Short Library Preparation

GENERAL NOTES

Following DNA isolation, samples with acceptable quality (90% of gDNA is >10 kb) for sequencing as assessed by bioanalyzer (or femto pulse), are used for SMRT library preparation using a modification of a PacBio protocol for short templates <3kb as indicated below. Samples can be multiplexed, but it is recommended that no more than 6 "short libraries" be ppooled for sequencing at 8M chip on Sequel2 at once. Unless otherwise mentioned, use Low Bind 1.5mL tubes. Keep samples on ice after incubations and during preparations, except bead purifications, which are done at room temperature. Follow bead purifications carefully, as care must be taken to separate (at the end) the 200bp library from SMRTbell prep adaptor dimers, which are close to the same size.

Preparation should be carried out using 0.5ug high quality gDNA. If gDNA is of lesser quality, an initial bead cleanup with 1x Ampure PB beads (elute with PacBio EB) should be conducted to remove lower MW gDNA, *after* which 0.5ug of gDNA can be used. DNA quantities as low as 0.2ug have been successfully used in this protocol, but this should only be attempted by experienced users.

SHEARING

1. Samples are sheared to ~175-200 bp in a Covaris LE220 Focused Ultrasonicator in a volume of 115ul (bring to volume with PacBio EB) in Covaris microtubes (520091) by manufacturer's instructions. Shearing settings are: 240sec, Peak 450, Duty 30%, Cycles 200, Avg Power 135.

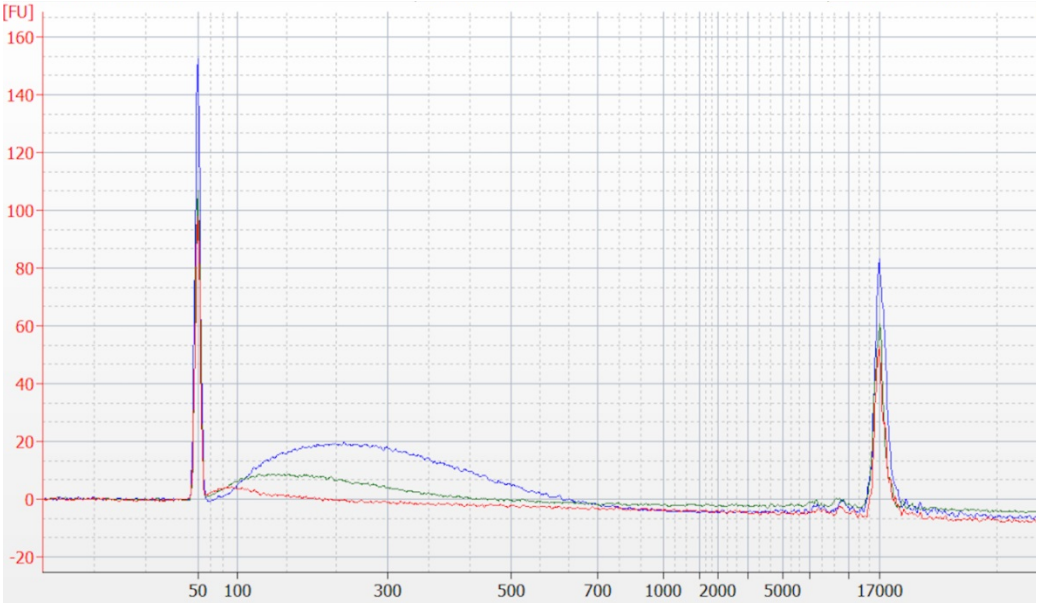
   The samples are organized by rows of 8 samples (in 96 well format, this is A-H of the same number [i.e.- A1- H1]. All samples in a row are sheared simultaneously. If we have less than 8 samples in a row we fill out the row with dummies (tubes with water or buffer). Up to 96 samples may be sheared in one run this way, though program will need to be modified to adjust the number of rows used. Save a small aliquot of DNA before and after shearing to assess the size distribution on the Bioanalyzer (see step 3 below).

2. Following shearing, transfer samples from Covaris tubes to Low Bind 1.5mL tubes. Volume is important, so keep track of final volumes.

3. Assess shearing by Bioanalyzer (Agilent DNA12000 chip). Shearing should produce a broad peak centered around 200bp. If the peak is centered too short (<150bp), no band is observed larger than 250 bp and the same short distribution was present in the unsheared sample this one should not be used for the further prep.

   In the representative BA trace below, we can see an example of acceptable shearing (blue and green lines), and a poor result (red). The poor result not only has less material, but even more importantly, the peak is centered too low (~100bp) for use. A new original aliquot of the sample shown in red should be sheared again.

