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(Please reference this article for use of the method)

TGIRT template-switching

The TGIRT template-switching reaction was originally developed as an efficient method for simultaneously reverse transcribing and adding an RNA-seq adapter to RNAs of all sizes and structures in a less biased manner than other methods (Mohr et al. 2013; Qin et al. 2016; Nottingham et al. 2016). Here, we described a variation of the method for use in single-stranded DNA sequencing.

I. RNA and DNA oligonucletoide (order PAGE or HPLC purified)

R2 RNA: 5’ rArGrA rUrCrG rGrArA rGrArG rCrArC rArCrG rUrCrU rGrArA rCrUrC rCrArG rUrCrA rC/3SpC3/ (Other blockers such as 3’ Amino Modifier C6 dT (3AmMC6T) from IDT are also effective)

R2R DNA: 5’ GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TN (N = equimolar A, T, G, C; hand mixed recommended)

R1R DNA: 5’ /5Phos/GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG/3SpC3/
A unique molecular identifier sequence (UMI) of sufficient complexity for the experimental purpose can be added at the 5’ end of the oligonucleotide. If desired, the UMI can be preceded by a short constant sequence (see Wu and Lambowitz, 2017).

NOTE: The Read 1 (R1) sequence corresponds to the small RNA sequencing priming site used in the NEBNEXT Small RNA Library Prep Set for Illumina.

Illumina multiplex PCR primer: 5' AAT GAT ACG GCG ACC ACC GAG ATC TAC ACG TTC AGA GTT CTA CAG TCC GAC GAT C

Illumina barcode PCR primer: 5' CAA GCA GAA GAC GGC ATA CGA GAT ***BARCODE*** GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC T

NOTE: The barcode sequence in the primer should be the reverse complement of the actual barcode listed on the Illumina website (*e.g.*, CGTGAT in the primer for TSBC01 ATCACG).

II. R2 RNA/R2R DNA annealing reaction

1. Start with 10X Primer Mix.

Component
10 x Primer Mix (1 μM R2 RNA and 1 μM R2R DNA in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA)

It may be useful to make aliquots of the Primer Mix in amounts needed for that subsequent experiments to avoid repeated freezing and thawing.

1. Thaw and then incubate at 82°C for 2 minutes in a thermocycler.

2. Cool down to 25°C with a 10% ramp or at a rate of 0.1°C/second.

III. Phosphatase treatment and denaturation of double-stranded DNA (RXN-1)

1. Set up the following reaction components in a sterile PCR tube adding the FastAP (ThermoFisher EF0654) last.

Components	Volume (final concentration)
Reaction Buffer I (21 mM $MgCl_2$, 86 mM Tris-HCl, pH 7.5)	3 μl (5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5)
DTT (500 mM; avoid excessive freezing and thawing)	0.2 μl (7.5 mM)
DNA sample[a]	1-50 ng[b]
FastAP (ThermoFisher EF0654)	1 μl (1 unit)
Nuclease-free H_2O	to 13 μl

[a] A template-switching reaction using the TGIRT®-III enzyme and FastAP to a 3' phosphorylated ssDNA oligonucleotide can be carried through the procedure as a positive control.
[b] Low DNA concentrations should be measured by Qubit or Bioanalyzer.

2. Incubate at 37°C for 20 minutes for dephosphorylation and then denature by heating at 95°C for 3 min.

IV. Pre-incubation step for formation of the TGIRT-template switching complex (RXN-2)

1. Set up the following reaction components in a sterile PCR tube adding the TGIRT®-III enzyme last.

Components	Volume (final concentration)
Reaction Buffer II (4.2 M NaCl, 17.4 mM $MgCl_2$, 69.9 mM Tris-HCl, pH 7.5)	2 µl (1.4 M Na Cl, 5.8 mM $MgCl_2$, 23.3 mM Tris-HCl, pH 7.5)
10 x mix of annealed R2 RNA/R2R DNA heteroduplex	2 µl (100 nM R2 RNA/R2R DNA)
TGIRT®-III enzyme (10 µM)	2 µl (3 uM final)

2. Incubate at room temperature for 30 min.

V. Template-switching reaction

1. Add 0.8 µl of 25 mM dNTPs (an equimolar mixture of dATP, dCTP, dGTP, and dTTP at 25 mM each; RNA grade) to RXN-1 and transfer RXN-1 to the tube containing RXN-2.

