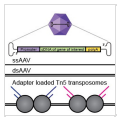

Apr 13, 2019

Working

Fast-Seq, a universal method for rapid and inexpensive genomic validation of rAAV vectors in preclinical settings

Lucy Maynard[1], Olivia Smith[1], Nicolas Tilmans[2], Eleonore Tham[1], Shayan Hosseinzadeh[3], Ryan Leenay[1], Weilun Tan[1], Nicole Paulk[3]

[1]Chan Zuckerberg Biohub, [2]Anagenios, [3]University of California San Francisco, Chan Zuckerberg Biohub

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Nicole K. Paulk
University of California, San Francisco

ABSTRACT

Sanger sequencing is the standard preclinical method used for sequence verification of AAV transfer vector cloning constructs and eventual packaged viral genomes. While Sanger sequencing does provide high quality data in a relatively short turnaround time, the data analysis requires manual evaluation of sequencing reads, rendering this approach incredibly low-throughput. Additionally, Sanger sequencing only provides 1-2X coverage, which results in low confidence single nucleotide polymorphism (SNP) and indel calling. It is imperative for preclinical gene therapy labs and core facilities to sequence both the transfer vector plasmids that go into viral production and the eventual packaged genomes to ensure no recombinations or mutations have occurred, and to validate that the genomes were not truncated during capsid packaging.

This protocol outlines a simple, fast, and inexpensive methodology geared toward preclinical environments for sequencing packaged AAV genomes via NGS, using paired-end Tn5 tagmentation-based library preparation. This protocol was optimized for use on the commonly available illumina MiSeq instrument, but other instruments would also work and should be chosen based on the number of samples and depth required (iSeq 100, MiniSeq, MiSeq, NextSeq, etc.).

MATERIALS

NAME	CATALOG #	VENDOR
5M NaCl solution	AM9759	Thermo Fisher Scientific
HEPES 1M	15630106	Thermo Fisher Scientific
Potassium hydroxide solution 8N	P4494	Millipore Sigma
0.5M EDTA solution	15575020	Thermo Fisher Scientific
Dithiothreitol (DTT)	R0861	Thermo Fisher Scientific
Triton X-100	85112	Fisher Scientific
Glycerol	17904	Thermo Fisher Scientific
1M Tris pH 7.0	AM9850G	Thermo Fisher Scientific
1M MgCl2 solution	AM9530G	Thermo Fisher Scientific
NN-Dimethylformamide (DMF) solution	D4551	Millipore Sigma
KAPA HiFi PCR kit with dNTPs	NC0142652	Fisher Scientific
10% SDS solution	15553027	Thermo Fisher Scientific
Nextera i5/i7 indexing primers	View	
DNA LoBind 1.5mL microcentrifuge tubes	13-698-791	Fisher Scientific
Tn5 transposase with Nextera adapters loaded	UC-Macro-Tn5-Nextera adapter	
Elution Buffer (EB)	19086	Qiagen

NAME	CATALOG #	VENDOR
Baseline-ZERO DNase Endonuclease	DB0715K	Lucigen
Plasmid-Safe DNase Exonuclease	E3101K	Lucigen
QIAamp MinElute Virus Spin Kit	57704	Qiagen
DNA Polymerase I Large (Klenow) Fragment	M0210L	New England Biolabs
Millex-GP Syringe Filter Unit 0.22 µm	SLGP033RS	Emd Millipore
Protein LoBind 1.5mL microcentrifuge tubes	0030108116	Eppendorf
Random primer mix	S1330S	New England Biolabs
Deoxynucleotide (dNTP) Solution Mix	N0447S	New England Biolabs
Qubit assay tubes	Q32856	Thermo Fisher Scientific
Qubit 1X dsDNA High Sensitivity Assay Kit	Q33230	Thermo Fisher Scientific
200 Proof Ethanol pure	E7023	Sigma Aldrich
SPRIselect Reagent	B23317	Beckman Coulter
UltraPure™ DNase/RNase-Free Distilled Water	10977015	Thermo Fisher Scientific
Illumina Library Quantitation Complete kit (Universal)	KK4824	Kapa Biosystems
MiSeq Nano v2 300-cycle kit	MS-102-2002	Illumina, Inc.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Millex-GP Syringe Filter Unit, 0.22 µm	SLGP033RS	Emd Millipore
Qubit 1X dsDNA High Sensitivity Assay Kit	Q33230	Thermo Fisher Scientific
Qubit assay tubes	Q32856	Thermo Fisher Scientific
Agilent High Sensitivity DNA Kit	5067-4626	Agilent Technologies
Illumina Library Quantitation Complete kit (Universal)	KK4824	Kapa Biosystems
MiSeq Reagent Kit V2 (300-cycles)	MS-102-2002	illumina
KAPA HiFi PCR kit with dNTPs	NC0142652	Fisher Scientific
DNA LoBind Tube 1.5ml	022431021	Eppendorf
SPRIselect reagent kit	B23317	Beckman Coulter
Elution buffer	19086	Qiagen
Qubit assay tubes	Q32856	Thermo Fisher Scientific
Qubit 1X dsDNA High Sensitivity Assay Kit	Q33230	Thermo Fisher Scientific
Plasmid-Safe ATP-Dependent DNase Exonuclease	E3101K	Epicentre
Baseline-ZERO DNase Endonuclease	DB0715K	Epicentre
UltraPure DNase/RNase-Free Distilled Water	10977015	Teknova
Protein LoBind Tubes, 1.5 mL	0030108116	Eppendorf
QIAamp MinElute Virus Spin Kit	55704	

