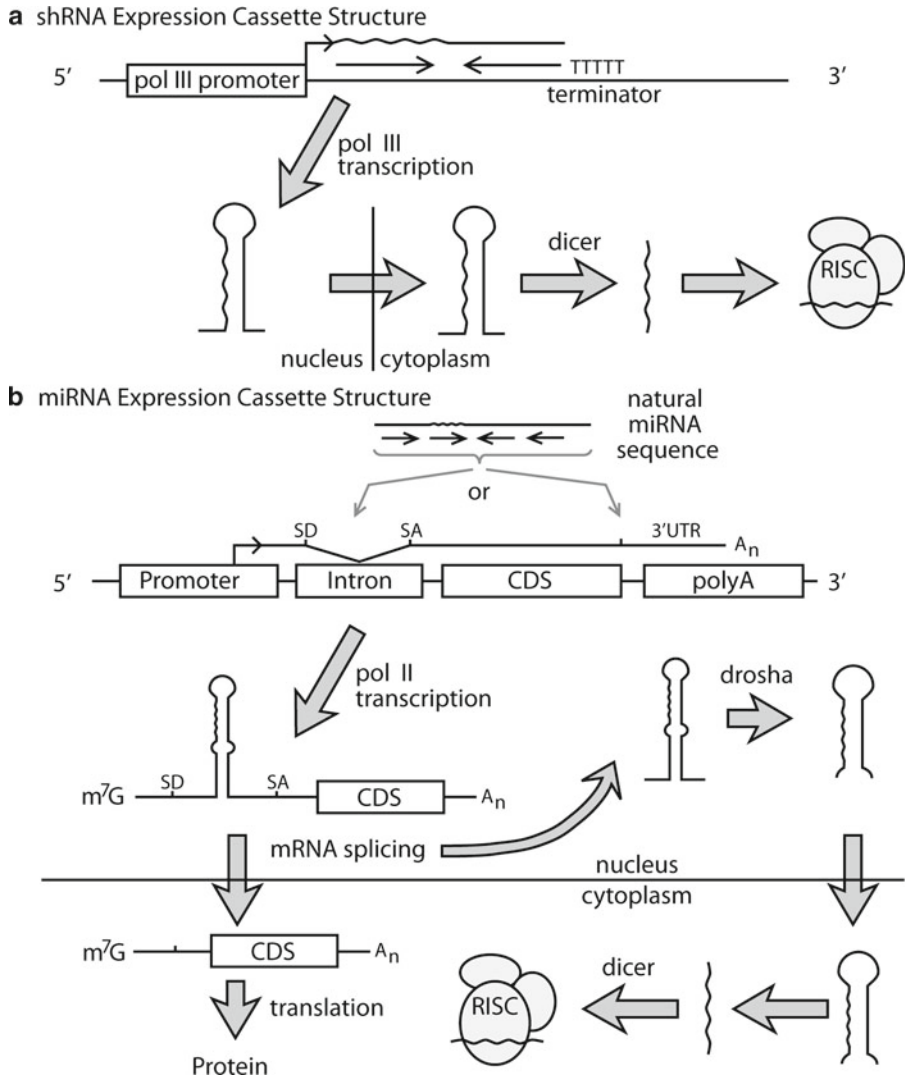

Fig. 2. Schematic diagram of two forms of non-coding RNA expression cassettes. Expression of siRNA for targeted knockdown of gene expression. *Wavy lines* indicate portion of primary RNA transcript that will be incorporated into the RISC. *Thin black arrows* indicate complementary arms of a hairpin. (**a**) Pol III promoter-driven expression of short hairpin RNAs (shRNAs). (**b**) Pol II-driven expression cassette for simultaneous expression of protein coding RNA and miRNA mimics.

expression cassette. The specific 21–22-nt RNA targeting sequence and its complement are grafted into the primary miRNA fragment native sequence in place of the miRNA sequence. The natural flanking sequences are recognized by the nuclear enzyme Drosha, which acts upstream of Dicer during miRNA biogenesis by cleaving the extended hairpin at bulged nucleotides distal of the loop. Hairpins processed in this way appear to be more efficiently transported to the cytoplasm and processed by the Dicer enzyme, apparently leading to reduced toxicity (21). Additionally, miRNA mimics

provide the added benefit that a wide range of well-characterized RNA Pol II promoters and expression components can be used, including regulated and tissue specific promoters (19). Additionally, some naturally occurring miRNA primary transcripts are processed to yield multiple miRNAs, which can be utilized to engineer a single vector encoded transcript that generates multiple miRNA mimics (22).

