Generation of tiled FIV amplicons for MiSeq sequencing

Version 1.0 (2018.03.23)

Adapted from: Quick, J. *et al*. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature Protocols 12 (6), 1261-1276*
https://www.nature.com/nprot/journal/v12/n6/abs/nprot.2017.066.html

Generation of tiled amplicons

Reagents:

NEB Q5 DNA High-fidelity Hot Start Polymerase
Custom primers
Agarose gel
Qubit High Sensitivity DNA kit

Library preparation and quantification

Reagents:

BIOO Scientific NEXTflex Rapid DNA-Seq Kit
Beckman Coulter Genomics Ampure XP beads
BIOO Scientific NEXTflex Dual-Indexed DNA Barcodes
Qubit High Sensitivity DNA kit
Agilent BioAnalyzer DNA 1000 kit
MiSeq Reagent kit v2 (500 cycle output)

Data analysis

Software:

Trimmomatic v0.36 - http://www.usadellab.org/cms/index.php?page=trimmomatic
Bowtie 2 v. 2.3.3.1 - http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools v1.3.1 - http://www.htslib.org/download/

Notes

Add no-template water controls at each of the cDNA and PCR steps to monitor for "amplicon jumping".

Primers were designed following Quick etal. 2017 guidelines using Primal Scheme http://primal.zibraproject.org.

1. Prepare two custom primer pools by mixing equal volumes of each 10 µM primer.

2. Prepare two PCR reactions for each sample (one for each primer pool):

Component	**Volume in 25 µL reaction**
5x Q5 reaction buffer	5 µL
10 mM dNTPs	1 µL
Q5 DNA Polymerase	0.5 µL
Nuclease-free water	15 µL
Primer pool (#1 or #2)	1 µL
DNA or cDNA	2.5 µL

3. Run the following cycles on a thermocycler:

Temperature	**Time**
98°C	30 seconds
95°C	15 seconds
68°C	5 minutes → -0.5°C per cycle
Repeat steps 2 & 3 x 6	
98°C	15 seconds
65°C	5 minutes
Repeat steps 4 & 5 x 28 (**35 total cycles**)	
4°C	6 minutes

4. Run 5 µL of each product on a 1% agarose gel. Each should produce a visible 400 bp band.

Post PCR cleanup (1.8:1 ratio of beads to sample) and quantification

1. Allow AMPure XP beads to equilibrate to **room temperature, vortex** until homogenous.
2. Bring PCR product volume up to 25 µL with water (if not at volume already).
3. Add 45 µL of beads to 25 µL of PCR product, mix well, and incubate at room temperature for 10 minutes.
4. Place tubes on a magnetic stand and incubate until solution appears clear.
5. Discard supernatant without disturbing the beads.
6. While tubes are on the magnet, add 200 µL of **freshly prepared** 80% EtOH, incubate for 30 seconds, and discard the EtOH wash.
7. Repeat previous 80% EtOH wash and remove as much EtOH as possible.
8. Leave tubes on magnet and air dry for 5 minutes.
9. Remove tubes from magnet and add 20 µL of nuclease-free water. Mix well by pipetting.
10. Place tubes on magnet stand. When solution appears clear, remove supernatant without disturbing the beads and place into new tubes.
11. Quantify the DNA concentration using the Qubit High Sensitivity DNA kit (or equivalent) from 1 µL of each product. Expected range = 10-100 *ng/µL* DNA.

Library preparation

End-repair

1. Combine 100 ng of PCR-amplified DNA from primer pool 1 and 2 together for a total of 200 ng in 50 µL of nuclease-free water.
   If yield is <100 ng in either or both pools, maximize DNA concentration by combining 8 uL of each pool and proceed to step 2.

2. Combine the following components from the Kapa Hyper prep kit for end repair:

Component	Volume in 25 µL reaction
End Repair & A-tailing buffer	7.5 µL
End Repair & A-tailing enzyme mix	1.5 µL
PCR-amplified DNA (200 ng)	16 µL

3. Run the following cycles on a thermocycler:

Temperature	Time
22°C	20 minutes
72°C	20 minutes
4°C	∞

Adaptor ligation

1. Select unique NEXTflex Dual-Indexed DNA Barcodes for each sample. Try not to repeat barcodes from recent runs.
   Note: Be careful to not cross-contaminate the adaptors by centrifuging all liquid from the caps and only opening one cap at a time.

