Construction of diverse adeno-associated viral libraries for directed evolution of enhanced gene delivery vehicles

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Rational design of improved gene delivery vehicles is a challenging and potentially time-consuming process. As an alternative approach, directed evolution can provide a rapid and efficient means for identifying novel proteins with improved function. Here we describe a methodology for generating very large, random adeno-associated viral (AAV) libraries that can be selected for a desired function. First, the AAV2 *cap* gene is amplified in an error-prone PCR reaction and further diversified through a staggered extension process. The resulting PCR product is then cloned into pSub2 to generate a diverse (>10^6) AAV2 plasmid library. Finally, the AAV2 plasmid library is used to package a diverse pool of mutant AAV2 virions, such that particles are composed of a mutant AAV genome surrounded by the capsid proteins encoded in that genome, which can be used for functional screening and evolution. This procedure can be performed in approximately 2 weeks.

INTRODUCTION

Gene therapy offers much promise for the treatment of acquired and inherited genetic diseases. While significant progress has been made in identifying therapeutic genetic cargo capable of treating disease, efforts have been limited by difficulties in developing safe and efficient gene delivery vectors[1,2]. Many viruses have been used to deliver therapeutic genetic material, but limitations of the viral vectors, such as activation of an immune response and nonspecific cell tropism, have reduced the therapeutic efficacy of the treatment. Various rational modifications, both chemical and genetic, have met with success; however, the lack of structure–function relationships for many of these viruses places limitations on these approaches. Therefore, alternative methods for improving the gene transfer properties of these viruses are needed.

AAV vectors have proven to be safe and efficient gene delivery vehicles in clinical trials for hemophilia and Parkinson Disease[3,4]. AAV contains a 4.7-kb single-stranded DNA genome with two open reading frames, *rep* and *cap*, packaged inside a protein shell, or capsid[5,6]. *Rep* encodes four proteins (Rep78, Rep68, Rep52 and Rep40) required for viral replication, whereas *cap* yields three proteins (VP1–VP3) that self-assemble into the viral capsid. Over 100 homologous human and nonhuman serotypes have been isolated, but only AAV2 has been extensively characterized and clinically utilized as a gene delivery vector[7–9]. In addition, the recent discovery of the crystal structure of AAV2 (ref. 10), combined with extensive mutagenesis studies[11–13], have allowed for an understanding of some structure-function relationships of the virus. Such mutagenesis studies have successfully mapped amino acids critical to AAV2 function[11–13], though serial site-directed mutagenesis can be a laborious process. In addition, insertion of peptide sequences into precise locations in the AAV2 capsid, guided by crystal structure data, has led to the creation of recombinant AAV2 vectors with some cell-specific gene delivery properties[14–17], though many peptides can drastically reduce viral production or infectivity or not be efficiently displayed on the capsid surface[14–17]. The molecular basis for many viral properties, such as virus–cell interactions[13,18] and antibody recognition[19,20], involves a complex interaction of amino acid residues that are distributed throughout the viral capsid and hence difficult to rationally design. Thus, more rapid approaches to elucidate synergistic effects of multiple point mutations could aid both basic investigations as well as vector engineering.

We have therefore developed a new directed-evolution approach to generate and select large, diverse AAV2 libraries for novel gene-delivery properties[21]. Directed evolution of proteins in the laboratory provides a rapid and efficient means to generate proteins with novel functions. Briefly, the gene encoding a protein of interest is mutated through one of several techniques, such as error-prone (EP) PCR, the staggered extension process (StEP)[22] and/or DNA shuffling[23], whereby the level of diversity and mutagenic frequency can be tuned by adjusting various parameters. The mutated forms of the gene are subsequently used to produce a random pool or library of protein variants, which can then be selected or screened for desired properties, and iterative rounds of gene diversification and selection can yield substantial improvements in protein fitness or function. The process should be designed to overcome a number of challenges, including (i) the introduction of deleterious mutations that mask beneficial mutations at high error rates, (ii) limitations of the screening/selection process on the size and region of sequence/function space that can be sampled and (iii) the requirement for a carefully designed and high-throughput selection/screening process that yields mutants with the desired new properties. When effectively designed, however, directed evolution allows for the generation of one or even many different solutions to a problem with no *a priori* knowledge of protein structure or function.