1.4. Special Considerations for Constructing Baculoviruses Containing AAV Vector Genomes

Although a baculovirus vector-mediated manufacture of rAAV in insect Sf9 cells is a scale-up method of choice, it is not usually recommended for a routine production of research vectors used for the initial screening at titers of 10^{12}–10^{13} DNase Resistant Particles (drp). Initially, rAAV vectors can be made by transient transfection at small scale to evaluate performance in rodents (23). Once the transgene expression cassette is optimized and the most efficient serotype is determined, the next step usually involves production of high titer vector stocks for pre-clinical studies in a large-animal model. At this stage, it might be worth investing effort in construction of the baculovirus expression vector (BEV) carrying rAAV cassette. The latter could be combined with the existent and published (24–26) Rep-, and VP-expressing helpers in a full complement set required for rAAV production.

Currently, there are three comparable systems to provide *rep* and *cap* helper functions in Sf9 cells. The first one is a modified BEV expressing both Rep and VP (26). A similar system expressing Rep and VP from two separate BEVs had been described by Chen (25). Yet another system utilizes stable insect cell lines expressing inducible Rep and VP upon infection with BEV-rAAV (24). Regardless of the approach, constructing rAAV-carrying BEV is a prerequisite step and it is described below in detail.

BEVs are prepared using Invitrogen's Bac-to-Bac system. The system utilizes a bacmid, an intermediate shuttle plasmid vector of 143 kbp incorporating complete BV genome, as well as Km^R gene and bacterial *ori*. It also contains a Tn7 attachment site engineered in-frame within a *lacZ* α peptide sequence. The rAAV cassette is first cloned into a Bac-to-Bac plasmid, flanked by Tn7R and Tn7L sequences. This is then transformed into the *Escerichia coli* DH10BAC that contains the bacmid, and a helper plasmid encoding the transposase. The rAAV cassette is transposed on to the bacmid and selected by growth in the presence of gentamicin, kanamycin and tetracycline. The parental bacmid encodes the LacZ α peptide which complements with the chromosomal β peptide to form a fully functional β-galactosidase which cleaves X-gal and produces blue colonies. The transposed cassette disrupts the *lacZ* α gene, and thus recombinant bacmid clones are white. Bacmid DNA is prepared from the putative recombinant clones, and the transposition is confirmed by PCR or Hirt DNA analysis. The recombinant bacmid is transfected into Sf9 cells, and several days later infectious BEV is harvested from the medium.

2. Materials

2.1. Construction of AAV Vector Plasmids Suitable for Transient Transfection of 293 Cells

1. AAV vector plasmid backbone: Although many plasmids are available from academic and commercial sources, the specific sites provided in this chapter are selected for pAAV-GFP and pscAAV-GFP, which have been deposited to AddGene (AddGene.org/John_T_Gray) and linked to this article (Fig. 3). pAAV-GFP contains two intact ITRs flanking a

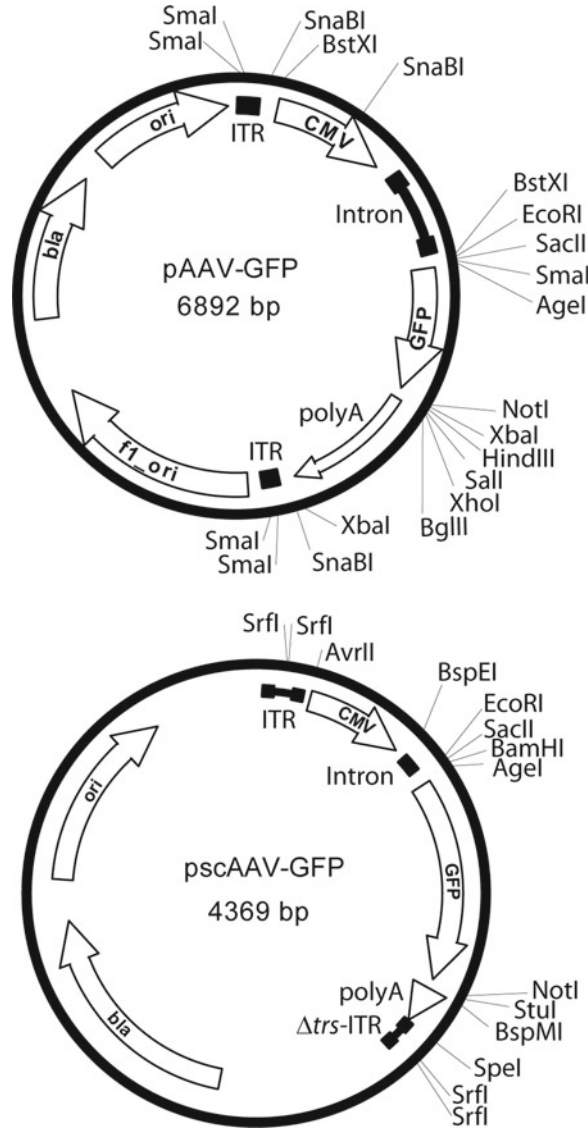

Fig. 3. Maps of single-stranded and Δ*trs* AAV vectors available from AddGene, showing relevant features and restriction sites. Additional details on the structure of these plasmids can be downloaded from the AddGene records linked to this article.

