Library Preparation for Sequencing of Assembled Gene Clusters

Molly Miranda

Abstract

This method describes the preparation of Illumina sequencing libraries with a 1/20 scale Nextera protocol. Typically, yeast or E. coli plasmid DNA is the input.

Method derived from:

Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony R (2015) Inexpensive Multiplexed Library Preparation for Megabase-Sized Genomes. PLoS ONE 10(5): e0128036. doi:10.1371/journal.pone.0128036

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Guidelines

Materials:

Hard shell pcr plates (E&K Scientific Framestar EK-75012 suggested)
PCR film (E&K Scientific T796150)

2 mL Deep square 96 well plates (Axygen P-2ML-SQ-C-S or equivalent)

8-strip PCR tube, 0.2 mL (E&K Scientific, 690030)

1.2 mL Deep 96 well plates (E&K Scientific, OX1262 or equivalent)

Exonuclease V (NEB M0345L)

NEB Buffer 4, 10x (NEB P0756S), supplied with Exonuclease V

ATP 10 mM (NEB M0440S), supplied with Exonuclease V
EDTA, 0.33 M
1.5 mg/mL Sera-mag magnetic particles in PEG/NaCl

10 mM Tris-HCl (pH 8.0 - 8.5)

Nextera® DNA Sample Preparation Kit, 96 samples (Illumina FC-121-1031)

Nextera® Index Kit, 96 indexes, 384 samples (Illumina FC-121-1012) and

Additional index primer/adapter set (IDT suggested vendor, sequences listed in Before Start section)

KAPA HiFi Library Amplification Kit (KAPA Biosystems KK2611/KK2612)

Sage Science Pippin Prep gel cassette 100 - 600 bp (Sage Science CSD2010 or CDF2010)
Sage Science Pippin Prep Marker B for CSD2010
Sage Science Pippin Prep loading solution for CSD2010

Sage Science Pippin Prep Marker Mix L for CDF2010

Agilent High Sensitivity DNA kit (5067-4626)
KAPA Illumina Platform Library quantification kit (ABI Prism) (KAPA Biosystems KK4835)

Equipment:

Thermal cycler
96-well magnet plate

Magnet stand capable of holding 1.5 - 2 mL eppendorf tubes, such as the DynaMag-2 from Thermo-Fischer, 12321D
Sage Science Pippin Prep

Instrument capable of small volume quantification such as a Nanodrop ND-1000 or

a Qubit fluorometer
Agilent Bioanalyzer
qPCR instrument
Illumina MiSeq

Illumina NextSeq
High throughput robotic liquid handler (suggested)

Multichannel pipettes, 10 µL and 20 µL

96-well plate capable centrifuge

Before start

NEB buffer 4 and ATP are supplied with Exonuclease V

Sera-mag magnetic particles should be made up beforehand. Alternatively, Agencourt AMPure XP reagent can be purchased from Beckman Coulter.

Sage Science Pippin Prep 2% marker B and loading solution are supplied with gel cassette kit. These reagents can also be purchased separately.

Primer/adapter/index oligos sufficient for 384 samples are listed in the table below. 12 N7xx type and 8 S5xx type are needed for each set of 96 samples.

Index#	5' --> 3' sequence
N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
N707	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
N708	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGG
N709	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGG
N710	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG
N711	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG
N712	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG
N713	CAAGCAGAAGACGGCATACGAGATATTACAATGTCTCGTGGGCTCGG
N714	CAAGCAGAAGACGGCATACGAGATGAATGATCGTCTCGTGGGCTCGG
N715	CAAGCAGAAGACGGCATACGAGATCGATCGGTGTCTCGTGGGCTCGG
N716	CAAGCAGAAGACGGCATACGAGATAATAACGGGTCTCGTGGGCTCGG
N717	CAAGCAGAAGACGGCATACGAGATTAGAAGAAGTCTCGTGGGCTCGG
N718	CAAGCAGAAGACGGCATACGAGATGTCAGGTAGTCTCGTGGGCTCGG
N719	CAAGCAGAAGACGGCATACGAGATGCGGTCCTGTCTCGTGGGCTCGG