2. Combine the following components:

Component	Volume in 50 µL reaction
Ligation enzyme mix	23.75 µL
NEXTflex DNA Barcodes (stock concentration)	1.25 µL
End repair reaction product	25 µL

3. Incubate at 20°C for 15 minutes.
4. **Proceed immediately to cleanup.**

Post ligation cleanup (0.8:1 ratio of beads to sample)

1. Allow AMPure XP beads to equilibrate to room temperature, vortex until homogenous.
2. Add 40 µL of beads to 50 µL of ligation product, mix well, and incubate at room temperature for 10 minutes.

3. Place tubes on a magnetic stand and incubate until solution appears clear.
4. Discard supernatant without disturbing the beads.
5. While tubes are on the magnet, add 200 µL of 80% EtOH, incubate for 30 seconds, and discard the EtOH wash.
6. Repeat previous 80% EtOH wash and remove as much EtOH as possible.
7. Leave tubes on magnet and air dry for 5 minutes.
8. Remove tubes from magnet and add 20 µL of nuclease-free water. Mix well by pipetting.
9. Place tubes on magnet stand. When solution appears clear, remove supernatant without disturbing the beads and place into new tubes.

Library amplification

1. Combine the following components:

Component	**Volume in 50 µL reaction**
NEXTflex PCR master mix	12 µL
NEXTflex primer mix	2 µL
Nuclease-free water	16 µL
Adaptor-ligated library	20 µL

2. Run the following cycles on a thermocycler:

Temperature	**Time**
98°C	2 minutes
98°C	30 seconds
65°C	30 seconds
72°C	60 seconds
Repeat steps 2-4 for a total of **7 cycles**	
72°C	4 minutes
4°C	∞

3. Proceed directly to cleanup or store at 4°C.

Post amplification cleanup (0.8:1 ratio of beads to sample)

1. Allow AMPure XP beads to equilibrate to room temperature, vortex until homogenous.
2. Add 40 µL of beads to 50 µL of amplified product, mix well, and incubate at room temperature for 10 minutes.

3. Place tubes on a magnetic stand and incubate until solution appears clear.
4. Discard supernatant without disturbing the beads.
5. While tubes are on the magnet, add 200 µL of 80% EtOH, incubate for 30 seconds, and discard the EtOH wash.
6. Repeat previous 80% EtOH wash and remove as much EtOH as possible.
7. Leave tubes on magnet and air dry for 5 minutes.
8. Remove tubes from magnet and add 30 µL of **NEXTflex resuspension buffer**. Mix well by pipetting.
9. Place tubes on magnet stand. When solution appears clear, remove supernatant without disturbing the beads and place into new tubes.

Library quantification and pooling

1. Quantify the DNA concentration **of each sample** using the Qubit High Sensitivity DNA kit (or equivalent).
2. Convert DNA libraries from weight to moles:

   Molecular weight [nM] = Library concentration [ng/µL] / ((ave. library size x 660)/1,000,000)
   ([ng/µL]/(580 x 660)) x 1000000

3. Normalize to **4 nM or higher**. If concentration of the lowest individual sample exceeds 4 nM, normalize to the concentration of the lowest sample.

   Volume of Index = **(V(*f*) x C(*f*)) / # x C(*i*)**

   where V(*f*) is the final desired volume of the pool,
   C(*f*) is the desired final concentration of all the DNA in the pool
   # is the numbers of indexes, and
   C(*i*) is the initial concentration of each indexed sample.

4. Scale to appropriate pipetting volumes and pool library.
   Note: Indexed samples lower than 4 nM can be spiked into the pool. However, too much volume of low concentration samples may dilute the library and alter sequencer clustering and/or sequence coverage.

Post pooling cleanup (0.66:1 ratio of beads to sample)

1. Allow AMPure XP beads to equilibrate to room temperature, vortex until homogenous.
2. Add **0.66 µL of beads per 1 µL of pooled library**, mix well, and incubate at room temperature for 10 minutes.
3. Place tubes on a magnetic stand and incubate until solution appears clear.
4. Discard supernatant without disturbing the beads.
5. While tubes are on the magnet, add 200 µL of 80% EtOH, incubate for 30 seconds, and discard the EtOH wash.
   For larger library volumes, increased volume of EtOH proportionately to fully cover beads during wash.
6. Repeat previous 80% EtOH wash and remove as much EtOH as possible.
7. Leave tubes on magnet and air dry for 5 minutes.
8. Remove tubes from magnet and add 30 µL of **NEXTflex resuspension buffer**. Mix well by pipetting.
   For larger library volumes, increased volume of resuspension buffer up to 50 µL.

