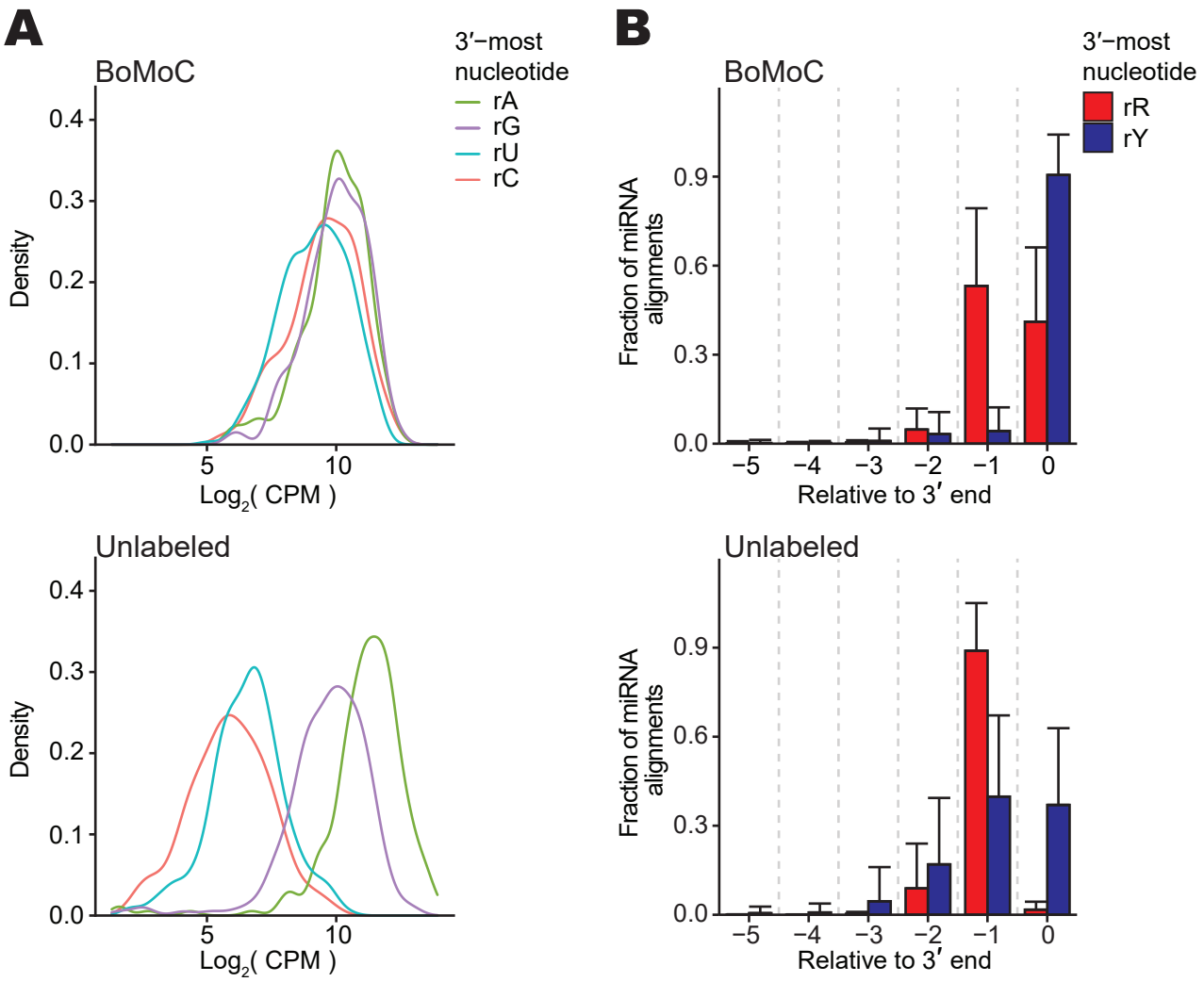
SI Figure 1

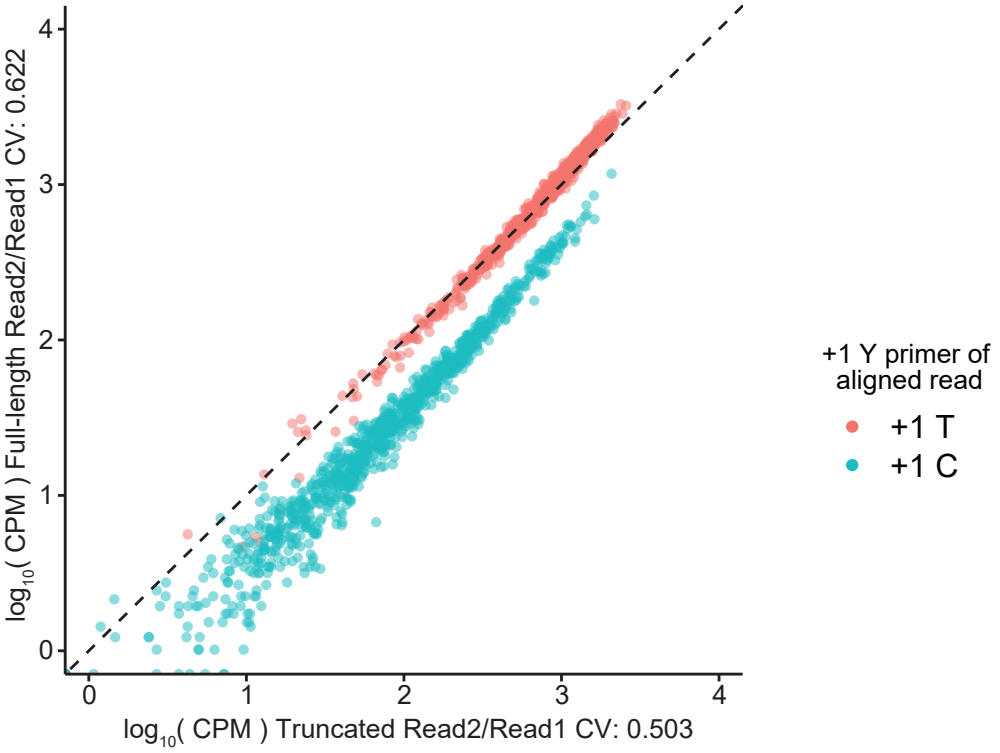


Supplemental Figure 1: Comparison of 3′ precision with and without the step of input RNA 3′ labeling.