BIOANALYZER TRACE OF 3 SHEARED SAMPLES



4. Concentrate the sample with 2.5x volume Ampure PB Beads following PacBio's recommendations. Bead cleanup requires a high bead volume since the fragment size is small. Briefly, after allowing 5 minutes of mixing of bead and sample at 400 RPM, wash 2 times with 1mL 70% ethanol, briefly dry, then elute in 39ul EB at 400RPM for 10 minutes. Run Qubit to assess concentration with 1ul.

DAMAGE REPAIR

5. Using SMRTbell Template 1.0 kit (PacBio 100-259-100), prepare a master mix (MM1) for all samples as detailed below. For multiple samples, add 10% extra to account for loss during pipetting.

   MM1, mix gently, keep on ice:

	1 sample
DNA Damage Repair Buffer	5ul
ATP-Hi	5ul
dNTP Mix	0.5ul
NAD+	0.5ul
DNA Damage Repair Mix	2ul

6. Add 13ul of MM1 to 37ul of each sample and mix by flicking the tube. Incubate 30 minutes at 37°C.

END REPAIR

7. To each 50ul sample, add 2.5ul of End Repair Buffer, and mix gently by flicking the tube. Incubate 15 minutes at 25°C.
8. Following End Repair, bead cleanup is carried out at a bead volume of 2.25x, but otherwise following bead purification as in step 4, above. For a 52.5ul sample, this requires 118ul beads. Elute in 29ul EB buffer.

LIGATE BLUNT END ADAPTERS

9. For these steps, use PB barcoded adapter kits 8A/8B (101-081-300, 101-081-400) to provide barcodes. Prepare a master mix (MM2) for all samples as detailed below. For multiple samples, add 10% extra to account for loss during pipetting.

   Prepare MM2, mix gently, keep on ice:

	1 sample
Template Prep Buffer	4ul
ATP-Lo	2ul
Ligase	1ul

10. To each 29ul purified DNA sample, add 4ul unique barcode. Gently mix.
11. Next, add 7ul of MM2 to each sample and gently mix.
12. Incubate at 25°C for at least 3hours, but up to overnight. Samples can be stored at -20°C after this step, if they cannot be immediately processed.
13. Incubate at 65°C 10' to activate the ligase

EXONUCLEASE TREATMENT

14. Prepare MM3, mix gently, keep on ice:

	1 sample
Exonuclease III	0.75ul
Exonuclease VII	0.75ul

15. Add 1.5ul of MM3 to each 36ul sample. Incubate 1 hour at 37°C. Exonuclease digests the unligated DNA and unincorporated adapters except adapter dimers. The additional incubation will digest also significant amount of the sample reducing the yield. Also, if poorer quality DNA is used, with damages/nicks, etc, you will need to do this step for a shorter time.

FINAL CLEANUPS

These not only get rid of buffer and other components, but also are successively more strict to get rid of the 100bp adapter dimer. Cleanup results need to be assessed by BA and the 100bp adapter dimer peak (or loss of this peak) can be clearly seen on BA. Do 3 rounds of cleanup with the following AMPure bead concentrations: 2x, 1.9x, 1.8x. Three rounds at 1.8x *could* be done, but that will get rid of a bit more desired product, too.

16. Bead cleanups should be carried out as per step 4, but with the indicated concentrations of beads detailed above. Elute the first two cleanups in 40ul EB; the last in 12ul EB.
17. Assess quantity by Qubit (1ul) and Assess quality by BA (1ul) for each sample to verify that the~100bp peak has been removed. In addition, use the BA to assess molarity of each sample. If ~100bp peak is still present, a 4th bead cleanup at 1.8x bead concentration should be carried out on the sample(s) that were not fully cleaned. Assess by BA again.

POOLING

18. Pool all cleaned samples together equally (300fmols each), and clean again at 1.8x AMPure beads as described above. Elute in 10ul EB.

SEQUENCING

19. Sequencing primer v4 (REF 101-654-600) was first conditioned according to the PacBio instructions (heating up to 80°C for 2 min then cooling down to 20°C and transferring to 4°C) annealed to the SMRTbells and incubated with the template for 1 hour at 20°C using a primer/template ratio of 20:1.
20. The Sequel 2.1 polymerase was then bound to the annealed template complex using a ratio of 30:1 polymerase to SMRTbell at 0.5 nM for 4 hours at 30°C.
21. The binding complex was cleaned up (using ProNex beads according to the PacBio recommendations from Sample Setup module of SMRTLink 10.0), and diluted to the final loading concentration according to the newest PacBio recommendations and held at 4°C until diffusion-based loading on the Sequel II instrument.
22. Diluted binding complex at concentration of 125-175pM was loaded on the 8M sequencing chip and 30 hour movies were recorded according to the manufacturer's guidelines.