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Transposon-Based Mutagenesis Generates Diverse Adeno-Associated Viral Libraries with Novel Gene Delivery Properties

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Summary

The engineering of novel properties and functions into viral vectors for improved gene delivery remains a barrier to the development of efficient, customized gene delivery vehicles. Rational methods for designing improved viral vectors are often experimentally challenging and laborious, particularly when knowledge of viral structure–function relationships is limited. As an alternative, high-throughput libraries may be rapidly and efficiently selected for viral variants with a desired function. Here we describe a transposon-based insertional mutagenesis approach to generate large diverse adeno-associated viral (AAV) libraries containing a randomly located peptide. Briefly, a selectable marker is randomly inserted throughout the AAV2 *cap* gene and the resulting "bookmarked" AAV *cap* gene is cloned into an AAV packaging vector. The selectable marker is then replaced with a defined oligonucleotide, and the final AAV library is used to package a diverse pool of AAV virions, which can used for functional selection.

Key Words: Adeno-associated virus; transposase; mutagenesis; gene therapy; protein engineering.

1. Introduction

Adeno-associated viral (AAV) vectors show tremendous potential for the safe and effective treatment of a range of genetic disorders, including Alzheimer's disease, hemophilia, and Parkinson's disease ***(1,2)***. AAV is a non-pathogenic parvovirus with a 4.7-kb single-stranded DNA genome that contains two viral genes: *rep* and *cap* ***(3,4)***. While *rep* encodes four proteins (Rep78, Rep68,

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Rep52, and Rep40) essential for viral replication, *cap* encodes three structural proteins (VP1-3) that self-assemble as a 60-mer to form the viral protein shell or capsid. The extensive safety record coupled with the high efficiency of AAV vectors has furthered their use to deliver genes to various dividing and nondividing cell types in vivo ***(5–7)***. While natural evolution has generated numerous alternatives to the well-characterized and most clinically utilized AAV serotype 2 (AAV2) ***(8,9)***, numerous remaining challenges, such as a robust universal purification platform and engineering cell-specific tropism, limit the gene delivery potential of these viral vectors.

Genetic engineering has greatly extended our knowledge of AAV biology and enhanced its gene delivery properties. Site-directed mutagenesis studies have identified amino acids critical to AAV2 function ***(10–13)***, and the insertion of some peptides in defined positions in the AAV capsid, guided by sequence alignment with related parvoviruses ***(14,15)*** and crystal structures ***(12,16)***, has conferred recombinant AAV vectors with some cell-specific delivery properties. However, these rational design approaches can be laborious, and results are often highly variable, as evidenced by large differences in functional peptide display and viral infectivity for various peptides inserted into the same location ***(10,12)***. Alternative high-throughput library approaches have selected AAV vectors with novel cell-targeting peptides inserted at a defined capsid location ***(17,18)***, altered receptor binding properties ***(19)***, and the ability to evade antibody neutralization ***(19)***. However, the same insertion location is likely not optimal for displaying all functional peptide inserts, and directed evolution approaches do not typically involve the insertion of a peptide of defined function into a protein. Therefore, inserting known peptides or domains randomly throughout the entire primary amino acid sequence of the viral structural proteins may generate viral capsids with novel functions. Endonuclease methods have been used to engineer both bacterial and mammalian proteins ***(20,21)***, but such techniques fail to yield large diverse random libraries because of low DNA ligation efficiencies and biased insertions. Alternatively, transposases, enzymes capable of moving or copying DNA sequences randomly from one DNA template to another (*see* **Fig. 1A**), have greatly facilitated prokaryotic and eukaryotic evolution through random DNA insertions into an organism's genome ***(22)***. Insertional mutagenesis using such transposases has improved functional genomics studies of viral genomes ***(23–25)***, and we have recently built on this approach to identify novel peptide insertion sites within the vesicular stomatitis virus protein for retroviral and lentiviral vector engineering ***(26)***.