2. Incubate at 60°C for 20-40 minutes. The optimal incubation time may be determined experimentally for different DNA templates.

3. Add 1 µl of 5 M NaOH and incubate at 95°C for 3 minutes or at 65°C for 15 minutes.

   NOTE: This step is an important because the TGIRT®-III enzyme binds nucleic acids very tightly and might impede the next step if not completely removed.

4. Cool to room temperature and neutralize with 1 µl of 5 M HCl.

5. Add 50-78 µl nuclease-free water to bring up the final volume to ≤100 µl.

6. Clean up the DNA product with a MinElute Reaction Cleanup Kit (QIAGEN, Cat. No. 28204) or a Nucleospin Gel and PCR Clean-up (Clontech 740609.50 if second strand DNA product is >150 nt), and elute in 10 µl QIAGEN elution buffer or 20 ul Clontech elution buffer (incubating the column with elution buffer at room temperature before centrifugation is recommended).

7. Proceed with R1R adenylation, thermostable ligation, and PCR amplification.

VI. R1R DNA adenylation (NEB, Cat. No. E2610S)

1. Set up the following reaction components in a sterile PCR tube:

Components (from NEB)	Volume
10 x reaction buffer	2 µl
1 mM ATP	2 µl
100 µM 5'p/3'SpC3 R1R DNA	1 µl
Mth RNA Ligase	2 µl
Nuclease-free water	To 20 µl

2. Incubate at 65°C for 1 hour.

3. Incubate at 85°C for 5 minutes to inactivate the enzyme.

4. Clean up with a Nucleospin Gel and PCR Clean-up (Clontech 740609.50) with binding buffer NTC (Clontech 740654.100), and elute in 10 µl double-distilled water to give a final concentration of 10 µM 5'-end adenylated R1R DNA.

   Note: Doing multiple adenylation reactions in separate PCR tubes and then combining reactions for cleanup is recommended because higher elution volume helps with consistent and efficient recovery of adenylated oligonucleotides.

5. Proceed with the thermostable ligation.

VII. Thermostable single stranded DNA ligation (NEB, Cat. No. M0319S)

1. Set up the following reaction components in a sterile PCR tube:

Components (from NEB)	Volume
10 x reaction buffer (NEBuffer 1)	2 µl
50 mM $MnCl_2$	2 µl
cDNA from template-switching reaction[a]	Up to 10 µl
Thermostable 5' AppDNA/RNA Ligase	2 µl
10 µM 5'-end adenylated R1R DNA	4 µl
Nuclease-free water	To 20 µl if using less than 10 µl cDNA

[a] If elution from TGIRT reaction clean up has volume > 10 ul, split to multiple single stranded DNA (ssDNA) ligation reaction.

2. Incubate at 65°C for 1-2 hours.

3. Incubate at 90°C for 3 minutes to inactivate the enzyme.

4. Clean up the ligated cDNAs with a MinElute Reaction Cleanup Kit (QIAGEN 28204) or a Nucleospin Gel and PCR Clean-up (Clontech 740609.50 if ssDNA ligation product is

>150nt), and elute in 20 µl QIAGEN/Clontech elution buffer (incubating the column with elution buffer at room temperature before centrifugation is recommended).

5. Proceed with PCR amplification.

VIII. PCR amplification (KAPA Biosystems, Cat. No. KK2611)

1. Set up the following reaction components in a sterile PCR tube:

Components	Volume (final concentration)
2x KAPA HiFi HotStart ReadyMix	25 µl
10 µM Illumina Mutliplex primer	2.5 µl (500 nM)
10 µM Illumina Barcode Primer	2.5 µl (500 nM)
ssDNA ligation product	Up to 20 µl
Nuclease-free water	To 50 µl if using less than 20 µl ligation product

2. PCR cycles:
   i. 98°C 30 sec, 1 cycle
   ii. Up to 12 cycles of 98°C 45 sec, 60°C 15 sec, 72°C 30 sec, hold at 4°C.

3. Use Agencourt AMPure XP beads (Beckman, Cat. No. A63880) to clean up the adapter dimers and to enrich for desired DNA sizes in the sample. The ratio of beads to sample volume can be adjusted depending on the size profile of DNA.

4. To check library quality and quantity, analyze 1 µl on a Bioanalyzer with a High Sensitivity DNA Analysis Kit (Agilent, Cat. No. 5067-4626).

References

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