Chapter 2

Delivering Transgenic DNA Exceeding the Carrying Capacity of AAV Vectors

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Abstract

Gene delivery using recombinant adeno-associated virus (rAAV) has emerged to the forefront demonstrating safe and effective phenotypic correction of diverse diseases including hemophilia B and Leber's congenital amaurosis. In addition to rAAV's high efficiency of transduction and the capacity for long-term transgene expression, the safety profile of rAAV remains unsoiled in humans with no deleterious vector-related consequences observed thus far. Despite these favorable attributes, rAAV vectors have a major disadvantage preventing widespread therapeutic applications; as the AAV capsid is the smallest described to date, it cannot package "large" genomes. Currently, the packaging capacity of rAAV has yet to be definitively defined but is approximately 5 kb, which has served as a limitation for large gene transfer. There are two main approaches that have been developed to overcome this limitation, split AAV vectors, and fragment AAV (fAAV) genome reassembly (Hirsch et al., Mol Ther 18(1):6–8, 2010). Split rAAV vector applications were developed based upon the finding that rAAV genomes naturally concatemerize in the cell post-transduction and are substrates for enhanced homologous recombination (HR) (Hirsch et al., Mol Ther 18(1):6–8, 2010; Duan et al., J Virol 73(1):161–169, 1999; Duan et al., J Virol 72(11):8568–8577, 1998; Duan et al., Mol Ther 4(4):383–391, 2001; Halbert et al., Nat Biotechnol 20(7):697–701, 2002). This method involves "splitting" the large transgene into two separate vectors and upon co-transduction, intracellular large gene reconstruction via vector genome concatemerization occurs via HR or nonhomologous end joining (NHEJ). Within the split rAAV approaches there currently exist three strategies: overlapping, trans-splicing, and hybrid trans-splicing (Duan et al., Mol Ther 4(4):383–391, 2001; Halbert et al., Nat Biotechnol 20(7):697–701, 2002; Ghosh et al., Mol Ther 16(1):124–130, 2008; Ghosh et al., Mol Ther 15(4):750–755, 2007). The other major strategy for AAV-mediated large gene delivery is the use of fragment AAV (fAAV) (Dong et al., Mol Ther 18(1):87–92, 2010; Hirsch et al., Mol Ther 21(12):2205–2216, 2013; Lai et al., Mol Ther 18(1):75–79, 2010; Wu et al., Mol Ther 18(1):80–86, 2010). This strategy developed following the observation that the attempted encapsidation of transgenic cassettes exceeding the packaging capacity of the AAV capsid results in the packaging of heterogeneous single-strand genome fragments (<5 kb) of both polarities (Dong et al., Mol Ther 18(1):87–92, 2010; Hirsch et al., Mol Ther 21(12):2205–2216, 2013; Lai et al., Mol Ther 18(1):75–79, 2010; Wu et al., Mol Ther 18(1):80–86, 2010). After transduction by multiple fAAV particles, the genome fragments can undergo opposite strand annealing, followed by host-mediated DNA synthesis to reconstruct the intended oversized genome within the cell. Although, there appears to be growing debate as to the most efficient method of rAAV-mediated large gene delivery, it remains possible that additional factors including the target tissue and the transgenomic sequence factor into the selection of a particular approach for a specific application (Duan et al., Mol Ther 4(4):383–391, 2001; Ghosh et al., Mol Ther 16(1):124–130, 2008;

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Hirsch et al., Mol Ther 21(12):2205–2216, 2013; Trapani et al., EMBO Mol Med 6(2):194–211, 2014; Ghosh et al., Hum Gene Ther 22(1):77–83, 2011). Herein we discuss the design, production, and verification of the leading rAAV large gene delivery strategies.

Key words Large gene delivery, Split AAV method, Overlapping, Trans-splicing, Hybrid, Fragment, Adeno-associated virus, Concatemer, Vectorcapacity

1 Introduction

1.1 AAV Vectorology

Adeno-associated virus (AAV) is a non-autonomous parvovirus that requires a helper virus, such as adenovirus, to complete its life cycle. AAV remains the smallest known virus and is comprised of a protein capsid (20–24 nm) and a single-strand DNA genome of approximately 4.7 kb. The AAV genome contains at least three open reading frames encoding replication, capsid, and capsid assembly proteins [14]. An important feature of the AAV genome is that it is flanked on both sides by 145-nucleotide inverted terminal repeats (ITRs) which are necessary for viral replication, packaging, and site-specific integration [14]. In the 1980s, the AAV genome was cloned from nature and the controlled production of WT AAV was demonstrated in cell culture in the presence of adenovirus [15]. Soon thereafter, it was discovered that the AAV genome could be substituted for a sequence of choice. Thus, recombinant AAV (rAAV) particles could be produced having transgenic genomes [15, 16]. The only viral DNA elements necessary for replication and packaging during rAAV production are the ITRs flanking the transgenic DNA in *cis*, as the *rep* and *cap* genes can be supplied in *trans* using a separate plasmid [16]. Shortly after these seminal observations it was demonstrated that adenovirus could be substituted by its partial genome in plasmid form, which allowed the production of rAAV in the absence of contaminating adenovirus [17]. Despite over 25 years of rAAV optimizations for diverse applications, this method of rAAV production predominantly remains unchanged.

Regarding the transduction efficiency, rAAV has proven the most efficient and safe method of gene delivery for sustained mammalian cell transduction. The favorable attributes of rAAV include (1) its non-pathogenicity, (2) ability to transduce nondividing and dividing cells, (3) its broad tissue tropism conferred by various natural and mutant serotypes, (4) the persistence of rAAV genomes as primarily episomes with very low levels of integration into the host chromosome, and (5) the ability to confer long-term transgene expression following a single injection [14]. Given these favorable attributes, well over 100 rAAV clinical trials have been performed to date for diverse diseases with notable successes for the treatment of hemophilia B and Leber's congenital amaurosis [18, 19].