A, Estimated kernel-density distribution of $\log_2$ CPM of miRNAs grouped by 3′ RNA nucleotide, with (top) or without (bottom) 3′ labeling.

B, Mean fraction and standard deviation of alignment coverage to miRNA 3′ ends grouped by 3′ rR (red) and 3′ rY (blue), with (top) or without (bottom).

SI Figure 2

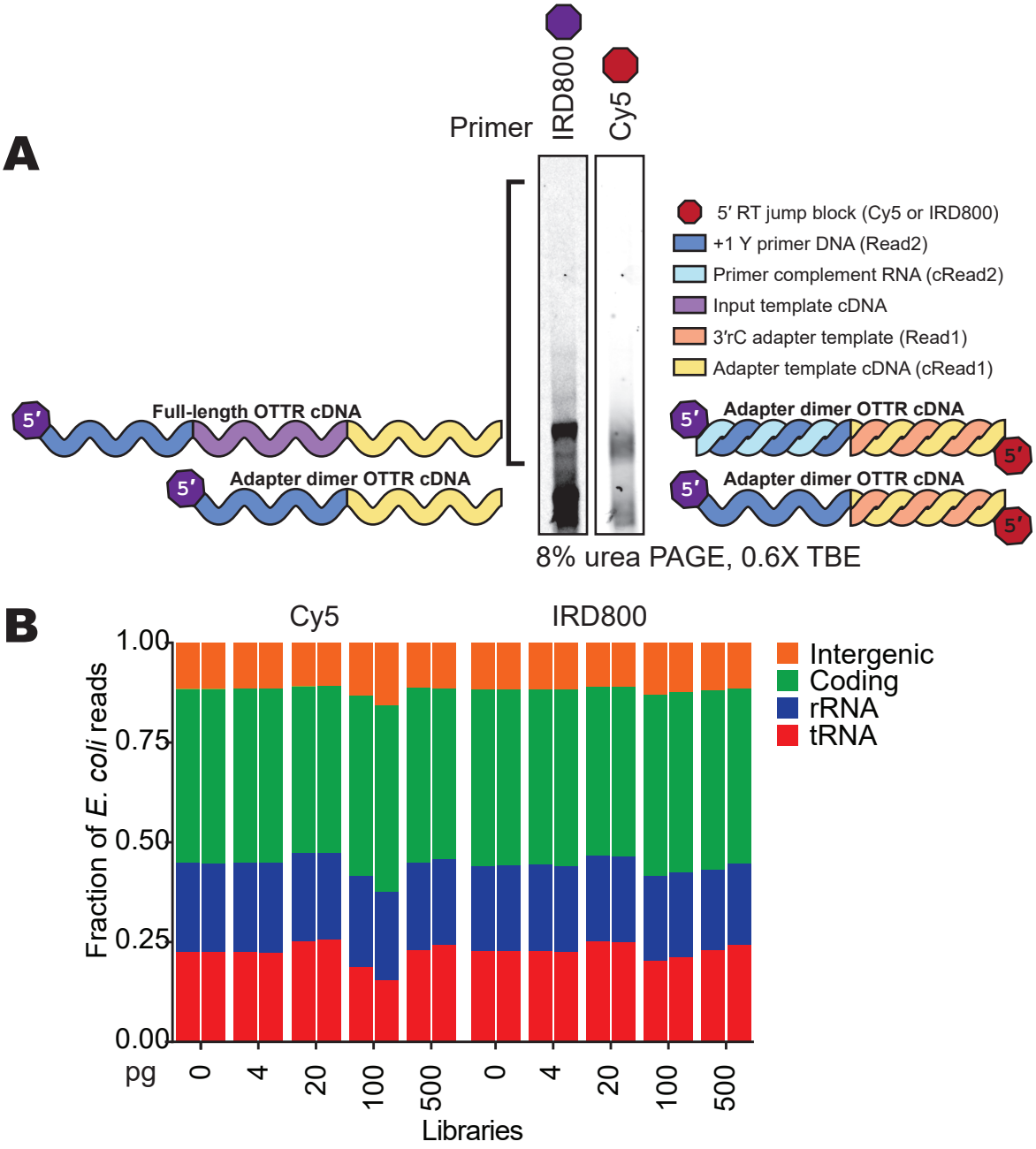


Truncated Read2/Read1 indexing primer pair example
CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTG
AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGAC

Full-length Read2/Read1 indexing primer pair example
CAAGCAGAAGACGGCATACGAGAT[i7]GTGACTGGAGTTCAGACGTG**TGCTCTTCCGATC*T**
AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGAC**GCTCTTCCGATC*T**

Supplemental Figure 2: Under-amplification of cDNA initiated using primer duplex +1 C using some primer sets for library PCR.
Comparison of miRNA CPM based on how the input RNA was captured, *i.e.*, either by +1 T primer duplex or +1 C primer duplex (**Table 3**, +1T.v2.Cy5 and +C.v2.Cy5). Replicate cDNA libraries were split and PCR-amplified by either full-length (n=2) or truncated (n=2) Read1 and Read2 indexing primers (examples below the graph, with the portions of the Read1 and Read2 sequences excluded from the truncated primer set shown in bold). The [i7] and [i5] indicate the index sequences (not shown).

