RNAConnect

Full-length RNA library preparation using the uMRT template switching reaction

1. INTRODUCTION

UltraMarathonRT (uMRT) undergoes an efficient and highly selective form of template switching that can be used to introduce a specific primer binding site to the 5'-end of RNAs within a library. This activity is initiated by a useful “side reaction”, in which uMRT catalyzes nontemplated addition of three adenosines (AAA) to the 3'-end of cDNA primer extension products. This ‘AAA’ overhang behaves as a “sticky end”, enabling base-pairing of a complementary template switching oligonucleotides (TSO) that terminate in ‘TTT’. Using the TSO as its new template, uMRT then adds a section of known sequence to the 3' end of the resulting cDNA. In this way, cDNA products are tagged at each end, enabling efficient PCR amplification and RNA-seq library preparation.

Importantly, reverse transcription and template switching use different reaction conditions for optimal performance. In this protocol, we describe a two-step reaction that carries out reverse transcription and template switching in successive stages, allowing implementation of optimal reaction conditions for each step in the process.

2. INFORMATION ON KEY REAGENTS USED IN THIS PROTOCOL

2.1 Custom oligonucleotides:

Oligo-dT primer, TSO and primer used for full-length cDNA preamplification.

- uMRT-dT_{18}(RT primer): CCCTCTCTCTCTCTTTCCTCTCTCTTTTTTTTTTTTTTTTT
- uMRT-TSO (TSO): CCCTCTCTCTCTCTTTCCTCTCTCTTTT
- AmpPCR (primer for cDNA amplification): CCCTCTCTCTCTCTTTCCTCTCTC

Note: the TSO is a proprietary DNA oligonucleotide that has been chemically modified to promote highly efficient template switching reaction while reducing TSO concatemerization and nonspecific extension.

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2.2 RT buffer (2x)

2.3 Template switching buffer (5x)

2.4 uMRT Boost:

20x concentration.

Note: The template switching buffer cannot be used alone for the template switching reaction. It must be combined with the RT buffer in the proportions indicated in order to promote optimal template switching reaction conditions.

3. STEP-BY-STEP PROCEDURE FOR CDNA LIBRARY PREPARATION USING UMRT TEMPLATE SWITCHING

3.1 Anneal RT primers to RNA templates:

- Combine the components as indicated in the table below to a nuclease-free PCR tube.
- Mix gently by tapping the tube. Collect the contents by brief centrifugation. Incubate at 95°C for 30 sec and then snap cool on ice to anneal the primer to the template.

Components		Final amount	Volume
Primer	uMRT-dT_{18} (5 µM)	2.5 pmol	0.5 µL
Template RNA	Total RNA	1 ng–20 ng	variable
	poly(A)-RNA	0.1 ng–20 ng	variable
dNTP mix stock, 10 mM each (e.g., NEB, Cat# N0447S)		0.5 mM final concentration	0.5 µL
Nuclease-free water			added to total 3 µL

3.2 Prepare the RT reaction mix:

- Combine the components as indicated in the table below in a nuclease-free PCR tube.
- Mix the contents gently by tapping the tube and collect the contents by brief centrifugation.

Components	Volume
Reaction Buffer (2x)	5 µL
UltraMarathonRT (20 U/µL)	0.5 µL
RNaseOUT™ (40 U/µL) (optional) (e.g. Thermo Fisher, Cat# 10777019)	0.5 µL
uMRT Boost (20x)	0.5 µL
Nuclease-free water	0.5 µL
Total volume	7 µL

3.3 Carry out the reverse transcription reaction:

- Add the RT reaction mix (7 µL, prepared in Section **3.2**) to the annealed primer and RNA template (3 µL, prepared in Section **3.1**) to make a 10 µL reaction.
- Mix gently by tapping the tube and collect the contents by brief centrifugation.
- Incubate the mixture at 42°C for 45 minutes and then hold the tubes at 4°C in a PCR thermocycler. Use a heated lid (55°C) to avoid water evaporation during the reaction. Do not inactivate the enzyme.

3.4 Prepare the template switching reaction mix:

- Combine the components as indicated in the table below in a nuclease-free microcentrifuge tube (for a total volume of 10 µL).
- Mix the contents gently by tapping the tube and collect the contents by brief centrifugation.

Components	Volume
5x template switching buffer	4 µL
UltraMarathonRT (20 U/µL)	2 µL
uMRT-TSO (10 µM)	2 µL
dATP (10 mM) (e.g., NEB, Cat# N0440S)	2 µL

3.5 Carry out the template switching reaction

- Add the template switching reaction mix (10 µL, prepared in Section **3.4**) to the RT reaction tube (containing 10 µL, from Section **3.3**) to make a 20 µL reaction.
- Mix gently by tapping the tube and collect the contents by brief centrifugation.
- Incubate at 42°C for 45 minutes to catalyze the template switching reaction and then 95°C for 1 min to inactivate the uMRT enzyme. Hold the tube at 4°C in a PCR thermocycler. Use a heated lid (55°C) to avoid water evaporation during the reaction.

The resulting cDNA that contains adapters at both ends can be stored at -20°C until use.

4. PRE-AMPLIFY THE FULL-LENGTH CDNA USING KAPA HIFI DNA POLYMERASE (ROCHE, CAT# KK2102)

4.1 Prepare the PCR amplification mix:

- Add the components as indicated in the table below in a nuclease-free microcentrifuge tube.
- Mix the contents gently by tapping the tube and collect the contents by brief centrifugation.

Components	Volume
Unpurified cDNA product (from Section **3.5**)	20 µL
PCR primer AmpPCR (20 µM)	10 µL
5x GC enhanced buffer	20 µL
dNTP mix (10 mM each)	3 µL
KAPA HiFi DNA polymerase (1 U/µL)	2 µL
Nuclease-free water	45 µL
Total volume	100 µL

4.2 Run the below PCR program in a thermocycler:

Cycle	Denature	Anneal	Extend	Hold
1	98 °C, 2 min	–	–	–
12 - 15*	98 °C, 15 s	62 °C, 30 s	72 °C, 6 min	–
1	–	–	72 °C, 5 min	–
	–	–	–	4 °C

*12 cycles are suggested for 10 ng total RNA or 1 ng mRNA input. 15 cycles are suggested for 1 ng total RNA or 0.1 ng of mRNA.

The preamplified cDNA library should be purified by (e.g. AMPure XP beads, Beckman Cat# A63881) or stored at -20°C until use.

5. OPTIMIZATION AND TROUBLESHOOTING

Potential Issue	Likely Cause	Solution
DNA yields are low after PCR amplification	RNA quality is low, which results in low cDNA yield	Use high quality RNA
	Not enough PCR cycles	Consider using more PCR cycles for cDNA amplification
	PCR efficiency is low	Consider using all cDNA product (20 µL from Section **3.5**) for a 100 µL PCR reaction
Pre-amplified DNA products are short	RNA quality is low (fragmented)	Use high quality RNA and check your RNA input
	Template switching buffer is used for reverse transcription	Make sure the correct reaction buffers are used for reverse transcription and template switching reaction

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