PROTOCOL: Illumina PCR-free Indexed Paired-End Library Construction

This protocol provides instructions for manual preparation of PCR-free indexed paired-end whole genome shotgun (WGS) libraries for Illumina sequencing. The entire process described in this protocol includes the following steps:

1. **DNA fragmentation**
2. **End repair**
3. **3'-end adenylation**
4. **Ligation with indexed Illumina PE adaptor**
5. **QC the final PCR-free library**

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Reagents

Item
NEBNext End-Repair Module, Cat# E6050L, NEB
NEBNext dA-Tailing Module, Cat# E6053L, NEB
NEBNext Ligation Module, Cat# E-6056L, NEB
Illumina indexed PE adaptor oligos All these oligos need to be HPLC purified. The copy right of these oligo sequences belongs to Illumina, Inc. * phosphothioate bond. Oligo name Sequence Illu-A-ID 1 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ATC ACG ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 2 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CGA TGT ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 3 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TTA GGC ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 4 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TGA CCA ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 5 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ACA GTG ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 6 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GCC AAT ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 7 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CAG ATC ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 8 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ACT TGA ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 9 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GAT CAG ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 10 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC TAG CTT ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 11 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GGC TAC ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 12 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CTT GTA ATC TCG TAT GCC GTC TTC TGC TTG Illu-A-ID 13 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC AGT CAA CAA TCT CGT ATG CCG TCT TCT GCT TG

Illu-A-ID 14 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC AGT TCC GTA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 15 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ATG TCA GAA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 16 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CCG TCC CGA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 18 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GTC CGC ACA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 19 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GTG AAA CGA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 20 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GTG GCC TTA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 21 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GTT TCG GAA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 22 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC CGT ACG TAA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 23 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC GAG TGG ATA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 25 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ACT GAT ATA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-ID 27 /5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC ATT CCT TTA TCT CGT ATG CCG TCT TCT GCT TG Illu-A-universal AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC* T
Agencourt AMPure XP beads(60ml), Cat# A63882, Beckman Coulter

Consumables

Item	Source	Catalog #
1.2% FLASH DNA gel cassette	Lonza/VWR	57023
Flash Gel Loading Dye (5X)	Lonza/VWR	50462
Flash Gel Marker 100 – 4kb	Lonza/VWR	50473
EtOH, 200 proof	Aaper	Stock room
1.7 ml microTubes (MCT-175-B)	VWR	10011-720
10 ul Pipet tips	VWR	47449-868
200 ul pipet tips	VWR	22234-016
1000 ul pipet tips	VWR	16466-008
Shearing microtube	Covaris	520045
Nuclease-free Water	Qiagen	129114

Equipments and Devices

Item	Source	Catalog #
Covaris S2 Acoustic Shearer	Covaris	600028
P1000 pipette	Rainin	L-1000
P200 pipette	Rainin	L-200

P10 pipette	Rainin	L-10
Flashgel Starter Kit	VWR	95015-612
Gel Imager PC 2000	BioRad	1708195
Thermomixer	Eppendorf	0022670000
Agilent Bioanalyzer 2100	Agilent	G2940CA
Microcentrifuge 5424	Eppendorf	5424000410
Minifridge Chillers	Boekel/VWR	260009
DynaMag2 particle concentration rack	Invitrogen	123-21D

Before you start, make sure that you read carefully the following safety information:

- Wear lab coat, gloves, and protective goggles.

- Protect bench working area with absorbent bottom plasticized pad.

- Discard all materials in Biohazard bag. Discard all liquid in a Biohazard labeled plastic bottle that should be tightly closed and disposed of in the Biohazard bag when full.

References:

- Illumina Multiplexing_SamplePrep_Guide_1005361_D.