Directed evolution has led to the development of enzymes with expanded catalytic functions[22,23], antibodies with higher binding

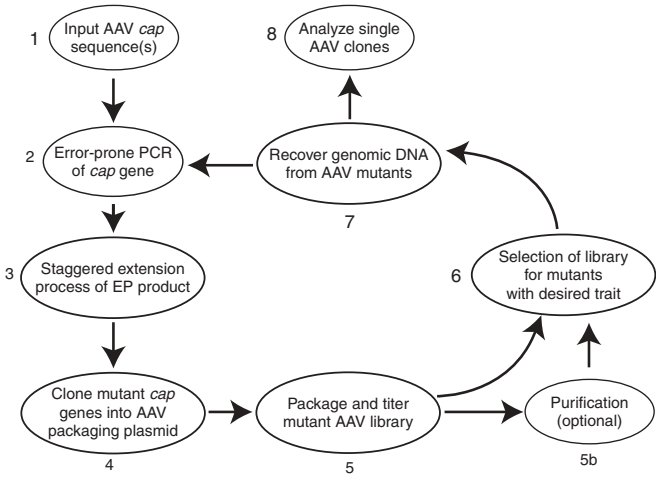

Figure 1 | Directed evolution approach to generating customized AAV vectors. Input AAV *cap* genes (1) are diversified through error-prone PCR (2) and subsequent StEP PCR (3). The mutant *cap* genes are cloned into an AAV packaged plasmid (4) to produce a large, diverse AAV library (5). After an optional purification step (5b), the library may be selected for variants with enhanced function (6), and the genomic DNA from the remaining virions may be recovered (7). Finally, the process may be repeated until the desired phenotype is achieved (8).

affinities[24,25] and retroviruses with altered cell tropism[26]. Recently, we have shown that a directed evolution approach allows for the development of AAV2 variants with altered receptor binding affinities and the ability to evade antibody neutralization[21]. Here we present a detailed protocol outlining the construction of diverse AAV libraries using EP and StEP PCR (**Fig. 1**). This protocol is easily extended to alternative AAV serotypes and other viruses to generate customized viral gene delivery vectors.

MATERIALS

REAGENTS

- pSub201, AAV plasmid vector or pAV2 (ATCC)
- pHelper, adenovirus helper plasmid (Stratagene)
- pBluescript or other inert DNA (Stratagene)
- Taq and Vent DNA polymerases with supplied Thermopol buffer (NEB)
- DMSO **! CAUTION** DMSO is an exceptional solvent and is readily absorbed through the skin. When handling DMSO, wear gloves, safety glasses and a pipeting aid.
- Restriction endonucleases *Hin*dIII, *Cla*I and *Not*I (NEB)
- 100 mM dNTPs (Invitrogen)
- Primer oligonucleotides (see **Table 1**)
- PCR purification kit (e.g., QIAquick PCR Purification Kit, Qiagen)
- Glycogen (Roche)
- 70% (vol/vol) ethanol
- 3 M sodium acetate, pH 5.2
- *Escherichia coli*, TOP10 and ElectroMAX DH10B cells (Invitrogen)
- LB plates with 100 μg ml^{-1} ampicillin
- T4 DNA ligase with ligase buffer (NEB)
- Agarose, electrophoresis grade (Cambrex)
- Gel extraction kit (e.g., QIAEX II Gel Extraction Kit, Qiagen)
- HEK 293 cells (ATCC)
- 2.5 M $CaCl_2$

EQUIPMENT

- Bench-top cell electroporator (e.g., MicroPulser electroporator, Bio-Rad)
- Electroporation cuvette (e.g., Gene Pulser cuvette, Bio-Rad)

