Iyer Laboratory:
Gel-free Library Preparation Protocol

Originally developed by Amelia Weber Hall in October 2012, then modified to avoid usage of silica gel columns to improve yield.

Used for preparing libraries of DNA derived from chromatin immunoprecipitation experiments by the Iyer lab, and distributed to several other labs across Central Texas. Documented by Amelia Weber Hall, December 2012, March 2015, and June 2017.

This protocol should work well with any DNA that needs to be prepared into a library for high-throughput sequencing, however it is highly optimized for small (nanogram to pictogram) quantities of DNA.

Equipment

Microcentrifuge
PCR machine
Magnetic plate (ex: Beckman cat #A32782; Thermo-Fisher cat #12303D)
Micropipettors
Tapestation or Bioanalyzer to examine DNA size distribution

Materials and suppliers

Agencourt AMPure XP (Beckman-Coulter, cat #A63880)
NEBNext® ChIP-Seq Library Prep Master Mix Set for Illumina (tested with NEB cat # E6240L; should also work with the newer kit: cat #E7645S)
Bioo Illumina Compatible adapters (Bioo cat #NOVA-514103)
1.5mL microcentrifuge tubes
Ethanol
RNase, DNase free water
Buffer TE (optional)

Ampure XP protocol (use in place of column purification to increase yield)

Ampure beads are magnetic beads that bind DNA (using PEG) in specific size ranges. When the PEG solution (supernatant) is removed, DNA can be eluted off of the beads into water or buffer TE. The concentrations given here are best recommendations, but each bottle of AMPure XP beads will have a slightly different size distribution, so test each new bottle of AMPure beads with non-precious DNA (in the Iyer lab, we often use sheared/sonicated genomic DNA to test the size selection).

To exchange buffers and keep all DNA fragments, use Ampure at 1.8x concentration.

- With a 50ul reaction, use 90μl of Ampure using the protocol below to purify the reaction and keep any small fragments present.
- To remove PCR primers (and adaptor dimers) use Ampure at a 1.0-1.2x concentration (50-60μl Ampure for a 50ul reaction).
- In Iyer lab tests, 1.0x Ampure beads gives a lower cutoff around 250bp and 1.2x is closer to 200bp, but this will vary with each new bottle of beads.

- To remove large fragments, see the specific double-sided section at the end of this protocol.

Protocol for AMPure Purification

1. Vortex the bottle of Ampure beads to resuspend them in solution, then remove the amount you need from the bottle. Allow the aliquot to equilibrate to room temperature for 10-30 minutes (small volumes warm more quickly).
2. Make up fresh 80% ethanol using 100% ethanol and DNase free, RNase free water.
3. Add beads to your reaction at the desired concentration, pipet thoroughly to mix and let the tube incubate at room temperature for 10 minutes to allow the DNA to precipitate out of the solution and bind to the beads.
4. Place tubes in magnetic rack and wait 15 minutes (or until the solution is CLEAR, not brownish, but really clear).
5. Remove supernatant by placing the pipette tip in the bottom of the tube (don't touch the sides of the tube when removing supernatant).
    a. Note that you can retain the supernatant for analysis if you are using Ampure to exclude certain sizes of DNA)
6. Wash by pipetting 200μl of fresh 80% ethanol into the tube (while the tube is still in the magnet rack) and let sit for at least 30 seconds, then remove.
    a. Repeat for a total of two washes.
7. Allow the magnetic beads to dry for at least 10 minutes while in the magnetic rack.
8. Resuspend the beads in water or 1x TE and let sit at room temperature for at least two minutes to allow DNA to elute from beads.
9. Place tubes on the magnet rack and wait 15 minutes (or until the solution is clear)
10. Remove elucate and continue to next step, run on the tapestation/bioanalyzer, or store at -20°C.

Note: The following NEB specific protocols are from the 06/2012 Version 2.1 of the NEB protocol, please check the NEB website for protocol updates, as they are often helpful in obtaining maximum yield for libraries.