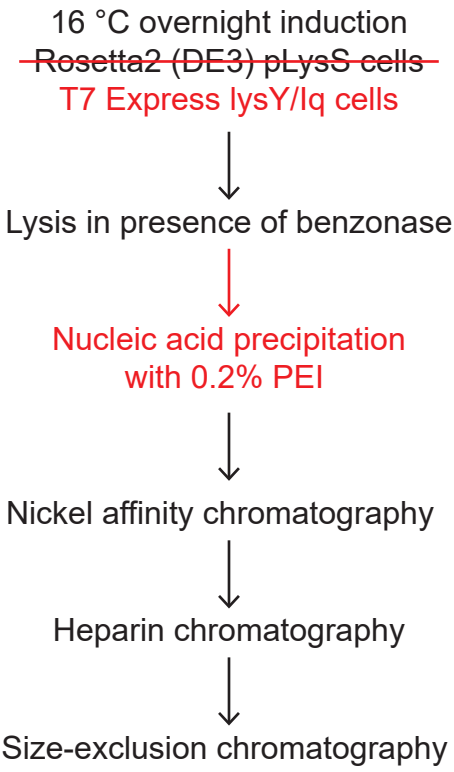
SI Figure 3



Supplemental Figure 3: dPAGE migration used for size-selection and results for subsequent sequencing of unwanted OTTR products.

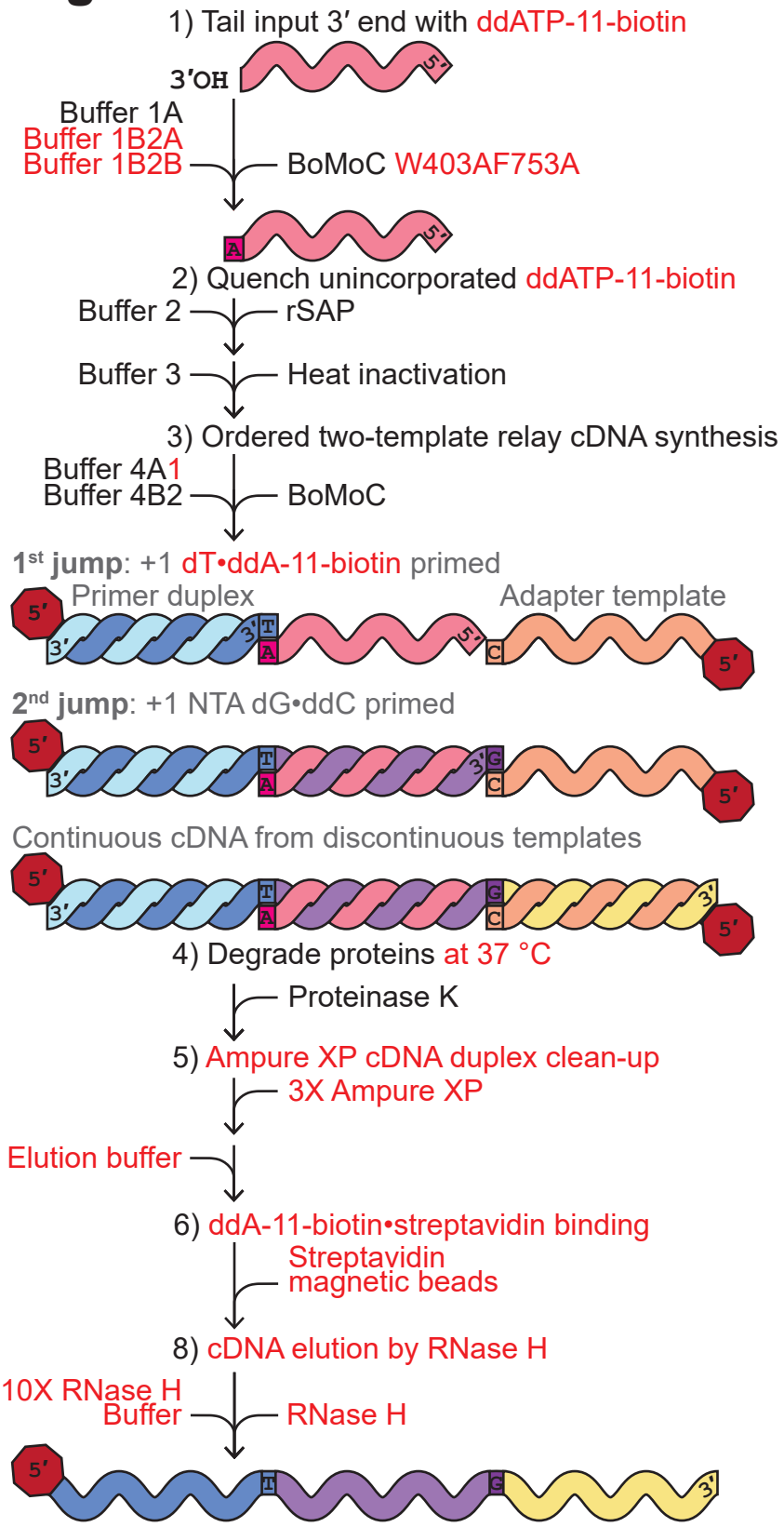
A, A single OTTR cDNA library constructed with a 5′ IRD800 primer fluorophore (from lane 10, **Fig. 5A**) was resolved by 8% dPAGE and imaged for both IRD800 and Cy5 emission. cDNA library product size-selection range is denoted by the left open bracket (black). Adapter-dimer and miRXplore OTTR cDNA products from primer (**Table 2**, +T.v2.IR and +C.v2.IR) with IRD800 emission signal are indicated with schematics. The Cy5 signal (red octagon) from the adapter template (**Table 5**, c3t_CY5N_6RNA) can have a slower migration than expected due to apparent reannealing with cDNA. This is schematized for adapter-dimer cDNA on the right. **B**, Subcategories of read mapping for the *E. coli* nucleic acid category of libraries in **Figure 5C**.

SI Figure 4



Supplemental Figure 4: Flowchart describing BoMoC purification steps. Modifications introduced in this work are added in red text, and a red line is drawn through previous features of the protocol that were replaced.