Transposon insertional mutagenesis relies on the transposase-facilitated transfer of a unique drug-resistance gene from a donor plasmid to a random location in an acceptor plasmid, containing the gene of interest, followed by

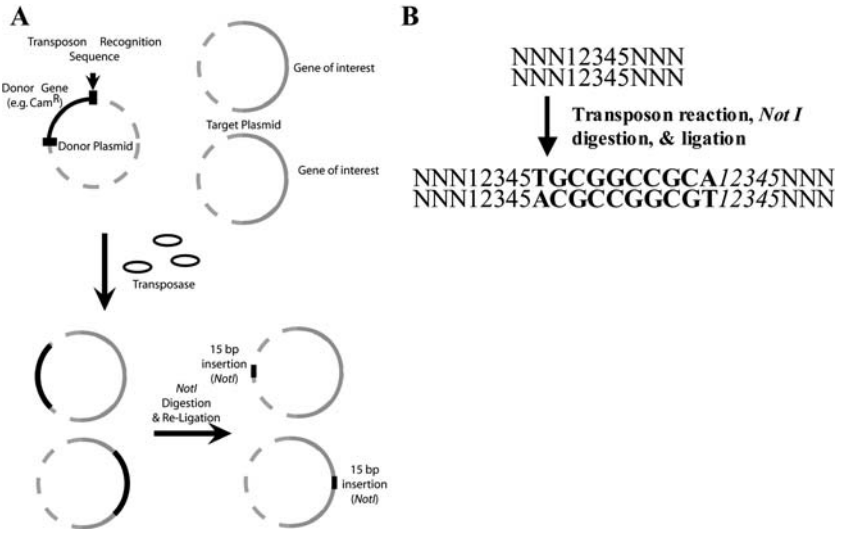

Fig. 1. Overview of transposon-based insertional mutagenesis reaction. (**A**) Donor plasmid containing the donor gene (black) and acceptor plasmids containing the gene of interest (gray) are mixed in the presence of a transposase, resulting in the transfer of the donor gene from the donor to the acceptor plasmid. The resulting pool of plasmids, which contains the donor gene randomly inserted throughout the acceptor plasmid, may be digested with *Not*I and re-ligated to yield a plasmid library containing a "bookmark" (i.e., *Not*I site) ideally covering every possible internucleotide position. (**B**) A random 5-bp sequence in the acceptor plasmid at the insertion site is duplicated (shown in italics) and placed after the inserted *Not*I site (shown in bold).

subsequent selection with the appropriate antibiotics. Subsequent replacement of the drug-resistance gene with an oligonucleotide encoding a desired peptide results in a diverse plasmid library, which can be used to produce virus containing the peptide randomly located within the viral capsid. Here we present a detailed protocol for employing a transposon-based system to randomly insert an oligonucleotide encoding for a peptide of interest [i.e., a hexahistidine (His_6) tag] throughout the entire AAV2 *cap* gene. Other motifs to modulate cell surface binding or other viral properties can also be inserted. Therefore, this general protocol is readily extended to other peptides, AAV serotypes, and viruses to generate customized viral gene delivery vectors.

2. Materials

1. 2.5 M calcium chloride ($CaCl_2$).
2. 2× HeBS: 1.5 mM Na_2HPO_4, 50 mM HEPES, 280 mM NaCl, pH 7.10.
3. AAV lysis buffer: 50 mM Tris–HCl, 150 mM NaCl, pH 8.5.

3. Methods

3.1. Construction of AAV cap Plasmid

1. To obtain AAV *cap* gene from viral genomic DNA or plasmid, mix in a PCR tube: 5 µl 10× Thermopol buffer, 5 µl dimethyl sulfoxide (DMSO), 1 µl 10 mM dNTP mix, ~0.1 pmol DNA template, 10 pmol of 5′ and 3′ primers containing suitable restriction sites for cloning, 5 U Vent DNA polymerase, and water to 50 µl. For example, to recover the AAV2 *cap* gene, use 5′-GCGG**AAGCTT**CGATCAACTACGC-3′ as the 5′ primer and 5′-GG**GGCGCGCC**GCAATTACAGATTACGAGTCAGGTATCTGGTG-3′ as the 3′ primer. These primers introduce *Hin*dIII and *Asc*I restriction sites (in bold), respectively, to facilitate cloning.
2. Using a QIAquick purification kit or similar spin column kit, purify the polymerase chain reaction (PCR).
3. The construction of a small plasmid vector (e.g., pBluescript) containing only the AAV *cap* gene ensures that more insertion events occur in the *cap* gene versus the vector backbone and thus reduces the final required library size, which should be significantly larger than the total number of base pairs in the template plasmid to ensure every possible position is well represented. However, the unique *Not*I site within pBluescript must first be eliminated because the transposon reaction introduces a *Not*I site. This may be accomplished by either mutagenesis or insertion of a small oligonucleotide linker at the *Not*I site to introduce a unique *Asc*I site (*see* **Note 1**).
4. Digest ~1 µg both the 2.6-kb AAV *cap* PCR product and modified pBluescript plasmid with *Hin*dIII and *Asc*I. Purify the digested products using a ~1% agarose gel and a commercial gel extraction kit, such as Qiagen QIAEX II Gel Extraction Kit.
5. Ligate 75–150 fmol of AAV *cap* PCR product and 25 fmol of pBluescript with 5 U of T4 DNA ligase in a 15 µl reaction.
6. Transform 10 µl of ligation into TOP10 bacteria and select for growth in the presence of ampicillin (100 µg/ml).
7. To screen for positive clones, digest plasmid DNA with *Hin*dIII and *Asc*I. Positive clones will yield two bands: 2.6 and 3.0 kb.

