cDNA library production by OTTR using low-cycle PCR for indexing

Use pipettes designed for low-volume measurements (e.g. a p2). If low-volume pipettes are not available, please double all volumes listed below for greater accuracy.

Sequential buffer additions to the OTTR reaction tube may be done at room temperature or on ice; ice should be used when performing many reactions in parallel.

STEP 1 Label template 3' ends using ddRTPs.

a. Adjust input single-stranded nucleic acid to 9 µL using sterile water.*
b. Add 2 µL Buffer 1A.
c. Add 1 µL Buffer 1B.
d. Add 1 µL **Tail Pol**.
e. Lightly mix and quick-spin.
f. Incubate at **30°C** 90 min.
g. Add 1 µL Buffer 1C.
h. Lightly mix and quickly spin.
i. Incubate at **30°C** 30 min.
j. Incubate at **65°C** 3-5 min, then place on ice.

Safe to stop; store at -20°C.

STEP 2 Eliminate unincorporated ddRTPs.

k. Add 1 µL Buffer 2
l. Add 1 µL diluted **rSAP**
m. Incubate at **37°C** 15 min, then place on ice.

STEP 3 Heat-neutralize phosphatase.

n. Add 1 µL Buffer 3.
o. Lightly mix and quick-spin.
p. Incubate at **65°C** 5 min, then place on ice.

Safe to stop; store at -20 ℃.

STEP 4 cDNA synthesis.

q. Add 1 µL Buffer 4A.**
r. Add 1 µL Buffer 4B.
s. Briefly vortex then quick-spin to ensure good mixing of viscous buffers.
t. Add 1 µL **Relay Pol**.
u. Lightly mix and quick-spin. *Place on ice if not rapidly transferred to 37°C.*
v. Incubate at **37°C** 15 min.
w. Incubate at **65°C** 5 min, then cool.

** Input RNA should not be strongly buffered and should lack >0.1 mM chelating agent (e.g. EDTA)*

*** **Buffer 4A is viscous**; check that the entire tube contents is collected & well mixed at the bottom of the kit buffer tube, and watch your pipet tip to not bring extra volume into OTTR reactions.*

EXAMPLE OF OPTIONAL cDNA CLEAN UP **Reagents not provided**

a. Add 0.5 μL RNase H (NEB Thermostable) + 0.5 μL RNase A (Sigma).

b. Incubate at 50°C ~10 min.

c. Add 80 μL stop mix (50 mM Tris pH 8, 20 mM EDTA, 0.2% SDS).

d. Extract with equal volume phenol:chloroform:isoamyl alcohol (25:24:1).

e. Move the upper aqueous phase to a new tube containing 10 μL 3 M NaOAc pH 5.2 and 20 μg glycogen. Mix. Add 350 μL 100% ethanol. Mix.

f. Chill in dry ice bath 2-3 min. Spin 15 min at max microfuge speed. Remove supernatant and wash pellet with 1 mL 70% ethanol. Spin again.

g. Remove all supernatant and dry pellet for 5 min. Resuspend for next use.

EXAMPLE OF PCR INDEXING FOR ILLUMINA NGS **Reagents not provided**

For 50 μL PCR reactions, make a master mix combining for each reaction:

16.5 μL sterile water

10 μL 5X Q5 buffer (NEB)

1 μL 10 mM dNTP mix

1 μL 10 μM P5(i5 index)R1 primer

1 μL 10 μM P7(i7 index)R2 primer (complement strand)

0.5 μL Q5 polymerase (NEB)

Add 30 μL master mix above to 20 μL cDNA + sterile water

Amplify in PCR machine with heated lid:
98°C, 1 min
8X Cycle (increase cycles for very low input)
98°C, 20 sec
65°C, 20 sec
72°C, 10 sec
72°C, 5 min

ILLUMINA NGS READ ANALYSIS

Read 1 orientation is input 5′ to 3′ with the first 7 nucleotides giving the adaptor template unique molecular identifier (UMI).
Read 2 orientation is input 3′ to 5′ with the first nucleotide from the adaptor primer 3′ end.

BUFFER COMPONENTS

Buffer 1A	140 mM Tris-HCl (pH 7.5), 1 M KCl, 14 mM DTT, 35% PEG-8000
Buffer 1B	28 mM $MnCl_2$, 3.5 mM ddATP
Buffer 1C	3.5 mM ddGTP
Buffer 2	80 mM $MgCl_2$
Buffer 3	100 mM EGTA
Buffer 4A	10 mM $MgCl_2$, 900 mM KCl, 40% PEG-6000; 4 mM dGTP, 0.8 mM dTTP, 0.8 mM dCTP, 0.04 mM dATP, 3 mM DAP-TP
Buffer 4B	Adaptor oligonucleotide mix: 1.8 μM annealed primer duplex mix **U5PD**, 3.6 μM adaptor template **U3T**

ADAPTOR OLIGONUCLEOTIDES

U5PD oligos c5pUNI_T (primer)
5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
c5pUNI_C (primer)
5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCC
c5pc (primer complement strand)
rGrArUrCrGrGrArArGrAmGmCmAmCmAmCmGmUmCmUmG
mAmAmCmUmCmCmAmGmU/3SpC3/

U3T oligo c3tUNI-UMI* (cDNA 3’ adaptor template)
5Cy5/ACACTCTTTCCCTACACGACGCTCTTCCGATCTrNrNrNr
NrNrYrC

ENZYMES

Tail Pol	BoMoC(ed) F753A	10 µM	AddGene Plasmid #185710
Relay Pol	BoMoC(ed) 10 µM		AddGene Plasmid #186461
rSAP	NEB M0371S diluted 1 vol: 1 vol into 50% glycerol, final 0.5 U/μL		

For reference:

Upton HE, Ferguson L, Temoche-Diaz MM, Liu XM, Pimentel SC, Ingolia NT, Schekman R, Collins K. *Low-bias ncRNA libraries using ordered two-template relay: Serial template jumping by a modified retroelement reverse transcriptase.* ***PNAS*** 118: e2107900118 (2021).

Pimentel SC, Upton HE, Collins K. *Separable structural requirements for cDNA synthesis, nontemplated extension, and template jumping by a non-LTR retroelement reverse transcriptase.* ***J Biol Chem*** 298: 101624 (2022).