96-well Illumina Library preparation for long PCR products

Adapter preparation

Custom adapters are required. These should be HPLC purified. Adapters are phosphorylated and annealed together, as described [1].

Ind_t	5’	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T	3’
Ind_b	5’	GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC	3’

* indicates phosphorothioate

Sample shearing

1. Take 1 µg of pooled long PCR products and make volume up to 75 µl with water.

2. Shear to approximately 200 bp by acoustic shearing: transfer to a 6 mm × 16 mm AFA fiber vial (Covaris cat. no. 520031).

3. Seal the tube with an 8 mm crimp seal cap (Covaris cat no. 520028) and crimping tool.

4. Shear with a Covaris, using the settings:

Duty cycle	20 %
Intensity	5
Cycle/burst	200
Time	150 sec

5. Transfer samples to 96-well PCR plate, gently spin and keep frozen until used.

End-repair

This protocol converts the overhangs resulting from fragmentation into blunt ends, using T4 DNA polymerase and E. coli DNA polymerase I Klenow fragment. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

1. Mix the reagents below (all NEB) in a 15ml Falcon Tube, and decant into a reagent reservoir.

	1X	104X
10x T4 DNA ligase buffer with 10 mM ATP	10 µl	1040 µl
10 µM dNTP mix	4 µl	416 µl
3 U / µl T4 DNA polymerase	5 µl	520 µl

5 U / µl Klenow DNA polymerase	1 µl	104 µl
10 U / µl T4 PNK	5 µl	520 µl

2. Add 25 µl of master mix to each sample using an electronic pipette. Cover the plate with transparent cover, vortex briefly and gently spin down.

3. Incubate plate for 30 min at 20 °C in a thermocycler.

4. While incubating, prepare SPRI beads for the reaction cleanup: allow SPRI beads to come to room temperature for at least 30 minutes. Mix well, and ensure that the beads appear homogeneous and consistent in colour.

5. Add 180 µl of SPRI beads per 100 µl of end-repaired DNA in a 1.5 ml Lo-Bind tube.

6. Vortex and leave at room temperature for 5 minutes.

7. Place tubes in a magnetic rack.

8. Leave for 5 minutes or until sample is clear.

9. Carefully remove the clear solution from the tubes and discard.

10. Dispense 700 µl of 70 % ethanol into each tube while in the magnetic rack taking care not to disturb the magnetic beads. Aspirate and discard ethanol.

11. Repeat the ethanol wash once again (total of two washes).

12. Dry the samples on a heat block (keep the lid of the tube open) at 37 °C for 5 to 10 minutes or until the residual ethanol has evaporated.

13. Add 32 µl of molecular biology grade water, vortex and incubate at room temperature for 2 minutes.

14. Place tubes into the magnetic rack and leave for 2-3 minutes or until sample is clear.

15. Carefully remove the water and retain in a new 1.5 ml Lo-Bind tube.

16. Centrifuge the eluates at 13,000 rpm in a bench top centrifuge for 10 minutes.

17. Transfer eluates to a 96-well plate leaving behind any precipitated beads.

18. Proceed immediately with A-tailing.

A-tailing

This protocol adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

1. Mix the reagents below (all NEB) in a 15ml Falcon Tube, and decant into a reagent reservoir.

	1X	104X
10x NEB buffer 2	5 µl	520 µl
1 mM dATP	10 µl	1040 µl
5 U / µl Klenow fragment (3' to 5' exo$^-$)	3 µl	312 µl

2. Add 18µl to each sample using an electronic pipette. Cover the plate with transparent cover, vortex and gently spin.

3. Incubate plate for 30 min at 37 °C in a thermocycler. Clean using SPRI beads, in the 96-well reaction plate (see End-Repair protocol above. Use 90 µl beads for each 50µl reaction, and elute in a mixture of 10 µl EB + 8.5 µl water).

