Preparing DNA Samples for Sequencing [July 29, 2009]

We will be using the New England Biolabs NEBNext™ DNA Sample Prep Reagent Set 1 (P/N E6000S) and Illumina oligonucleotides

A. DNA Fragmentation

1. Dilute up to 5 ug of genomic DNA in 80 ul of water or TE buffer in a 1.5 ml microcentrifuge tube.
[NOTE: for ChIP-seq dilute entire ChIP in 75 ul instead of 80 ul and skip steps 2 and 3 below]

2. Using a Diagenode Bioruptor UCD-200 sonicate for 15 minutes on the highest power setting with pulses of 30 seconds ON and 30 seconds OFF.
[NOTE: These conditions may vary depending on your organism and nature of the input material so optimization may be required]

3. Run 5 ul of fragmented DNA alongside a 100 bp DNA ladder on a 1.5% agarose gel to confirm that the mean fragment size is approximately 500 bp and the majority of fragments are 800 bp or less.

B. End Repair

1. Prepare the following reaction mix:

- Fragmented DNA (75 ul)
- Phosphorylation Buffer (10 ul)
- dNTP Solution Mix (4 ul)
- T4 DNA polymerase (5 ul)
- DNA Pol I (Klenow LF) (1 ul)
- T4 Polynucleotide Kinase (5 ul)

The total volume should be 100 ul.

2. Incubate in the thermal cycler for 30 minutes at 20ºC.

3. Follow the instructions in the AMPure PCR Purification Kit to purify the reaction product, eluting in 32 ul of EB (Note: You can purify the reaction product using the Qiagen QIAquick PCR Purification kit instead if desired).

a. Vortex AMPure beads briefly to resuspend
b. Using a 0.5 ml tube, add 180 ul (1.8x volume) Ampure beads to sample and mix by pipetting 10 times or vortexing for 30 seconds.
c. Let the mixed sample incubate for 3-5 minutes and make sure beads stay resuspended by flicking tube throughout incubating period.
d. Quick spin and place tube on magnet for 2 minutes.
e. Aspirate clear solution and discard.
f. Dispense 200 ul of 70% ethanol and (OPTIONAL) resuspend beads.
g. Place tube on magnet for a short period until solution is clear and remove ALL solution and discard.
h. Repeat f and g without resuspension.
i. Quick spin to get the last of the liquid to the bottom.
j. Place tube on magnet and aspirate the very last of solution.
k. Leave tube open to air dry a couple of minutes, liquid should be gone.
l. Add the appropriate amount of EB mentioned above and resuspend beads by vortexing for 30 seconds.
m. Quick spin and place tube on magnet until solution is clear.
n. Pipette off clear solution and keep for use in next step.

C. A-tailing

1. Prepare the following reaction mix in a 0.2 ml PCR tube:

- DNA sample from above (32 ul)
- NEBuffer 2 (5 ul)
- dATP Solution Mix (10 ul)
- Klenow exo^{-} (3 ul)

The total volume should be 50 ul.

2. Incubate for 30 minutes at 37°C.

3. Follow the instructions in the AMPure PCR Purification Kit to purify the reaction product, eluting in 19 ul of EB (Note: You can purify the reaction product using the Qiagen QIAquick PCR Purification kit instead if desired).

D. Adapter ligation

1. Prepare the following reaction mix:

- DNA sample (19 ul)
- 2X Quick Ligation Buffer (25 ul)
- Adapter oligo mix (1 ul)
- Quick T4 DNA Ligase (5 ul)

The total volume should be 50 ul.

[NOTE: for paired end libraries use PE adapter oligo mix, for SR use genomic adapter oligo mix {in the illumina nomenclature}. The amount of adapter is not super critical, but it is possible to use too much. This can lead to excess adapters and contamination of the final library with adapter product. This is bad because these will generate clusters and sequence. We keep adapter amounts low, using only 1 ul {or 10 ul of a 1:10 dilution} to be on the safe side.]

2. Incubate for 15 minutes at room temperature.

3. Follow the instructions in the AMPure PCR Purification Kit to purify the reaction product, eluting in 10 ul of EB (Note: You can purify the reaction product using the Qiagen MinElute PCR Purification kit instead if desired).

E. Gel-Purify Ligation Products

1. Prepare a 50 ml, 1.8% agarose gel with 1X final concentration of TAE.

2. Add ethidium bromide (EtBr) just before pouring to warm agarose at a final concentration 400 ng/ml (i.e., add 20 ug EtBr to 50 ml of agarose).

3. Add 2 ul of 80% glycerol loading buffer (1X TAE, 80% Glycerol, 0.15% Xylene Cyanol) to the 10 ul of purified ligation reaction.

4. Load 2 ul of 100 bp ladder to one lane of the gel.

5. Slowly load half of the sample in another lane of the gel, leaving a gap of at least one empty lane between ladder and sample. Wait three minutes then slowly add the other half of the sample in the same well. The number of wells used depends on the amount of starting material. Starting with the full 5 ug will result in a lot of DNA for one lane, so splitting into 2 or 3 lanes may give better results.