Table 1
Useful restriction enzyme sites in AddGene AAV vectors

	Vector plasmid			
	pAAV-GFP		pscAAV-GFP	
Component to be replaced	5′ Enzymes	3′ Enzymes	5′ Enzymes	3′ Enzymes
GFP	*Eco*RI, *Sac*II, and *Age*I	*Not*I, *Hin*dIII, *Sal*I, *Xho*I, and *Bgl*II	*Eco*RI, *Sac*II, *Bam*HI and *Age*I	*Not*I, *Stu*I, and *Bsp*MI (*Xho*I compatible)
CMV promoter (with intron)	*Bst*XI	*Bst*XI, *Eco*RI, *Sac*II, and *Age*I	*Avr*II	*Eco*RI
PolyA sequence	*Xba*I	*Xba*I	*Not*I, *Stu*I, and *Bsp*MI	*Spe*I
Entire expression cassette	*Sna*BI	*Sna*BI	*Avr*II	*Spe*I

GFP expression cassette, which allows for production of single stranded AAV vectors. In pscAAV-GFP, the right ITR contains a terminal resolution site mutation (Δ*trs*), which prevents Rep-mediated nicking and forces packaging of dimer or self-complementary genomes. Pay strict attention to the integrity of the vector ITRs in your plasmid preparations (see Note 1). The restriction enzyme combinations used to replace individual components of the expression cassette in these plasmids are outlined in Table 1. Modular expression cassette components: Table 2 provides a brief list of *cis*-acting vector sequence components that can be assembled into an AAV vector expression cassette.

2. PCR cloning kit, provided by commercial vendor (pGEM-T-Easy, Promega, Topo, Invitrogen, etc.).
3. Restriction endonucleases, high fidelity PCR enzymes and kits (Pfu Turbo, Stratagene, Santa Clara, CA or Phusion, NEB, Beverly, MA or the like), transformation competent *E. coli* (SURE strain, Stratagene), and standard molecular cloning equipment.
4. Sterile 50% glycerol, in water, for freezing *E. coli* stocks (use at a final glycerol concentration of 25%).

2.2. Construction of Transfer and Bacmid Plasmid Vectors

1. SURE *E. coli* electrocompetent cells (Stratagene).
2. Bac-to-Bac Baculovirus Expression System and pFastBacDual vector (Invitrogen, Carlsbad, CA). All reagents required for the construction of transfer and bacmid vector are listed in the kit.

Table 2
Useful sequence elements for constructing AAV vector expression cassettes

Name	Length	Accession	Sequence	Comments
CMV immediate early promoter/ enhancer	42	X03922	CCATTGCATACGTTGTATC CATATCATAATATG… CGGGACCGATCCAGCCTCC	Strong expression in a wide range of tissues. Enhancer portion (first 454 bp) can be used with heterologous promoters. Shorter versions remove up to 321 bp from 5′ end, and 47 bp from the 3′ end, but have not been directly compared with the full length form
Murine PGK promoter	513	M18735	AATTCTACCGGGTAGGGGAGGCG… CATCTCCGGGCCTTTCGACCT	Mild but consistent expression in a wide variety of cell types
Human EF1α promoter	1,169	HUMEF1a	CGTGAGGCTCCGGTGCCC… TTTTTCTTCCATTTCAG GTGTCGTGAA	Strong, consistent expression in a wide range of tissues. This fragment includes the first intron (positions 237–1,159), which enhances expression but is often removed to create a 'short' version
Human ApoE hepatic control region (HCR)	192	HSU32510	CCCTAAAATGGGCAAACATTGCA… GCGTGGTTTAGGTAG TGTGAGAGGG	Strong liver-specific enhancer. Used with human α-1-antitrypsin promoter in 'LP1′ liver specific promoter (38)
Human α-1-antitrypsin promoter	256	HUMA1ATP	AATGACTCCTTTCGGTAAG TGCAGTG…CACCACTGACCTGGG ACAGTGAATC	Liver-specific promoter. Used with ApoE HCR in 'LP1′ liver-specific promoter (38)
Rat Synapsin promoter	1,108	RATSYN1A	GGGTTTTGGCTACGTCCAGAGC… ACCGACCCACTGCCCCTTGT	Neuron-specific promoter (39, 40)
Human muscle creatine kinase enhancer (MCK)	206	AF188002	CCACTACGGGTCTAGGCTGCCC… AAAAATAACCCTGTCCCTGG TGGATC	Muscle-specific enhancer, used in combination with MCK promoter fragment (41)
Human MCK promoter	366	AF188002	CAATCAAGGCTGTGGGGGACTG… GGCTGCCCCCGGGTCAC	Muscle-specific promoter, used in combination with MCK enhancer (41)