4. Proceed immediately with ligation.

Ligation

This protocol ligates adapters to the ends of the DNA fragments.

1. Mix the reagents below in a 15ml Falcon Tube, and decant into a reagent reservoir.

	1X	104X
2x Quick DNA ligase buffer (NEB)	25 µl	2.6 ml
Adapter oligo mix (see above)	1.5 µl	156 µl

2. Add 26.5 µl to each sample using an electronic pipette. Cover the plate with transparent cover, vortex and gently spin.

3. Pipette 5 µl Quick Ligase (NEB) into each well using a manual pipette. Mix by pipetting, do not cover, and after pipetting in the last column leave for 15 min at room temperature (20 °C).

4. Clean using AMPure SPRI beads as described above, eluting in 20 µl EB.

5. Run an DNA 1000 chip on an Agilent BioAnalyzer 2100 on a small selection of samples. You should detect smear between 300-1000 bp.

Indexing Enrichment PCR

Indexes can be added to the central region of the reverse PCR primer. The general sequence is:

5' CAAGCAGAAGACGGCATACGAGAT-INDEX-GAGATCGGTCTCGGCATTC 3'

where INDEX represents an oligonucleotide sequence that is used to identify the sample. Indexes should be selected so that they are maximally different from one another. We typically use 8-base indexes. Indexed primers must be PAGE purified. For an example of a 96-plex set of error-correcting barcodes, and primer sequences, see **Supplementary Table 1**.

The common forward primer (HPLC purified) has the sequence:

5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

1. Dilute indexed primers to 10 µM with water.

2. Pipette 2 µl of each library into a well of a 96-well plate.

3. Add 6 µl of 10 µM indexed primer to each well.

4. Prepare a master mix of all other reagents:

	1X	52X
10X PFx buffer (Invitrogen)	10µl	520µl
2.5 mM dNTPs	20µl	1040µl
50 mM $MgSO_4$	4µl	208µl
10 µM Forward primer	6µl	312µl
Platinum PFx Polymerase (Invitrogen)	1µl	52µl
Water	51µl	2652µl

5. Mix, and add 92 µl master mix to each well. The PCR is performed using the following program :

    94 °C for 2 min

94 °C for 15 sec
68 °C for 45 sec x 12 cycles

4°C indefinitely

6. Clean PCR reactions SPRI beads, as described above, eluting in 18 µl EB.

7. Run an DNA 1000 chip on an Agilent BioAnalyzer 2100 on a small selection of samples. You should detect smear between 300-1000 bp.

Normalization

Normalize PCR products by qPCR before pooling. The primers used for the qPCR are locus-specific, designed to amplify one of the long PCR products in the original pool. In this way, adapter dimers are not problematic. As a concentration standard, the most concentrated library from the 6 random libraries quantified by Agilent Bioanalyzer 2100 in the preceding step, is used.

1. Dilute 2µl of the chosen concentration standard 10x, 100x and 1,000x with EB buffer.

2. Dilute all other libraries 80x in EB.

3. Perform qPCRs in duplicate, using a 2x SybrGreen master mix.

4. Pool products in equimolar ratios.

Size selection

1. Load sample pools on a 2% agarose gel in 1X TBE, and are electrophoresed against a Low Molecular Weight ladder (NEB), at 5V cm^{-1} for approximately 1 hour.

2. From each lane, cut a 250-450 bp gel slice is cut and extract the DNA.

3. Quantify pooled libraries by SYBRGreen qPCR [2].

4. Sequence using standard primers for reads 1 and 2. Indexes are sequenced after read 1, using a custom primer.

Ind_seq 5’ AAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC 3’

References

1. Kozarewa I. *et al.* Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. *Nat Methods* **6**, 291-295 (2009).

2. Quail M.A., Swerdlow H. & Turner D.J. Improved protocols for the illumina genome analyzer sequencing system. *Curr Protoc Hum Genet* **Chapter 18**, Unit 18 12 (2009).