Total RNA-seq Protocol Using the TGIRT®-III Kit

Qin Y, Yao J, Wu DC, Nottingham RM, Mohr S, Hunicke-Smith S, and Lambowitz AM. High-throughput sequencing of human plasma RNA by using thermostable group II intron reverse transcriptases. RNA *22*, 111-128, 2016.

Nottingham RM, Wu DC, Qin Y, Yao J, Hunicke-Smith S, and Lambowitz AM. RNA-seq of human reference RNA samples using a thermostable group II intron reverse transcriptase. RNA *22*, 597-613, 2016.

(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; 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 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 (Qin et al. 2016; Nottingham et al. 2016; Bazzini et al. 2016; Burke et al. 2016).

Here, we describe the total RNA-seq method, which is used in the TGIRT®-III 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. RNA fragments containing a 3’ terminal phosphate should be dephosphorylated to remove the 3’ phosphate, which inhibits TGIRT template-switching (Mohr et al. 2013). Variations of the method can also be used for single-stranded DNA-seq (Wu and Lambowitz, manuscript in preparation).

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

The TGIRT®-III enzyme initiates from a synthetic RNA/DNA heteroduplex consisting of a 34-nt RNA oligonucleotide that contains the primer binding site for Illumina Read 2 (R2) sequence and is annealed to a complementary 35-nt DNA primer that leaves a single-nucleotide 3' overhang (an equimolar mixture of A, T, G, and C, denoted N). The TGIRT®-III kit contains the RNA/DNA heteroduplex pre-mixed with the 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) sequence 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 oligonucletoide sequences (For oligonucleotides NOT included in the kit, order them separately with PAGE or HPLC purification)

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)

R1R DNA: 5' /5Phos/GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG/3SpC3/ (***NOT included in the TGIRT®-III kit)
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: 5' AAT GAT ACG GCG ACC ACC GAG ATC TAC ACG TTC AGA GTT CTA CAG TCC GAC GAT C (***NOT included in the TGIRT®-III kit)

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 (***NOT included in the TGIRT®-III kit)
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. 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 (50 mM; made fresh or from frozen aliquots)	2 μl (5mM)
RNA sample[a]	1[b]-50 ng or <100 nM
10 x mix of R2 RNA/R2R DNA heteroduplex and TGIRT®-III enzyme	2 μl (100 nM R2 RNA/R2R DNA; 500 nM TGIRT®-III enzyme)
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). An optional second MinElute cleanup step (which can be done on the same MinElute column used in the first cleanup step) may be useful to further decrease R2R DNA contamination of cDNAs in experiments that start with small amounts of RNA templates. For alternative size selection step, see Section VI.

8. Proceed with 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 microfuge 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 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 an Bioanalyzer with a High Sensitivity DNA Analysis Kit (Agilent, Cat. No. 5067-4626).

VI. Optional size-selection step after the template-switching reaction

For RNA-seq of specific size classes of RNAs, an optional size-selection step can be added by 5' end-labeling the cDNA products with phage T4 polynucleotide kinase and [γ-^{32}P]-ATP after NaOH treatment and MinElute cleanup (see steps 4-7 in Section II). The labeled cDNAs are then purified in a denaturing polyacrylamide gel, as described in Katibah et al. (2014), Shen et al. (2015) and Zheng et al. (2015). After PAGE purification, the cDNA is ligated to a 5'-end adenylated R1R DNA, followed by PCR amplification with the overlapping multiplex and barcode primers that add all the sequences necessary for Illumina sequencing, as described above in Section III, IV and V.

VII. References

Bazzini A, Viso FD, Moreno-Mateos MA, Johnstone TG, Vejnar CE, Qin Y, Yao J, Khokha MK, and Giraldez AJ. Codon identity regulates mRNA stability and translation efficiency during the maternal-to-zygotic transition. The EMBO Journal *35*, 2087-103, 2016.

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