NAME	CATALOG #	VENDOR
DNA LoBind Tubes, 1.5 mL	0030108051	Eppendorf
Random primer mix	S1330S	New England Biolabs
Deoxynucleotide (dNTP) Solution Mix	N0447S	New England Biolabs
DNA Polymerase I Large (Klenow) Fragment	M0210L	New England Biolabs

SAFETY WARNINGS

N,N-Dimethylformamide (DMF) is a volatile carcinogen and should be handled with appropriate safety precautions in a chemical fume hood. When handling stock DMF, wear appropriate PPE including a lab coat, goggles, closed toe shoes and double gloves. Dispose of any pipette tips that contact DMF in a labeled solid waste container. Clean pipettes with 70% ethanol after use with DMF.

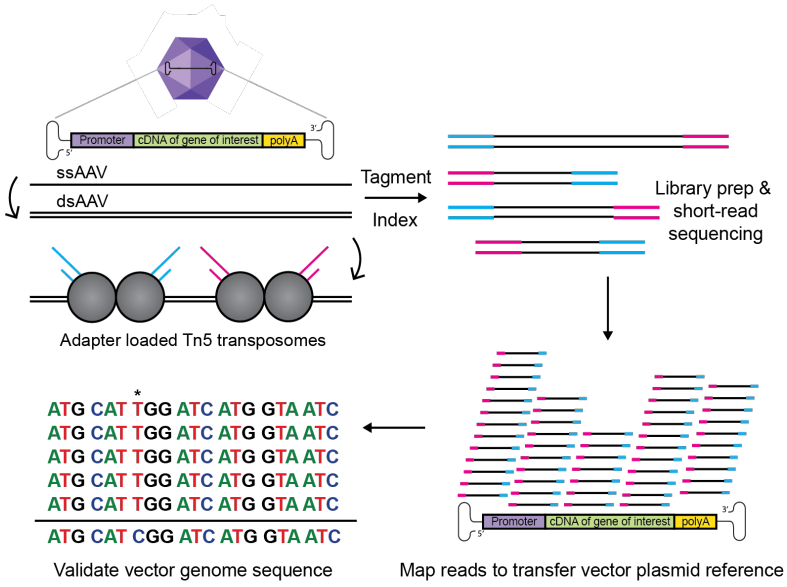
Per NIH Biosafety Guidelines, recombinant AAV vectors are Biosafety Level 1 so long as the encoded transgene is not a potentially tumorigenic gene product or toxin molecule, and provided they were produced in the absence of a helper virus. Otherwise, it should be handled under Biosafety Level 2 containment.

BEFORE STARTING

Ensure that you have all the necessary reagents and equipment prior to starting.

ISOLATING PURE ssDNA AAV GENOMES FROM VECTOR LOT

1



Overview of Fast-Seq, a Tn5-based packaged rAAV genome sequencing method. Schematic of the Fast-Seq protocol. Preparations of rAAV undergo: ssDNA extraction, second-strand synthesis to generate dsDNA, tagmentation with adapter-loaded Tn5 transposomes, indexing, QC validation, short-read sequencing on Illumina MiSeq, mapping reads to the transfer vector plasmid used to generate the input rAAV, and validation of the packaged genome. An example packaged genome mutation (*) is shown at position 7. ssAAV = single-stranded AAV genome; dsAAV = double-stranded AAV genome; Tn5 = transposase.

STEPS

1. We recommend extracting total gDNA from 1E11 full rAAV particles. However, this protocol has been successfully validated for inputs as low as 1E9 full rAAV particles. We suggest including a known pre-validated control vector with replicates for every run. To meet the minimum input DNA requirement for NGS, we recommend you prepare at least 8 samples (this can be composed of biological/technical replicates or dilutions of your sample and controls) for sequencing.