SI Figure 5



Buffer recipes

Input (9 µL)
Up to 0.9 pmole template in water

Buffer 1A (2 µL)
140 mM Tris-HCl pH 7.5, 1 M KCl,
14 mM DTT, 35% PEG-8000

Buffer 1B2A (1 µL)
28 mM MnCl2, 28 mM $(NH_4)_2SO_4$,
10 mM sodium acetate pH 5.5

Buffer 1B2B (1 µL)
1.0 mM ddATP-11-biotin

Buffer 2 (1 µL)
80 mM $MgCl_2$

Buffer 3 (1 µL)
100 mM EGTA

Buffer 4A1 (1 µL)
15 mM $MgCl_2$, 900 mM KCl,
15% PEG-6000, 4 mM dGTP,
1.5 mM dDAP-TP, 3.2 mM dCTP,
6.4 mM dTTP, 0.04 mM dATP

Buffer 4B2 (1 µL)
1.8 µM +1 T DNA/RNA.v2.1
primer duplex, 3.6 µM
3′ddCRNNNNN adapter template

Washing/Binding Buffer
500 mM NaCl, 20 mM Tris-HCl
pH 7.5, 1 mM EDTA

Other reagents & enzymes
Ampure XP
Hydrophilic Streptavidin
Magnetic Beads
10X RNase H Buffer
rSAP
Proteinase K
RNase H

Figure legend

- Unlabeled template
- ddA-11-biotin labeled template
- 5′ RT jump block (Cy5 or IRD800)
- +1 T primer DNA (Read2)
- Primer complement RNA (cRead2)
- Input template cDNA
- Template cDNA w/3′G NTA
- 3′ddC adapter template (Read1)
- Adapter template cDNA (cRead1)

Supplemental Figure 5: Workflow for gel-free, automation-compatible OTTR library synthesis
Starting from the optimized gel-based OTTR v1.3 conditions (**Figure 1**), which are indicated in black or gray text, OTTR v2 workflow differences are indicated in red text and mark-out of black text with a red line. The left side explains the steps, while the right side gives buffer recipes.

OTTR v1.3 Protocol
Ordered Two-Template Relay
Features of the OTTR v1 series relative to other OTTR protocols:

- Input 3′ labeling using ddATP
- Recommended cDNA size selection by gel electrophoresis and IRD800 imaging

Description
This OTTR v1 update introduces TurboTail Pol, engineered from the previously used Tail Pol for improved 3′-labeling activity.

Also included is a protocol for AMPure XP bead clean-up of OTTR cDNA product duplexes. AMPure XP is a convenient cDNA purification alternative to organic extraction and precipitation or column-based purification for input amounts near the upper limit (see below). If AMPure XP reagents are not available, other purification approaches can be used prior to gel electrophoresis (see below).

Note that OTTR protocols preferentially capture input nucleic acids with a 3′-terminal 3′ hydroxyl (3'OH) group.[1]

Input for OTTR
Recommended maximal input is limited by adapter oligonucleotide concentrations in the standard reaction volume. Do not use more than 0.9 pmol of input nucleic acid per final 20 µL reaction, equivalent to 5.8 ng of ~20 nt miRNA. Table 1 below can be used to evaluate whether an input sample should be diluted prior to an OTTR reaction, *i.e.*, if it exceeds the "Upper Limit" amount. If a sample is less than the "Lowest Limit" amount, there may be >10% of sequencing reads derived from cDNA synthesis on the trace *E. coli* nucleic acid contaminants that co-purify with OTTR polymerases. As a guideline for maximizing productive sequencing reads, we recommend using input amounts within the range of Upper Limit to "Lower Limit".

Table 1: OTTR Input Calculations

	Upper Limit	**Lower Limit**	**Lowest Limit**
	0.9 pmol	20 fmol (.02 pmol)	3 fmol (.003 pmol)
Input size (nt)	Mass (ng)	Mass (ng)	Mass (ng)
20	5.8	0.13	0.02
30	8.7	0.19	0.03
40	12	0.26	0.04
50	15	0.32	0.05
60	17	0.39	0.06
70	20	0.45	0.07
80	23	0.52	0.08
90	26	0.58	0.09
100	29	0.64	0.10

[1] For capture of input nucleic acids independent of the nature of their 3′-end, a different OTTR-based protocol should be implemented.

OTTR reagents (per reaction) **PROVIDED IN KIT**

2 µL	Buffer 1A	140 mM Tris-HCl pH 7.5, 1 M KCl, 14 mM DTT, 35% PEG-8000
1 µL	Buffer 1B2	3.5 mM ddATP, 10 mM Na acetate pH 5.5, 28 mM $(NH_4)_2SO_4$, 28 mM $MnCl_2$
1 µL	Buffer 2	80 mM $MgCl_2$
1 µL	Buffer 3	100 mM EGTA
1 µL	Buffer 4A2	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; 15% PEG-6000
1 µL	Buffer 4B3	Oligonucleotides: 1.8 µM primer duplex, 3.6 µM 3′ adapter template
1 µL	rSAP	1:1 mixture of shrimp alkaline phosphatase (rSAP) from New England Biolabs and 50% glycerol.
1 µL	TurboTail Pol	
1 µL	Relay Pol	

Oligonucleotide sequences

Buffer 4B3 Primer duplex:

IR_c5pUNI_T (34 nt DNA)

5IRD800/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

c5pc_v1.1 (17 nt RNA, 16 nt 2′-O-Methyl RNA)

rGrArUrCrGrGrArArGrAmGmCmAmCmAmCmGmUrCrUrGrArArCrUmCmCmAmGmUmCmAmC/3SpC3/

Buffer 4B3 3′ adapter template:

RddC_c3tUNI-UMI (39 nt DNA, 1 ddC)

5Cy5/ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNR/3ddC

OTTR cDNA purification reagents **OPTIONAL, NOT PROVIDED**

Which of these reagents to use is dependent on the choice of sample clean-up procedure

Proteinase K (PK) (ThermoFisher Scientific, EO0491)

RNase A (Sigma Aldrich, 10109142001)

Thermostable RNase H (New England Biolabs, M0523S)

AMPure XP (Beckman Coulter, A63880)

Oligo Clean and Concentrate column (Zymo Research, D4061)

Phenol:chloroform:isoamyl alcohol (25:24:1) at near pH 6.7

Glycogen 20 mg/mL (ThermoFisher Scientific, R0551)

