TGIRT ssDNA-seq

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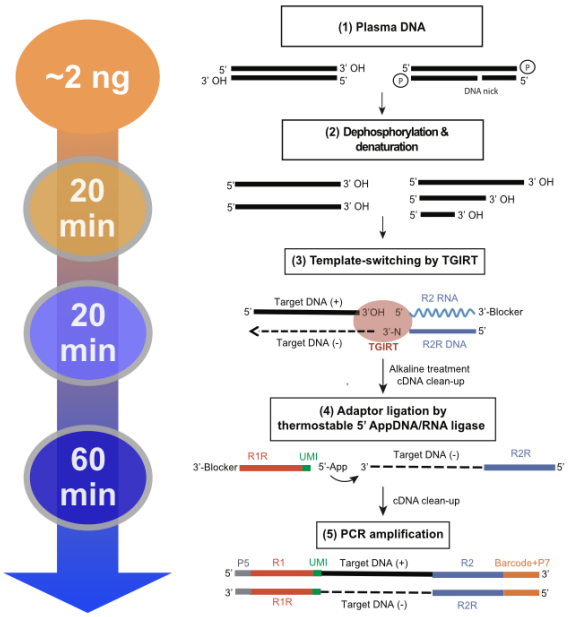
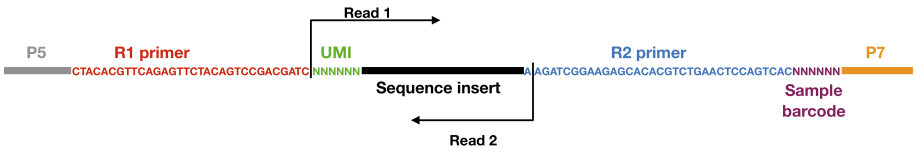
Overview

Procedures



Final sequence structure



Reagents for TGIRT-DNA-seq

Buffers

You can scale up accordingly.

DNA TS Buffer I

Material	Volumn	Final concentration
2M $MgCl_2$	105 uL	21 mM
2M Tris-HCl pH 7.5	430 uL	86 mM
H_2O	9.465 mL	
Total	10 mL	

DNA TS Buffer II (420 mM)

Material	Volumn	Final concentration
2.4M NaCl	8.4 mL	200uM
2M $MgCl_2$	87.5 uL	17.5 mM
2M Tris-HCl pH 7.5	350 uL	70 mM
H_2O	1162.5 uL	
Total	10 mL	

TE Buffer

Material	Volumn	Final concentration
2 M Tris HCl pH 7.5	50 uL	10 mM
0.5 M EDTA pH 8	20 uL	1 mM
H_2O	9.93 mL	
Total	10 mL	

Primers

DNA primers

- R2R (TTN): GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TTN
- 6N UMI R1R: /5'Phos/ NNN NNN GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG /3'C3Sp/
- Illumina Barcoded PCR primer: CAA GCA GAA GAC GGC ATA CGA GAT NNNNNN GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T
- Multiplex PCR primer: AAT GAT ACG GCG ACC ACC GAG AT C TAC ACG TTC AGA GTT CTA CAG TCC GAC GAT C

RNA primer

- R2 (TTN): AAG AUC GGA AGA GCA CAC GUC UGA ACU CCA GUC AC/3'C3Sp/

Preparation of 5'App-R1R

5´ DNA Adenylation Kit (NEB E2610S)

Material	Volumn
H_2O	13 uL
10X rxn buffer	2 uL
1mM ATP	2 uL
100 uM 6N UMI R1R	1 uL
Mth RNA ligase	2 uL
Total	20 uL

- Incubate at 65^{o}C for 1 hr
- Incubate at 85^{o}C for 5 min
- Cleanup using NucleoSpin Gel and PCR Clean-up (use Binding buffer NTC, Macherey-Nagel^{TM} 740654.100)

Protocol

Pre-anneal template-switching duplex

Material	Volumn
TE buffer	12 uL
10uM R2R primer	1.5 uL
10uM R2 primer	1.5 uL
Total	15 uL

* 15 uL is enough for 7 rxns, can scale up if needed

- Incubate at 82^{o}C for 2 min (start preparing DNA template)
- Cool down to 25^{o}C with 10% ramp or 0.1^{o}C/second

Prepare DNA template

Material	Volumn
DNA TS Buffer I	3 uL
0.5M DTT	0.2 uL
DNA sample	9 uL
FastAP	1 uL
Total	13.2 uL

- Incubate at 37^{o}C for 20 min (start preparing template-switch complex)
- Denature at 95^{o}C for 3 min

Prepare template-switch complex

Material	Volumn
DNA TS Buffer II	2 uL
Pre-annealed duplex	2 uL
TGIRT	2 uL
Total	6 uL

- Incubate at room temperature for 30 min

R2-adapter addition and cDNA synthesis

- Add 6 uL template-switch complex into 13.2 uL of DNA template
- Add 0.8 uL dNTP (25 mM) to the mixture
- Incubate at 60^{o}C for 20 min
- Add 1 ul of 5 M NaOH and incubate at 95^{o}C for 3 min
- Add 1 ul 5 M HCl to neutralize pH
- Clean-up with Qiagen MinElute Reaction Cleanup Kit

R1-adapter ligation

Thermostable 5´ AppDNA/RNA Ligase (NEB M0319S)

Material	Volumn
10X NEB-1 buffer	2 uL
50mM $MnCl_2$	2 uL
10uM 5'App-R1R	4 uL
cDNA	10 uL
5'App thermostable ligase	2 uL
Total	20 uL

- Incubate at 65^{o}C for 1 hr
- Incubate at 90^{o}C for 3 min
- Clean-up with Qiagen MinElute Reaction Cleanup Kit

PCR amplification and addition of sequencing primers

KAPA HiFi HotStart ReadyMix (KAPA KK2602)

Material	Volumn
Barcoded PCR primer	2.5 uL
Multiplex PCR primer	2.5 uL
Ligation product	20 uL
KAPA hotsart master-mix	25 uL
Total	50 uL

- PCR cycles:
  - 98^{o}C 30 sec, 1 cycle
  - 98^{o}C 45 sec, 60^{o}C 15 sec, 72^{o}C 30 sec 11 cycles
  - 72^{o}C for 5 min, 1 cycle
  - hold at 4^{o}C

Final cleanup

- Add 65 uL Agencourt AMPure XP beads into the PCR product and pipette the mixture to a 2 mL microcentrifuge tube
- Incubate at room temperature for 10 min
- Put the tube on magnetic rack and wait 5 min
- Remove all liquid
- Do twice: Add 200 uL 80% EtOH, wait 30 sec and remove
- Quick spin down, and put the tube on magnetic rack, remove residual EtOH using 20P pipette
- Add 31 uL H_2O, mix well and incubate in room temperature for 10 min
- Put the tube on magnetic rack and wait 5 min
- Pipette 30 uL of elute to a clean tube