SAFETY INFORMATION

Per NIH Biosafety Guidelines, recombinant AAV vectors are Biosafety Level 1 so long as the encoded transgene is not a potentially tumorigenic gene product or toxin molecule, and provided they were produced in the absence of a helper virus. Otherwise, it should be handled under Biosafety Level 2 containment.

NIH Guidelines.pdf

2. To remove unincorporated DNA, incubate rAAV samples with 40-U exonuclease and 10-U endonuclease for 30-min at 37°C in the provided 10X buffer, supplemented with 1 mmol/L of the provided ATP in a final volume of 200-µL in a protein low-binding microcentrifuge tube.

REAGENT	[STOCK]	[FINAL]	VOLUME NEEDED
10X Baseline ZERO buffer	10X	1X	20 µL
Baseline ZERO endonuclease	1U/uL	10U	10 µL
Plasmid-safe exonuclease	10U/µL	40U	4 µL
ATP	1mmol/L	25mM	8 µL
AAV 1E11 full viral particles	-	1E11	x µL
PCR-certified water			x µL
Total Volume			200 µL

NUCLEASE DIGESTION MIX

Plasmid-Safe ATP-Dependent DNase Exonuclease
by Epicentre
Catalog #: E3101K

Baseline-ZERO DNase Endonuclease
by Epicentre
Catalog #: DB0715K

UltraPure DNase/RNase-Free Distilled Water
by Teknova
Catalog #: 10977015

Protein LoBind Tubes, 1.5 mL
by Eppendorf
Catalog #: 0030108116

ThermoMixer
Benchtop Incubator
Eppendorf 5382000023
Any heat block will suffice

3. Incubate samples at 65°C for 10-min to stop the nuclease digestion.

4. Continue with rAAV gDNA extraction with Qiagen's MinElute Virus Spin Kit following the manufacturer's protocol. Use the entire 200-µL reaction from Step 3 as the input for the kit. Additional reagents and equipment required for the kit: 100% ethanol, 1.5-mL microcentrifuge tubes, heat block, microcentrifuge, vortexer. Elute the final DNA into 28-µL, into a DNA low-binding tube and either store short-term (<24-hr) at 4°C, or long-term (>24-hr) at -20°C.

QIAamp-MinElute-Virus-Spin-Handbook.pdf

QIAamp MinElute Virus Spin Kit
Catalog #: 55704

DNA LoBind Tubes, 1.5 mL
by Eppendorf
Catalog #: 0030108051

This is a safe stopping point

SECOND STRAND DNA SYNTHESIS

2 1. Heat the extracted gDNA input from above for 5-min at 95°C on a heat block then quench on ice.

2. Transfer the entire cold gDNA solution to a PCR tube and add a mix containing 6-µM of random primers, 2-mM of each dNTP and 10-U of DNA polymerase I (Klenow) in the provided buffer in a final volume of 50-µl.

REAGENT	[STOCK]	[FINAL]	VOLUME NEEDED
Random primers	60µM	6µM	5 µL
dNTPs	10mM each	2mM	10 µL
Klenow	5U/µL	10U	2 µL
10X NEB2 buffer	10X	1X	5 µL
gDNA from Step 1	-	-	28 µL
Total Volume			50 µL

Random primer mix
by New England Biolabs
Catalog #: S1330S

Deoxynucleotide (dNTP) Solution Mix
by New England Biolabs
Catalog #: N0447S

DNA Polymerase I Large (Klenow) Fragment
by New England Biolabs
Catalog #: M0210L

3. Add sealed tubes to PCR to randomly prime DNA synthesis with a ramp of 0.1°C/second until 37°C, followed by 1 hour of incubation at 37°C.

SimpliAmp Thermal Cycler
PCR
Applied Biosystems A24811
Any standard PCR thermocycler will suffice

4. If you are ready to go on to the next step, maintain your dsDNA AAV genomes on ice. If you won't be preparing your AAV libraries in the next 24 hours, store the tubes wrapped in parafilm at -20°C.

This is a safe stopping point

SOLUTIONS TO PREPARE IN ADVANCE OF AAV LIBRARY PREP AND STORE

3 The dilution of each new batch of adapter-loaded Tn5 enzyme should be empirically determined based on activity. We recommend making a dilution series of loaded enzyme to determine the optimal yield and fragment size. Dilute the Tn5 enzyme in Tn5 Storage Buffer. For a starting point reference for your dilution series, we have found that adapter-loaded Tn5 at 1-ng/µL in storage buffer works best in our hands, however working concentration is subject to the activity of the batch. We suggest performing 2-fold serial dilutions in a range of concentrations both above and below this when qualifying your initial batch of Tn5. Adapter-loaded Tn5 lots can be custom ordered from the UC Berkeley QB3 MacroLab here: (http://qb3.berkeley.edu/macrolab/servicesrates/).