RNaseP H1 fragment RNA polymerase III promoter	221	NC_00014.8 Range 20811229.. 20811569	GAATTCGAACGCTGACGTC ATCA… GGAATCTTATAAGTTCTGTATGAG ACCAC	Promoter used for the expression of short, hairpin RNAs for targeted knockdown of genes, as used in the pSUPER vectors (17). Insert hairpin followed by 'TTTTT' (for pol III termination) downstream of this sequence. For hairpin design algorithms, see http://www.ambion.com
Human β-globin intron, RsaI deleted	547	GQ370762	GATCCTGAGAACTTCAGGGTGAGT CTATGG…GGCCCATCACTTTGG CAAAGAATT	Large, but provides significantly enhanced expression in many cell types. RsaI digestion of native human genomic sequence removes an internal 374 bp fragment to generate this 547 bp element
SV40 intron	93	J02400	CTCTAAGGTAAATATAAAATTTTT AAGTGTATAATGTGTTAAACTAC TGATTCTAATTGTTTCTCTCTTTTAG ATTCCAACCTTTGGAACTGA	This very short sequence has been modified to enhance similarity to consensus splice site signals
Internal ribosome entry site (IRES) from ECMV	556	NC_001479	CCCCCCCCCTAACGTTACTGG… CGTGGTTTTCCTTTGAAAAACACGAT	Used for creating bicistronic vectors by placing this sequence between two coding sequences. For optimal translation of the downstream ORF, position ATG within 10 bp of the end of this sequence
Porcine teschovirus-1 2A peptide	57	AJ011380	GCCACGAACTTCTCTCTGTTAAA GCAAGCAGGAGACGTGGAAGAAA ACCCCGGTCCC	This sequence must be fused in frame between two open reading frames, with no intervening stop codons, to allow efficient cotranslation of two coding sequences (13)
SV40 late polyadenylation sequence	133	J02400	TGCTTTATTTGTGAAATTTGT GATGCTATTGCTTTATTTGTAAC CATTATAAGCTGCAATAAACAAGTT AACAACAACAATTGCAT TCATTTTATGTTTCAGGTTCAG GGGGAGGTGTGGGAGGTTTTTTAAA	Compact, relatively strong polyadenylation sequence

(continued)

Table 2 (continued)

Name	Length	Accession	Sequence	Comments
Bovine growth hormone polyadenylation sequence	225	M57764	CTGTGCCTTCTAGTTGCCAGCCA…GGGATGCGGTGGGCTCTATGG	Very strong polyadenylation sequence
Human β-globin polyadenylation sequence	760	GQ370762	AATTCACCCCACCAGTGCAGGC…TGCCTCCCCCACTCACAGTGAC	Very strong longer polyadenylation sequence
Human miR-30a primary microRNA sequence fragment	324	Human Chr. 6, 72113127-72113449	TGTTTGAATGAGGCTTCAGTACTT TACAGAA…CAAAGCTGAAT TAAAATGGTATAAATTAAATCACTTT	This primary miRNA transcript sequence is processed to generate the miR-30a microRNA. Substitution of nts 129–154 (left arm) and 170–193 (right arm) with your targeting sequence and its complement can lead to efficient loading of the inserted sequence into the RISC (18)

Each element in the table is provided with a GenBank accession number and sequence tags to uniquely identify the sequence that can be used, along with either the entire sequence for short segments, or the beginning and end sequence for longer sequences (>100 bp), separated by an ellipsis. The length of the beginning and end sequences of longer sequence entries were chosen such that oligonucleotide primers of the provided sequence and length will have a melting temperature of >60°C, to facilitate the design of PCR primers with enzyme sites chosen for compatibility with the above or other plasmid backbones

3. PCR primers: pUC/M13 forward or reverse primer (New England Biolabs, Beverly, MA) and a vector insert specific primer.
4. A real-time quantitative SYBRgreen PCR mixture (e.g., iQ SYBR Green Supermix, Bio-Rad, Hercules, CA), or RT² Real-Time™ SYBR Green PCR master mix (SuperArray Bioscience Corporation, Frederick, MD).

2.3. Generation of Primary BEV Stocks

1. Sf9 insect cells (Invitrogen).
2. Sf9 cell culture medium (e.g., Sf-900 II SFM (1×) liquid, Invitrogen).
3. Reagents suitable for transfection of insect cells. For example: cationic liposomes (Cellfectin – Invitrogen, ESCORT – Sigma-Aldrich, St. Louis, MO, *Tfx*™ – Promega, Madison, WI, FectoFly™ – Polyplus-Transfection Inc., New York, NY); polyethyleneimine/PEI (ExGen 500 – Fermentas, Glen Burnie, MD); or calcium phosphate (Baculovirus Expression Vector System – BD Biosciences, San Jose, CA). Any one of these reagents could be utilized to transfect cells with bacmid DNA following manufacturer's protocol.
4. Rep78/68-expressing BEV, such as Bac-Rep (27) or similar.
5. DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA).
6. Reagents for standard agarose gel electrophoresis.