N720	CAAGCAGAAGACGGCATACGAGATAATCGGACGTCTCGTGGGCTCGG
N721	CAAGCAGAAGACGGCATACGAGATAACTCGTGGTCTCGTGGGCTCGG
N722	CAAGCAGAAGACGGCATACGAGATGGCCGTGGGTCTCGTGGGCTCGG
N723	CAAGCAGAAGACGGCATACGAGATTTACATGTGTCTCGTGGGCTCGG
N724	CAAGCAGAAGACGGCATACGAGATAGTTAACAGTCTCGTGGGCTCGG
S502	AATGATACGGCGACCACCGAGATCTACAC CTCTCTAT TCGTCGGCAGCGTC
S503	AATGATACGGCGACCACCGAGATCTACAC TATCCTCT TCGTCGGCAGCGTC
S504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC
S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC
S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
S509	AATGATACGGCGACCACCGAGATCTACACGGCTACTCTCGTCGGCAGCGTC
S510	AATGATACGGCGACCACCGAGATCTACACCCTCAGACTCGTCGGCAGCGTC
S511	AATGATACGGCGACCACCGAGATCTACACTCCTTACGTCGTCGGCAGCGTC
S512	AATGATACGGCGACCACCGAGATCTACACACGCGTGGTCGTCGGCAGCGTC
S513	AATGATACGGCGACCACCGAGATCTACACGGAACTCCTCGTCGGCAGCGTC
S514	AATGATACGGCGACCACCGAGATCTACACTGGCCATGTCGTCGGCAGCGTC
S515	AATGATACGGCGACCACCGAGATCTACACGAGAGATTTCGTCGGCAGCGTC
S516	AATGATACGGCGACCACCGAGATCTACACCGCGGTTATCGTCGGCAGCGTC
S517	AATGATACGGCGACCACCGAGATCTACACGCGTAAGATCGTCGGCAGCGTC

Protocol

Prepare Samples

Step 1.

Arrange 30 - 384 DNA samples in 96-well plates

Yeast plasmid DNA is treated with Exonuclease V before fragmenting

Prepare Samples

Step 2.

Check concentration of several wells.

Exonuclease V (yeast plasmid DNA only)

Step 3.

Per well, add together:

1.5 µL 10 U/µL Exonuclease V (NEB M0345L)

3.0 µL NEB Buffer 4 (NEB B7004S)

3.0 µL 10 mM ATP (NEB P0756S)

22.5 uL **yeast** plasmid DNA, ≤ 1 µg per 10 U ExoV

See external link for calculation table.

seal plate, centrifuge briefly @ 200g

LINK: https://docs.google.com/spreadsheets/d/1jcTqSR6wFUZfvbZixamz7FcuniFnAuPUBBVdYrrt1tM/edit#gid=0

Exonuclease V (yeast plasmid DNA only)

Step 4.

Thermal cycle at 37˚C for 60 minutes

Exonuclease V (yeast plasmid DNA only)

Step 5.

To each well, add 1 µL of 0.33 M EDTA

Exonuclease V (yeast plasmid DNA only)

Step 6.

Thermal cycle 70˚C for 30 minutes. Hold at 4˚C if bead cleaning same or next day. Store at -20˚C if time to bead clean will be more than a day.

Bead clean

Step 7.

With high throughput liquid hander or manually, clean Exonuclease V treated sample with 1.5 mg/mL Sera-mag magnetic particles. Bead/sample ratio = 1.0. Elute in 25 uL 10 mM Tris-Cl.

Bead clean

Step 8.

Check concentration of several wells.

Index/adapter PCR master mix

Step 9.

Prepare PCR master mixes for index adapter addition in labeled 200 µL strip tubes, a PCR plate, or a 1 mL half block. Master mixes can be made ahead and frozen. There will be a total of 20 master mixes, one set of 12 and one set of 8. Each of the 20 tubes will contain a primer/adapter with a unique index.

For one 96-well plate:

n=12 master mix

57.2 µL - Kapa HiFi HotStart ReadyMix (2x)

45.6 µL - 5 µM PCR adapter, index 1 (700s)

n=8 master mix

85.8 µL - Kapa HiFi HotStart ReadyMix (2x)

68.0 µL - 5 µM PCR adapter, index 2 (500s)

For two 96-well plates, double the above volumes. Set master mixes aside at 4˚C if using the same day or at -20˚C if using at a later date.

REAGENTS

Kapa HiFi Hotstart ReadyMix (2x) KK2612 by Kapa Biosystems

Tagmentation`

Step 10.

Pre-heat thermal cycler to 55˚C.

Tagmentation

Step 11.

If PCR master mix is frozen, thaw, then mix gently before starting tagmentation.

Per well, add together

1.25 µL - Nextera TD buffer

0.25 µL - Nextera TDE1 enzyme

1.0 µL - clean plasmid DNA, 0.5 - 2 ng/µL

See external link for calculation table.