3.2. Transposon-Based Insertion

1. The use of commercially available transposon kits, such as the Mutation Generation System used here, permits efficient generation of a diverse library containing restriction site markers located randomly throughout the plasmid template (*see* **Note 2**).
2. To perform the transposition reaction, mix in a PCR tube: (40 ng × plasmid size in kilobases) plasmid template, 4 µl 5× reaction buffer for MuA Transposase, 1 µl Entraceposon (M1-Cam^R or M1-Kan^R) (*see* **Note 3**), 1 µl MuA Transposase, and water to 20 µl.

3. In a thermal cycler, incubate the reaction at 30ºC for 1 h, followed by a 10 min incubation at 75ºC. This results in either the *Cam*^{R} or *Kan*^{R} gene, flanked by *Not*I sites and with a 5 bp duplication, being randomly inserted throughout the plasmid template (*see* **Fig. 1** and **Note 4**).
4. Purify the DNA mixture by ethanol precipitation through addition of 2 µl 3M NaOAc, pH 5.2, and 50 µl 100% ethanol. We recommend adding glycogen (100 µg/ml final concentration) carrier as a pellet marker. Thoroughly wash the pellet with 70% ethanol and air dry. Resuspend the dried pellet in no more than 10 µl water.
5. Transform the purified ligation reaction into ElectroMAX DH10B bacteria through electroporation according to the manufacturer's instructions. Take a small fraction of the electroporation reaction and streak on a bacterial agar plate with ampicillin and chloroamphenicol. Estimate the initial plasmid diversity of the library from the number of bacterial colonies. Typical initial library diversity should be on the order of 10^6 independent bacterial clones.
6. Inoculate a 100 ml TB culture with the remaining reaction and shake culture at 250 rpm (1.3 × g) for 13 h at 37ºC in the presence of 100 µg/ml ampicillin and 10 µg/ml chloroamphenicol.
7. Purify DNA from culture using a standard DNA purification method such as polyethylene glycol (PEG) precipitation or a commercial purification kit. Quantify purified DNA by measuring the absorbance at 260 nm using a UV-Vis spectrophotometer.

3.3. Construction of AAV Plasmid Library

1. To transfer the AAV *cap* gene containing the *Cam*^{R} gene, digest ~1 µg pBS *cap*-Cam^R and the appropriate modified AAV packing vector, such as pSub2 ***(19)*** with *Hin*dIII and *Asc*I. The pSub2 packaging vector contains the entire AAV2 genome except for the *cap* gene, which has been replaced with unique *Hin*dIII and *Not*I sites. Here, the *Not*I site in pSub2 has been replaced with an *Asc*I site through mutagenesis to create pSub2Asc. Purify the digested products using a 1% agarose gel. Gel extract the 3.7-kb band from the pBS *cap*-Cam^R sample and 5.7 kb linearized pSub2Asc plasmid.
2. Ligate 75–150 fmol of *cap*-Cam^R fragment and 25 fmol of pSub2Asc with 5 U of T4 DNA ligase in a 15 µl reaction (*see* **Note 5**).
3. Purify the ligation reaction as in **step 4**, **Subheading 3.2** and transform into ElectroMAX DH10B bacteria as in **step 5**, **Subheading 3.2**. Estimate the library diversity size as in **step 5**, **Subheading 3.2**.
4. Inoculate a large-scale TB culture in the presence of ampicillin and chloroamphenicol and purify as in **steps 6 and 7**, **Subheading 3.2**. A diagnostic restriction digest screen with *Age*I (which cuts near the start of the Cam^R gene) and *Asc*I (which cuts at end of *cap* gene) should yield a smear ranging from ~1.2 kb to 3.8 kb in size.
5. To replace the chloroamphenicol-resistance gene with a desired oligonucleotide, design oligonucleotides such that the sequences at the 5´ and 3´

