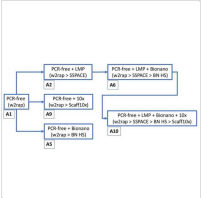

Oct 25, 2022

Library Construction Protocols

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ABSTRACT

Amplification Free Paired End Library Construction Protocol.

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PCR-free Illumina 2500

1 A total of **600 ng** of DNA was sheared in a **60 µL** volume on a Covaris S2 (Covaris, Massachusetts, USA) for 1 cycle of **00:00:40** with a duty cycle of 5%, cycles per burst of 200 and intensity of 3.

2 The fragmented molecules were then end repaired in **100 µL** volume using the NEB End Repair Module (NEB, Hitchin, UK) incubating the reaction at **22 °C** for **00:30:00** .

3 Post incubation **58 µL** beads of CleanPCR beads (GC Biotech, Alphen aan den Rijn, The Netherlands) were added using a positive displacement pipette to ensure accuracy and the DNA precipitated onto the beads.

4 This is then washed twice with 70% ethanol and the end repaired molecules eluted in **25 µL** Nuclease free water (Qiagen, Manchester, UK).

5 End repaired molecules were then A tailed in **30 µL** volume using in the NEB A tailing module (NEB) incubating the reaction at **37 °C** for **00:30:00** .

6 To the A tailed library molecules **1 µL** of an appropriate Illumina TruSeq Index adapter (Illumina, San Diego, USA) is added and mixed, then **31 µL** of Blunt/ TA ligase (NEB) is added and incubated at **22 °C** for **00:10:00** .

7 Post incubation **5 µL** of stop ligation is added and the reaction incubated at **Room temperature** for **00:05:00** .

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8 Following this incubation **67 µL** beads of CleanPCR beads (GC Biotech, Alphen aan den Rijn, The Netherlands) were added and the DNA precipitated onto the beads.

9 The samples are then washed twice with 70% ethanol and the end repaired molecules eluted in **100 µL** nuclease free water.

10 Two further CleanPCR bead based purifications were undertaken to remove any adapter dimer molecules that may have formed during the adapter ligation step. The first with 0.9x volume beads, the second with 0.6x and the final library eluted in **25 µL** Resuspension Buffer (Illumina).

11 Library QC was performed by running a **1 µL** aliquot on a High Sensitivity BioAnalyser chip (Agilent, Stockport, UK) and the DNA concentration measured using the High Sensitivity Qubit (Thermo Fisher, Cambridge, UK).

12 To determine the number of viable library molecules the library was subjected to quantification by the Kappa qPCR Illumina quantification kit (Kapa Biosystems, London, UK) and a test lane run at 10pM on a MiSeq (Illumina) with 2x300bp reads to allow the library to be characterised prior to generation of the 60x coverage required on the Hiseq2500s (Illumina) with a 2x250bp read metric.

Illumina NovaSeq 6000

13 The libraries for this project were constructed at the Earlham Institute, Norwich, UK using the KAPA High Throughout Library Prep Kit (Roche Part No: KK8234/07961901001) on the Perkin Elmer Sciclone NGS Workstation liquid handling platform.

1µg of genomic DNA was sheared to 350bp using the Covaris LE220 Sonicator (Covaris and Life Technologies), the ends of the DNA were repaired; 3' to 5' exonuclease activity removed the 3' overhangs and the polymerase activity filled in the 5' overhangs creating blunt ends. A single ‘A’ nucleotide was added to the 3’ ends of the blunt fragments to allow for the ligation of barcoded adapters (12bp - Perkin Elmer NEXTFLEX-HT (NOVA-51474/5/6/7)) at a concentration of 6µM prior to a double sided clean up using Beckman Coulter AMPure XP beads (A63882). Adaptor ligated DNA was then enriched with 6 cycles of PCR (45 secs at 98°C, 6 cycles of: 15 secs at 98°C _30 secs at 60°C _30 secs at 72°C, 60 secs at 72°C, final hold at 4°C).

14 The resulting libraries were QC’d using the Perkin Elmer GX Touch DNA High Sensitivity assay (DNA High Sensitivity Reagent Kit CLS760672) and the concentrations determined with a high sensitivity plate reader Quant-iT™ dsDNA Assay Kit, (ThermoFisher Q-33120). The resulting libraries were then equimolarly pooled and q-PCR was performed on the pool prior

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to sequencing using the KAPA qPCR kit which quantifies full-length library fragments by using primers that anneal to the p5 and p7 sequences.

15 The library pool was diluted down to 0.65 nM using EB (10mM Tris pH8.0) in a volume of 18ul before spiking in 1% Illumina phiX Control v3 (Illumina, FC-110-3001). This was denatured by adding 4ul 0.2N NaOH and incubating at room temperature for 8 mins, after which it was neutralised by adding 5ul 400mM tris pH 8.0. The ExAmp master mix was prepared by combining EPX1, EPX2, and EPX3 from the NovaSeq Xp 2-lane kit v1.0 as per the manufacturer's instructions before loading onto a NovaSeq SP flow cell which was loaded onto the NovaSeq 6000 along with a NovaSeq 6000 SP cluster cartridge, buffer cartridge, and 300 cycle SBS cartridge (Illumina, 20027465). The NovaSeq had NVCS v1.6.0 and RTA v3.4.4 and was set up to sequence 150bp PE reads. The data was demultiplexed and converted to fastq using bcl2fastq2.

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