2.4. Expansion, Validation, and Titration of Plaque Purified BEV Stocks

1. Sf9 insect cells (Invitrogen).
2. Sf9 cell culture medium (e.g., Sf-900 II SFM (1×) liquid, Invitrogen).
3. Alkaline PEG 200 solution, pH 13.5 (28).
4. PCR primers: Bac-F: 5′-CCGTAACGGACCTCGTACTT-3′; Bac-R: 5′-CCGTTGGGATTTGTGGTAAC-3′.
5. A real-time quantitative SYBRgreen PCR mixture (e.g., iQ SYBR Green Supermix, Bio-Rad), or RT² Real-Time™ SYBR Green PCR master mix (SuperArray Bioscience Corporation).

3. Methods

3.1. Construction of AAV Vector Plasmids Suitable for Transient Transfection of 293 Cells

1. Design the expression cassette and the assembly process. In the simplest form, this can be the ligation of your cDNA into one of the plasmids shown in Fig. 1. after removing GFP (e.g., with *Eco*RI and *Not*I digestion). However, by using a modular design strategy, it is straightforward to generate a small library of fragments that can be combined in multiple configurations

to create a series of vectors with alternative genes and regulatory elements. For considerations of AAV vector size constraints and clinical AAV vector backbones see Notes 2 and 3, respectively.

2. Using a high fidelity polymerase (such as *Pfu* Turbo, Stratagene or Phusion, New England Biolabs), PCR amplify the individual components using primers containing the necessary restriction enzyme sites and ligate them each into a PCR cloning plasmid (see Note 4). Alternatively, using the sequences provided in Table 2 and the referenced GenBank record, one can order the entire sequence to be commercially synthesized and provided cloned in a plasmid (see Note 5).
3. Fully sequence the component fragment plasmids. These plasmids should be saved and over time become part of a valuable library for future constructions (á la, the BioBricks™ model (29)).
4. Purify the fragments from parental plasmids after restriction digestion, ligate them together, and transform *E. coli* (see Note 1).
5. Screen small scale plasmid preparations for structural integrity.
   (a) After your small scale *E. coli* culture has grown overnight, save a small frozen glycerol stock of your cells prior to purifying the plasmid DNA from the remaining culture. Leaving bacterial cells at 4°C for extended periods increases the likelihood that the ITRs will be deleted (see Note 1).
   (b) In addition to whatever digestions are necessary to confirm proper assembly of the fragments in the ligation, always screen your plasmid preparations with an appropriate enzyme to monitor for ITR deletion, such as SmaI or SrfI (see Note 1).
6. Using the frozen cell stock for the individual clone that meets all screening criteria, prepare a large-scale plasmid preparation by re-streaking the frozen cells to obtain fresh, individual colonies and inoculating a single colony into a larger culture.
7. After the large-scale culture has grown to late log phase, and prior to lysing the cells for DNA purification, remove another aliquot of culture liquid to prepare several 0.5–1.0 ml glycerol aliquots for long-term storage. If the purified DNA from this culture proves to be significantly free of ITR-deleted plasmid, then all future plasmid preparations should be generated from this validated stock to reduce the risk of re-introducing ITR deletions.

3.2. Construction of Transfer and Bacmid Plasmid Vectors

1. First, modify Invitrogen's pFastBacDual plasmid by StuI and XhoI double digest, blunting with Klenow, and religation. This step deletes both *p10* and *polh* promoters, which are not

required for rAAV cassette rescue. The truncated polylinker contains 12 unique restriction sites that could be utilized to insert your recombinant AAV vector genomes.

2. Transfer each AAV vector genome into the plasmid created in Subheading 3.2 (step 1), including both ITRs flanking the expression cassette. Again, pay strict attention to the integrity of your ITRs (see Note 1).
3. Transform *E. coli* DH10BAC electrocompetent cells with the pFastBacDual-derived vector plasmid containing your AAV vector genome. Incubate plates at 37°C for at least 24 h followed by overnight incubation at RT. The extended time is required for colonies to grow and to develop light blue color. Clones containing recombinant transposed bacmids are white and usually slightly bigger in size. Pick 6–8 white colonies, expand in 2 ml LB cultures, and purify the bacmid DNA for further screening.
4. At this stage, candidate bacmids can be optionally screened by PCR to confirm the presence of the vector genome. Using one primer in the backbone (either pUC/M13 forward or reverse primers) and one primer in your vector cassette, PCR amplify each bacmid preparation to confirm successful introduction of the vector genome. Although the ITR inhibits the PCR and can lead to amplification of truncated products, it can still be a useful tool to exclude recombinants that do not contain a vector genome. The integrity of the AAV ITRs is screened at a later step by testing replicative fitness.

