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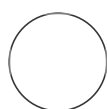
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## AAV DNA library generation

Forked from a private protocol

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### ABSTRACT

This protocol describes how to generate a DNA library of AAV capsid variants diversified by insertion of a randomized sequence encoding 7 amino acids between AA588 and AA589 of AAV9.

**Protocol status:** Working  
We use this protocol and it's working

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81604

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## Generation of Library Fragments

### 1 Design primers for the randomized insertion.

#### Note

We use a randomized heptamer codon insertion ([NNK] x 7) based on the NNK saturation mutagenesis strategy. This uses degenerate primers containing mixed bases (Integrated DNA Technologies). N can be A, C, G or T; K can be G or T. This strategy yields combinations of all 20 amino acids at each position of the heptamer peptide using 33 codons, resulting in a theoretical library size of 1.28 billion amino acid combinations.

To introduce genetic diversity to the Round 1 library, we use a reverse primer containing 21 degenerate nucleotides ([NNK] x 7) inserted between amino acids 588 and 589 (VP1 numbering) of the *cap* gene.

The forward primer contains a 20 bp 5' overhang near the XbaI restriction enzyme sequence.

Reverse primers contain a 20 bp 5' overhang near the AgeI restriction enzyme sequence.

Our primer sequences:

XF (forward): ACTCATCGACCAATACTTGTACTATCTCTCTAGAAC

7xMNN-588i (reverse):

GTATTCCTTGTTTGAACCAACCGGTCTGCGCCTGTGCMNNMNNMNNMNNMNNMNNM  
NNTTGGGCACTCTGGTGGTTTGTG

### 2 Generate the AAV capsid library fragments by PCR using the AAV9 *cap* gene as template with



Q5 Hot Start High-Fidelity 2X Master Mix - 500 rxns [New England Biolabs Catalog](#)  
#M0494L

and forward and reverse primers.

#### Note

To avoid PCR-induced biases resulting from point mutations, recombination, and template switching, limit PCR amplification to 10-15 cycles and scale up to get the required yield.

3 Run PCR products on a 1% agarose gel.

4 Purify the 480 bp band with



Zymoclean™ Gel DNA Recovery Kit [Zymo Research Catalog](#)  
#D4001

#### Note

It is critical to avoid AAV contamination during this step by taking precautionary measures like using a clean gel-running box and freshly prepared 1× TAE buffer.

## Library Assembly



5 Linearize the rAAV-ΔCap-in-cis-Lox plasmid by restriction digest with AgeI and XbaI


6 Insert the amplified library fragments into the linearized vector in a 1:2 molar ratio using






NEBuilder HiFi DNA Assembly Master Mix - 50 rxns [New England Biolabs Catalog](#)  
#E2621L

## Library Purification

- 7 Treat with either  Plasmid Safe ATP-Dependent DNase Epicentre Catalog #E3105K or  Exonuclease V (RecBCD) - 5,000 units New England Biolabs Catalog #M0345L to degrade non-assembled DNA fragments remaining in the mixture.

- 8 Purify assembled library with  DNA Clean & Concentrator-5 (Capped) 50 Preps Zymo Research Catalog #D4013

## Library Quality Validation

- 9 Transform 1 ng assembled library into  SURE 2 Supercompetent Cells Agilent Technologies Catalog #200152 and check for colonies after overnight incubation at 37°C on LB-agar plates containing carbenicillin.
- 10 Sequence the DNA library around the insertion site. A non-biased library should match the diversity of the NNK/NNM motif (N=25% each of A, T, G and C; K=50% each of G and T; M=50% each of A and C) with minor fluctuations.
- 11 To verify that the ITRs are intact, digest with  SmaI - 2,000 units New England Biolabs Catalog #R0141S
- 12 Transfect  293T cell line ATCC Catalog #CRL-3216 with 10 ng of library. Uniform expression of mNeonGreen should be observed across cells. Measuring the average yield per dish will inform scaling for full production.

### Note

Our typical yields are on the order of 0.1 - 1E11 v.g. per 150 mm dish.