100%, 80%, 70% (v/v) ethanol in nuclease-free water

PCR multiplexing reagents **NOT PROVIDED**

5X Q5 buffer and Q5 polymerase (New England Biolabs, M0491S)

10 mM dNTP solution mix (New England Biolabs, N0447S)

NEBNext® Multiplex Oligos for Illumina® (New England Biolabs, E6609S)

OTTR reaction

Step 1: label each template 3' end using ddATP

1. Resuspend or dilute up to 0.9 pmol nucleic acid in 10 µL nuclease-free water [2].
2. Add 2 µL **Buffer 1A**[3].
3. Add 1 µL **Buffer 1B2**.
4. Add 1 µL **Turbo Tail Pol**.
5. Mix by pipetting. Incubate 30°C, 120 min.
    - Safe to stop after this step if stored at -80°C.
6. Add 1 µL **Buffer 2**[4].
7. Add 1 µL **rSAP**[4].
8. Mix by pipetting. Incubate 37°C, 15 min.
9. Add 1 µL **Buffer 3**.
10. Mix by vortex. Incubate 65°C, 5 min.
11. Immediately cool on ice, then do a quick spin to collect liquid from lid.
    - Safe to stop here if reaction is stored at -80°C

Step 2: cDNA library synthesis

Do not use temperatures greater than 37°C, to maintain primer duplex annealing. Do not vortex to mix. Continue to maintain RNase-free working conditions.

1. Add 1 µL **Buffer 4A2**[5].
2. Add 1 µL **Buffer 4B3**[6].
3. Add 1 µL **Relay Pol**[6]. The final reaction volume is now 20 µL.
4. Mix by pipetting. Incubate 37°C, 30 min.
    - Safe to stop after this step if stored at -80°C.

OTTR cDNA purification

Step 3: Post-cDNA synthesis reaction clean-up

Option 1: cDNA duplex purification by AMPure XP

1. Add 1 µL **Proteinase K**.
2. Mix by pipetting. Incubate 37°C, 15 min.
    - Safe to stop after this step if stored at -80°C.
3. Add 63 µL (3X) of pre-warmed **AMPure XP beads**[7]. Mix well and incubate at room temperature for 10 min.

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[2] Low concentrations of stabilizing agents for nucleic acid purification in neutral pH buffers will not interfere. However, metal-chelator concentration should be minimal.
[3] Bring Buffer 1A to room temperature shortly before use for accurate pipetting.
[4] Buffer 2 and rSAP can be combined as a master-mix.
[5] Buffer 4A2 needs to be pre-warmed to room temperature and mixed by repeated pipetting using a filter-tipped pipette to ensure that the dNTPs are entirely solubilized.
[6] Buffer 4B3 and Relay Pol can be combined as a master-mix.
[7] Pre-warm AMPure XP beads to room temperature for 30 min. prior to use.

4. Bind **AMPure XP beads** with magnetic tube-rack and wash the beads three times with 200 µL of freshly prepared **80% ethanol**[8,9]. Allow 30 sec. between each wash step to allow beads to rebind to the magnet.
5. After washing, completely remove all traces of **80% ethanol** and let the beads airdry for up to 10 min.
6. Add 10-20 µL (see step 8 below) nuclease-free water to dried **AMPure XP beads**, remove sample tube from magnet, resuspend beads, and incubate for 10 min.
7. Bind **AMPure XP beads** with magnetic tube-rack and transfer eluent to a pre-labeled tube.
8. Most cDNA pools, especially those with input closer to the Lower Limit than Upper Limit (see Table 1), benefit from cDNA size-selection to remove adaptor dimer. This can be done using denaturing (d) or native (n) PAGE.
    - For cDNA size-selection by 8% dPAGE (see below), add an equal volume of 2X dPAGE loading buffer (95% formamide, 0.02% (w/v) bromophenol blue, 0.002% (w/v) xylene cyanol, 5 mM EDTA). We recommend eluting in 10 µL in step 6 above so the final volume is 20 µL. Mix by pipetting. Can store at -80°C.
    - For cDNA duplex size-selection by 8-10% native PAGE, add 6X nPAGE loading buffer (*e.g.*, New England Biolabs, B7024S) to a final concentration of 2X. Mix by pipetting. Can store at -80°C.

Option 2: cDNA purification by Zymo Clean and Concentrate

1. Add 1 µL of **RNase A** and 1 µL **Thermostable RNase H**.
2. Mix by pipetting. Incubate 50°C, 15 min.
3. Add 1 µL **Proteinase K** and 27 µL of 0.1% (w/v) SDS, 50 mM Tris-HCl pH 7.5, 20 mM EDTA[10]. The final volume is now 50 µL.
4. Mix by pipetting. Incubate 50°C, 15 min.
5. Perform **Zymo Oligo Clean and Concentrate** as described in the manufacturer's protocol. At the end, elute in 6 µL of nuclease-free water, and then elute again with 8 µL of 2X dPAGE loading buffer (95% formamide, 0.02% (w/v) bromophenol blue, 0.002% (w/v) xylene cyanol, 5 mM EDTA). Pool both eluents.
6. Proceed to gel purification (see below) or store at -20°C.

Option 3: cDNA purification by organic extraction

1. Add 1 µL of **RNase A** and 1 µL **Thermostable RNase H**.
2. Mix by pipetting. Incubate 50°C, 15 min.
3. Add 1 µL **Proteinase K** and 77 µL of 0.1% (w/v) SDS, 50 mM Tris-HCl pH 7.5, 20 mM EDTA. The final volume is now 100 µL.
4. Mix by pipetting. Incubate 50°C, 15 min.
5. Incubate 95°C, 5 min.

[8] 80% ethanol should be made fresh on the day of the experiment.
[9] Do not remove sample tubes from the magnetic rack during the 80% ethanol washes.
[10] Proteinase K and 0.1% (w/v) SDS, 50 mM Tris-HCl pH 7.5, 20 mM EDTA can be combined as a master-mix. SDS will precipitate if stored <25°C.

