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(Please reference these papers for use of the method)

Overview of TGIRT template-switching for RNA-seq (TGIRT-seq)

The TGIRT template-switching reaction is 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). The method makes it possible to obtain full-length reads of tRNAs and other structured non-coding RNAs, which are difficult to reverse transcribe by using conventional reverse transcriptases. There are two different variations of the method, one for RNA-seq of **small RNAs ONLY** in which PAGE-purified cDNAs of selected sizes are circularized with CircLigase II (Katibah et al. 2014; Shen et al. 2015; Zheng et al. 2015; Clark et al. 2016; Liu et al. 2016), and the other for RNA-seq of **total RNAs of ALL size classes** in a single RNA-seq reaction (Qin et al. 2016; Nottingham et al. 2016; Bazzini et al. 2016; Burke et al. 2016; Shurtleff et al. 2017).

Here, we describe the total RNA-seq method, which is used in the TGIRT®-III template-switching modular kit. This method can be used for RNA-seq of whole-cell, exosomal, microvesicle, or plasma RNAs, as well as for analysis of protein- or ribosome-bound RNA fragments in procedures like HITS-CLIP/CLIP-seq, RIP-seq, CRAC, or ribosome profiling.

A modified protocol with some differences from that described below and employing a different buffer than is provided in the kit can be used for single-stranded DNA-seq and bisulfite DNA-seq (Wu and Lambowitz 2017).

Important Notes

RNA fragments containing a 2',3'-cyclic phosphate or a 3'-terminal phosphate should be treated by T4 Polynucleotide Kinase with 3'-phosphatase activity to produce a **3'-hydroxyl group**, which is critical for efficient TGIRT template-switching reaction (Mohr et al. 2013).

Total RNA-seq protocol using the TGIRT®-III kit

The TGIRT®-III enzyme initiates from a synthetic RNA/DNA heteroduplex consisting of a 35-nt RNA oligonucleotide that contains the primer binding site for Illumina Read 2 (R2) sequencing primer and is annealed to a complementary 36-nt DNA primer that leaves a single-nucleotide 3' overhang (a hand-mixed equimolar mixture of A, T, G, and C, denoted N). The TGIRT®-III kit contains a mixture of the R2 RNA/R2R DNA oligonucleotides (Primer Mix) that can be annealed by heating and cooling to form the R2 RNA/R2R DNA heteroduplex used for initiating the template-switching reaction with the provided TGIRT®-III enzyme. After cDNA synthesis and cleanup, the cDNA product is ligated to a 5'-end adenylated DNA oligonucleotide containing the reverse complement of an Illumina Read 1 (R1R) sequencing primer binding site using a thermostable ligase (NEB, Cat. No. M0319S), followed by PCR amplification using Phusion DNA polymerase (Thermo Fisher Scientific, Cat. No. F531S) with overlapping multiplex and barcode primers that add all the sequences necessary for Illumina sequencing. The RNA and the adenylated DNA oligonucleotides have blocked 3' ends (*e.g.*, 3'SpC3, IDT) to inhibit template-switching or ligation to that end.

I. RNA and DNA oligonucleotide sequences (For oligonucleotides NOT included in the kit, order them separately with PAGE or HPLC purification)

R2 RNA
5' rArArG rArUrC rGrGrA rArGrA rGrCrA rCrArC rGrUrC rUrGrA rArCrU rCrCrA rGrUrC rArC/3SpC3/

NOTE: 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 TTN (N=equimolar A, T, G, C)

NOTE: The R2R DNA used in the current TGIRT®-III v3 kit contains a single nucleotide change (insertion of the underlined T residue at -3 position) which reduces recovery of R1R-R2R adapter dimers after the PCR step by 82-89% (Xu et al. 2018). The complementary nucleotide is inserted in R2 RNA (see above, underlined A).

R1R DNA (NOT included in the TGIRT®-III kit)
5' /5Phos/GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG/3SpC3/

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

Illumina multiplex PCR primer (NOT included in the TGIRT®-III kit)
5' AAT GAT ACG GCG ACC ACC GAG ATC TAC ACG TTC AGA GTT CTA CAG TCC GAC GAT C

Illumina barcode PCR primer (NOT included in the TGIRT®-III kit)
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).

I. 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)

The kit contains 50 μl of 10X primer mix sufficient for 25 reactions (2 μl per reaction). After initial thawing, it may be useful to make aliquots of the Primer Mix that can be stored frozen in amounts needed for 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.

II. Template-switching reaction

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

Components	Volume (final concentration)
5 x Reaction Buffer (2.25 M NaCl, 25 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5)	4 μl (450 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5)
10 x DTT (100 mM; avoid excessive freezing and thawing)	2 μl (10 mM)
RNA sample[a]	1[b]-50 ng or <100 nM
10 x mix of annealed R2 RNA/R2R DNA heteroduplex (1 μM)	2 μl (100 nM final)

TGIRT®-III enzyme (10 μM)	1 μl (500 nM final)
Nuclease-free H_2O	to 19 μl

[a] A template-switching reaction using the TGIRT®-III enzyme to a commercial RNA ladder or other RNA standards can be carried through the procedure as a positive control.
[b] Low RNA concentrations should be measured by Qubit or Bioanalyzer.

2. Pre-incubate at room temperature for 30 minutes, then add 1μl of 25 mM dNTPs (an equimolar mixture of dATP, dCTP, dGTP, and dTTP at 25 mM each; RNA grade).

3. Incubate at 60°C for 5-15 minutes (for small RNAs) or up to 60 minutes (for long or heavily modified RNAs). The optimal incubation time may need to be determined experimentally for different RNA templates.

4. 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 very important because the TGIRT®-III enzyme binds RNA very tightly and might impede the next step if not removed.

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

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

7. Clean up the cDNAs with a MinElute Reaction Cleanup Kit (QIAGEN, Cat. No. 28204) or a MinElute PCR Purification Kit (QIAGEN, Cat. No. 28004) and elute in 10 μl QIAGEN elution buffer (incubating the column with elution buffer at room temperature before centrifugation is recommended). For alternative size selection step, see Section VI.

8. Proceed with R1R adenylation, thermostable ligation, and Phusion PCR amplification.

III. 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 an Oligo Clean & Concentrator™ Kit (Zymo Research, Cat. No. D4060) 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.

IV. Thermostable 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	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

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 MinElute PCR Purification Kit (QIAGEN 28004) and elute in 23 µl QIAGEN elution buffer (incubating the column with elution buffer at room temperature before centrifugation is recommended).

5. Proceed with Phusion PCR amplification.

V. PCR amplification (Thermo Fisher Scientific, Cat. No. F531S)

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

Components	Volume (final concentration)
2x Phusion High-Fidelity PCR Master Mix with HF buffer*	25 µl
10 µM Illumina Mutliplex primer	1 µl (200 nM)
10 µM Illumina Barcode Primer	1 µl (200 nM)
cDNA from thermostable ligation	Up to 23 µl
Nuclease-free water	To 50 µl if using less than 23 µl cDNA

*KAPA HiFi HotStart ReadyMix (KAPA Biosystems) is also a recommended option for PCR amplification.

2. PCR cycles:
   i. 98°C 5 sec, 1 cycle
   ii. Up to 12 cycles of 98°C 5 sec, 60°C 10 sec, 72°C 15-30 sec/kb, 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 (Total RNA-seq method)

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