3.3. Generation of Primary BEV Stocks

1. Transfect Sf9 cells with bacmid DNA samples using any one of the reagents described in Subheading 2.2 (item 2) following manufacturer's protocol (see Note 6). The supernatant (4 ml from 60 mm cell culture dish) harvested at day 5 post-transfection contains BEV-rAAV stock, albeit at a lower titer of 10^7–10^8 pfu/ml. Multiple bacmids for each recombinant vector are typically transfected separately to allow screening of the resulting BEV for replicative fitness.
2. Each supernatant can at this point be screened for replicative fitness in an in vivo AAV genome replication assay. This allows the best stock of BEV to be used in the plaque purification, and can reduce the number of plaques that must be similarly screened.
   (a) To do this assay, add 0.5 ml of each supernatant to 60-mm dish with Sf9 cells plated at ~75% confluence. Knowing the precise titers and multiplicity of infection (MOI) is not important for this step as long as all BEV stocks derived from the individual transfected bacmid DNAs are treated the same way. To rescue rAAV cassette, co-infect cells with

Rep78/68-expressing BEV, such as Bac-Rep (27) or similar vector at MOI of 5.

(b) Forty-eight hour postinfection, isolate Hirt DNA according to the following procedure (modified from ref. 30). Harvest cells in the culture media from each of the dishes, transfer into 15-ml conical tubes and pellet by centrifugation at 1,000 × *g* for 5 min. Resuspend cell pellets in 1 ml PBS, transfer into Eppendorf tubes, centrifuge at 5,000 × *g* for 5 min, and discard the supernatants. Resuspend cell pellets in 250 µl 50 mM Tris–HCl, pH 7.5, 10 mM EDTA containing 100 µg/ml RNase A and lyse by the addition of 250 µl 1.2% SDS. Gently mix the suspension by inverting the tube several times and then incubate at RT for 5 min. Precipitate cellular debris and chromosomal DNA by the addition of 350 µl solution containing 3 M CsCl, 1 M potassium acetate and 0.67 M acetic acid. Immediately, gently mix the tubes and place them on ice for 15 min. After centrifugation for 15 min at 14,000 × *g*, load the supernatants onto a silica-based matrix spin column, such as those supplied with DNA Clean & Concentrator™-5 Kit (D4004, Zymo Research). Wash the column twice with the wash buffer (supplied in a kit) and elute the DNA in 20 µl H_2O. Utilizing spin columns greatly facilitates the DNA purification while reducing the sample-to-sample yield variability.

(c) Load half of the DNA sample (10 µl) onto 1.2% agarose TAE gel containing 10 µg/ml EtBr and separate the DNA during 1.5 h electrophoresis. The pattern of UV light-visualized bands should display a major band corresponding to rAAV-cassette double-stranded monomer, as well as several bands corresponding to higher order concatemers, a replication intermediate (Fig. 4). Comparing all DNA samples on the gel allows identifying one or two clones with higher yield of replicating AAV vector genomic DNA. These clones presumably contain BEV-rAAVs with higher content of intact ITRs.

3. Perform plaque purification using the best supernatant. Assuming that the starting titer of the original, bacmid-derived BEV-rAAV is 10^8 pfu/ml, use tenfold serial dilutions to derive a plate with no more than ten isolated plaques (see Note 7).

3.4. Expansion, Validation, and Titration of Plaque Purified BEV Stocks

1. Pick 3–4 well isolated plaques (called P0) and expand them by serial infection of naïve Sf9 cells. Optimally this is achieved by titrating each amplification supernatant so as to determine the quantity of supernatant necessary to achieve 0.1 MOI during

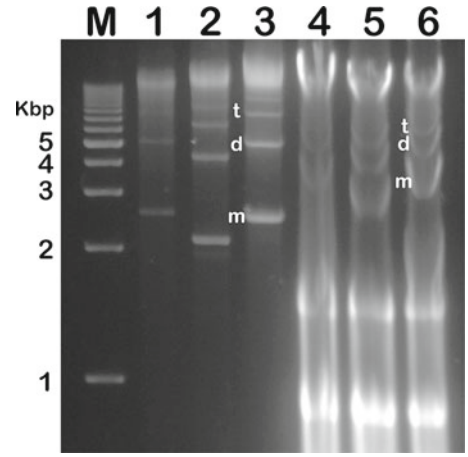

Fig. 4. Agarose gel analysis of replicating rAAV DNA isolated using modified Hirt procedure. *Lane M* contains 1-kbp DNA ladder. *Lanes 1* and *4*, *2* and *5*, *3* and *6* contain same rAAV expression cassettes but purified using modified Hirt protocol (*lanes 1–3*) or standard Hirt protocol (*lanes 4–6*). Letters "*m,*" "*d,*" and "*t*" indicate rAAV mono-, di-, and trimer replicating forms, respectively. Because of the excessive contaminating RNA and DNA in *lanes 4–6*, the mobility of rAAV forms is abnormally retarded. *Lanes 2* and *5* contain a smaller size self-complementary (sc) rAAV cassette.

