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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; Xu et al. 2019). 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; Reinsborough et al. 2019; Temoche-Diaz et al, 2019).

Here, we describe the total RNA-seq method, which is also used in the InGex TGIRT®-III template-switching modular kit. This method can be used for RNA-seq of whole-cell, extracellular vesicle/exosome, 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 can be used for single-stranded DNA-seq and bisulfite DNA-seq (Wu and Lambowitz 2017).

## Important Note

RNA fragments containing a 2',3'-cyclic phosphate or a 3'-terminal phosphate should be treated with T4 polynucleotide kinase with 3'-phosphatase activity to remove 3' phosphates, which can impede the TGIRT template-switching reaction (Mohr et al. 2013).

## Total RNA-seq protocol using TGIRT®-III

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). 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 R2 RNA oligonucleotide and the adenylated R1R DNA oligonucleotides have blocked 3' ends (*e.g.*, 3'SpC3, IDT) to inhibit template-switching or ligation to those ends.

**I. RNA and DNA oligonucleotide sequences (oligonucleotides should be PAGE or HPLC purified).**

**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 protocol contains a single nucleotide change (insertion of the underlined T residue at -3 position), which decreased reovery of R1R-R2R adapter dimers after the PCR step by 82-89% (Xu et al. 2019). The complementary nucleotide is inserted in R2 RNA (see above, underlined A).

**R1R DNA**
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 sequencing.

**Illumina multiplex PCR primer**
5’ AAT GAT ACG GCG ACC ACC GAG AT BARCODE C TAC ACG TTC AGA GTT CTA CAG TCC GAC GAT C

**NOTE:** The barcode sequence in the primer is the sense strand (e.g., ATCACG in the primer for the TSBC01 on the Illumina website). This barcode is optional but recommend to provide unique dual indices (UDI) for libraries sequenced on a Novaseq instrument.

**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. Preparation of 10X Primer Mix

1. For prepartion of 10X R2R mix containing an equimolar ratio of A, C, G, and T R2R 3' overhangs, hand-mix equal volumes of 10 μM of each individual R2R DNA in a 1.5 ml Eppendorf Lobind microcentrifuge or equivalent tube and vortex. This stock can be aliquoted and stored at -20°C for future use.

2. Set up the 10X Primer Mix.

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

50 μl of 10X primer mix is sufficient for 25 reactions (2 μl per reaction). The volume of the reaction can be scaled up or down according to the needs of the experiment.

## III. R2 RNA/R2R DNA annealing reaction

1. Pre-heat the thermocycler to 82°C. Then, incubate the 10X Primer Mix reaction 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.

## IV. 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 or 2 µl (500 nM or 1 µM 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.

**NOTE:** The high salt concentration (450 mM NaCl) used in the TGIRT template-switching reaction suppresses multiple end-to-end template switches by the TGIRT enzyme, but also decreases the efficiency of the template-switching reaction. Multiple template switches are particularly problematic for miRNA sequencing. Recent experments suggest that 200 mM NaCl can be used for some applications (*e.g.,* TGIRT-seq of human plasma RNA) with acceptable levels of multiple template switches detected as fusion reads (0.5-4% fusion reads, which include multiple template switches; Lentzsch et al., 2019). For new applications, we suggest testing both 200 and 450 mM NaCl.

2. Pre-incubate at room temperature for 30 minutes, then add 1 µl of 20 mM dNTPs (an equimolar mixture of 20 mM each dATP, dCTP, dGTP, and dTTP; 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
   **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).

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

## V. 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. If desired, adenylation can be monitored by PAGE or by using an Agilent small RNA Bioanalyzer kit.

5. Proceed with the thermostable ligation.

## VI. 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).
**Note:** Ligation can be monitored by gel electrophoresis prior to PCR.

5. Proceed with Phusion PCR amplification.

## VII. 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 default ratio is 1.4X v/v (70 µl beads / 50 µl PCR reaction). 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).
**NOTE:** If the desired product is not observed, absence of a ~120-bp adapter dimer peak may indicate failure of the adenylation and/or ligation reactions. Bioanalyzer traces showing only the adapter dimer peak could indicate failure of cDNA synthesis or low input amounts of RNA. However, libraries whose Bioanalyzer traces show very small peaks can typically yield good data sets. Further PCR amplification (or qPCR) of 1 µl of the library can reveal if the desired product is present.

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