Keep plate in ice while pipetting to minimize evaporation.

Seal plate well with PCR film, mix briefly by tapping plate on a vortexer, centrifuge briefly @ 200g

REAGENTS

PCR film T796150 by E&K Scientific

LINK:

https://docs.google.com/spreadsheets/d/1FAqNHN6zNiac0FETlJ7UiYn_XrCCWJw8V2QrYBE36MI/edit#gid=0

NOTES

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DNA concentration suggested is as listed in referenced paper. In this protocol, samples are used as is, without normalization. Several wells per plate/library are spot-checked in order to have an idea of the overall amounts in the plate. Our actual input amounts are much more varied and probably higher than 2 ng/µL.

Tagmentation

Step 12.

Thermal cycle @ 55˚C for 10 minutes. Place plate on ice and go directly to adapter addition.

Adapter addition and library amplification

Step 13.

Add 2 x 10 µL of PCR master mix from step 7. With a multichannel pipette, add 10 µL of the n=12 master mix to each row and 10 µL of the n=8 master mix to each column.

The resulting mixture will be 22.5 µL total and will contain fragmented, tagged DNA, PCR mix, and 2 oligos.

Seal plate well with PCR film, mix briefly by tapping plate on a vortexer, centrifuge briefly @ 200g

REAGENTS

PCR film T796150 by E&K Scientific

NOTES

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Please keep very accurate and detailed notes about sample, plate name, index combination, and sequencing run, for each sample. A permanent sample-tracking database is recommended.

Adapter addition and library amplification

Step 14.

Thermal cycle:

72˚C, 3 min.

98˚C, 5 min.

13 cycles @

[98˚C, 10 sec.

65˚C, 30 sec.

72˚C, 30 sec.]

72˚C, 5 min.

4˚C, hold

NOTES

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After this step, your library has sequencing adapters attached. Use extra caution to avoid contaminating equipment and lab benches with library. Clean before, after, and often with 70%

Ethanol, or move subsequent stages to a different room if feasible.

Pooling samples

Step 15.

Pool all wells together. Samples are pooled in sets of 30 to 120 to make 1 library. Usually, each 96-well plate of samples is made into 1 library. Samples with widely varying input concentrations, for example E. coli plasmid DNA vs yeast plasmid DNA, should be pooled into separate libraries.

Bead clean

Step 16.

Clean and concentrate pooled samples with 1.5 mg/mL Sera-mag magnetic particles. Bead/sample ratio = 1.0 - 1.8. Elute in 60 uL 10 mM Tris-Cl. Pooled library may be split among several 1.5 or 2 mL tubes and all tubes eluted into 1 volume.

NOTES

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Maximum amount of concentration when cleaning with 1.5 mg/mL beads is about 30x. For example, total pooled volume of 1000 µL (sample + beads) could not be eluted in a volume lower than 17 µL because of the volume of bead solids. Greater reduction in sample volume may be possible depending on the bead ratio.

Bead/sample ratio may need to be adjusted depending on bead type, home-made or commercial. With home-made Sera-mag preps, a bead/sample ratio of 1.0 is most commonly used.

Concentration and amount to size select

Step 17.

Determine concentration of clean pool. Adjust volume if necessary, so that the 30 µL input to the Pippin will contain ≤ 10 µg

NOTES

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Size selection on the Pippin may work just fine with larger input amounts, ~ 1.5x maximum load listed.

Size select

Step 18.

Prepare 30 uL of input library at a concentration of ≤ 333 ng/µL. With a Sage Science Pippin Prep, and 2% gel cassettes, size-select each library between 200 and 600 bp. Sage Science gel cassette product CSD2010 or CDF2010 can be used. http://www.sagescience.com/

NOTES

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Size selection on the Pippin may work just fine with larger input amounts, ~ 1.5x maximum load listed.

Quality check

Step 19.

Assess library quality and concentration with both an Agilent Bioanalyzer High Sensitivity kit and by qPCR. For qPCR, the KAPA Biosystems library quantification kit, KK4835 is suggested. Library should appear on the Agilent as a broad 200 - 600 bp band. The bulk of the size may be skewed to one end. This is acceptable. Calculate a weighted average for the bp size to use for the qPCR size correction.

NOTES

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An Agilent DNA 1000 kit can also be used if your library yields are routinely high enough to see with that kit.

READY

Step 20.

The library is now ready to sequence. Store at -20˚C.