each phase of expansion. As plaque assay is impractical at such steps, a quick quantitative PCR assay is described below. Alternatively, one can simply dilute each supernatant 1:100 into a naïve Sf9 culture for each amplification.

2. To perform a quick QPCR assay to estimate a relative plaque forming unit (pfu) titer, use this adaptation of an alkaline PEG-based method for direct PCR (28). Briefly, add 5 µl of baculovirus stock to 95 µl of alkaline PEG solution. After vortexing, incubate the sample at room temperature for 15 min and then dilute fivefold by adding 0.4 ml H_2O. Use 5 µl of this diluted mixture directly in QPCR 25 µl reaction mixture containing 12.5 µl SybrGreenER and 1.5 µl of 5 µM universal BEV Bac-F and Bac-R primers each (see Note 8).
3. Early in the amplification process (P1 or P2), perform an assay of the AAV vector genome replicative fitness, as described in Subheading 3.3 (step 2). Select the best replicator for the master seed virus stock for future expansions.
4. Propagate and expand the selected plaque purified clone by inoculating fresh Sf9 cells at an MOI of 0.1 (see Note 9). The large-scale production and purification of the insect cell-derived rAAV vectors are not covered in this chapter. Follow the detailed procedures described elsewhere (26, 31–36).

4. Notes

1. AAV vector plasmids are notorious for the instability and frequent deletion of the ITRs when propagated in *E. coli*. For this reason it is important to carefully confirm the integrity of every preparation used for vector construction. Even the best plasmid preparations contain significant quantities (5–15%) of DNA containing small deletions in the ITR, which during cloning procedures can be randomly selected and amplified. The ITRs have such strong secondary structure they cannot be sequenced with standard automated protocols, and so integrity of the ITRs is typically monitored by restriction enzyme analysis sufficient to identify small deletions (20 bp). Most commonly this is achieved with digestion with *Sma*I or *Srf*I, which cut twice within an unstable portion of the ITR, although an ideal strategy must also consider the ability of the resulting fragments to be separated and accurately sized on gels (Fig. 5). As long as this type of careful screening technique and optimal microbiological practice is maintained, then significant modifications to standard molecular cloning techniques are not absolutely necessary. Once a validated, frozen glycerol stock of *E. coli* is archived, subsequent preparations of plasmids from those stocks rarely contain high levels of deleted DNA, in

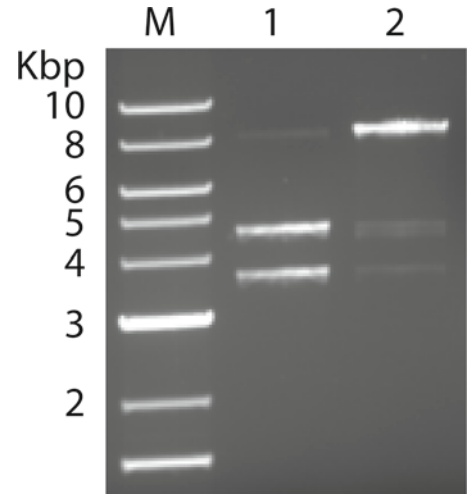

Fig. 5. Agarose gel analysis of SrfI digested AAV vector plasmid DNA. A complete digestion product would be expected to generate two bands for each plasmid, with sizes of 3.7 and 4.7 kbp. *Lane 1*, AAV vector plasmid preparation with minimal ITR deletion, cut with SrfI. Note that this successful preparation still appears as a partial digestion, with an 8.4 kbp, singly cut plasmid band visible at ~5% of total. *Lane 2*, an ITR deleted preparation with the proportions reversed, and ~5% of the DNA digested at both ITRs.

contrast to frequent deletions observed when screening fresh transformants. Because of these stability issues, most investigators utilize a recombination deficient strain, such as SURE for AAV vector plasmid propagation and cloning.