6. Transfer to a 1.5 mL tube with 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1). Mix by vortex for 10 sec. Incubate at room temperature for 10 min.
7. Centrifuge at 20,000 x g for 10 min.
8. Transfer the upper aqueous phase to a new tube containing 10 µL 3 M Na acetate pH 5.2 and 20 µg glycogen. Mix by vortex for 10 sec.
9. Add 350 µL 100% ethanol, Mix by vortex for 10 sec.
10. Either chill in dry ice bath for 10 min., flash freeze in liquid nitrogen, or place at -80°C for an hour.
11. Centrifuge at 20,000 x g for 30 min.
12. Remove supernatant and wash pellet with 1 mL 70% ethanol. Vortex for 10 sec.
13. Chill at -20°C for 20 min.
14. Centrifuge at 20,000 x g for 30 min.
15. Remove supernatant completely and let the pellet dry for 5 min.
16. Resuspend in 5 µL 2X dPAGE loading buffer (95% formamide, 0.02% (w/v) bromophenol blue, 0.002% (w/v) xylene cyanol, 5 mM EDTA). Proceed to gel purification (see below) or store at -20°C.

Step 4: Denaturing polyacrylamide gel purification

Reagents: **NOT PROVIDED**

2X dPAGE loading buffer: 95% formamide, 0.02% (w/v) bromophenol blue, 0.002% (w/v) xylene cyanol, 5 mM EDTA
1X TBE, 8% urea denaturing polyacrylamide gel
1X TBE gel running buffer
cDNA elution buffer: 300 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA
Glycogen 20 mg/mL (ThermoFisher Scientific, R0551)
100% and 70% ethanol

Protocol:

Step A: loading, running, and cutting slices from a gel

1. Heat samples in dPAGE loading buffer for 5 min. at 95 °C then place on ice.
2. Pre-run an 8%[11] denaturing urea polyacrylamide gel for 15 min. Rinse the wells of the gel using a needle and syringe immediately before sample loading.
3. Ideally, load a positive control OTTR library in a middle lane to have a size marker. It can reduce cross-contamination to load samples in wells separated by spacer lanes, but the spacer lanes should be loaded with 2X dPAGE loading buffer to promote even sample migration across the gel.
4. Run the gel until the xylene cyanol dye front is near the bottom of the gel. By 1X TBE 8% dPAGE, the xylene cyanol migrates at roughly the same rate as the unwanted adapter dimer cDNA.
5. Disassemble gel and transfer to plastic wrap. Carefully cut the gel above the xylene cyanol dye front and discard the bottom portion of the gel to reduce adapter dimer contamination.

[11] We recommend use of an 8% 19:1 (acrylamide:bis-acrylamide) denaturing 7 M urea polyacrylamide gel. Size-select for cDNA lengths greater than 80 nt to exclude adapter dimer cDNA side products of ~74-77 nt, which using recommended gel conditions co-migrate with xylene cyanol dye.

6. Options for detecting OTTR cDNA:
    a. Direct IR800 detection: capture IR-long using an emission filter of 810 – 840 nm.
    b. SYBR Gold nucleic acid staining: Combine 5 µL of SYBR Gold nucleic acid stain with 5 – 10 mL of 1X TBE and cover the gel with this buffer for no more than 3 min. Agitate the gel while it stains by gentle shaking. Rinse briefly with 1X TBE and remove as much residual buffer from the gel as possible before proceeding. Transfer gel to a scanner and image using a Cy2 emission filter of 515 – 535 nm.
7. Print the scanned gel image at the actual size of the gel with signal adjusted so that it is easy to see cDNA products. Transfer the gel to the printed image and perfectly align.
8. Using a clean razor blade, excise the desired size range of cDNA. Use a clean edge of the blade for each cDNA sample. Transfer gel slice to a pre-labeled tube. Can store at -20°C.
9. Crush the gel slices with a clean 1 mL pipette tip. Add 450 µL of cDNA elution buffer. Incubate at 70°C for 1 hour. Can store at -20°C.

Step B: cDNA recovery from gel slices

10. Centrifuge for 10 min. at maximum speed.
11. Transfer 375 µL of eluate to a new tube. If necessary, centrifuge and transfer again to completely remove residual gel fragments.
12. Add 1 µL of 20 mg/mL glycogen, vortex, then add 3 volumes of 100% ethanol. Split across two tubes if necessary. After adding ethanol, vortex and any remaining gel fragments will immediately become opaque and flocculate. Typically, a narrow pipette tip can be used to fish these bits out before centrifuging, but this can be challenging if your sample tube is completely full.
13. Either chill in dry ice bath for 10 min., flash freeze in liquid nitrogen, or place at -80°C for an hour.
14. Centrifuge at 20,000 x g for 30 min.
15. Remove supernatant and wash pellet with 1 mL 70% ethanol. Mix by vortex for 10 sec.
16. Chill at -20°C for 20 min.
17. Centrifuge at 20,000 x g for 30 min.
18. Remove supernatant completely and let the pellet dry for 5 min.
19. Resuspend cDNA pellet in 20-30 µL nuclease-free water.

cDNA PCR for Illumina sequencing

Step 5: PCR for Illumina P5/P7 addition and indexing

1. Perform qPCR quantification of the library cDNA before library multiplexing PCR amplification[12].

[12] qPCR quantification of purified cDNA using Read1 and Read2 primers can be used to inform how much cDNA to include and how many cycles of PCR amplification to perform. See “cDNA quantification by qPCR” section in the ribosome profiling protocol introduced in Ferguson *et al.,* 2023 (*Nature Methods*) or Section 5.8 in McGlincy *et al.*, 2017 (*Methods*).