ends are compatible with *Not*I sites (*see* **Fig. 1B**). For example, the following oligonucleotides were used for insertion of a His_6 tag (histidine codons shown in bold): 5´-GGCCGGT**CACCACCACCACCACCAC**TC-3´ and 5´-GGCCGA**GTGGTGGTGGTGGTGGTG**ACC-3´ (*see* **Note 6**). Mix equal amounts of single-stranded oligonucleotides in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with 50 mM NaCl, heat to 94ºC, and gradually cool to the anneal the oligonucleotides. Phosphorylate the resulting double-stranded oligonucleotide by mixing 300 pmol DNA, 5 µl T4 DNA ligase buffer, 1 mM dATP, and 10 units T4 polynucleotide kinase. Incubate the reaction at 37 ºC for 30 min and inactivate the enzyme by incubation at 65 °C for 20 min.

6. Digest ~1 µg pSub2Asc *cap*-Cam^R with *Not*I and gel extract the 8.3-kb DNA fragment.
7. Ligate 75–150 fmol of phosphorylated oligonucleotide and 25 fmol of pSub2Asc *cap* fragment with 5 U of T4 DNA ligase in a 15-µl reaction. Incubate at 14 °C for at least 6 h.
8. Purify the ligation reaction as in **step 4**, **Subheading 3.2** and transform into ElectroMAX DH10B bacteria as in **step 5**, **Subheading 3.2**. Estimate the library diversity size as in **step 5**, **Subheading 3.2**.
9. Inoculate a large-scale TB culture in the presence of ampicillin and purify as in **steps 6–7**, **Subheading 3.2**. A diagnostic restriction digest screen with *Eag*I (which cuts once in the oligo insert and once at end of the *cap* gene) should yield a smear ranging from 20 bp to 2.6 kb in size (*see* **Fig. 2**).

3.4. Production of Viral Library

1. Plate ~10^7 HEK 293 cells in 25 ml of DMEM onto a 15-cm tissue culture dish such that cells are ~75% confluent after 24 h.
2. After ~24 h, transfect cells by calcium phosphate precipitation ***(19)***. Briefly, mix 7 ng pSub2Asc library, 25 µg pBluescript, and 25 µg pHelper with 120 µl 2.5

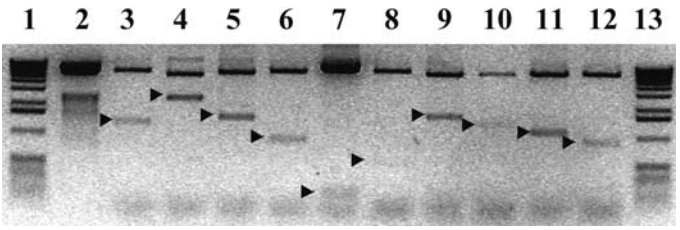

Fig. 2. Analysis of plasmid library diversity. The His_6 AAV insertion library and single clones contain two *Eag*I sites, one that cuts once in the His_6 insertion and one at 3´ end of the AAV *cap* gene. Successful insertions into *cap* will yield a large band ranging from 8.3 to 5.7 kb, along with a smaller band ranging up to 2.6-kb in size (indicated by arrow). Lanes: 1, 1-kb ladder; 2, His_6 AAV library; 3–12, 10 randomly selected single His_6 AAV clones; 13 1-kb ladder.

M $CaCl_2$ and water to 2.5 ml total volume. The pBluescript helps to maintain a constant DNA : calcium phosphate ratio, which we have found necessary for maintaining high efficiency DNA transfection and viral packaging. Add DNA/$CaCl_2$ solution dropwise to 2× HeBS solution. Mix once and add mixture dropwise to cells. Remove media after 6–8 h and replace with 25 ml DMEM. This 1:2 × 10^{-4} molar ratio of plasmid DNA to pSub2Asc library was calculated such that >90% of cells received approximately one member of pSub2Asc library, assuming each cell receives ~50,000 total plasmids ***(27)***. This helps to ensure that most virions contain a viral genome with a *cap* gene encoding their capsid (*see* **Note 7**).

