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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(Please reference these papers for use of the method)

Small RNA/CircLigase RNA-seq method

In this method, the TGIRT® reverse transcriptase initiates from a synthetic template-primer substrate consisting of a 41-nt RNA oligonucleotide that contains primer binding sites for both the Illumina Read 1 and Read 2 sequences and is annealed to a complementary 42-nt DNA primer that leaves a single-nucleotide 3' overhang (an equimolar mixture of A, C, G, or T). The Read 1 sequence corresponds to the small RNA sequencing primer site used in the NEBNEXT Small RNA Library Prep Set for Illumina. The RNA oligonucleotide has a blocked 3' end (*e,g.*, 3'SpC3 or 3AmMC6T, IDT) to inhibit template switching to that end, and the DNA primer is 5'-end labeled with T4 polynucleotide kinase (Epicentre P0503K) and [γ-^{32}P]ATP, so that the cDNAs containing the linked primer can be purified from a denaturing polyacrylamide gel. After gel electrophoresis, the cDNAs are eluted from gel slices using D-Tube™ Dialyzers Maxi, MWCO 6-8 kDa (Novagen 71509-3) and precipitated in the presence of carrier (glycogen or linear acrylamide). The cDNAs are then circularized using CircLigase II (Epicentre CL9021K) and amplified by PCR using Phusion DNA polymerase (Thermo Fisher Scientific F531S) with overlapping multiplex and barcode primers that add all the sequences necessary for Illumina sequencing.

RNA and DNA oligonucleotide sequences (order PAGE or HPLC purified):

R1/R2 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)

R1/R2 comp DNA: 5' GAT CGT CGG ACT GTA GAA CTA GAC GTG TGC TCT TCC GAT CTN (N = equimolar A, C, G, T)

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).

Protocol for template/primer annealing

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

Components	Volume
10 x reaction buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA	3 μl
10 μM R1/R2 DNA	3 μl
10 μM R1/R2 RNA	3 μl
Nuclease free water	To 30 μl

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

3. Cool down to 25°C with a 10% ramp.

Protocol for template-switching reaction using TGIRT®-III enzyme

1. Set up the following reaction components in a sterile microfuge tube adding RNA sample and 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)
10 x labeled R1/R2 template-primer substrate	2 μl (100 nM)
RNA sample	> 20 ng optimal or <100 nM
TGIRT®-III RT (10 μM stock; InGex)	1 μl (500 nM final)
Nuclease-free water	To 19 μl

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

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

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, since 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 cDNAs on a 6% denaturing PAGE.

7. After size selection, the cDNA is eluted from the gel using D-Tube™ Dialyzers Maxi, MWCO 6-8 kDa (Novagen 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 AM9520) or glycogen (Thermo Fisher Scientific 10814010) carrier.

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

Protocol for the CircLigase II ligation (CircLigase II ssDNA ligase, Epicentre CL9021)

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

Components (from Epicentre)	Volume
10 x Reaction buffer	2 μl
50 mM $MnCl_2$	1 μl
Gel-purified cDNAs	0.5 pmol/μl
CircLigase II	1 μl
5 M betaine	4 μl
Nuclease free water	To 20 μl

2. Incubate at 60°C for 1 hours 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 AM9520) or glycogen (Thermo Fisher Scientific 10814010) carrier.

5. Proceed with Phusion PCR amplification.

Protocol for PCR amplification (Phusion High-Fidelity PCR Master Mix with HF buffer, Thermo Fisher Scientific F531S)

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

Components	Volume
2x Phusion High-Fidelity PCR Master Mix	25 μl
10 μM Illumina Mutliplex primer	1 μl
10 μM Illumina Barcode Primer	1 μl
cDNA from ligation	Up to 23 μl
Nuclease-free water	To 50 μl if using less than 23 μl cDNA

2. PCR cycles:
   i. 98°C 5 sec, 1 cycle
   ii. 10-15 cycles of 98°C 5 sec, 60°C 10 sec, 72°C 15-30 sec/kb, hold at 4°C

3. Clean up the library with 1.3-1.4X Agencourt AMPure XP beads (Beckman A63880) to get rid of primer dimer products and submit for sequencing. The ratio of AMPure beads can be adjusted depending on the size profile of PCR products.

4. To check library quality and quantity, analyze 1 μl on an Bioanalyzer with a High Sensitivity DNA Analysis Kit (Agilent 5067-4626). TGIRT template-switching reaction to a commercial RNA ladder or other RNA standards can be carried through the procedure as a positive control.

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