OTTR cDNA Library Workflow ***Store all reagents at -20°C until use***

Input can be less than 10 pg to a maximum of 10 ng of a pool of size-selected <100 nt RNAs. If RNA lengths in the input sample are predominantly much greater than the small RNAs of interest (e.g. using total RNA or RNA from purified ribosomes), up to 40 ng input RNA can be used. If you exceed recommended RNA input, the cDNA library will be biased by preferential capture of some RNA 3' ends. RNA samples should lack chelating agents like EDTA or EGTA. Do not use more >10 µL input per reaction. If all of your input samples are less than 10 µL, a consistent needed volume of water can be pre-mixed with Buffer 1A. Add the volume of extra water + 2 µL Buffer 1A at step 1 below.

Shortly before use, place Buffer tubes on ice. Collect liquid at the bottom of each tube by quick centrifugation and mix by pipetting or very gentle vortexing. If there is a precipitate, use a well-suspended slurry. Be aware that Buffers 1A and 4A are particularly viscous; they can be brought to room temperature before use for easier pipetting. We strongly recommend using a P2 or other low-volume pipette for this protocol. When pipetting liquid from the tube, visually check for the intended volume in the pipet tip and inspect the outside of the pipet tip for unwanted clinging liquid.

If the kit has a tube labeled MIR1, it is a 22 nt positive control miRNA oligonucleotide. Running a positive control reaction in parallel with each sample set is useful to generate a reference lane for cDNA size selection. Other sizes of small RNA can be substituted (e.g., 30 and 40 nt oligonucleotides for ribosome profiling with P1 nuclease). Use no more than 0.9 pmoles RNA as input (e.g. 9 µL of 100 nM stock).

It is safe to pause the protocol after any 65°C incubation by placing tubes at -20°C.
Reagents are not provided for cDNA purification or PCR indexing of NGS libraries.

Step 1: label each template 3' end using ddATP

- Resuspend input nucleic acid in 10 µL sterile, nuclease-free water.
- Add 2 µL **Buffer 1A**. (Bring Buffer 1A to room temperature shortly before use.)
- Add 1 µL **Buffer 1B2**. (OK to pre-mix 1A+1B2 immediately before use)
- Add 1 µL **Tail Pol.** Mix by pipetting. Incubate 30°C, 120 min.
- Add 1 µL **Buffer 2**. (OK to pre-mix Buffer 2 and Phosphatase)
- Add 1 µL **Phosphatase**. Mix by pipetting. Incubate 37°C, 15 min.
- Add 1 µL **Buffer 3**. Incubate 65°C, 5 min. Quick spin to collect liquid.

Step 2: cDNA library synthesis

- Add 1 µL **Buffer 4A**. (Bring Buffer 4A to room temperature shortly before use.)
- Add 1 µL **Buffer 4B2**. (OK to pre-mix 4B2+Pol, but DO NOT pre-mix 4A+4B2)
- Add 1 µL **Relay Pol**. Mix by pipetting. Incubate 37°C, 20 min; then 65°C, 5 min.

Reagents for Ordered Two-Template Relay (OTTR)

Reagent composition and volumes used per 20 µL reaction:

2 μL	Buffer 1A	140 mM Tris-HCl pH 7.5, 1 M KCl, 14 mM DTT, 35% PEG-8000
1 μL	Buffer 1B2*	10 mM Na acetate pH 5.5, 28 mM $(NH_4)_2SO_4$, 28 mM $MnCl_2$, 3.5 mM ddATP
1 μL	Buffer 2	80 mM $MgCl_2$
1 μL	Buffer 3	100 mM EGTA
1 μL	Buffer 4A	10 mM $MgCl_2$, 900 mM KCl, 4 mM dGTP, 0.8 mM dTTP, 0.8 mM dCTP, 0.04 mM dATP, 3 mM dDAP-TP; 40% PEG-6000 (optional)
1 μL	Buffer 4B2*	Oligonucleotides: 1.8 μM primer duplex, 3.6 μM 3′ adaptor template
1 μL	rSAP	1:1 dilution of shrimp alkaline phosphatase (rSAP) from New England Biolabs.
1 μL	Tail Pol	
1 μL	Relay Pol	

**Comments:*

Original Buffer 1B manganese oxidized over time. Low pH of Buffer 1B2 inhibits oxidation.

Original Buffer 1C to chase ddA 3′ labeling with ddG is unnecessary with improved ddA labeling.

Original Buffer 4B

(a) +1C overhang primer was incompatible with some commercial i7 multiplexing primers, and increased adaptor dimer formation. Buffer 4B2 contains only +1T overhang primer.
(b) primer 5′ dye was changed from Cy5 to IR800 for discrimination from 5′-Cy5 adaptor template and to enable cDNA library gel purification at higher sensitivity.
(c) 3′ adaptor template ribonucleotides were replaced by deoxynucleotides, and the 3′ end was changed to R_ddC for increased template jumping precision (R = 50:50 A:G).

Buffer 4B2 oligonucleotides, **with changes in bold**

- **IR**_c5pUNI_T (34 nt DNA)

5IRD800/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

- c5pc (10 nt RNA, 20 nt 2′OMethyl RNA)

rGrArUrCrGrGrArArGrAmGmCmAmCmAmCmGmUmCmUmGmAmAmCmUmCmCmAmGmU/3SpC3

- **RddC**_c3tUNI-UMI (39 nt DNA, 1 ddC)

5Cy5/ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNR/3ddC**

Original Buffer 4B oligonucleotides

- c5pUNI_C (34 nt DNA)

5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCC

- c5pUNI_T (34 nt DNA)

5Cy5/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

- c5pc (10 nt RNA, 20 nt 2′OMethyl RNA)

rGrArUrCrGrGrArArGrAmGmCmAmCmAmCmGmUmCmUmGmAmAmCmUmCmCmAmGmU/3SpC3

- c3tUNI-UMI (33 nt DNA, 7 nt RNA)

5Cy5/ACACTCTTTCCCTACACGACGCTCTTCCGATCTrNrNrNrNrNrYrC