3. After 48 h, scrape cells from the plate and centrifuge at 1000 × *g* for 2 min. Aspirate medium and resuspend cell pellet in 1 ml PBS or AAV lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.5).
4. Freeze/thaw three times using a dry ice/ethanol bath, or sonicate the cell suspension, to lyse the cells.
5. Centrifuge the lysate at 13,000 × *g* for 10 min to clarify, or pellet cell debris. The resulting supernatant contains the AAV viral library, which can be quantified by standard protocols such as dot blotting, ELISA, or quantitative PCR. If necessary, the library can be purified by density ultracentrifugation, such as with iodixanol or CsCl ***(19)***. A representative titer from two independent viral productions is shown in **Fig. 3A**. After this stage, the viral library can be selected for variants with a desired enhanced function (*see* **Note 8**). For example, to select for the functional display of His_6 tags on the viral surface, a mixture of 1 volume of cell lysate containing ~10^{11} viral particles, 0.5 volume binding buffer (10 mM Tris-HCl pH 8.0, 300 mM NaCl, and 20 mM imidazole), and 500 µl of 50%

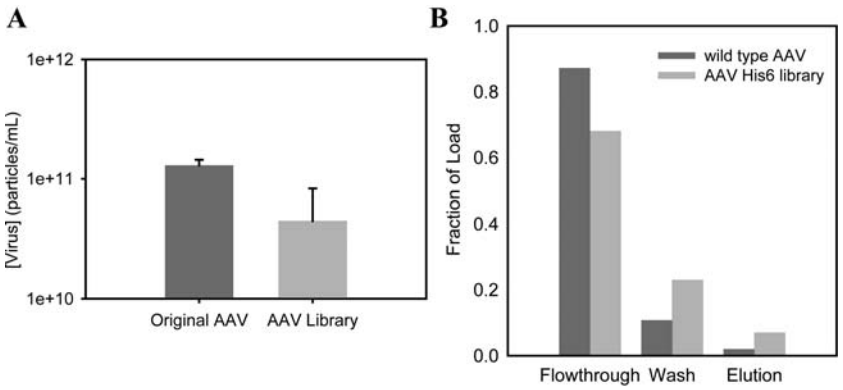

Fig. 3. Characterization of His_6 AAV Library. (**A**) Representative titers of both wildtype AAV2 and the His_6 AAV library from two independent preparations. (**B**) Chromatogram of viral binding fractions to Ni-NTA resin for both wildtype AAV2 and His_6 AAV library.

Ni-NTA agarose (Qiagen, Valencia, CA, USA) was agitated gently overnight at 4 °C. This slurry was then loaded onto a plastic column (Kontes, Vineland, NJ, USA) before washing with 3 ml of wash buffer (10 mM Tris-HCl pH 8.0, 50 mM imidazole) and eluting with 3 ml of elution buffer (10 mM Tris-HCl pH 8.0, 500 mM imidazole). Characterization of the binding profile of the viral library showed elevated levels of Ni-NTA binding relative to the wild type AAV2 control (*see* **Fig. 3B**).

4. Notes

1. Alternative restriction sites may be used for the construction of this plasmid, provided the sites do not occur within the inserted drug-resistance gene or the acceptor plasmid (map provided with transposon kit).
2. Other commercial kits may result in different final insertion sizes, alternate reaction conditions, or alternate antibiotic selections. Be sure to thoroughly review the transposon kit's instructions before use.
3. Choose a drug-resistance gene that differs from gene present in the plasmid template.
4. Include a control reaction without either MuA Transposase or Entraceposon to verify the transfer of the drug-resistance gene.
5. The ratio of the amount of the plasmid insert to plasmid backbone may be varied to identify the optimal cloning condition.
6. Alternate oligonucleotides or gene fragments may be used provided the ends contain restriction sites compatible with *Not*I overhangs. Alternatively, the *Not*I overhangs may be blunted or filled in with Klenow, and the oligonucleotides may be cloned into the modified site.
7. Alternative transfection methods such as electroporation or lipofection may be used. In all cases, high transfection efficiency and proper plasmid amounts are essential for producing a diverse viral library that can be easily functionally selected.
8. For selection protocols involving infection of a cell line, such as HEK293 ***(19)***, care should be taken to avoid infection with a large (>1000) number of virions per cell [i.e., multiplicity of infection (MOI)]. The presence of multiple AAV genomes with different *cap* genes in one cell leads to potential recombination between the *cap* genes or production of several different versions of the VP1-3 proteins, yielding chimeric or mosaic virions ***(28,29)***. Hence, isolation of the genomes from these mosaic virions will fail to recover the genotype responsible for the novel property or phenotype.

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