Despite rAAV's popularity and clinical success, it does have a major limitation in that the AAVcapsid cannot package sequences greater than about 5 kb [1]. This packaging limitation is an obstacle for treatment of genetic diseases requiring larger transgenes such as Duchenne muscular dystrophy, hemophilia A, and cystic fibrosis. However, to overcome the packaging limitations, creative intracellular large gene reconstruction strategies have been developed including the split vector approaches (overlapping, trans-splicing, and hybrid vectors) and fAAV vector transduction [1].

1.2 Split rAAV Large Gene Delivery

Split vector large gene delivery approaches take advantage of the observation that rAAV genomes undergo concatemerization via non-homologous end joining (NHEJ) and intermolecular HR. Previous reports have shown that rAAV genomes form concatemer in liver, muscle, and cell lines [9, 20–23], which likely aids in the persistence and long-term expression of the transgenic cassette. Following uncoating, rAAV genomes readily circularize through intramolecular ITR linkages and over time dimer and multimeric concatemers are generated that persist as episomes [2, 3, 9, 21, 22]. This inherent tendency for intermolecular genome associations serves as a requirement for all rAAV large gene delivery approaches herein. Split rAAV vectors rely on the division of a large transgene (and expression requirements) into two rAAV vectors (termed vector A and vector B herein) that are distinct and produced independently [4, 5]. Within the split vector approaches there is a primary division of overlapping and trans-splicing rAAV vectors [4–6].

1.3 Overlapping Vector Approach

In the overlapping vector approach, vectors A and B display a region of sequence homology to promote intermolecular HR, thus generating the large transgene by recombining vectors A and B as depicted (Fig. 1). The efficiency of homologous recombination correlates with the degree of sequence overlap, and the actual sequence, of the vector A and vector B genomes. Increased size of the overlapping sequence can lead to an increase in homologous recombination [4]. This increased homologous recombination can also lead to a slight hindrance in the overall objective of rAAV large gene therapy, as increasing the overlapping sequence effectively decreases the overall transgene capacity [23]. Therefore the limitation for the overlapping vector approach is largely dependent on the recombination efficiency of the overlapping sequence and the propensity for episomal homologous recombination in the transduced cell.

1.4 Trans-splicing Technique

The trans-splicing technique also relies on a co-transduction approach in which the transgene is split such that one vector (vector A) contains a promoter, a 5′ portion of the gene, followed by a splice donor sequence (Fig. 2). The downstream vector (vector B) contains a splice acceptor sequence the remaining 3′ cDNA (or