REAGENT SETUP

Mutagenic buffer 70mM $MgCl_2$, 500 mM KCl, 100 mM Tris (pH 8.3), 0.1% (wt/vol) gelatin. Combine 70 μl of 1 M $MgCl_2$ (20.3 g $MgCl_2 \cdot 6H_2O$ per 100 ml deionized H_2O), 500 μl of 1 M KCl (7.46 g KCl per 100 ml deionized H_2O), 100 μl of 1 M Tris (pH 8.3; 12.1 g Tris base per 100 ml deionized H_2O), 100 μl of 1% wt/vol gelatin (1 g gelatin per 100 ml deionized H_2O) with deionized H_2O to a final volume of 1 ml. Store at –20 °C.

10× EP dNTPs 2mM ATP, 2 mM GTP, 10 mM CTP, 10 mM TTP. Add 4 μl of 100 mM dATP, 4 μl of 100 mM dGTP, 20 μl of 100 mM dCTP and 20 μl of 100 mM dTTP to 120 μl of deionized H_2O. Mix and store at –20 °C.

AAV lysis buffer 50mM Tris, 150 mM NaCl (pH 8.0). Dissolve 3.03 g of Tris base and 4.38 g NaCl in deionized H_2O to a final volume of 500 ml. Adjust the solution pH to 8.5 with HCl and store at 4-30 °C.

2× HEPES-buffered saline 280mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES (pH 7.10). Dissolve 1.64 g NaCl, 1.19 g HEPES and 1 ml 150 mM Na_2HPO_4 (2.13 g Na_2HPO_4 per 100 ml deionized H_2O) in deionized H_2O to a final volume of 100 ml. Adjust the solution pH to 7.1 with NaOH and store at room temperature (4–30 °C).

TABLE 1 | Primer sequences for relevant AAV plasmids.

Primer	Sequence	Function
A	5′- GCGAAGCTTACGCGGCCGCTTGTTAATCAATAAACCGTTTAATTCG-3′	Binds at polyA site after AAV *cap* gene; introduces *Hin*dIII and *Not*I restriction sites
B	5′- GATGCCGGGAGCAGACAAGCCCGTCAGGGC-3′	Binds inside backbone of pSub201
C	5′- GCGGAAGCTTCGATCAACTACGC-3′	Binds *Hin*dIII site in AAV *rep* gene
D	5′- GGGGCGGCCGCAATTACAGATTACGAGTCAGGTATCTGGTG-3′	Binds *Not*I site before polyA tail following AAV *cap* gene

PROCEDURE

1| This protocol requires an AAV vector plasmid with restriction sites suitable for cloning of the entire *cap* gene/library. Previously, we generated such a construct, called pSub2, which contains unique *Hin*dIII and *Not*I sites flanking an insertion site for *cap* (**Fig. 2**). If another AAV vector plasmid is used, the primer sequences and PCR conditions should be adjusted to match the AAV cloning vector.

Mutagenesis of AAV *cap* gene

2| Use the following error-prone PCR reaction protocol to mutate the entire *cap* sequence: 1 cycle of 5 min at 95 °C, 24 cycles of 30 s at 95 °C, 30 s at 62.5 °C, 2.5 min at 72 °C and finally 1 cycle of 10 min at 72 °C.

Reagent	Amount
Mutagenic buffer, 10×	5 μl
5 mM $MnCl_2$	1.5 μl
10× EP dNTP mix	5 μl
50 mM Primer C	1 μl
50 mM Primer D	1 μl
pSub201 template, 10 ng μl^{-1}	1 μl
Taq DNA polymerase, 5 U μl^{-1}	1 μl
Water	34.5 μl

▲ **CRITICAL STEP** Several factors such as the concentration of $MnCl_2$ and choice of DNA polymerase may be varied to yield a range of mutagenic frequencies.