2. For 50 µL PCR reactions, make a master mix combining for each reaction:
    16.5 µL sterile water
    10 µL 5X Q5 buffer
    1 µL 10 mM dNTP mix
    1 µL 10 µM P5(i5 index)R1 primer
    1 µL 10 µM P7(i7 index)R2 primer
    0.5 µL Q5 polymerase
2. Add 30 µL master mix to 20 µL cDNA + sterile water.
3. Perform the PCR reaction using a thermocycler with 105°C heated lid: 98°C, 1 min.; 6X or more cycles [98°C, 20 sec.; 65°C, 20 sec.; 72°C, 10 sec.]; 72°C, 5 min.; cool to room temperature.
    - cDNA over amplification will result in “daisy chain” PCR products due to insufficient amounts of primers at later amplification cycles. Use qPCR quantification of the cDNA to precises control both the cDNA input amount and PCR cycle number[13]. Alternatively, empirically determine cycle number by titrating PCR cycle numbers in scaled-down reactions to identify the number of cycles needed to sufficiently amplify cDNA without the formation of “daisy chain” PCR products.

OTTR v2.1 Protocol
Automation-Compatible Ordered Two-Template Relay

- Input 3′ labeling using biotinylated ddATP
- cDNA duplex enrichment from adapters and side products using streptavidin

Description
Libraries made using OTTR version 1 (v1) protocols benefit from cDNA size-selection by denaturing polyacrylamide gel electrophoresis. OTTR version 2 (v2) protocols replace gel-based cDNA size-selection with a biotin-streptavidin interaction that enriches only cDNA duplexes containing an input template. This enrichment removes adapter-only side products in a manner agnostic to input nucleic acid length and RNA versus DNA content. Note that OTTR v2 protocols will capture only input nucleic acids with a 3′-terminal 3′ hydroxyl (3'OH) group.[1]

The OTTR v2 initial step of input nucleic acid 3′-labeling uses a biotinylated dideoxynucleotide. After steps of excess biotinylated nucleotide inactivation by phosphatase treatment and subsequent cDNA duplex synthesis, biotinylated nucleosides are removed by a magnetic bead-based clean-up. Next, cDNA duplexes containing 3′-labeled input nucleic acid are recovered by binding to streptavidin resin, removing unused adapter oligonucleotides and reaction products containing only adapter sequences. After streptavidin binding and washing, RNase H digestion fragments the primer-complement strand of primer duplex and liberates cDNA from input RNA. Subsequent library PCR can use eluted cDNAs with or without additional nucleic acid purification.

The protocol uses reaction incubation times that maximize enzymatic activity. Many reaction steps can be shortened with a modest probability of compromised input capture. If workflow time to completion should be minimized, use the alternative reaction incubation times in the "rapid workflow" adaptation. Stages are indicated at points that the protocol can be paused, if cDNA duplex is stabilized by storage at -80°C.

Input for OTTR
Recommended maximal input is limited by adapter oligonucleotide concentrations in the standard reaction volume. Do not use more than 0.9 pmol of input nucleic acid per final 20 µL reaction, equivalent to 5.8 ng of ~20 nt miRNA. Table 1 below can be used to evaluate whether an input sample should be diluted prior to an OTTR reaction, *i.e.*, if it exceeds the "Upper Limit" amount. If a sample is less than the "Lowest Limit" amount, there may be >10% of sequencing reads derived from cDNA synthesis on the trace *E. coli* nucleic acid contaminants that co-purify with OTTR polymerases. As a guideline for maximizing productive sequencing reads, we recommend using input amounts within the range of Upper Limit to "Lower Limit".

When using input close to the "Lower Limit" amount, consider implementing additional cDNA-duplex washing steps after streptavidin binding. For low-input samples less than 20 fmol, it may be advantageous to size-select the post-PCR sequencing library if streptavidin washing steps have not adequately depleted adapter-only cDNAs.

[1] For capture of input nucleic acids independent of the nature of their 3′-end, a different OTTR-based protocol should be implemented.

Table 1: OTTR Input Calculations

	Upper Limit	**Lower Limit**	**Lowest Limit**
	0.9 pmol	20 fmol (.02 pmol)	3 fmol (.003 pmol)
Input size (nt)	Mass (ng)	Mass (ng)	Mass (ng)
20	5.8	0.13	0.02
30	8.7	0.19	0.03
40	12	0.26	0.04
50	15	0.32	0.05
60	17	0.39	0.06
70	20	0.45	0.07
80	23	0.52	0.08
90	26	0.58	0.09
100	29	0.64	0.10

OTTR reagents (per reaction) **PROVIDED IN KIT**

2 µL	Buffer 1A	140 mM Tris-HCl pH 7.5, 1 M KCl, 14 mM DTT, 35% PEG-8000
1 µL	Buffer 1BAC	10 mM Na acetate pH 5.5, 28 mM $(NH_4)_2SO_4$, 28 mM $MnCl_2$
1 µL	Buffer 1CAC	1 mM biotin-11-ddATP
1 µL	Buffer 2	80 mM $MgCl_2$
1 µL	Buffer 3	100 mM EGTA
1 µL	Buffer 4AC	15 mM $MgCl_2$, 900 mM KCl, 4 mM dGTP, 6.4 mM dTTP, 3.2 mM dCTP, 0.04 mM dATP, 1.5 mM dDAP-TP; 15% PEG-6000
1 µL	Buffer 4B3	Oligonucleotides: 1.8 µM primer duplex, 3.6 µM 3′ adapter template
1 µL	rSAP	1:1 dilution of shrimp alkaline phosphatase (rSAP) from New England Biolabs and 50% glycerol.
1 µL	TurboTail Pol[2]	
1 µL	Relay Pol	

Oligonucleotide sequences

Buffer 4B3 Primer duplex:

lR_c5pUNI_T (34 nt DNA)
5IRD800/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

c5pc_v1.1 (17 nt RNA, 16 nt 2′-O-Methyl RNA)
rGrArUrCrGrGrArArGrAmGmCmAmCmAmCmGmUrCrUrGrArArCrUmCmCmAmGmUmCmAmC/3SpC3/

Buffer 4B3 3′ adapter template:

RddC_c3tUNI-UMI (39 nt DNA, 1 ddC)
5Cy5/ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNR/3ddC

OTTR cDNA duplex purification reagents (per reaction) **NOT PROVIDED**

60 µL	AMPure XP	(Beckman Coulter, A63880)
1 mL	80% ethanol	
50 µL	Binding/Washing Solution	10 mM Tris HCl pH 7.5, 1 mM EDTA, 1 M NaCl
30 µL	Hydrophilic Streptavidin Magnetic Beads	4 mg/mL (New England Biolabs, S1421S)
1 µL	Proteinase K	(ThermoFisher Scientific, EO0491)
2 µL	10X RNase H Buffer	(optional, New England Biolabs, M0297S)
1 µL	RNase H	(optional, New England Biolabs, M0297S)

[2] TurboTail Pol has improved 3′-labeling activity relative to Tail Pol, which is significant in protocols using biotinylated dideoxynucleotide.