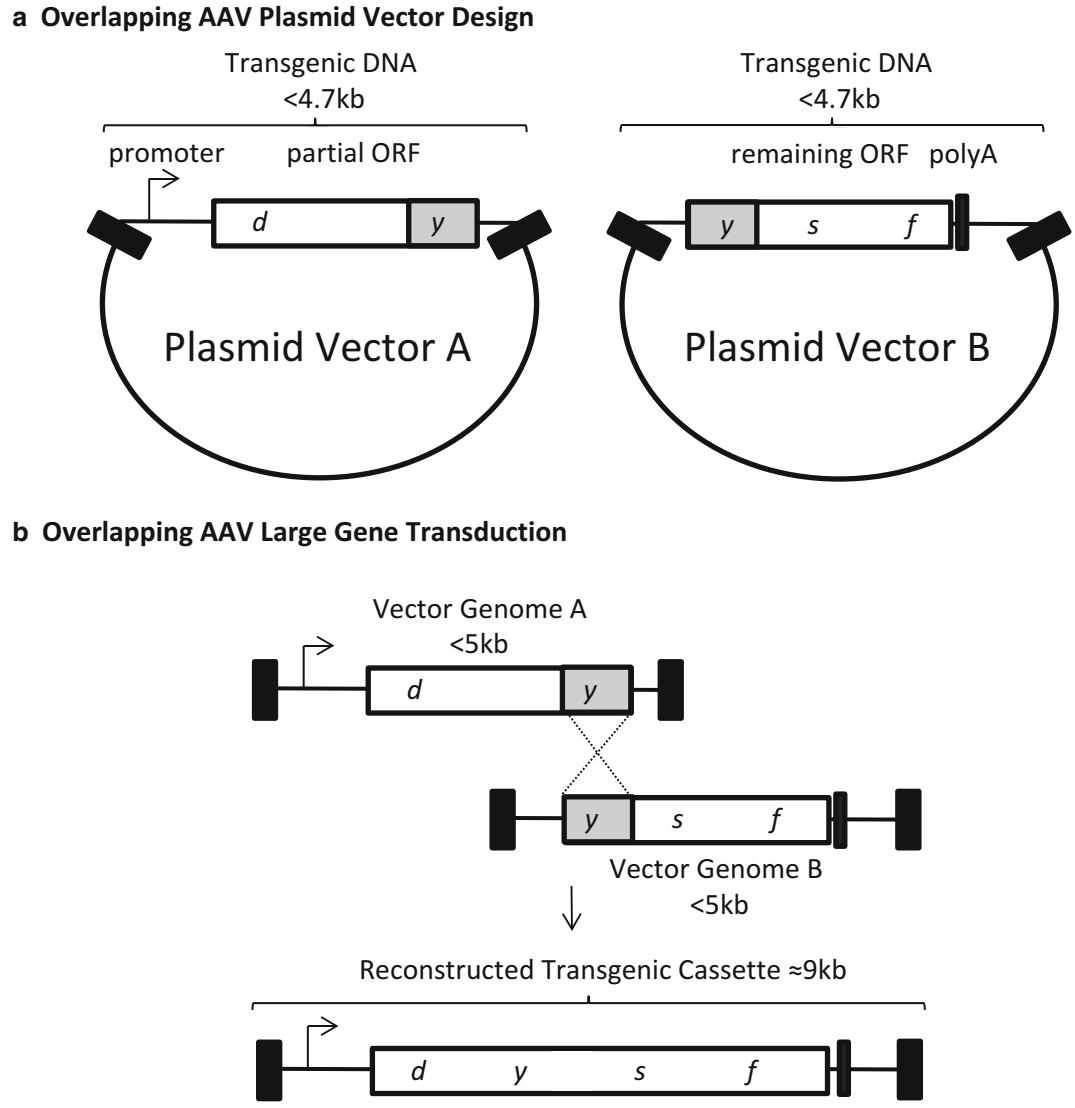

Fig. 1 Overlapping AAV genome design and transduction. (**a**) The transgenic cassette, containing the depicted elements, is split into two separate vectors such that there is a homologous sequence overlap (*gray*). The total size of the transgenic DNA must be less than 4.7 kb. (**b**) Following co-transduction of vector genomes A and B, homologous recombination at the overlapping sequence occurs to reconstruct the depicted large transgenic DNA cassette of approximately 9 kb. *Black box* AAV inverted terminal repeat

gene), and a poly A tail [4]. A concatemerization event in the correct orientation (theoretically about a 16 % chance) generates a single DNA molecule containing the intended large transgene expression cassette (Fig. 2) [4, 24]. Upon production of the pre-mRNA the intron is spliced out along with the ITR recombination junction, establishing an intact open reading frame large than can be packaged in a single vector [23, 25]. The trans-splicing method has been used in several Duchenne muscular dystrophy mouse

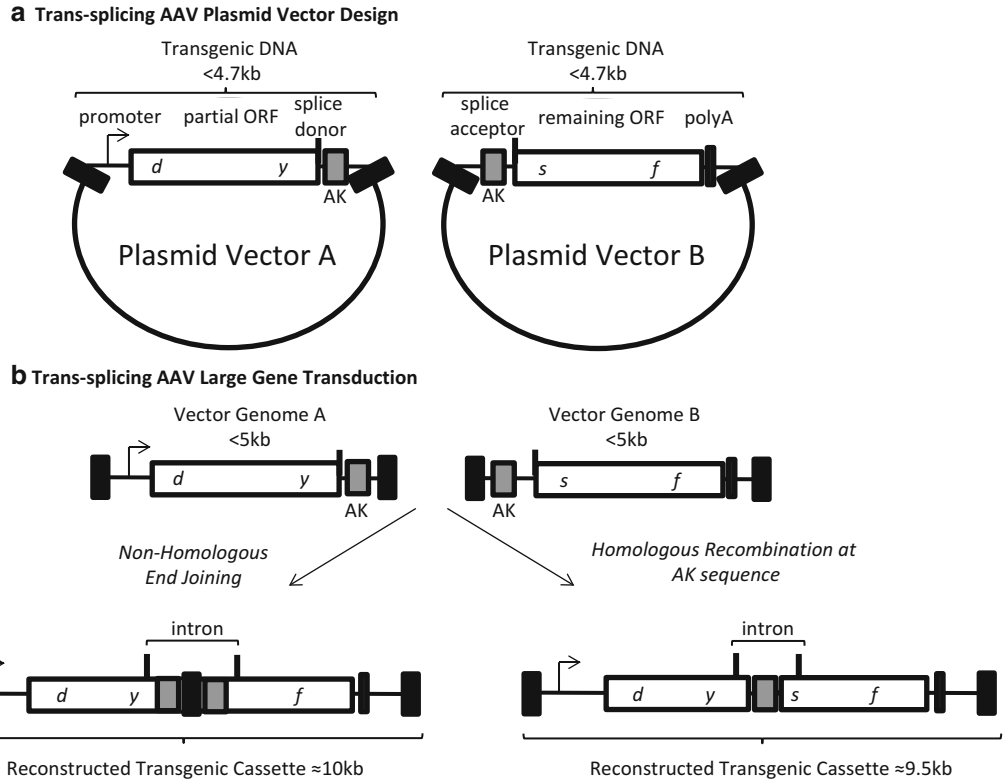

Fig. 2 Trans-splicingAAV vector design and transduction. (**a**) The genetic elements of the two required AAV plasmids are depicted. Plasmid A contains a promoter, a partial open reading frame terminated at a splice donor site, followed by a recombinogenic sequence (hybrid vectors only, AK). The vector B plasmid contains a recombinogenic sequence (hybrid vectors only, AK), a splice acceptor site, the remaining ORF followed by a poly A sequence. The transgenic DNA in each plasmid is less than 4 kb. (**b**) Following co-transduction vector genomes A and B can undergo end joining (*left*) or homologous recombination at the AK sequence (*right*) to reconstruct a large transgenic cassette containing the depicted intron. *Black box* AAV inverted terminal repeat

models and has shown detectable restoration levels of dystrophin post-transduction [26–28]. There are also studies showing efficient whole-body and retina transduction that may also be promising for efficiently restoring large transgene expression [7, 29]. Even though efficient transduction is noticed in these experiments the transduction efficiency of these trans-splicing vectors is decreased compared to that of a single intact vector in skeletal muscle, eye, and liver [9]. The reason for this decreased transduction efficiency is due to several factors including (1) the efficiency of multiple vector transduction of a single cell, (2) the efficiency of the cellular machinery to reconstruct the large transgene in the desired orientation, (3) the decreased accumulation of mRNA due to the instability of the pre-mRNA, and (4) the gene splitting site [30]. The use of synthetic introns, including exonic splicing enhancers, can be used to at least partially counteract these rate-limiting steps [26, 31, 32].