(A) Preparation of 50-mL of **Tn5 Storage Buffer**
Combine all reagents for the Storage Buffer *except for the glycerol*, pH the buffer to 7.2, and then bring to a final volume of 20-mL. Sterile filter the buffer with a 0.22-µm filter. Then add 30-mL of sterile glycerol up to a final volume of 50-mL. Store at -20˚C.

REAGENT	[STOCK]	[FINAL]	VOLUME NEEDED
NaCl	5 M	100 mM	1 mL
EDTA	0.5 M	0.1 mM	10 µL
HEPES-KOH, pH 7.2	1 M	50 mM	2.5 mL
DTT	100 mM	1 mM	0.5 mL
Triton X-100	98%	0.1%	51 µL
Glycerol	100%	60%	30 mL
PCR-certified water	-	-	16 mL
Total volume	-	-	50 mL

PREPARATION OF 50-ML OF Tn5 STORAGE BUFFER

NOTE: To accurately pipette the Tn5 storage buffer to make dilutions of the Tn5 enzyme, heat the buffer in a 42˚C water bath, pipette the desired volume for the dilution, then cool the buffer on ice before making the dilution.

Millex-GP Syringe Filter Unit, 0.22 µm
by Emd Millipore
Catalog #: SLGP033RS

(B) Preparation of 50-mL of **5X Tn5 Reaction Buffer**
Combine the reagents for the Reaction Buffer, adjust the pH to 7.5, and bring to final volume of 50-mL. Sterile filter the buffer with a 0.22-µm filter. Store at -20˚C.

REAGENT	[STOCK]	[FINAL]	VOLUME NEEDED
Tris, pH 7.0	1 M	50 mM	2.5 mL

MgCl2	1 M	50 mM	2.5 mL
PCR-certified water	-	-	45 mL
Total Volume	-	-	50 mL

(C) Preparation of **Neutralization Buffer**
The neutralization buffer is used to inactivate the Tn5 enzyme and quench the tagmentation reaction to stop over-tagmentation of the AAV libraries.

Neutralization Buffer is a 0.1% Sodium Dodecyl Sulfate (SDS) solution in water. Heat the solution in a 37˚C water bath to completely dissolve the SDS and then cool to room temperature. Store at room temperature.

(D) Qualify each new batch of Nextera adapter-loaded **Tn5 enzyme**
The dilution of each new batch of adapter-loaded Tn5 enzyme should be empirically determined based on activity. We recommend making a dilution series of loaded enzyme to determine the optimal yield and fragment size. Dilute the Tn5 enzyme in Tn5 Storage Buffer. For a starting point reference for your dilution series, we have found that adapter-loaded Tn5 at 1-ng/µL in storage buffer works best in our hands, however working concentration is subject to the activity of the batch. We suggest performing 2-fold serial dilutions in a range of concentrations both above and below this when qualifying your initial batch of Tn5. Adapter-loaded Tn5 lots can be custom ordered from the UC Berkeley QB3 MacroLab here: (http://qb3.berkeley.edu/macrolab/servicesrates/)

SOLUTIONS TO PREPARE FRESH FOR AAV LIBRARY PREP

4 NOTE BEFORE PREPARING SOLUTIONS:
Before beginning: We've provided volumes for **1** sample. As these are small volumes susceptible to loss and evaporation, we recommend using PCR strip tubes rather than PCR plates, and making master mixes **at least 1.5x** the number of samples. For example, if making libraries of 25 samples, prepare master mixes that are suitable for 1.5 * 25 = 30 samples. This will ensure you don't run out of master mix.

(A) **1.6X Tris-DMF Buffer**
If this will be a regular assay, we recommend pre-mixing and aliquoting the 5X Tn5 Reaction Buffer and water and storing as aliquots. Importantly, DMF should be added fresh, right at the time of the assay.

REAGENT	1 SAMPLE (µL)
5X Tn5 Reaction Buffer	0.64
PCR-certified water	1.2
100% DMF	*0.16*
Total Volume	2

1.6X TRIS-DMF BUFFER

SAFETY INFORMATION
N,N-Dimethylformamide (DMF) is a volatile carcinogen and should be handled with appropriate safety precautions in a chemical fume hood. When handling DMF stock, wear appropriate PPE including a lab coat, goggles, closed toe shoes and double gloves. Dispose of any pipette tips that contact DMF in a labeled solid waste container. Clean pipettes with 70% ethanol after use with DMF.

(B) **Tagmentation Reaction Mix**

REAGENT	1 SAMPLE (µL)
1.6X Tris-DMF Buffer	2
Tn5 Enzyme (adapter loaded)	0.4
Total volume	2.4

TAGMENTATION REACTION MIX

(C) **Index PCR Master Mix**
This can be prepared while the AAV samples are tagmenting.