[NOTE: At this step it is not uncommon to see the product “swirl out” of the well, which is why such a high glycerol concentration is used and why the material is added slowly]

6. Run the gel at 85-95 mA for 60 minutes, or untill the XC is about 1/3 of the way down.

7. Pre-weigh a 1.5 ml microcentrifuge tube.

8. View the gel on a transilluminator and use a clean scalpel or a GeneCatcher tip (Gel Co.) to excise the region of gel containing the desired molecular weight material and transfer to the pre-weighed tube. NEVER use a scalpel directly on a transilluminator surface.

[NOTE: The desired MW will vary depending on your application. For many kinds of libraries, 250-300 is a good range. Going too low, 200 or less, increases the possibility of getting a bit of adapters and this is to be avoided.]

9. Follow the instructions in the Nucleotrap kit to extract the DNA from the agarose slice, eluting in 30 ul EB for a genomic library or 24 ul EB for a ChIP-seq library (Note: You can extract the DNA from the agarose using the Qiagen QIAquick Gel Extraction Kit instead if desired).

a. Add 3 ul NT1/mg of agarose, but always at least 300 ul NT1 to agarose in tube.
b. Add 4 ul of silica matrix/ug of DNA, but always at least 10 ul.
c. Periodically flick tube until agarose is fully dissolved. Manufacturer recommends 50° incubation at this step but some reports indicate this biases the base composition of the library, so we are using room temp
d. Spin at 10,000xg for 1 min. Aspirate clear solution and discard.
e. Add 500 ul of NT2 and vortex to resuspend silica.
f. Spin at 10,000xg for 1 min. Aspirate clear solution and discard.
g. Add 500 ul of NT3 and vortex to resuspend silica.
h. Spin at 10,000xg for 1 min. Aspirate clear solution and discard.
i. Repeat f and g.
j. Repeat spin at 10,000xg for 1 min. Aspirate the very last of clear solution and discard.
k. Leave tube open to air dry for 10 to 15 minutes minutes.
l. Add the appropriate amount of EB mentioned above and resuspend beads by vortexing.
m. Incubate at room temp for at least 10', then spin at 10,000xg for 1 min.
n. Pipette off clear solution and SAVE for use in next step.

F. PCR Enrichment

1. Prepare the following PCR reaction mix on ice:

Genomic library:

- DNA (1 to 3 ul)
- Water (23 ul)
- 2X Phusion HF Master Mix (25 ul)
- Genomic PCR Primer 1.1 (0.25-0.33 ul) Note: for paired end libraries use PCR Primer PE 1.0
- Genomic PCR Primer 2.1 (0.25-0.33 ul) Note: for paired end libraries use PCR Primer PE 2.0

The total volume should be 50 ul.

ChIP-seq library:

- DNA (24 ul)
- 2X Phusion HF Master Mix (25 ul)
- Genomic PCR Primer 1.1 (0.25-0.33 ul)
- Genomic PCR Primer 2.1 (0.25-0.33 ul)

The total volume should be 50 ul.

2. Amplify using the following PCR protocol with a hot start:

a. 30 seconds at 98°C
b. 10 cycles of:
10 seconds at 98°C
30 seconds at 65°C

30 seconds at 72°C

c. 5 minutes at 72°C

d. Hold at 10°C (don't set your PCR machines to 4°)

[NOTE: The number of cycles to amplify is an ongoing debate. The original protocol used 18, but now says 10. Groups are trying to develop no-amplify libraries as well. If 10 is too low one can go to 14.]

3. Follow the instructions provided with the Ampure beads to purify the PCR reaction, eluting in 15 _l of EB (Note: You can purify the reaction product using the Qiagen MinElute PCR Purification kit instead if desired).

4. Check 1 ul by nanodrop, but also run a 1 ul aliquot of the purified product on an Agilent Bioanalyzer DNA 1000 Chip or a 1.5% agarose gel as described in section G below. If the library is not detectable proceed as follows:

Genomic Library: Perform step 1 with 5 ul of sample, 19 ul of water, and 18 cycles of PCR

ChIP-seq library: Dilute the entire remaining PCR product to 24 ul in water and use as template for a second round of amplification (14 cycles) as described in steps 1, 2 and 3 above.

G. Library Validation

1. Determine the concentration of the library by measuring its absorbance at 260 nm.

2. Measure the 260/280 ratio. It should be approximately 1.8.

3. Load 10% of the volume of the library alongside a 100 bp ladder on a 2% agarose gel and check that the size range is as expected. It should be similar in size to the size-range excised during the gel purification step.

4. To determine the molar concentration of the library, examine the gel image and estimate the median size of the library smear.

a. Multiply this size by 650 (the molecular mass of a base-pair) to get the molecular weight of the library.

b. Use this number to calculate the molar concentration of the library.

PCR Primer sequences

Single Read:

1.1 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

2.1 5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCT 3'

Paired End:

PE 1.0 5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

PE 2.0 5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT 3'

Working stocks are supplied by Illumina at 20 uM concentration.