3| Analyze a 3-μl aliquot of the PCR reaction on a 1% agarose gel. Successful reactions should yield a single band at 2.6 kb with possibly a faint smear (**Fig. 3a**). If successful, run two more of the same PCR reaction.

4| Pool the three PCR reactions and purify with a PCR purification kit. Quantify the amount of DNA present in the PCR product by measuring the absorbance at 260 nm using a UV-Vis spectrophotometer.

■ **PAUSE POINT** PCR products may be stored at −20 °C for several weeks.

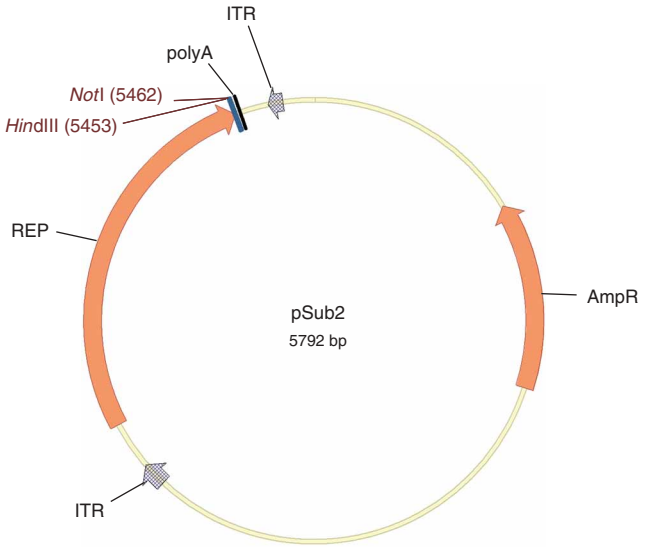

Figure 2 | Map of the AAV cloning vector, pSub2. This construct contains unique *Hin*dIII and *Not*I restriction sites, allowing for the insertion of a portion the AAV *rep* gene and the entire *cap* gene.

5| Set up the following StEP PCR reaction to further diversify the entire *cap* gene using a protocol such as 1 cycle for 5 min at 95 °C, 149 cycles of 1 s at 95 °C, 5 s at 55 °C, 2 s at 72 °C and 1 cycle of 7 min at 72 °C. Always include a negative control containing water instead of DNA template.

Reagent	Amount
Thermopol buffer	5 μl
DMSO	5 μl
10 mM dNTP mix	1 μl
10 mM Primer C	2 μl
10 mM Primer D	2 μl
EP PCR DNA template, >100 ng	1 μl
Vent DNA polymerase, 5 U μl^{-1}	0.5 μl
Water	33.5 μl

▲ **CRITICAL STEP** Several parameters such as template amount and annealing temperature may require optimization to obtain the desired recombined PCR product.

? TROUBLESHOOTING

See **Table 2**.

6| Analyze a 3-μl aliquot of PCR reaction on a 1% agarose gel. Successful reactions contain a 2.6-kb band, often within a smear of DNA (**Fig. 3b**).

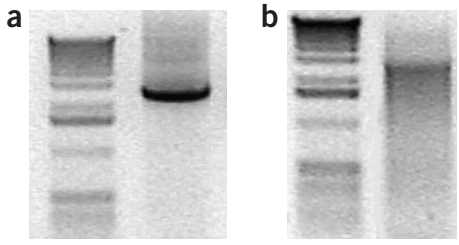

Figure 3 | Expected PCR products from EP and StEP PCR reactions. (**a**) Products of EP PCR reaction with AAV2 *cap* as the template were screened on an agarose gel. The lanes were loaded as follows: (1) 1-kb DNA ladder; (2) EP PCR reaction. (**b**) Products from StEP PCR reaction with AAV2 *cap* EP PCR products as template were screened on an agarose gel. The lanes were loaded as follows: (1) 1-kb DNA ladder; (2) StEP PCR reaction.