1.5 Hybrid Trans-splicing Technique

The hybrid trans-splicing technique is essentially a combination of the overlapping and trans-splicing large gene delivery approaches described above. These hybrid vectors are designed in the same manner as trans-splicing vectors with one key difference: the inclusion of an overlapping sequence within the intron of both the 5′ and 3′ vectors (Fig. 2) [6, 13]. To increase the likelihood of the desired large gene reconstruction, particular sequences that are considered "recombinogenic" can be used as the overlapping sequence [6, 12, 13, 33]. Following co-transduction, the full-length transgene can either be formed by HR at the overlapping sequence or the ITR homology. Alternatively, the ITRs may join via NHEJ [9, 20]. Then, transcription, splicing and translation generate the desired protein as described above for trans-splicing vectors. Current reports comparing different overlapping "recombinogenic" sequences within the hybrid trans-splicing strategy suggest that a 77 nt sequence derived from the F1 phage genome is the most efficient in promoting large transgene reconstruction, at least in the tested cells [12]. This hybrid AK vector context, as well as the normal trans-splicing approach, has demonstrated relevance for the treatment of retinal diseases including Stargardt's disease and Usher syndrome type 1B [12].

1.6 Fragment AAV Large Gene Delivery

Regarding rAAV large gene delivery, reports have demonstrated the ability of the AAVcapsid to package genomes over 5 kb [34–37]. For instance, Allocca et al. surveyed the packaging capacity of different capsid serotypes [34]. In the eye, it was demonstrated that AAV serotype 5 capsid, in particular, could package an expression cassette containing the adenosine triphosphate-binding cassette (ABCA4) at an unprecedented size of 8.9 kb, which was verified by alkaline gel electrophoresis [34]. Importantly, this AAV5-ABCA4 vector mediated effective transduction and resulted in the correction of disease phenotypes observed in the retina of a Stargardt's disease mouse model [34]. The excitement for this groundbreaking report was later tempered by the reports of several groups that concluded that rAAV genomes over 5 kb were not packaged in their entirety, but instead DNA "fragments" of the intended oversized cassette were encapsidated (Fig. 3). Characterization of this process, demonstrated that oversized genome packaging initiates from the 3′ end and the external 5′ end is truncated when the capacity of the capsid is reached [8, 10, 11]. However, this process remains largely uncharacterized as heterogeneous DNA species (all <5 kb) are packaged in the apparently intact AAV capsids [8–11, 38]. Interestingly, these fAAV preparations retained their ability to mediate large gene transduction, albeit at a reduced efficiency compared to intact AAV in a tissue- and administration-dependent manner. A mechanistic eval-

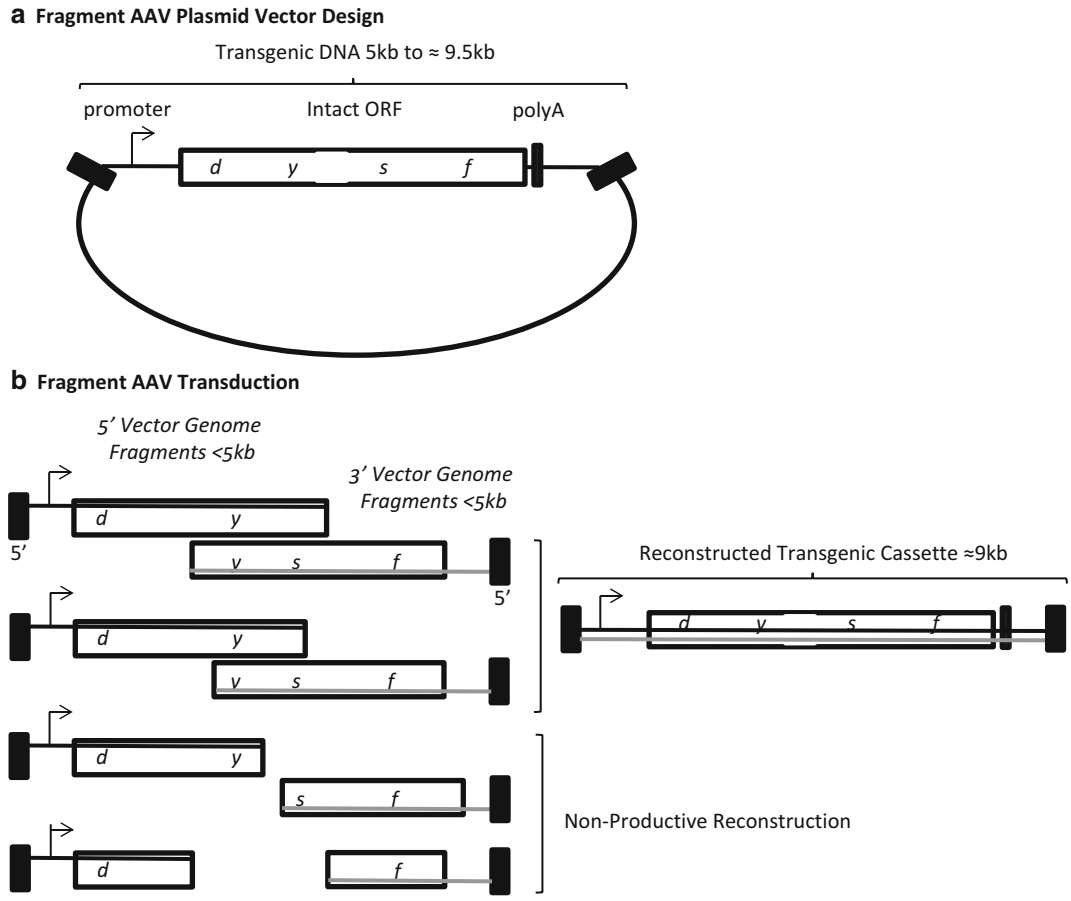

Fig. 3 Fragment AAV vector design and transduction. (**a**) A single plasmid with an intact transgenic cassette is depicted. (**b**) During AAV vector production different sized genome fragments of the over-sized transgenic cassette are packaged. Fragments of both polarities are packaged (*gray and black lines*) starting at the 3′ end. Opposite polarity fragments containing regions of complementarity can anneal followed by strand synthesis to generate a large transgenic cassette. Contaminating fragment genome species below half of the intended transgenic cassette do not contain strand complementarity and are non-productive for transduction. *Black box* AAV inverted terminal repeat