REAGENT	1 SAMPLE (µL)

5X KAPA HiFi Fidelity Buffer	1.6
dNTP Mix	0.24
PCR-certified water	0.4
KAPA HiFi Polymerase	0.16
Total Volume	2.4

INDEX PCR MASTER MIX

KAPA HiFi PCR kit with dNTPs
by Fisher Scientific
Catalog #: NC0142652

AAV TAGMENTATION WITH Tn5

5 Background: The tagmentation reaction is a vital step for producing AAV libraries of uniform size. There are several buffer components necessary for this reaction. The Tn5 Storage Buffer is used to dilute the adapter-loaded Tn5 enzyme to the appropriate concentration. Tn5 enzyme is diluted to 1-ng/µL in Storage Buffer. Reaction Buffer is prepared as a 5X stock and is combined with a crowding agent, N,N-Dimethylformamide, to create the buffer that the Tn5 enzyme is added to for tagmenting.

NOTE: The dilution of the adapter-loaded Tn5 should be empirically determined for every new batch of Tn5 enzyme based on activity. It is recommended to make a dilution series of pre-loaded Tn5 enzyme to determine optimal yield and fragment size.

FOR 1 REACTION (multiply volumes to scale for more reactions):

1. Dilute your double-stranded AAV gDNA to 1-ng/µL and verify the concentration with a Qubit. Be sure to use Qubit thin-walled assay tubes and calibrate with the Qubit dsDNA High Sensitivity Assay Kit. We do not recommend using a NanoDrop to measure DNA concentration here, as these readings can be inaccurate given the low concentrations.

Qubit
Flurometer
Invitrogen Q33228

Qubit 1X dsDNA High Sensitivity Assay Kit
by Thermo Fisher Scientific
Catalog #: Q33230

Qubit assay tubes
by Thermo Fisher Scientific
Catalog #: Q32856

2. Add 2.4-µL of **Tagmentation Reaction Mix** per PCR tube.
3. Add 1.6-µL of DNA solution per PCR tube.
4. Briefly spin down to pull all reagents to the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher S67601B

Any standard mini centrifuge with adapters for different tube sizes will suffice

5. Tagment PCR tubes in a thermocycler with the following conditions: 1 cycle of 55˚C for 10 min; 1 cycle of 72˚C for 3 min; 1 cycle of 10˚C kept at an infinite hold.

SimpliAmp Thermal Cycler
PCR

Applied Biosystems A24811

Any standard PCR thermocycler will suffice

NEUTRALIZATION

6 1. Add 0.8-µL of **Neutralization Buffer** per PCR tube.
2. Spin down in a benchtop centrifuge/platefuge at room temp for 5-min at 4,000 rpm.

Centrifuge
Benchtop Centrifuge

Eppendorf 5405000441

Any benchtop centrifuge will suffice

INDEX PCR

7 1. Add 2.4-µL of **Index PCR Master Mix** per PCR tube/well.
2. Add 1.6-µL of premixed Illumina Nextera i5/i7 indexing primers previously diluted to 5-µM.

illumina-adapter-sequences.pdf

Index 1 (i7);

CAAGCAGAAGACGGCATACGAGAT *NNNNNNNNNNNN*GTCTCGTGGGCTCGG

Index 2 (i5):

AATGATACGGCGACCACCGAGATCTACAC *NNNNNNNNNNNN*TCGTCGGCAGCGTC

bold = P5/P7 adapter sequence

italics = unique 12-bp barcode index

underline = primer for mosaic ends added during tagmentation

Additional help designing adapters can be found here: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-09.pdf

3. Briefly spin down to pull reagents to the bottom of the tube.

Mini-centrifuge
Centrifuge
Fisher S67601B
Any standard mini centrifuge with adapters for different tube sizes will suffice

4. Run the tubes in a thermocycler with the following conditions: 1 cycle of 72˚C for 3 min; 1 cycle of 95˚C for 30 sec; 12 cycles of (98˚C for 10 sec; 67˚C for 30 sec; 72˚C for 1 min); 1 cycle of 10˚C for an infinite hold.

SimpliAmp Thermal Cycler
PCR
Applied Biosystems A24811
Any standard PCR thermocycler will suffice

▯ This is a safe stopping point ▯

POOLING AND CLEANING AAV LIBRARIES

8 1. Pool 8-µL of every well into a 1.5-mL DNA LoBind microcentrifuge tube.

DNA LoBind Tube 1.5ml
by Eppendorf
Catalog #: 022431021

2. To clean the pooled libraries of primer dimers and undesired fragment sizes, perform two SPRI-bead washes.
a. Wash 1: 0.6X volume of your sample, followed with 2X wash with 80% ethanol, and elute into 100-µL EB.
b. Wash 2: 0.7X volume of your sample, followed with 2X wash with 80% ethanol, and elute in 30-µL EB.