Construction of plasmid library

7| Purify PCR product with a PCR purification kit. Quantify the amount of DNA present in the PCR product by measuring the absorbance at 260 nm using a UV-Vis spectrophotometer. Digest ~1 μg of PCR product and pSub2 with *Hin*dIII and *Not*I.
■ **PAUSE POINT** PCR products may be stored at −20 °C for several weeks.

8| Run digests on 1% agarose gel and gel-extract the 2.6-kb PCR product and the 5.7-kb linearized pSub2 plasmid.

9| Set up the ligation including 75–150 fmol of PCR product and 25 fmol of pSub2 and T4 DNA ligase in a 15-μl reaction. Incubate the reaction at 14 °C for at least 6 h.
▲ **CRITICAL STEP** Several parameters such as relative concentrations and purity of gel-extracted products can significantly influence the ligation efficiency.

10| Transform 3 μl of the ligation product into TOP10 bacteria and select for growth in the presence of ampicillin (100 μg ml^{-1}). This step tests the ligation efficiency before moving to the electrocompetent ElectroMAX DH10B bacteria.

11| According to the manufacturer's instructions (Invitrogen) regarding preparation of ligation reactions for electroporation, add 12 μl water and 12 μl phenol/chloroform (1:1 vol/vol) to the remaining 12 μl of ligation. Mix well and centrifuge for 2 min at 16,000*g*.

12| Remove 20 μl of upper aqueous phase containing the ligation products. Recover the DNA by precipitation with ethanol in the presence of 0.3 M NaOAc (pH 5.2). Thoroughly wash the pellet with 70% ethanol. Resuspend the dried pellet in no more than 10 μl water. We recommend adding glycogen (100 μg ml^{-1} final concentration) carrier as a pellet marker.

13| Transform the purified ligation reaction into ElectroMAX DH10B bacteria via electroporation according to the manufacturer's instructions. Dilute a small fraction of experimental reaction as necessary and select for growth in the presence of ampicillin. 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 colonies.

14| Inoculate a 100-ml TB culture with the remaining reaction and shake culture at 250 r.p.m. for 13 h at 37 °C in the presence of 100 μg ml^{-1} ampicillin.

15| Purify DNA from culture using a standard DNA purification method such as PEG precipitation or a commercial purification kit. Quantify purified DNA by measuring the absorbance at 260 nm using a UV-Vis spectrophotometer.
■ **PAUSE POINT** Purified DNA stock may be stored at −20 °C indefinitely.

Packaging of viral library

! **CAUTION** Adeno-associated viral library work should be performed in accordance with BioSafety Level 2 procedures.

16| 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.

17| After ~24 h, transfect cells by calcium phosphate precipitation[27]. Briefly, mix 7 ng pSub2 library (see below for further details), 25 μg pBluescript and 25 μg pHelper with 120 μl 2.5 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× HEPES-buffered saline solution. Mix once and add mixture dropwise to cells. Remove media after 6–8 h and replace with 25 ml DMEM.

The $1{:}2\times10^{-4}$ molar ratio of plasmid DNA to pSub2 library was calculated such that >90% of cells received approximately one member of the pSub2 library, assuming each cell receives ~50,000 total plasmids[28]. This helps to ensure that most virions contain a viral genome with a *cap* gene encoding their capsid.
▲ **CRITICAL STEP** Proper dilution of DNA and a high transfection efficiency (>80%) are required for production of a large, diverse AAV viral library.

18| After 48 h, scrape cells from plate and centrifuge at 1,000*g* for 2 min. Aspirate media and resuspend cell pellet in 1 ml AAV lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0).

19| Freeze/thaw three times using a dry ice/ethanol bath or sonicate the cell suspension to lyse the cells.

20| Centrifuge mixture at 13,000*g* for 10 min. The supernatant contains the AAV viral library, which can be quantified via standard protocols such as dot blot, ELISA or quantitative PCR. If necessary, the library can be purified by density ultracentrifugation, such as with iodixanol or CsCl[21]. After this stage, the viral library can be selected for variants with a desired enhanced function.

