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 gel-purified cDNAs are circularized with CircLigase II (Katibah et al. 2014; Shen et al. 2015; Zheng et al. 2015), and the other for RNA-seq of total RNAs without size selection (Qin et al. 2016; Nottingham et al. 2016). Here, we describe the total RNA-seq method, which is used in the InGex 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 (ss) DNA-seq (Wu and Lambowitz, manuscript in preparation).

Total RNA-seq method

For preparation of RNA-seq libraries from pools of RNAs or RNA fragments without size selection, the TGIRT template-switching reaction is carried out as described in the protocol below for 5 to 60 minutes depending on the size and extent of modification of the RNAs being reverse transcribed. The initial template-primer substrate in the InGex kit consists of a 34-nt RNA containing an Illumina Read 2 sequence (R2 RNA) with a 3' blocker (3' SpC3, IDT) annealed to a complementary 35-nt DNA oligonucleotide (R2R) to leave a single nucleotide 3' overhang (an equimolar mixture of A, C, G or T, denoted N). The InGex kit contains the annealed template-primer substrate bound to TGIRT®-III enzyme (a derivative of GsI-IIC reverse transcriptase; Mohr et al. 2013). After cDNA synthesis and clean-up, the cDNA is ligated to an adenylated DNA oligonucleotide containing the complement of an Illumina Read 1 sequence (R1R; purchased separately) using a thermostable ligase (Thermostable 5' AppDNA/RNA ligase, NEB M0319S) and then amplified with the overlapping multiplex and barcode primers, as described in the protocol. The Read 1 sequence corresponds to small RNA sequencing primer site used in the NEBNEXT Small RNA Library Prep Set for Illumina.

RNA and DNA oligonucletoide sequences (Note – The InGex kit contains annealed R2 RNA/R2R DNA template-primer substrate bound to TGIRT®-III reverse transcriptase (a derivative of GsI-IIC reverse transcriptase). The R1R DNA oligonucleotide must be purchased separately). The purchased oligonucleotide should be PAGE or HPLC purified.

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, C, G, T)

R1R DNA: 5' /5Phos/GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG/3SpC3/ (not included in the InGex template-switching kit)

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 InGex template-switching 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 InGex template-switching kit).
*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

****NOTE: If using stand-alone enzyme and separately purchased template-primer oligonucleotides instead of the InGex kit.**

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 R2R DNA	3 μl
10 μM 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

****NOTE: If using stand-alone enzyme and separately purchased template-primer oligonucleotides instead of the InGex kit.**

1. Set up the following reaction components in a sterile microfuge tube or PCR 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 aliquots)	2 μl (5 mM)
10 x annealed R2 RNA/R2R DNA (1μM)	2 μl (100 nM)
RNA sample	1*-50 ng or <100 nM
TGIRT®-III RT (10 μM; InGex)	1 μl (500 nM)
Nuclease-free H_2O	to 19 μl

*Low RNA concentrations should be measured by Qubit or Bioanalyzer.

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. 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 28204) or a MinElute PCR Purification Kit (QIAGEN 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 clean-up step (which can be done on the same MinElute column used in the first clean-up step) may be useful to further decrease primer contamination of cDNAs in experiments that start with small amounts of RNA sample. Agencourt AMPure XP beads (Beckman A63880) can be used as an alternative for clean-up of longer cDNAs instead of the MinElute kit.

8. Proceed with thermostable ligation step (Thermostable 5' App DNA/RNA Ligase, NEB M0319S; all sizes, recommended because of high efficiency) and PCR amplification.

Protocol for template-switching reaction using TGIRT®-III template-switching kit (InGex kit)

1. Set up the following reaction components in a sterile microfuge tube or PCR tube adding RNA sample and enzyme/template-primer mix 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	1*-50 ng or <100 nM
10 x TGIRT®-III RT/template-primer substrate mix	2 μl (500 nM TGIRT®-III RT; 100 nM template-primer)
Nuclease-free H_2O	to 19 μl

*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 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. 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 28204) or a MinElute PCR Purification Kit (QIAGEN 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 clean-up step (which can be done on the same MinElute column used in the first clean-up step) may be useful to further decrease primer contamination of cDNAs in experiments that start with small amounts of RNA sample. Agencourt AMPure XP beads (Beckman A63880) can be used as an alternative for clean-up of long cDNAs instead of the MinElute kit.

8. Proceed with thermostable ligation step (Thermostable 5' AppDNA/RNA Ligase, NEB M0319S; all sizes, recommended because of high efficiency) and PCR amplification.

Protocol for oligo adenylation of R1R DNA (5' DNA Adenylation Kit, NEB E2610S)

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

Components	Volume
10 x reaction buffer	2 μl
1 mM ATP	2 μl
100 μM R1R DNA, 5'p/3'SpC3 (IDT, PAGE-purified)	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 the adenylated R1R DNA with an Oligo Clean & Concentrator™ (Zymo Research D4060), and elute in 10 μl double-distilled water to give a final concentration of 10 μM R1R DNA.
   **Note: Doing multiple adenylation reactions in separate PCR tubes and then combining reactions for clean-up is recommended because higher elution volume helps with consistent and efficient recovery of adenylated oligos.

5. Proceed with the thermostable ligation.
   R1R DNA: 5'-/5Phos/GAT CGT CGG ACT GTA GAA CTC TGA ACG TGT AG/3SpC3/-3'

Protocol for thermostable ligation (Thermostable 5' AppDNA/RNA Ligase, NEB M0319S)

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

Components	Volume
10 x reaction buffer (NEBuffer 1)	2 μl
50 mM $MnCl_2$	2 μl
cDNA from template-switching	Up to 10 μl
Thermostable 5' AppDNA/RNA Ligase	2 μl
10 μM R1R DNA, 5'App/3'c3SpC3	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 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 PCR tube:

Components	Volume
2x Phusion High-Fidelity PCR Master Mix*	25 μl
10 μM Illumina multiplex 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

*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. 8-15 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.

Optional size-selection step using TGIRT®-III template-switching kit (InGex kit)

For RNA-seq of specific size classes of RNAs (e.g., miRNAs, tRNAs, gel-purified RNA fragments), an optional size-selection step can be added to the InGex kit by 5' end-labeling the cDNAs with phage T4 polynucleotide kinase and [γ-^{32}P]ATP after NaOH treatment and MinElute clean-up of the cDNAs (see above). 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 gel-purification, the cDNA is ligated to an adenylated DNA oligonucleotide complementary to Read 1 sequences (R1R; purchased separately) using a thermostable ligase (Thermostable 5' App DNA/RNA Ligase, NEB M0319S), followed by amplification with the overlapping multiplex and barcode primers using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Life Technologies, F-531S), as described above.

An alternative method for TGIRT-seq of small RNAs, in which cDNAs are size-selected via denaturing PAGE and circularized with CircLigase II, requires a different initial template-primer substrate than is provided in the InGex kit and is described separately (Small RNA/CircLigase protocol).

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.

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.

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.

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.

Shen PS, Park J, Qin Y, Li X, Parsawar K, Larson M, Cox J, Cheng Y, Lambowitz AM, Weissman JS, Brandman O, and Frost A. Protein synthesis. Rqc2p and the 60S

ribosomal subunits mediate mRNA-independent elongation of nascent chains. Science *347*, 75-78, 2015.

Zheng G, Qin Y, Clark WC, Dai Q, Yi C, He C, Lambowitz AM, and Pan T. Efficient and quantitative high-throughput tRNA sequencing. Nature Methods *12*, 835-837, 2015.