SPRIselect reagent kit
by Beckman Coulter
Catalog #: B23317

Elution buffer
by Qiagen
Catalog #: 19086

Magnetic Stand
Magnetic Stand
Thermo Scientific MR02
Any magnetic rack that fits your tubes will suffice.

3. Measure the final DNA concentration with a Qubit. Be sure to use Qubit thin-walled assay tubes and calibrate with the Qubit dsDNA High Sensitivity Assay Kit. NOTE: To have enough sample to run on an Illumina MiSeq, ensure that your final DNA concentration is at least 300-pM at this step to continue.

Qubit
Flurometer
Invitrogen Q33228

Qubit assay tubes
by Thermo Fisher Scientific
Catalog #: Q32856

Qubit 1X dsDNA High Sensitivity Assay Kit
by Thermo Fisher Scientific
Catalog #: Q33230

4. If you're submitting samples to a core facility or external sequencing CRO for validation and sequencing, submit the samples at this stage (skip ahead to 'DATA ANALYSIS' once you've received the raw data back from your sequencing service provider). If you're planning on doing the validation and sequencing yourself, continue on to the next step.

This is a safe stopping point

QC LIBRARIES AND LOAD SEQUENCER

9 1. QC pooled and cleaned libraries on a bioanalyzer for fragment size verification and sample integrity. Follow the manufacturer instructions for your bioanalyzer, and use their recommended "high sensitivity" DNA kit. QC pooled and cleaned libraries on a bioanalyzer for fragment size verification and sample integrity. Follow the manufacturer instructions for your bioanalyzer, and use their recommended "high sensitivity" DNA kit. The absolute shape and bp size of the peaks in the traces are less important than having a large smooth proportion of inserts in the 200 bp – 800 bp range. Inserts in this size range from AAV genomes are optimal for cluster formation on Illumina flow cells.

Agilent High Sensitivity DNA Kit
by Agilent Technologies
Catalog #: 5067-4626

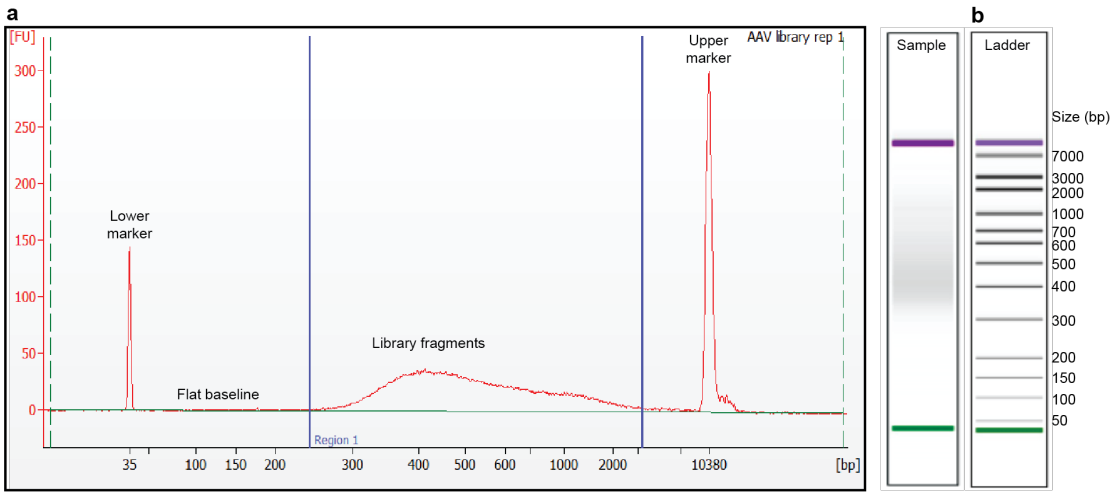
Bioanalyzer
Bioanalyzer
Agilent G2991AA
Any bioanalyzer will suffice.

Agilent High Sensitivity DNA Kit Guide.pdf

2. The fragment size range should be between 200 and 800 bp to effectively sequence the AAV genome. If this is not the case, review the AAV tagmentation step and determine if the generated library is over- or under-fragmented. Set up a qPCR to determine the library concentration. Although you can estimate the concentration with a Qubit, we highly recommend using qPCR instead as a gold standard method to determine the library concentration. We recommend following the detailed illumina protocol here:

Library-qpcr-quantification-guide.pdf

Illumina Library Quantitation Complete kit (Universal)
by Kapa Biosystems
Catalog #: KK4824



Example of a clean bioanalyzer run for fragmented DNA following successful library preparation. (a) The fragmented library trace shown was generated from pooling 16 AAV library samples following the Fast-Seq protocol, and which generated successful sequencing results. Ideally your electropherogram will have a flat baseline (indicated) preceding the sample peak, and a gradual curve centered on your library product for a gel-free library run on a high sensitivity DNA chip. FU = arbitrary fluorescence intensity signal; bp = base-pair. **(b)** Example of a good ladder showing adequate separation of the control size bands.