1. DNA Fragmentation

a) Fill a Covaris™ water bath to the level of 12 in the Covaris S2 device and degas for 30-45 minutes prior to shearing your gDNA in the S2. When a microtube is placed in the tube holder, the water level should be at the base of the cap and the glass portion of the tube should be completely submerged. The water bath temperature should be between 6 ºC and 8 ºC.

b) Dilute 500ng of DNA (pico-green or Qubit reading) into 80 µl of TE in a microcentrifuge tube.

c) Place a Covaris™ microTube (6X16mm) into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the 100 µL of DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube. Bubbles will interfere with the acoustic field.

d) Shear the DNA using the following Covaris™ S2 System shearing conditions.
   1. Number of Cycles: **2 (32 seconds per cycle)** (if sample quality is poor, start with 1 cycle and check on a gel).
   2. Batch Temperatue: **6-8 ºC**
   3. Bath Temperature Limit: **<15 ºC**
   4. Mode: **Frequency sweeping**
   5. Water Quality Testing Function: **Off**
   6. Duty cycle: **10%**

7. Intensity:**4**
8. Cycles/burst: **200**
9. Time: **64 s**

e) Place the Covaris™ microtube into the loading station. While keeping the snap-cap on, insert a pipette tip through the pre-split septa, slowly remove the sheared DNA and transfer it into a new 1.7 ml tube.

f) Load 4 µl of each sample on a 1.2% FlashGel DNA Cassette (Lonza, Cat#: 57023). Gel running condition set as the following: Voltage 270, running time: 4 minutes 30 sec. Gel imager (Bio-Rad) setting should be uniformed to the condition as described here. Condition of Bio-Rad gel imager: Filter 1 for Eth Bromide, auto expose of 0.6 s. Image transform: Higher: 3000, low: 200, Gamma: 1.00, Min: 79, Max: 4095.

Note: The average size should be approximately 350-400 bp and the majority of fragment sizes should be smaller than 800 bp (see Figure 1 gel picture for example).

g) Purify by adding 0.8X Agencourt AMPure XP beads (64µl) and binding by rotating on a thermomixer for about 5 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant. Wash 2 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash and add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 2 minutes to allow the beads to briefly dry. Elute DNA in 78 µl of Nuclease free water.

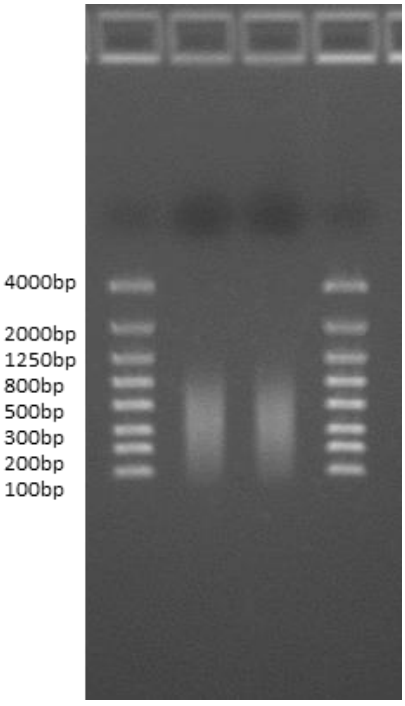

Figure 1 Post-Covaris DNA size distribution on 1.2% Flash Gel. Marker lane: FlashGel DNA marker (100 bp – 4000 bp, Cat. No. 50473, Lonza).

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2. DNA End Repair

a) Combine and mix the following components in a 1.7 ml tube:

Component	Volume (µL)
Sheared DNA	76.0
End-repair 10× buffer*	9.0
End-repair enzyme mix*	5.0
Total	90.0

Pipet Mix. Gentle Quick Vortex. Quick Spin.

*From NEBNext End-Repair Module (Cat. No. E6050L).

b) Incubate the mixture at 20°C (or RT) for 30 minutes at bench top cooler.

c) Purify by adding 1.8X Agencourt AMPure XP beads (162µl) and binding by rotating on a thermomixer for about 5 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant. Wash 2 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash and add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 2 minutes to allow the beads to briefly dry. Elute DNA in 53 µl of Nuclease free water.

3. 3'-end Adenylation

a) Combine and mix the following components in a 1.7 ml tube:

Component	Volume (µL)
End-repaired DNA	51.0
NEBNext™ dA-Tailing Reaction Buffer (10X)*	6.0
Klenow Fragment (3'-5' exo$^-$)*	3.0
Total	60.0

Pipet Mix. Gentle Quick Vortex. Quick Spin.

*From NEBNext dA-Tailing Module (Cat. No. E6053L).

b) Incubate the mixture at 37ºC for 30 min in a calibrated thermomixer.

c) Purify by adding 1.8X Agencourt AMPure XP beads (108ul) and binding by rotating on a thermomixer for about 5 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant. Wash 2 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash and add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 2 minutes to allow the beads to briefly dry. Elute DNA in 64 µl of Nuclease free water.