uation of fAAV transduction in cell culture suggested that a large gene reconstruction event relies on HR, as the repair proteins Rad51C and XRCC3 were necessary while the NHEJ mediator DNA-PKcs was dispensable [9]. Despite the lack of an explanation for the earlier report demonstrating AAV5 large gene packaging [34], what is clearly evident is that a new rAAV large gene strategy emerged (fAAV) which, as in the other strategies, relies on the intracellular reconstruction of the oversized genome fragments, perhaps by canonical homology directed repair, at least in vitro [1, 9].

2 Materials

2.1 rAAV Production

1. 2× HEPES-buffer saline: 0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na_2HpO_4, titrate to pH 7.05 with 5 N NaOH.
2. Polyethylenimine (PEI) linear MW 25,000: 1 mg of PEI in 1 ml of 1× PBS, Adjust pH to 4 or 5 with 12 N HCl (*see* **Note 1**).
3. DMEM: 10 % FBS, 1× penicillin/streptomycin.
4. DNaseI digestion buffer: 10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 U/ml DNase I.
5. Sonicator (Branson Sonifier 250, or equivalent).
6. Beckman Quick-Seal polypropylene (16 mm×76 mm) centrifuge tubes (Beckman, Indianapolis IN, USA).
7. Ultracentrifuge (Sorvall Discovery 90SE centrifuge, or equivalent).
8. Ultracentrifuge rotor (Beckman NVT65 rotor, or equivalent).
9. 15 cm tissue culture plates.
10. 1.7 ml polystyrene microfuge tubes.
11. Slide-A-Lyzer dialysis cassette with 20,000 (MWCO) and 0.5–3.0 ml capacity.
12. HEK 293 cells.

2.2 Southern Dot Blot

1. DNaseI digestion buffer (6 ml): 5.808 ml ddH_2O, 60 µl DNase (10 µg/µl), 60 µl 1 M Tris pH 7.5, 60 µl $MgCl_2$, 12 µl 1 M $CaCl_2$.
2. Proteinase K solution (10 ml solution): 6.9 ml ddH_2O, 2 ml 5 M NaCl, 1 ml 10 % Sarkosyl, 100 µl proteinase K (10µg/µl).
3. 0.5 M NaOH (10 ml): 1 ml 5 M NaOH, 9 ml ddH_2O.
4. 0.4 M Tris pH 7.5 (100 ml): 40 ml 1 M Tris pH 7.5, 60 ml ddH_2O.
5. Hybond-XL nylon membrane (GE Healthcare Life Sciences, Pittsburgh, PA, USA).
6. Filter paper.
7. 96-well dot blot manifold (or equivalent).
8. Church buffer.
9. Radioactive probe labeling kit (*see* **Note 2**).
10. High-salt wash (500 ml solution): 445 ml ddH_2O, 50 ml 20 % SSC, 5 ml 10 % SDS.
11. Low-salt wash (500 ml system): 492.5 ml ddH_2O, 2.5 ml 20× SSC, 5 ml 10 % SDS.

2.3 Alkaline Gel Southern Blot

1. Loading dye (6×): 80 µl 5 M NaOH, 10 µl 0.5 M EDTA, 0.18 g Ficoll, 730 µl H_2O, xylene cyanol for color.
2. Alkaline gel: Add 0.5 g agarose to 50 ml water (1 %), microwave to dissolve, let cool then add 500 µl 5 M NaOH and 100 µl 0.5 M EDTA (*see* **Note 3**).
3. Gel running buffer: 10 ml 5 M NaOH, 2 ml 0.5 M EDTA, 988 ml H_2O.
4. Transfer buffer: 80 ml of 5 M NaOH in 920 ml of deionized water.
5. Hybond-XL nylon membrane the size of the gel.
6. Two sheets of filter paper slightly bigger than the size of the gel.
7. One sheet of filter paper the width of the gel and about 1½ ft long for the transfer bridge.
8. A pan that can hold the transfer buffer.
9. A stack of paper towels.

2.4 Quantitative Polymerase Chain Reaction

1. DNaseI mixture (listed above in Subheading 2.2, Southern Blot materials).
2. Proteinase K solution (listed above).
3. 10 mM Tris (pH 8).
4. 2× SYBR mix (Life Technologies, Grand Island, NY, USA).
5. Forward primer (20 µM) (*see* **Note 4**).
6. Reverse primer (20 µM) (*see* **Note 4**).
7. Sterile H_2O.

3 Methods

3.1 Split rAAV Vectors: Construct Design

1. Overlapping technique: To design your two split overlapping vectors, first construct plasmid vector A by placing a promoter followed by the transgene such that the total transgenic sequence is <4.7 kb (excluding the 2 ITR sequences which are each about 150 nucleotides) (Fig. 1). Next, place the remaining transgene sequence on plasmid vector B followed by a poly-adenylation sequence (Fig. 1). Again, the total size of the transgenic DNA needs to be <4.7 kb. Realistically, the overall size of the transgene cassette will dictate the maximum region of vector genome overlap (which is a requirement), with the basic understanding that increased region of homology will be more likely to stimulate intermolecular recombination, and thus the reconstructed large transgene cassette [23].
2. Trans-splicing technique: Divide the transgene approximately near the middle, ideally at a conserved splicing junction.