3. Load samples for sequencing according to the manufacturer's protocol from the instrument you're using. We have confirmed that samples prepared following this protocol run successfully on Illumina MiSeq using Nano V2 and iSeq instruments. For use on a MiSeq, prepare samples using a MiSeq Reagent Nano V2 kit, for 300 cycles, and run 2 x 75bp paired-end settings.

Additional information on MiSeq specifications and expected outputs and run times can be found here: https://www.illumina.com/systems/sequencing-platforms/miseq/specifications.html

MiSeq
Sequencer
illumina SY-410-1003

MiSeq Reagent Kit V2 (300-cycles)
by illumina
Catalog #: MS-102-2002

10 NGS DATA ANALYSIS

We have developed a free, open-source computational workflow for analysis of NGS data. Please go to the Paulk Lab Github repository to download our code and find instructions on how to run the pipeline in the README:

https://github.com/paulk-lab/FastSeq-pipeline

Summary: NGS fastq data files are filtered using Trimmomatic to remove adapter sequences, low quality reads (PHRED score <20, or length <50-bp) and unpaired reads. Trimmed reads will then be aligned to the rAAV transfer vector plasmid reference sequence (including both flanking ITRs) with BWA v0.7.17, using the mem algorithm. Alignments will be saved as BAM files, which will then be used to generate VCF files using GATK Haplotype Caller algorithm. SNPs and indels identified in VCF files will be filtered using BCFtools filter algorithm, with a 15X depth threshold and a user-specified allele fraction requirement. A consensus sequence will be generated using BCFtools consensus algorithm. Alignment and fragment distribution statistics will be obtained with Picard tools. Feel free to modify the code as needed. The code and tooling is licensed under the Creative Commons with Attribution license.

ANTICIPATED OUTCOMES

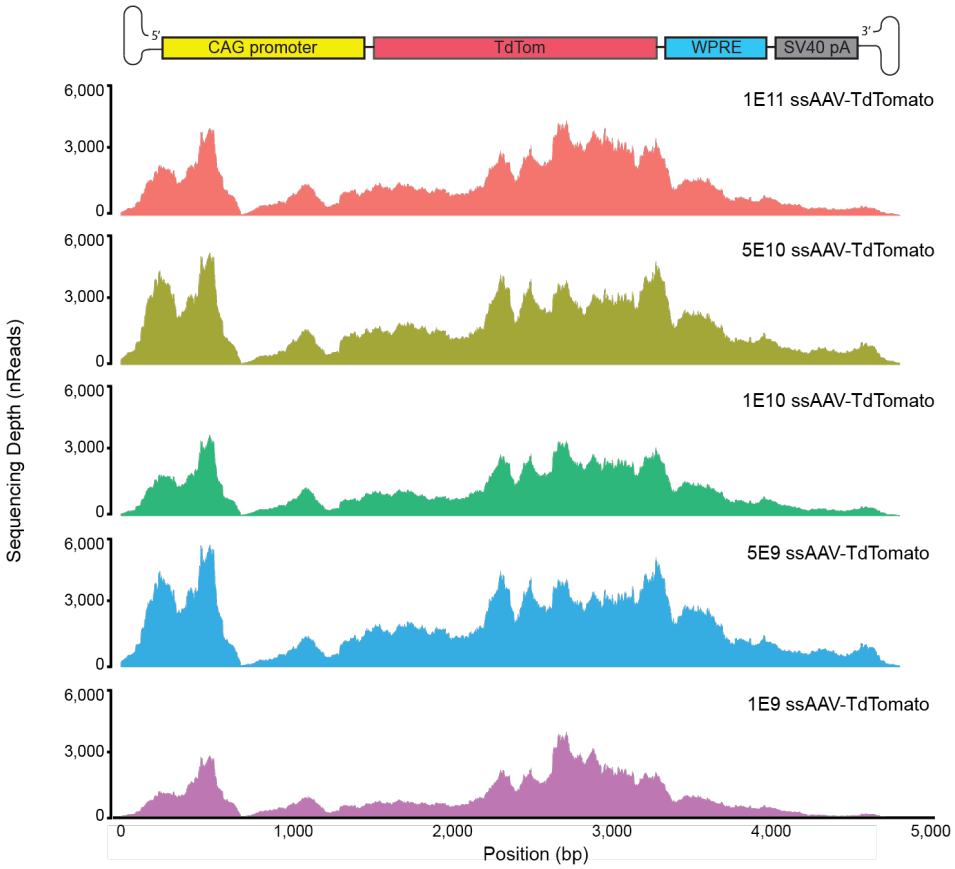
This code outputs an Output folder in your data directory, containing sample-specific folders and a compiled .csv file containing NGS statistics for all samples. Each sample-specific folder contains all the files necessary for obtaining alignments and statistics. Of note, please take a look at the following files:

