Heat fragmented genomic DNA before Illumina library preparation for sequencing GC rich fraction of heterogeneous genome

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Abstract

This protocol was developed with the aim of sequencing the GC rich part of heterogeneous genomes with the Illumina technology. To this aim, fragmented genomic DNA is denaturized up to 90°C before library preparation. This procedure enriches the library in GC-rich fragments and leads to a substantial increase in average GC content of sequence reads.

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Guidelines

Reagents:

Illumina library protocol is an adaptation to publication Meyer and Kircher 2010 (Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb Protoc 2010:pdb.prot5448. doi: 10.1101/pdb.prot5448).

DNeasy Blood and Tissue Kit (Qiagen)

Agencourt AMPure XP beads (Beckman Coulter, France S.AS)

10X Tango buffer (Thermo Fisher Scientific, Waltham, MA, USA)

25mM dNTPs (New England Biolabs, Ipswich, MA, USA)

100mM dATP (New England Biolabs)

T4 DNA polymerase (New England Biolabs)

T4 polynucleotide Kinase (New England Biolabs)

T4 DNA ligase (New England Biolabs)

Bst polymerase large fragment (New England Biolabs)

2X KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS, Wilmington, MA, USA)

Phusion High-Fidelity DNA Polymerase (New England Biolabs)

Dimethylsulfoxyde 100% (Thermo Fisher scientific)

50 µM adapters mix primers (see table 1) (Eurofins, Vergèze, France) HPLC purification (see recipes)

10X Oligo Hybridation Buffer (see recipes)

Names	Sequences
IS1_adapter P5.F	A*C*A*C*TCTTTCCCTACACGACGCTCTTCCG*A*T*C*T
IS2_adapter P7.F	G*T*G*A*CTGGAGTTCAGACGTGTGCTCTTCCG*A*T*C*T
IS3_adapter P5+P7.R	A*G*A*T*CGGAA*G*A*G*C
IS4_ind PCR P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
I1_ind PCR P7	CAAGCAGAAGACGGCATACGAGATcctgcgaGTGACTGGAGTTCAGACGTGT

* PTO = phosphothioate oligonucleotide bond

Recipes:

Oligo hybridization buffer (10X)
500 mM NaCl
10 mM Tris-Cl, pH 8.0
1 mM EDTA, pH 8.0

Adapter mix ready to use.

Reagent	Volume (µL)	[Final]	[Combined]
IS1_adapter_P7.F (500 µM)	20	200 µM	**100uM each**
IS3_adapter_P5+P7.R (500 µM)	20	200 µM	**100uM each**
Oligo hybridization buffer (10X)	5	1X	1X
H2O	5	-	-

Mix and incubate each reaction in a thermal cycler for 10 s at 95°C, followed by ramp from 95°C to 12°C decreasing of -0,1°C/s. Pool both reactions to obtain adapters mix ready to use.

Protocol

Step 1.

1/ DNA extraction

Genomic DNA was extracted using DNeasy Blood and Tissue Kit, according the manufacturer's instructions.

Step 2.

Shearing DNA

Shear (tube 0,2 ml) 500ng (10ng/ul) of extracted genomic in 200bp to 500bp using Ultrasonic cleaning unit fill in with 250ml of 4°C sterilized water for 20 min.

Step 3.

3/ Denaturized fragmented DNA

Preheat thermocycler to 90°C and heat the tube for 5 min.

Without allowing the tube to cool, add 90ul (ratio volume fragmented DNA / volume beads was 1,8) Agencourt beads for sizing fragmented genomic DNA following Illumina library protocol.

Elute in 32ul of ultra pure water.

Be careful: at this step, the loss of DNA is about 60%. To obtain a higher amount of DNA, use several 500 ng samples rather than one of 1µg. Indeed, denaturation at 90 ° C is less effective with larger amounts of DNA

Step 4.

4/ Illumina library preparation

4-1/ Blunt-end repair

Prepare the master mix for the required number of samples.

Add 8 µl of master mix to each sample. Mix gently by pipetting.

Incubate 15 min at 25°C, followed by 5 min at 12°C.

MASTER MIX:	**[Stock]**	**[Final]**	**Units**	**1X vols**
10X Buffer Tango (Fermentas)	10	1	X	**4,00**
dNTPs (25 mM each)	25000	100	µM	**0,16**
dATPs (100 mM)	100	1	mM	**0,40**
T4 polynucleotide kinase (10 U/µL) (NEB)	10	0,5	U/µL	**2,00**
T4 DNA polymerase (3 U/µL) (NEB)	3	0,1	U/µL	**1,33**

H2O	**0,11**
DNA	**32,0**

Clean the samples: mix 64 µl of AMPure XP beads to each tube and follow the manufacturer's instructions.

Elute in 32 µl of ultra pure water.

4-2/ Adapter ligation

Prepare the master mix for the required number of samples.

Add 6 µl of master mix to each sample. Mix gently by pipetting.

Incubate at 16°C overnight.

MASTER MIX:	**[Stock]**	**[Final]**	**Units**	**1X vols**
T4 DNA ligase buffer (10X)	10	1	X	**4,00**
Adapter mix (100 µM each)	100	2,5	µM	**1**
T4 DNA ligase (5.97U/µL) (NEB)	5,97	0,125	U/ µL	**0,84**
H2O				**0,16**
DNA				**34,0**

Clean the samples: mix 64 µl of AMPure XP beads to each tube and follow the manufacturer's instructions.

Elute in 34 µl of ultra pure water.

4-3/ Fill in

Prepare the master mix for the required number of samples.

Add 6 µl of master mix to each sample. Mix gently by pipetting.

Incubate 20 min at 37°C.

MASTER MIX:	**[Stock]**	**[Final]**	**Units**	**1X vols**
ThermoPol rxn buffer (10X)	10	1	X	**4,00**
dNTPs (25 mM)	2500	250	uM	**0,40**
Bst polymerase, lg frag (8 U/µL)	8	0,3	u/ul	**1,50**
H2O				**0,1**

DNA	**34,0**

Clean the samples: mix 64 µl of AMPure XP beads to each tube and follow the manufacturer's instructions.

Elute in 30 µl of ultra pure water.

Step 5.

5/Indexed PCR

MASTER MIX:	[STOCK]	[FINAL]	UNITS	1X VOLS.
2X KAPA HiFi Hotstart ReadyMix	2	1	X	25,00
Dimethylsulfoxyde (DMSO)	1	3%	µL	1,50
Forward primer IS4	10000	200	nM	1,00
Reverse primer indexR_01	10000	200	nM	1,00
DI water				up to 50

Cycling protocol for library amplification: 12 cycles

Step		Temperature	Time
Initial denaturation		98°C	45s
Cycling :	Denaturation	98°C	15s
	Annealing	64°C	30s
	Extension	72°C	30s
Final extension		72°C	60s

Clean the samples: mix 80 µl of AMPure XP beads to each tube and follow the manufacturer's instructions.

Elute in 30 µl of ultra pure water.

Step 6.

Library quantification

Libraries were quantified with Nanodrop ND-800 and pooled in equimolar ratio before Illumina

sequencing.