Then, add a canonical intron donor sequence immediately following the vector A ORF (Fig. 2). Any sequence between this donor signal and the vector A 3′ ITR will be intronic (Fig. 2). To generate vector B, an intronic sequence is positioned downstream of the 5′ ITR and terminated by an intron acceptor sequence (Fig. 2). Then, the remainder of the ORF (prematurely truncated in vector A is positioned upstream of a poly-A sequence prior to the 3′ ITR of vector B (Fig. 2)). As with the overlapping AAV vectors, and the overall rationale of this methods chapter, the total size of intact vectors (including the ITRs) needs to be <5 kb. Designs of trans-splicing constructs in the literature provide examples of the genetic arrangements and intronic sequences used previously [4, 6, 7, 12, 13, 24, 27].

3. Hybrid trans-splicing technique: For this large gene AAV vector format, follow the design of trans-splicing vectors with a single exception, place the same overlapping sequence within the intron on the 3′ end of vector A and the 5′ end of vector B as depicted in Fig. 2. One consideration is the maintenance of a valid intronic branch point to facilitate splicing. Currently, there are only a few reports on overlapping sequences employed in hybrid AAV vectors and evaluation of the sequences used therein should be further investigated [6, 12, 13, 27].
4. Fragment AAV vectors: construct design: A key distinction between fAAV large gene transduction and the split AAV vectors described above is that for the later approaches two distinct plasmid vectors (A and B) need to synthesized and employed for independent vector preparations whereas, a single plasmid containing the ITR sequences is used for fAAV (Fig. 3). Since DNA strands of both polarity are truncated and packaged during the viral vector production, the single plasmid design format is the following (5′ to 3′): (1) ITR, (2) promoter, (3) the oversized transgene, (4) a poly-A sequence, and (5) the flanking ITR sequence (Fig. 3). As genomic fragments will be packaged in both strand orientations, and need to anneal at a region of sequence complementarity, the total fAAV plasmid size between the ITR sequences should not exceed 9 kb.

3.2 rAAV Production

1. 24 h prior to transfection, split a confluent 15 cm plate of 293 cells at a 1:3 ratio into 4–6, 15 cm plates [final volume of culture medium (DMEM 10 % FBS 1× Penn/Strep) is approximately 20 ml].
2. At approximately 70 % confluency, perform the triple transfection using polyethylenimine (PEI) using the plasmids and amounts described in Table 1 below and elsewhere [39].

Table 1
Transfection mixture for rAAV production

Transfection reagents	Amount per 15 cm plate
pXX680 (Ad helper functions)	12 μg
pRepCap (replication and capsid genes)	10 μg
pITR-transgene (packaged sequence)	6 μg
Serum-free DMEM	500 ml
PEI	100 μl

3. Add the three plasmids to a tube followed by the serum free DMEM and then the PEI. Immediately vortex the mixture and let sit at RT for 5 min. Add the transfection mixture dropwise to a single 15 cm plate of HEK 293 cells and gently swirl the plate to achieve uniform distribution.
4. 48–72 h post-transfection harvest the transfected 293 cells for intracellular vector purification (*see* **Note 5**).
5. Collect the HEK-293 cells from all four plates and place in a centrifuge tube (*see* **Note 6**).
6. Pellet cells by centrifuging at 2500 rpm for 5 min.
7. Remove the supernatant, wash the cells with 10 ml of 1×PBS, and spin again.
8. Resuspend the washed cell pellet in 10 ml of 1×PBS and keep on ice.
9. Sonicate each sample on ice for 25 pulses with a Branson Sonifier set at cycle 50 and output 5 (or equivalent).

3.3 rAAV CsCl Gradient Purification

1. Following sonication, add 100 μl of the DNaseI solution to the samples and incubate at 37 °C for 1 h.
2. Bring samples to a final volume of 11.7 ml using 1×PBS.
3. Add 6.6 g of CsCl to each sample, vortex immediately to dissolve, and place on ice.
4. Repeat the sonication (**step 7**) and proceed to the next step.
5. Load samples into a Beckman Quick-Seal polyallomer (16 mm×76 mm) centrifuge tubes (about 11.5 ml will fit with a little leftover which can be discarded) and seal the tube with heat (*see* **Note 7**).
6. Centrifuge at 65,000 rpm in a Beckman NVT65 rotor using a Sorvall Discovery 90SE centrifuge (or equivalent) for at least 12 h.

7. After the centrifugation, collect gradient fractions by placing a 21-gauge needle in the bottom of the tube and an 18-gauge needle in the top to let liquid flow (*see* **Note 8**). Collect 1 ml fractions (about 20 drops).
8. Determine your peak fractions containing the most packaged viral genomes using the Southern dot blot hybridization method described below. In the case of fAAV, CsCl gradient fractions should be analyzed on an alkaline gel to determine packaged genome size as reporter [9] (*see* **Note 9**).
9. Combine peak fractions (*see* **Note 10**) and dialyze in 1× PBS using a Slide-A-Lyzer dialysis cassette (or equivalent) at 4 °C with constant stirring for at least 12 h (approximately 1 L of PBS to 1 ml of vector fraction is sufficient).
10. Remove the samples from the dialysis cassettes, aliquot into micro-centrifuge tubes and store at −80 °C. All analyses of vector characterizations should be performed on thawed aliquots (*see* **Note 11**).