- BAM alignment file
- Inferred consensus sequence in FASTA format
- PDF depiction of your library prep fragment distribution
- Raw and filtered VCF file, containing all called SNPs and indels

Reported statistics include: mean coverage, standard deviation of coverage, number of SNPs/indels, mean fragment size, as well as other relevant metrics. Resultant BAM files can be aligned to your AAV transfer vector plasmid reference sequence and viewed in a genome browser, such as Integrative Genomics Viewer (IGV) which can be downloaded here:

https://software.broadinstitute.org/software/igv/download

Plots of sequencing depth and coverage can be generated in R using ggplot, with instructions here: http://ggplot.yhathq.com/install.html



AAV dilution series validates Fast-Seq packaged AAV genome sequencing method. NGS sequence validation of recombinant packaged ssAAV genomes, including ITRs, depicting the expected sequencing depth and coverage from a standard MiSeq run after generating libraries with Fast-Seq. Various serotypes and vector produced in both common manufacturing platforms have been run successfully using Fast-Seq.

11 TROUBLESHOOTING

- **Final DNA concentration is below 300-pM.**

  Remeasure your DNA concentration with a Qubit to ensure that concentration is indeed 1-ng/µL. If the concentration is 1-ng/µL, consider increasing the input AAV gDNA concentration to 2-5 ng/µL, or increasing the number of PCR cycles. Another alternative is to increase the number of samples to be sequenced (we recommend a minimum of 8 samples/replicates). If your concentration is less than 1-ng/µL, even without diluting, increase the amount of input AAV. You can add more samples to your library by completing the protocol a second time with more samples (be sure to use unique indices), pooling the two libraries together and performing a final 0.7X SPRI clean-up, eluting into 30-µL.

- **Fragment size as measured by the bioanalyzer is below 200 bp.**

  Your DNA is over-fragmented. Possible causes are: a) you started with low-quality material. QC your starting material to ensure it is high quality DNA by absorbance; b) not enough starting material. Quantitate your starting DNA again by Qubit; c) your ratio of DNA to Tn5 is too low. Remeasure your dsDNA concentration with a Qubit to ensure that concentration is indeed 1-ng/µL. If the concentration is 1-ng/µL, consider increasing the dsDNA to 2-5ng/µL or increasing the number of samples to be sequenced. If the issue persists, we recommend requalifying your adapter-loaded Tn5 enzyme.

- **Fragment size as measured by the bioanalyzer is above 800 bp.**

  Your DNA is under-fragmented. Possible causes are: a) you started with too much DNA. Quantitate your starting DNA again by Qubit; b) your ratio of DNA to Tn5 is too high. Remeasure your dsDNA concentration with a Qubit to ensure that concentration is indeed 1-ng/µL. If the concentration is 1-ng/µL, consider increasing the tagmentation time at Step 13 from 10 minutes at 55°C to 20 minutes, or decreasing the number of samples to be sequenced (a maximum of 50 AAV samples can be sequenced on a MiSeq chip); c) your Tn5 transposase enzyme is inhibited. Either requalify your adapter-loaded Tn5 enzyme or check your DNA purification kit (if you didn't use the suggested Qiagen MinElute Virus Spin kit) for inhibitors.

- **No library peak is present by bioanalyzer.**

  This library should not be sequenced. Possible causes are: a) sample lost during bead clean up; b) low quality or insufficient starting DNA; c) not using a heated lid on the thermocycler during the second strand synthesis; or Tn5 transposase enzyme has expired and/or

gone through multiple freeze thaws.

- **How to qualify each new batch of Nextera adapter-loaded Tn5 enzyme.**
  Make a dilution series of Nextera adapter-loaded Tn5 enzyme in Tn5 Storage Buffer. We have found that 1-ng/µL is optimal for Fast-Seq. Use this concentration as middle starting position and test concentrations above and below this when qualifying your initial batch of Tn5. To then assess the efficacy of your various dilutions following fragmentation, perform a bioanalyzer run on all your dilution samples and see which concentrations of Tn5 give you fragments between 200 and 800 bp. Choose the lowest concentration that reproducibly works.

  Additional help with troubleshooting common library preparation problems can be found here:

  https://www.illumina.com/content/dam/illumina-marketing/documents/products/technotes/nextera-xt-troubleshooting-technical-note.pdf