Selection of viral library

21| The last critical step involves careful design of a selection strategy, which encompasses several key features. First, an ideal selection permits those virions with a desired phenotype, but not the wild-type virions, to propagate. Alternatively, a gradual increase in the selective pressure over several rounds, coupled with additional mutagenesis between each round, provides a robust means for accelerating the improvement in the virions without complete removal of the wild-type virions. Additionally, since each virion contains a mutant AAV genome that encodes the surrounding capsid proteins, the mutant DNA sequences can be efficiently recovered from the successful AAV virions via PCR. Hence, large pools ($>10^6$) of mutant AAV virions can be simultaneously selected.

If a selection strategy involves replication of the mutant AAV variants within a cell line, such as HEK 293 (ref. 21), the number of viral particles that infect one cell, that is, the multiplicity of infection (MOI), should be constrained. The presence of more than one type of mutant AAV genome per cell will result in the production of AAV virions with mosaic capsids that contain a combination of VP proteins encoded by several mutant AAV *cap* genes[29,30]. Consequently, many of the virions recovered from the selection will fail to contain the mutant genome encoding the capsid proteins, thus decreasing the efficiency of the selection.

● **TIMING**

Step 1, 3 d (optional)
Steps 2–4, 3 h
Steps 5+6, 4–5 h
Steps 7–9, 1 d
Step 10, 12 h
Steps 11–14, 5–6 h
Step 15, 1 d
Steps 16–18, 4 d
Steps 19+20, 1 h

? TROUBLESHOOTING

See **Table 2**.

TABLE 2 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Step 5: The PCR reaction only produces a smear without a 2.6-kb band.	The primer:template ratio is critical for proper amplification.	Perform a series of PCR reactions with a range of primer:template ratios.
	Annealing temperature is not optimal for starting template and primers.	Perform a gradient PCR reaction varying the annealing temperature to identify the optimal temperature (e.g., vary from 50 °C to 65 °C).
	Polyermase processivity is too slow for proper extension to occur.	Add 0.2 μl of Taq DNA (5 U μl^{-1}) polymerase to enhance processivity.
Step 9: A small number of clones is obtained upon transformation into TOP10 bacteria.	Amount of pSub2 backbone and pSub2:PCR product ratio is critical.	Increase the amount of pSub2 backbone in ligation (>100 ng) and perform a series of ligations with varying pSub2:PCR product molar ratios.

ANTICIPATED RESULTS

AAV vectors have proven to be safe and efficient gene delivery vehicles. However, numerous challenges in vector design remain, including neutralizing antibody responses, tissue transport and infection of resistant cell types. Directed evolution has led to the development of numerous proteins with novel or enhanced functions[21–26]. Hence, the use of mutagenesis and recombination to generate diverse AAV libraries and subsequent selection steps should prove useful for designing AAV vectors with enhanced gene delivery properties (**Fig. 1**).

Mutagenesis via EP PCR and recombination via StEP often yield PCR products with a background DNA smear (**Fig. 2**). However, the use of commercial kits to purify the resulting products aids in the subsequent cloning to generate a diverse AAV plasmid vector library, which can be used to produce a large, random AAV library for a desired selection. We have employed such a selection strategy to evolve AAV2 virions capable of gene delivery in the presence of anti-AAV rabbit serum. By gradually

