gRNA Punch MiSeq Library Prep

I. Transposon Amplification

1) Follow standard DNA extraction protocol for purifying genomic DNA from gRNA clone punch.

2) Place punch in 50 uL DNA extraction buffer. Freeze or process immediately.

98 C, 10 min

4 C, 5 min

add 2.5 uL Proteinase K

55 C, 2 hours

98 C , 10 min

4 C, infinity

3) Amplify fragment of transposon with standard PCR

Reagent	1 rxn	18x
PCR Mix	18 uL	324 uL
3044 F primer (10 uM)	0.5 uL	9 uL
3267 R primer (10 uM)	0.5 uL	9 uL
Taq Polymerase	0.3 uL	5.4 uL
H20	3.7 uL	66.6 uL
DNA from punch	2 uL	
Total	25 uL	414 uL

4) Run amplicons out on well spaced 1.6 % agarose gel (i.e. one blank lane between each sample to avoid cross-contamination when gel extracting.

5) Purify gel extractions with Nucleo-Spin Gel and PCR Clean-Up Kit and elute in 15 uL of elution buffer

6) Nano-drop all samples to determine DNA concentration

II. End Repair and Ligation

1.Procedure

1) Prepare the following reaction mix

Reagent	**1rxn**	18x
T4 DNA ligase buffer with 10mM ATP (10X) (NEB B0202S)	2.5ul	45 uL
dNTP (1mM)	1ul	18 uL
T4 Polymerase(NEB, M0203S, good for 100rxns)	0.5ul	9 uL
T4 PNK (NEB M0201S, good for 100rxns)	0.5ul	9 uL
Taq Polymerase (NEB, M0267S,good for 160rxns)	0.5ul	9 uL
Total	5ul	90 uL

2) Add 5µl of reaction mix to 20uL volume containing 30-50 ng of amplified DNA from the end of step I.

3) Incubate 30 min at 25ºC.

4) Incubate 20 min at 75ºC

Add 5µl of adapter mix(1uM), and 0.8ul of T4 DNA ligase (NEB, M0202M, good for 62rxns) to each of the 25µl of reaction from step 4). Carefully pipet ligase and thoroughly mix 18 x mixture below before aliquoting to 25 uL rxns. Be sure to use separate tips for each sample to avoid cross contamination.

Reagent	1 rxn	18 x
1 uM Adapters	5 uL	90 uL
T4 DNA ligase	0.8 uL	14.4 uL
Total	5.8 uL	104.4 uL

5) Incubate 40min at 16ºC.

6) Incubate 10min at 65ºC.

7) Purify all 30.8 uL of reaction mixture with the MinElute PCR purification kit and elute in 10 uL of elution buffer.

III. PCR Enrichment of Size-Selected Ligation Products

- • Reagents needed:
  - 2X Phusion HF Master Mix (Finnzymes, #F-531)
  - Illumina Amplification Primer Mix, 10uM
- 1. Each amplification reaction will contain the following:

'	**1 Rxn (µl)**	18 x
2X Phusion HF Master Mix:	12.5	225 uL
Nuclease-free water:	9.5	171 uL
Illumina PCR Primer Mix (F+R), 10uM	1	
Gel-purified DNA	2	
Total	25ul	396 uL

- Thermocycler program:

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1. 98C 30sec (0:30)
  2. 98C 10sec (0:10)
  3. 65C 30sec (0:30)
  4. 72C 30sec (0:30)
  5. Go to #2, 17 times
  6. 72C 5min
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7. 4C forever

- Illumina PCR Primers:
  - Forward: 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 58
  - Reverse: 5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT 61

IV. Purification and Clean-up

1. Purify PCR products using the MinElute PCR purification kit, eluting in 15 uL of elution buffer

2. Quantify amount of DNA using NanoDrop. If 260/280 values are extremely high (above 2.2) or low (below 1.6) consider doing another NanoDrop to confirm concentration and purity

3. Dilute samples to 10 nM stocks for

In this case PCR product is ~300 bp, so a concentration of 1. 9 ng/uL = 10 nM.

4. Combine 1.5 uL of each 10 nM sample into one single tube, total volume 25.5 uL. Nanodrop final mixture of all libraries to confirm concentration is ~ 1.9 ng/uL = 10 nM.

5. Take 20 uL of this 10nM mixture of libraries and place in tube for submission into MiSeq spike-in.