4. Ligate Adaptors to the DNA

Oligonucleotide annealing:

Illu-A-universal and Illu-A-ID adapter oligos should be ordered as HPLC purified.

Mix the paired oligonuclieotides at final concentration of 300uM in 1x Ligase buffer (from NEBNext Ligation Module, Cat# E6056-L) and run with the following annealing program on thermocycler: 95°C for 5 min, 80°C for 3 min, 70°C for 3 min, 60°C for 3 min, 50°C for 3 min, 40°C for 3 min, 30°C for 3 min, 20°C for 3 min and 4°C hold. Dilute 20x to obtain 15uM working concentration. Aliquot annealed adaptor into eppendorf tubes and store them in -20°C freezer.

a) Combine and mix the components below

Component	Volume (µL)
A-Tailed DNA	62.0
Annealed Illumina indexed PE Adaptors (15 µM concentration)	5.0
Quick Ligase 5X buffer*	18.0
Quick Ligase Enzyme*	5.0
Total	90.0

Pipet Mix. Gentle Quick Vortex. Quick Spin.

*From NEB (Cat. No. E-6056L).

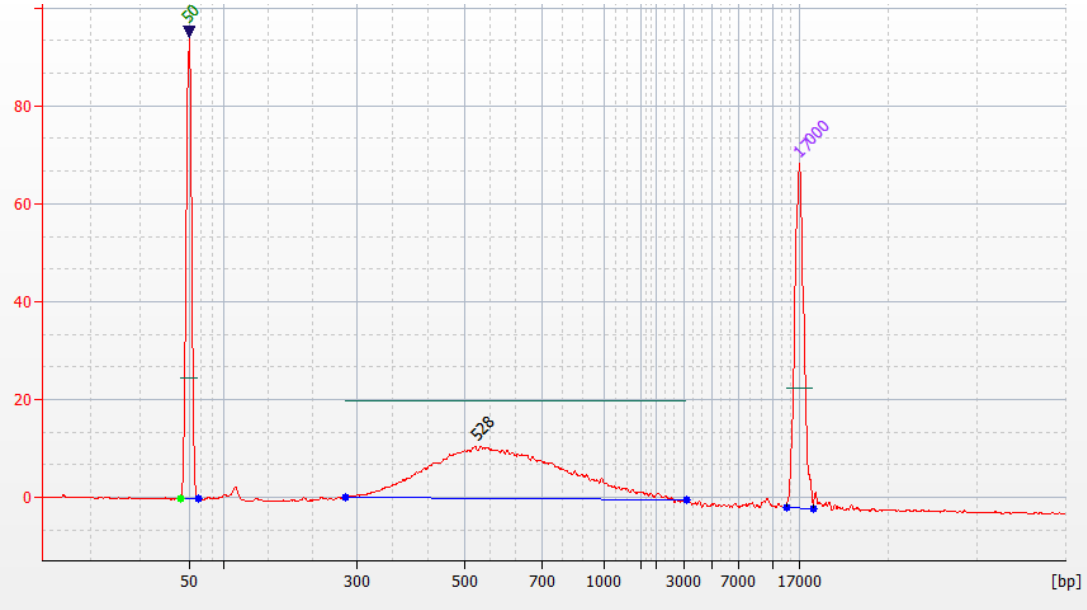
b) Incubate at room temperature (25 ºC) for 30 minutes.

c) Purify by adding 1.0X Agencourt AMPure XP beads (90µl) and binding by rotating on a thermomixer for about 5 minutes at RT before placing into the magnetic particle concentrator (MPC). Remove the supernatant. Wash 2 times with freshly prepared 70% ethanol. Keep the sample tube in MPC during the wash and add ethanol slowly to avoid disturbing the bead pellet. After wash, leave the tube in MPC for 2 minutes to allow the beads to briefly dry. Elute DNA in 15 µl of Nuclease free water.

5. QC the final PCR-free library

Assess yield and size of the final PCR-free WGS library by running Agilent Bioanalyzer 2100 DNA 7500 Chip (see Figure 2 for an example). Make sure there is no excess free adaptor's peak in the eluted DNA sample.

Figure 2 Agilent Bioanalyzer analysis of the final PCR-free library.



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