9. Place tubes on magnet stand. When solution appears clear, remove supernatant without disturbing the beads and place into new tubes.

Final quantification and preparation for sequencing

1. Check library fragment distribution using the BioAnalyzer DNA 1000 kit. Peak fragment size from 400 bp tiles amplicons with proper ligated adaptors should be ~520-580 nt. If ~180 bp bands (adaptor dimers) still exist, perform post pooling cleanup again or size select by running the pool on an agarose gel, followed by cleanup of excised band using Qiagen QIAquick Gel Extraction Kit.
2. Quantify library using the Qubit High Sensitivity DNA kit.
3. Follow MiSeq kit instructions for further preparing the pooled library for sequencing.
   Notes: Based on QC analysis, determine the average size of desired fragment (~520-580 nt) and the best estimate of concentration [pM] for the given fragment size based on reading for PhiX control. Denature library according to MiSeq kit instructions and ***load library at a final concentration of 10 pM****.*

Bioinformatics Pipeline

Trim with Trimmomatic:

```
java -jar /apps/Trimmomatic-0.36/trimmomatic-0.36.jar \
PE \
-phred33 \
x1936_S1_L001_R1_001.fastq.gz \
x1936_S1_L001_R2_001.fastq.gz \
x1936_S1_L001_R1_001_trimmed_PRIMERS_1P.fastq.gz \
x1936_S1_L001_R2_001_trimmed_PRIMERS_2P.fastq.gz \
x1936_S1_L001_R1_001_trimmed_PRIMERS_1U.fastq.gz \
x1936_S1_L001_R2_001_trimmed_PRIMERS_2U.fastq.gz \
  ILLUMINACLIP:/home/jmalmber/unixstff/ADAPTER_SEQS.fa:2:30:7 \
  LEADING:20 \
  TRAILING:20 \
  SLIDINGWINDOW:4:20 \
  AVGQUAL:20 \
```

Align with Bowtie 2:

Build index:

```
/apps/bowtie2-2.3.3.1-linux-x86_64/bowtie2-build
EF455605.fasta,EF455603.fasta,EF455604.fasta,EF455606.fasta,EF455607.fasta,EF455608.fasta,EF4556
09.fasta,EF455610.fasta,EF455611.fasta,EF455612.fasta,EF455613.fasta,EF455614.fasta,EF455615.fasta
,DQ192583.fasta,KF906185.fasta,KF906186.fasta,Pco732.fasta,P1123.fasta,P901.fasta,P1124.fasta,P109
4.fasta,P910.fasta,P1033.fasta,KF906191.fasta,KF906187.fasta,KF906189.fasta,KF906184.fasta,KF9061
88.fasta,KF906192.fasta,KF906190.fasta,KF906194.fasta,KF906179.fasta,KF906176.fasta,KF906177.fas
ta,KF906175.fasta,KF906181.fasta,KF906180.fasta,KF906178.fasta,KF906182.fasta, PLVB_index
```

Inspect index:

```
/apps/bowtie2-2.3.3.1-linux-x86_64/bowtie2-inspect -n PLVB_index
```

Align to index:

```
/apps/bowtie2-2.3.3.1-linux-x86_64/bowtie2 -x PLVB_index \
-q -1 x1936_R1_TRIMMED_1P.fastq -2 x1936_R1_TRIMMED_1U.fastq \
-S x1936_mapped_to_PLVB.sam
```

Sort with Samtools:
Convert .sam to .bam:
samtools view -S -b x1936_mapped_to_PLVB > x1936_mapped_to_PLVB.bam
Sort:
samtools sort x1936_mapped_to_PLVB.bam x1936_to_PLVB_sort
View header (if needed – must have header to sort):
samtools view -H x1936_sort.bam

View bam files in Geneious
Identify the index sequence to which the highest number of reads were allocated from the multiple sequences index. Now repeat steps beginning with **Align with Bowtie 2***, but build index for only that sequence and map ALL reads to the single, best fit reference sequence. Then convert to bam for downstream data analysis.*