2. AAV vector genomes cannot significantly exceed 5 kb without dramatically reducing yield. For single-stranded vectors, the size of the resulting genome can easily be measured as the length of the sequence between and including the ITRs. For double stranded genomes, the Δ*trs* ITR becomes a central hairpin in the middle of a dimer genome, and so the total length of the vector in the plasmid, including the ITRs, should be less than 2.4 kbp.
3. For clinical applications, additional modifications to the vector backbone plasmid are desired. Substitution of kanamycin resistance for ampicillin allows preparation of the plasmid without allergenic β-lactam antibiotics. Additionally, the backbone sequences of plasmids can occasionally be incorporated into vector particles by virtue of their proximity to the AAV ITR. Insertion of large fragments of stuffer DNA into the backbone can reduce this phenomenon (37).
4. In many cases PCR products can be ligated directly to plasmid backbone fragments after digestion with restriction enzymes; however, the efficiency is significantly reduced, often precluding successful assembly of more than two fragments at a time. Preliminary subcloning and sequencing is slower, but provides an easily archived reagent and allows for the most efficient ligation reaction.
5. The advent of lower cost gene synthesis services provides yet another avenue from PCR for getting your gene of interest into a vector. This also has the advantage of allowing the modification of coding sequences to "optimize" expression or other properties for the particular application you are interested in. The instability and strong secondary structure of AAV ITRs makes direct synthesis of whole vectors by this method much more challenging, and for that reason most investigators, even if they are synthesizing the gene and or regulatory elements, will subsequently ligate those elements into a plasmid backbone for large-scale DNA propagation necessary for vector production.
6. The efficiency of Sf9 cell transfection can be assessed using a plasmid with GFP reporter gene driven by baculovirus promoter, such as immediate early OpIE2 in pIZ/V5-His vector (Invitrogen). This reporter has to be constructed in advance using conventional cloning techniques. Late promoters, such as *polh* or *p10* in pFastBacDual cloning vector are not functional in Sf9 cells even if cells are infected with baculovirus.

These late promoters require *cis*-acting viral enhancer elements (homologous region, *hr*) to be fully activated.

7. For a plaque assay, it is important to plate Sf9 cells at no higher than 40–50% confluence at the time of infection. At higher densities, plaques are smaller in size, harder to visualize, and yield fewer viruses. The other important consideration is the quality of the BEV-rAAV. To improve the virus stability during propagation, it is critical to infect cells at low MOI of about 0.1. Most of the virus harvested in a plaque derives from the second round infection around the cell infected at time zero. At high initial seeding density cells grow confluent by the time of secondary infection and, thus, only few nearby cells are infected at significantly higher than optimal MOIs. This results in smaller plaques, lower yields, and higher ratios of re-arranged, ITR-less progeny virus, which, by the fourth passage, becomes the prevalent species.
8. The BEV sample is assayed side by side with a serially diluted reference standard, a BEV of a known titer. Universal BEV primers sequences are the following: Bac-F: 5′-CCGTAAC GGACCTCGTACTT-3′; Bac-R: 5′-CCGTTGGGATTTGTGG TAAC-3′. The amplified sequence is part of AcMNPV gene *Ac-IE-01*, locus tag – *ACNVgp142*, a putative early gene trans-activator; the size of the amplified DNA fragment is 103 bp. The sensitivity of RT PCT titering method is as low as 10^5 pfu/ml. One can use either universal BEV primers or transgene-specific primers. When using the latter, a shuttle plasmid with the cloned transgene is utilized to generate a serially diluted reference standard. Prior to PCR analysis, plasmid DNA should be linearized by a single-cutter restriction enzyme, phenol/chloroform purified, and stored at 1 μg/μl frozen. Serial dilution DNA standards are prepared at the time of the RT PCR experiment, preferentially after handling experimental samples. Titers derived by this method are identical to the titers obtained using standard plaque assay.
9. Using the Baculovirus system, one should expect to produce at least 10^{14} drp rAAV from 1 L Sf9 cells culture. This would require about 50 ml of BEV helper stock of a relatively low titer of 2×10^8 pfu/ml. If BEV harvested from the plaque designated as “passage zero” (P0), then propagating BEV exponentially to passage two (P2) will generate as much as 4–5 L of the BEV stock. BEV helpers were shown to be stable up to P5, thus generating sufficient amount of BEV does not constitute a serious technical challenge. Titration of viral stocks harvested at P1 through P3 is recommended to propagate the vector at the known MOI of 0.1. Once the BEV stock is produced, it could be stored for extended periods of time (up to a year) at 4°C. Adding FBS to 1% increases long-term stability

of the stored stock. Over time, the infectious titer of the stock is reduced necessitating re-titration assays. Because titers drop due to viral particle aggregation, the RT PCR assay described above might not be appropriate due to potential overestimation of the infectious titer. Instead, a regular plaque assay, although more laborious, will generate the up-to-date infectious titer information.

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Adeno-Associated Virus
Methods and Protocols
Snyder, R.O.; Moullier, P. (Eds.)
2011, XIII, 462 p. 43 illus., 20 illus. in color., Hardcover
ISBN: 978-1-61779-369-1
A product of Humana Press