increasing the concentration of antiserum present during the neutralization reaction, we successfully isolated AAV variants with >100-fold improved resistance *in vitro* and *in vivo* to serum that neutralizes wild-type AAV2 (ref. 21).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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1. Mulligan, R.C. The basic science of gene therapy. *Science* **260**, 926–932(1993).
2. Verma, I.M. & Weitzman, M.D. Gene therapy: twenty-first century medicine. *Annu. Rev. Biochem.* **74**, 711–738 (2005).
3. Kay, M.A. *et al.* Evidence for gene transfer and expression of factor IX in hemophilia B patients treated with an AAV vector. *Nat. Genet.* **24**, 257–261 (2000).
4. Manno, C.S. *et al.* AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* **101**, 2963–2972 (2003).
5. Srivastava, A., Lusby, E.W. & Berns, K.I. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* **45**, 555–564 (1983).
6. Samulski, R.J., Chang, L.S. & Shenk, T. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. *J. Virol.* **63**, 3822–3828 (1989).
7. Chiorini, J.A., Kim, F., Yang, L. & Kotin, R.M. Cloning and characterization of adeno-associated virus type 5. *J. Virol.* **73**, 1309–1319 (1999).
8. Gao, G. *et al.* Clades of Adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* **78**, 6381–6388 (2004).
9. Gao, G.P. *et al.* Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. USA* **99**, 11854–11859 (2002).
10. Xie, Q. *et al.* The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proc. Natl. Acad. Sci. USA* **99**, 10405–10410 (2002).
11. Rabinowitz, J.E., Xiao, W. & Samulski, R.J. Insertional mutagenesis of AAV2 capsid and the production of recombinant virus. *Virology* **265**, 274–285 (1999).
12. Wu, P. *et al.* Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J. Virol.* **74**, 8635–8647 (2000).
13. Lochrie, M.A. *et al.* Mutations on the external surfaces of adeno-associated virus type 2 capsids that affect transduction and neutralization. *J. Virol.* **80**, 821–834 (2006).
14. Bartlett, J.S., Kleinschmidt, J., Boucher, R.C. & Samulski, R.J. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat. Biotechnol.* **17**, 181–186 (1999).
15. Girod, A. *et al.* Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat. Med.* **5**, 1052–1056 (1999).
16. Muller, O.J. *et al.* Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat. Biotechnol.* **21**, 1040–1046 (2003).
17. Perabo, L. *et al.* In vitro selection of viral vectors with modified tropism: the adeno-associated virus display. *Mol. Ther.* **8**, 151–157 (2003).
18. Opie, S.R., Warrington, K.H., Jr., Agbandje-McKenna, M., Zolotukhin, S. & Muzyczka, N. Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. *J. Virol.* **77**, 6995–7006 (2003).
19. Moskalenko, M. *et al.* Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *J. Virol.* **74**, 1761–1766 (2000).
20. Wobus, C.E *et al.* Monoclonal antibodies against the adeno-associated virus type 2 (AAV-2) capsid: epitope mapping and identification of capsid domains involved in AAV-2-cell interaction and neutralization of AAV-2 infection. *J. Virol.* **74**, 9281–9293 (2000).
21. Maheshri, N., Koerber, J.T., Kaspar, B.K. & Schaffer, D.V. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol.* **24**, 198–204 (2006).
22. Zhao, H., Giver, L., Shao, Z., Affholter, J.A. & Arnold, F.H. Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.* **16**, 258–261 (1998).
23. Stemmer, W.P. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370**, 389–391 (1994).
24. Boder, E.T. & Wittrup, K.D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553–557 (1997).
25. Daugherty, P.S., Chen, G., Iverson, B.L. & Georgiou, G. Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc. Natl. Acad. Sci. USA* **97**, 2029–2034 (2000).
26. Soong, N.W. *et al.* Molecular breeding of viruses. *Nat. Genet.* **25**, 436–439 (2000).
27. Lai, K., Kaspar, B.K., Gage, F.H. & Schaffer, D.V. Sonic hedgehog regulates adult neural progenitor proliferation *in vitro* and *in vivo*. *Nat. Neurosci.* **6**, 21–27 (2003).
28. Batard, P., Jordan, M. & Wurm, F. Transfer of high copy number plasmid into mammalian cells by calcium phosphate transfection. *Gene* **270**, 61–68 (2001).
29. Rabinowitz, J.E. *et al.* Cross-dressing the virion: the transcapsidation of adeno-associated virus serotypes functionally defines subgroups. *J. Virol.* **78**, 4421–4432 (2004).
30. Gigout, L. *et al.* Altering AAV tropism with mosaic viral capsids. *Mol. Ther.* **11**, 856–865 (2005).