3.4 Split rAAV Vector Production

1. Vectors A and B are produced independently using this protocol (i.e., 2 separate transfections/vector preparations; Figs. 1 and 2). Final titer after dialysis can be determined by Q-PCR (*see* **Note 12**).

3.5 fAAV Vector Production

1. One ITR plasmid vector is used for production as described (single preparation). It is important to keep fractions one to nine for characterization of packaged genome size by alkaline gel Southern blotting (*see* **Note 13**) as the genome size of the transducing species must be over ½ of the total sequence to be packaged including the ITRs and sequence between them (Fig. 3) [9]. For fAAV preparations, the final titer should be performed by Q-PCR using multiple primers sets, as not all regions are packaged at similar efficiencies. An effective way to do this is to perform Q-PCR using a primer set designed to amplify the conserved ITRs, with the assumption that as only the 3′ ITR is packaged while the 5′ ITR was removed during production.

3.6 AAV Final Titer

1. Perform a DNAseI digestion to remove rAAV genomes and plasmidDNA that are not encapsidated. 10 µl of virus + 90 µl of DNaseI solution and vortex. Incubate for 1 h at 37 °C.
2. To inactivate the DNAseI, add 6 µl of 0.5 M EDTA and thoroughly vortex.
3. To degrade the AAVcapsid, add 120 µl of Proteinase K solution and vortex.
4. Incubate at 55 °C for ≥2 h (sample can be left at 55 °C overnight).

5. Heat the sample for 10 min at 95 °C to inactivate proteinase K.
6. Dilute the sample at least 100-fold in clean water or 10 mM Tris-HCl (pH 8) and use this solution as a template for Q-PCR.
7. Make plasmidDNA standards for the Q-PCR reaction. Dilute plasmid DNA (ITR plasmid used in the initial transfection) to 10 ng/μl in 10 mM Tris-HCl (pH 8) in 1.5 ml siliconized tubes. Vortex and spin briefly (to collect contents) in every step. Make standard serial 1:10 dilutions from 50 pg to 0.05 fg.
8. Prepare the Q-PCR SYBR master reaction (5 μl 2× SYBR mix, 0.25 μl forward primer (20 μM), 0.25 μl reverse primer (20 μM), 2.5 μl H_2O). The final volume of each reaction is 10 μl. Set up an 8 μl master mix per reaction as follows and use a twofold dilution series for the viral genome preparations (*see* **Note 14**). Although total amplified double-strand DNA as measured by SYBR stain is described herein, probe-specific methods for detection can also be used.
9. Pipette 8 μl of master mix into each well.
10. Add 2 μl of DNA (vector sample or plasmid standard**) to a well of a 96-well plate.
11. Add 2 μl of dH_2O or 10 mM Tris-HCl solution as a no template control.
12. Seal the plate and spin down @ 1800 rpm for 10 s.
13. Perform the Q-PCR in a thermocycler using the manufacturer's recommended conditions specific to the primer annealing temperature.

3.7 Southern Dot Blot for Vector Genome: Sample Preparation

1. In an appropriate tube or well add 10 μl of the CsCl vector fraction to 100 μl of DNaseI digestion buffer (solution 1). For final titer determination by this method a twofold dilution series can be used (in triplicate) starting with a max of 10 μl of the dialyzed vector preparation.
2. Vortex and incubate at 37 °C for 1 h to remove unpackaged DNA.
3. Stop the digestion by adding 6 μl of 0.5 M EDTA and vortex to mix thoroughly.
4. To digest the vector capsid, add 120 μl of the proteinase k solution (solution 2).
5. Vortex and incubate at 55 °C for a minimum of 2 h (up to 24 h is ok)
6. Add 144 μl of 0.5 M NaOH.
7. Vortex and incubate at RT for 30 min to denature the DNA.

3.8 Southern Dot Blot for Vector Genome: Plasmid Standard Preparation

1. Using the AAV ITR plasmid that contains the transgenic sequence, prepare a plasmid standard from 50 ng to 5 ng to generate a standard curve (*see* **Note 15**).
2. Denature the plasmid standard by add 300 μl of 0.5 M NaOH, and 1 M NaCl (solution 4) to each tube.
3. Incubate at RT for 30 min.

3.9 Southern Dot Blot for Vector Genome: Preparation of Dot Blot Apparatus

1. Cut a HyBond XL nylon membrane and two sheets of filter paper to fit the size of the dot blot apparatus such that all 96 wells are covered.
2. Wet membrane and the two sheet of filter paper in solution 5.
3. Place the two sheets of filter paper down first followed by the membrane such that sample loading will be directly on the membrane. Then place the top of the dot blot apparatus on tightly to prevent sample leakage.
4. Attach a vacuum to the apparatus for 5 min.
5. Stop the vacuum.
6. Pipette each sample and standard into an individual well.
7. Wait for 5 min.
8. Apply vacuum for 10 min.
9. Wash each well with 300 μl of H_2O.
10. Vacuum for an additional 10–15 min.
11. Remove the membrane from the apparatus and UV cross-link the DNA to the membrane.
12. Place the membrane in a glass hybridization tube containing Church buffer to cover the membrane and pre-hybridize for 30 min at 60 °C using a rotisserie to provide constant rotation.
13. Add a radio-labeled probe and let incubate with membrane overnight in the rotisserie at 60 °C (*see* **Note 16**).
14. Wash the membrane two times in the high-salt wash for 5 min.
15. Wash the membrane in the low-salt solution for 30 min.
16. Wrap membrane in saran wrap or a sheet protect.
17. Expose to film or a phospho-imaging screen for 2–3 h.
18. Develop film or scan the screen and quantify the dot intensity (*see* **Note 17**).
19. Prepare a curve using the dot intensities of the standards and determine the number of viral genomes/μl as previously described [39].

