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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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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):
GTATTCCTTGGTTTTGAACCCAACCGGTCTGCGCCTGTGCMNNMNNMNNMNNMNNMNNMNNTTGGGCACTCTGGTGGTTTGTG

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.