NEB End Repair Protocol

1. DNA should be quantified using Qubit, or another fluorescence based method (NOT nanodrop). Ideally the DNA should be checked for target region fold enrichment using qPCR.
2. Mix the following in a sterile microfuge tube:
    - 1-40μl ChIP DNA (ideally at least 10ng, using up to 50ng if possible)
    - 5μl NEBNext End Repair Reaction Buffer
    - 1μl NEBNext End Repair Enzyme Mix
    - Water to bring the final volume to 50μl
3. Incubate for 30 minutes in a thermal cycler at 20°C.
4. Purify the reaction using 1.8x Ampure beads as above and elute in 46μl water, remove 44μl for the dA tailing reaction

NEB dA-tailing protocol

1. Mix the following in sterile microfuge tube:
   - 44μl End Repaired DNA
   - 5μl NEBNext dA-Tailing Reaction Buffer
   - 1μl Klenow Fragment (3' → 5' exo-)
2. Incubate at 37°C for 30 minutes
3. Purify the reaction using 1.8x Ampure beads and elute in 21μl water, remove 19μl for the adaptor ligation reaction

NEB adapter ligation (user supplied adapters)

Note: we use the Bioo adaptors at a 25μM concentration – you may need to dilute the ordered adapters substantially (1:400) to achieve this concentration. Don't use too many adapters, as adapter dimers will accumulate and require additional AMPure purification steps for removal.

1. Mix the following in a sterile microfuge tube:
   - 19μl End Repaired, dA-Tailed DNA
   - 6μl Quick Ligation Reaction Buffer (5X)
   - 1μl Adaptor
   - 4μl Quick T4 DNA ligase
2. Incubate for 15 minutes in a thermal cycler at 20°C.
3. Purify **TWICE** with Ampure at 1.0-1.2x to remove adaptor dimers (120bp); elute the second purification in 23μl water or buffer TE.

PCR amplification

Use the NEBNext High-Fidelity 2X PCR Master Mix, this reduces polymerase bias for short DNA fragments and produces a more "normal" distribution of amplified fragments.

Set up the following reaction:

- 23μl Adaptor Ligated DNA
- 25μl NEBNext HF 2X PCR master mix
- 2μl 25μM Bioo adapter primers
  - Primer 1: AAT GAT ACG GCG ACC ACC GAG ATC TAC AC
  - Primer 2: CAA GCA GAA GAC GGC ATA CGA GAT

PCR Cycling Conditions:

Initial Denaturation: 98°C for 30s
15 cycles of:
    Denaturation: 98°C for 10s
    Annealing: 65°C for 30s
    Extension: 72°C for 30s
Final Extension: 72°C for 5m
Hold at 4°C

When the PCR is finished, purify once with Ampure at 1.2x and then purify again with the below Ampure large fragment removal protocol, to remove large fragments above ~500bp. Running the amplified library on the tapestation/bioanalyzer before doing any upper limit size selection is important to avoid loss of useful material in the final size selection.

AMPure XP for removal of large fragments (double sided size selection)

This section assumes you've already used Ampure to remove adaptor dimers and other small DNA molecules. If you haven't yet, perform two Ampure purifications at 1.0-1.5x, then come back to this section.

1. Dilute your library to 50ul with DNase free, RNase free water
2. Add 27.5μl (0.55x) Ampure beads and pipet to mix thoroughly.
3. Allow DNA to precipitate from solution and bind to beads at room temperature for 10 minutes
4. Place tubes in magnet rack and allow beads to separate from solution for 15 minutes or until clear.
5. Remove the supernatant and **SAVE IT** in a new tube.
    a. Ethanol wash and elute from the beads with large sizes bound if desired, otherwise proceed to the next step.
6. Add 1.0x Ampure beads to the supernatant (total Ampure amount should be 1.2-1.5x).

This works because the supernatant contains PEG, which causes DNA to drop out of solution in a size dependent manner. Adding more Ampure beads means you'll retain all of the DNA present in the supernatant (which should be mostly below the large size cutoff your introduced with the first Ampure selection at 0.55x). Don't worry about overloading the beads with DNA - 1μl of Ampure can bind at least 1μg of DNA, according to Beckman's literature.

7. Allow the beads and supernatant to sit at room temperature for 10 minutes so the lower sized DNA fragments can precipitate and bind to the beads.
8. Place tubes in magnet rack and allow beads to separate from solution for 15 minutes or until clear
    a. Wash 2x with 200μl fresh 80% ethanol
    b. Allow beads to dry on magnet rack for 10 minutes, don’t overdry!
9. Elute DNA from beads in 20-30μl of DNase free, RNase free water and let sit at room temperature for 2 minutes to allow for complete elution
    a. Place beads on magnet rack and allow to sit for 15 minutes or until solution is clear.
10. Remove 17-27μl of DNA eluted in water, but ensure you don't pull any beads into the final library as they could interfere with sequencing.
    a. Make a 1:5 dilution of the library and run on tapestation/bioanalyzer to see the final library size distribution.
    b. It should be ~200-600bp using this technique.