3.10 Packaged Genome Characterization: Alkaline Gel Southern Blot

1. Remove unpackaged DNA by adding 10 μl of the DNaseI solution (solution 1) to 10 μl of virus (≈1–5e^9 viral genomes; *see* **Note 19**).
2. Incubate for 30 min at 37 °C.

3. Add 4 μl of 0.5 M EDTA and vortex to inactivate the DNaseI.
4. Add the gel loading dye to make a 1× solution.
5. Add 2 μl of 10 % SDS to disrupt the AAVcapsid exposing the viral genome (*see* **Note 18**).
6. Vortex for 10 s and let sit at RT for 20 min.
7. Spin at 12,000 rpm for 20 min.
8. Load the alkaline gel along with a proper ladder/marker (*see* **Note 19**).
9. Normally two to three fractions are combined for dialysis. In general, fractions corresponding to the intensity of the 25 ng plasmid standard or greater are worth moving forward with.
10. Run the gel at 15 V overnight in alkaline gel electrophoresis buffer (*see* **Note 20**).
11. When gel has finished running cut the top wells off and any other part of the gel that may not be needed.
12. Soak the gel in transfer buffer for 10 min with mild agitation.
13. Measure the size of the gel, so you can cut your filter paper and HyBond-XL membrane accordingly.
14. Perform Southern transfer using standard procedures [39, 40].
15. Once the transfer is complete UV cross-link the membrane.
16. Hybridize the DNA on the membrane to a radio-labeled probe in Church buffer as described for the Southern dot blot method (*see* **Notes 16** and **21**). The wash and detection conditions are also the same as described above.
17. Intact AAV genomes (<5 kb) package primary a single DNA species the size of the transgenic DNA cassette and the ITRs, whereas fAAV packages heterogeneous DNA species of different sizes which are separated in the gradient based on density. For transduction competent fAAV preparations, the fractions containing the largest DNA species are the most desirable to pool, dialyze, and prior to determining the final titer (*see* **Note 9**). We have previously reported explicit examples of both intact and fAAV genome species found in the different fractions of the CsCl gradient and correlated them to both transduction efficiency and the refractive index of the CsCl fraction [9].

3.11 AAV Large Gene Transduction Verification

1. Following production and characterization of your AAV vector preparations large transgene synthesis will, of course, require verification. To do this follow standard procedures, such as Western and northern blots, but be sure to always included the proper controls. For instance, all split AAV vector approaches rely on co-transduction of AAV vectors. Therefore, it will be necessary to perform transduction with each vector independently, and in concert, to be certain that large transgene pro-

duction only occurs if both vector types are used. In the case of fAAV preparations, functional transduction can be performed on all fractions prior to dialysis to determine which fractions are competent for large transgene production [9]. Then, the size of the packaged genome fragments can be correlated to functional large transgene production and guide the decision of which fractions to dialyze (only packaged genome fragments greater than half the size of the transgenic cassette, theoretically, will mediate transduction).

4 Notes

1. PEI is a potent transfection reagent that is relatively inexpensive. A large batch can be made and stored in aliquots at –20 °C to provide consistency. We routinely store small aliquots at 4 °C for up to 1 month.
2. For most applications we use a random primed labeling kit and P^{32} CTP due to the strength of the signal. However, end labeling kits and other types of non-radioactive detection can be used as well.
3. It is important to add the 5 M NaOH and 0.5 M EDTA to the cooled alkaline gel solution prior to polymerization. If added when the solution is too hot it will turn a yellowish color and should be discarded.
4. Primer design for Q-PCR follows normal considerations in regards to length, nucleotide composition, etc.
5. At the time of harvest the cells should appear smaller and rounder in morphology as they approach the pXX680 induced cytopathic effect. If the cells appear completely healthy then it is likely that the transfection is likely poor. At this point the vector preparation should be terminated and repeated with further optimized transfection conditions.
6. Although not described herein, AAV vectors can also be purified from the culture medium at the time of cell harvest [41].
7. It is important to make sure that all tubes are precisely balanced in the centrifuge.
8. It is a good idea to use one needle to punch a hole in the bottom of the tube and then place a new in the created hole to ensure a constant flow.
9. Only genomes greater than ½ of the total cassette size retain the ability for single strand annealing and therefore productive transduction.

10. If the Proteinase K solution is added without the EDTA being added first it will lead to the degradation of the viral DNA.
11. Repeated freeze/thawing of viral vector aliquots should be avoided.
12. Determination of the exact AAV vector titer is difficult and therefore a Southern dot blot should be used to confirm the PCR data.
13. SYBR Gold (Life Technologies) nucleic acid staining of the alkaline gel can be used for AAV genome detection instead of transferring the DNA to a membrane and detecting via probe hybridization.
14. Primer design follows standard protocols and it is wise to target at least two positions within the transgenic vector cassette. If the signal is too strong, then you can let the membrane sit until the signal reduces in intensity.
15. Digestion of the pAAV-ITR plasmid to recover a plasmid fragment representing the exact sequence to be packaged is the preferred species to use as the standard.
16. Both end-labeled defined probes and those generated via random-primed labeling can be used and prepared using kits following the manufacturer's recommendation. If using a random-primed labeling kit a digested fragment representing the packaged transgene cassette is ideal as the reaction template. Nonradioactive probe detection is also acceptable.
17. Dot intensity for the Southern dot blot can be quantified using the Image J program.
18. The Proteinase K solution can be used in lieu of the 10 % SDS.
19. Linear fragments of the AAV ITR plasmid at several different sizes work nicely for this, although probe homology must be accommodated.
20. It is important to remove air bubbles between the membrane and the alkaline gel to not disrupt the transfer.
21. To decrease the nonspecific background, probes for the Southern blot applications can be column purified prior to hybridization.

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