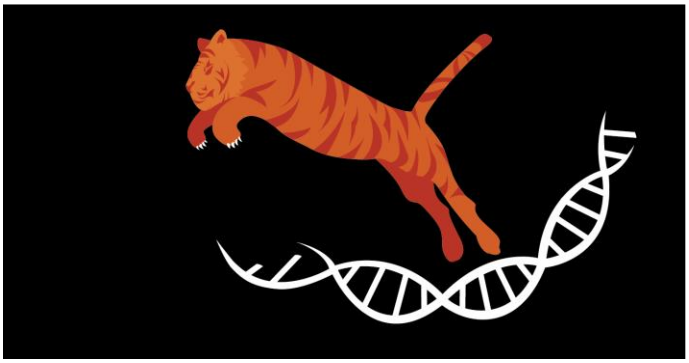

Katibah G, Qin Y, Sidote D, Yao J, Lambowitz AM, and Collins K. Broad and adaptable RNA structure recognition by the human interferon-induced tetratricopeptide repeat protein IFIT5. Proc Natl Acad Sci USA *111*, 12025-30, 2014.

Mohr S, Ghanem E, Smith W, Sheeter D, Qin Y, King O, Polioudakis D, Iyer VR, Hunicke-Smith S, Swamy S, Kuersten S, and Lambowitz AM. Thermostable group II intron reverse transcriptase fusion proteins and their use in cDNA synthesis and next-generation RNA sequencing. RNA *19*, 958-70, 2013.

(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 small RNA/CircLigase protocol, which can be used for RNA-seq of miRNAs, 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 the TGIRT template-switching reaction (Mohr et al. 2013).

Small RNA/CircLigase RNA-seq protocol using the stand-alone TGIRT®-III enzyme

The TGIRT®-III enzyme initiates from a synthetic RNA/DNA heteroduplex consisting of a 41-nt RNA oligonucleotide that contains primer binding sites for both the Illumina Read 1 (R1) and Read 2 (R2) sequences and is annealed to a complementary 42-nt DNA primer that leaves a single-nucleotide 3' overhang (an equimolar mixture of A, T, G, and C, denoted N). The RNA oligonucleotide has a blocked 3' end (*e.g.*, 3'SpC3, IDT) to inhibit template-switching to that end, and the DNA primer is 5'-end labeled with T4 polynucleotide kinase (Epicentre, Cat. No. P0503K) and [γ-^{32}P]-ATP, so that the cDNA products containing the linked DNA primer can be purified from a denaturing polyacrylamide gel. After gel electrophoresis, the cDNAs are size-selected and then eluted from gel slices using D-Tube™ Dialyzers Maxi (Novagen, Cat. No. 71509-3), or other methods preferred by the users, and precipitated in the presence of carrier (glycogen or linear acrylamide). The purified cDNAs are circularized using CircLigase II (Epicentre, Cat. No. CL9021K) and amplified by PCR 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.

I. RNA and DNA oligonucleotide sequences (PAGE or HPLC purified; NOT included, order separately by the users):

R1R2 RNA: 5'- rArGrA rUrCrG rGrArA rGrArG rCrArC rArCrG rUrCrU rArGrU rUrCrU rArCrA rGrUrC rCrGrA rCrGrA rUrC/3SpC3/ (Other blockers such as 3' Amino Modifier C6 dT (3AmMC6T) from IDT are also effective)
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.

R1R2 comp DNA: 5' GAT CGT CGG ACT GTA GAA CTA GAC GTG TGC TCT TCC GAT CTN (N = equimolar A, T, G, C)

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. R1R2 RNA/comp DNA annealing reaction

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

Components	Volume (final concentration)
10 x reaction buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA)	2 µl (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)
10 µM 5'-end labeled R1R2 comp DNA	2 µl (1 µM)
10 µM R1R2 RNA	2 µl (1 µM)
Nuclease free water	To 20 µl*****

*****The annealing reaction above contains labeled R1R2 RNA/comp DNA heteroduplex at a final concentration of 1 µM for use in a total number of 10 template-switching reactions. The volume of the annealing reaction can be adjusted proportionally for the number of template-switching reactions needed.

2. Incubate at 82°C for 2 minutes in a thermocycler.

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

III. 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 stock)	2 µl (5 mM)
1 µM labeled R1R2 RNA/comp DNA heteroduplex	2 µl (100 nM)
RNA sample[a]	<100 nM
TGIRT®-III enzyme (10 µM; InGex)	1 µl (500 nM final)[b]
Nuclease-free water	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]The final concentration of TGIRT®-III enzyme can be adjusted.

2. Pre-incubate at room temperature for 30 minutes, then add 1 µl 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. Size select the cDNA products on a denaturing PAGE.

7. After size selection, the cDNA is eluted from the gel using D-Tube™ Dialyzers Maxi (Novagen, Cat. No. 71509-3), followed by precipitation (0.3 M sodium acetate, pH 5.2 in ethanol) with 10-25 μg/ml linear acrylamide (Thermo Fisher Scientific, Cat. No. AM9520) or glycogen (Thermo Fisher Scientific, Cat. No. 10814010) carrier.

8. Proceed with CircLigase II ligation and Phusion PCR amplification (cDNAs <500 nt).

IV. CircLigase II ligation (Epicentre, Cat. No. CL9021)

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

Components (from Epicentre)	Volume
10 x Reaction buffer	2 μl
50 mM $MnCl_2$	1 μl
PAGE-purified cDNA from template-switching reaction	Up to 12 μl
CircLigase II ssDNA ligase	1 μl
5 M betaine	4 μl
Nuclease free water	To 20 μl if using less than 12 μl cDNA

2. Incubate at 60°C for 1 hour or overnight.

3. Incubate at 80°C for 10 minutes to inactivate the enzyme.

4. Clean up by precipitation (0.3 M sodium acetate, pH 5.2 in ethanol) with 10-25 μg/ml linear acrylamide (Thermo Fisher Scientific, Cat. No. AM9520) or glycogen (Thermo Fisher Scientific, Cat. No. 10814010) carrier.

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 CircLigase II 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 15 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. References

Katibah G, Qin Y, Sidote D, Yao J, Lambowitz AM, and Collins K. Broad and adaptable RNA structure recognition by the human interferon-induced tetratricopeptide repeat protein IFIT5. Proc Natl Acad Sci USA *111*, 12025-30, 2014.

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