PCR multiplexing reagents	**NOT PROVIDED**
5X Q5 buffer and Q5 polymerase	(New England Biolabs, M0491S)
10 mM dNTP solution mix	(New England Biolabs, N0447S)
NEBNext® Multiplex Oligos for Illumina®	(New England Biolabs, E6609S)

OTTR reaction

Step 1: label each template 3' end using biotinylated ddATP

1. Resuspend input nucleic acid in 9 µL sterile, nuclease-free water[3].
2. Add 2 µL **Buffer 1A**[4].
3. Add 1 µL **Buffer 1BAC**[5].
4. Add 1 µL **Buffer 1CAC**[5].
5. Add 1 µL **TurboTail Pol**.
6. Mix by pipetting. Incubate 30°C, 120 min.
    - 60 min. for rapid workflow.
    - Safe to stop after this step if stored at -80°C.
7. Add 1 µL **Buffer 2**[6].
8. Add 1 µL **rSAP**[6].
9. Mix by pipetting. Incubate 37°C, 15 min.
    - 10 min. for rapid workflow.
10. Add 1 µL **Buffer 3**.
11. Mix by vortex. Incubate 65°C, 5 min.
12. Immediately cool on ice, then do a quick spin to collect liquid from lid.
    - Safe to stop here if reaction is stored at -80°C

Step 2: cDNA library synthesis

Do not use temperatures greater than 37°C, to maintain primer duplex annealing. Do not vortex to mix. Continue to maintain RNase-free working conditions.

1. Add 1 µL **Buffer 4AC**[7].
2. Add 1 µL **Buffer 4B3**[8].
3. Add 1 µL **Relay Pol**[8]. The final reaction volume is now 20 µL.
4. Mix by pipetting. Incubate 37°C, 30 min.
    - 15 min. for rapid workflow.
    - Safe to stop after this step if stored at -80°C.

[3] Low concentrations of stabilizing agents for nucleic acid purification in neutral pH buffers will not interfere. However, metal-chelator concentration should be minimal.
[4] Bring Buffer 1A to room temperature shortly before use for accurate pipetting.
[5] Buffer 1BAC and 1CAC can be combined as a master mix, but TurboTail Pol and Buffer 1A cannot be combined as master-mix.
[6] Buffer 2 and rSAP can be combined as a master mix.
[7] Buffer 4AC needs to be pre-warmed to room temperature and mixed by repeated pipetting using a filter-tipped pipette to ensure that the dNTPs are entirely solubilized.
[8] Buffer 4B3 and Relay Pol can be combined as a master mix.

OTTR cDNA duplex purification

Step 3: Purification, enrichment, and elution of full-length OTTR cDNA

1. Add 1 µL **Proteinase K**.
2. Mix by pipetting. Incubate 37°C, 15 min.
    - 5 min. for rapid workflow.
    - Safe to stop after this step if stored at -80°C.
3. Add 63 µL (3X) of pre-warmed **AMPure XP beads**[9]. Mix well and incubate at room temperature for 10 min.
4. Bind **AMPure XP beads** with magnetic tube-rack and wash the beads three times with 200 µL of freshly prepared **80% ethanol**[10,11]. Allow 30 sec. between each wash step to allow beads to rebind the magnet.
5. After washing, completely remove all traces of **80% ethanol** and let the beads airdry for up to 10 min[12].
6. Add 20 µL nuclease-free water to dried **AMPure XP beads**, remove sample tube from magnet, resuspend beads, and incubate for 10 min. at room temperature.
7. Bind **AMPure XP beads** with magnetic tube-rack and transfer eluent to a pre-labeled tube.
    - Safe to stop after this step if stored at -80°C.
8. Wash 30 µL of **Hydrophilic Streptavidin Magnetic Beads** three times with equal volumes of **Binding/Washing Solution**. Allow 30 sec. between each wash step to allow beads to rebind the magnet.
9. After washing the beads, remove the **Binding/Washing Solution**, and then add 30 µL of **Binding/Washing Solution** and 20 µL of eluted OTTR cDNA to the beads.
10. Ensure beads are completely resuspended by pipetting.
11. Rotate for 120 min. at room temperature to bind OTTR cDNA duplexes.
    - 30 min. for rapid workflow.
12. Separate beads with magnet and remove supernatant[13].
13. Perform a single wash with 50 µL of **Binding/Washing Solution**[14].
14. Remove **Binding/Washing Solution** and add 17 µL of water, 2 µL of **10X RNase H Buffer**, and 1 µL of **RNase H**[15].
15. Mix by pipetting and incubate for 30 min. at 37°C.
    - 10 min. for rapid workflow.
16. Incubate at 95°C for 5 min.
17. Separate beads with magnet and transfer eluent to a labeled tube.

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[9] Pre-warm Ampure XP beads to room temperature for 30 min. prior to use.
[10] 80% ethanol should be made fresh on the day of the experiment.
[11] Do not remove sample tubes from the magnetic rack during the 80% ethanol washes.
[12] Failure to completely remove all traces of 80% ethanol before the next step will result in non-specific binding of cDNA duplex to the Hydrophilic Streptavidin Magnetic Beads.
[13] Save the supernatant from this step until it is determined that the experiment was a success. If desired, this supernatant can be used to investigate the efficiency of bead binding, following a step of nucleic acid purification from the supernatant *e.g.*, by Zymo Oligo Clean and Concentrate.
[14] For lower-input <0.1 pmol, especially for inputs <0.02 pmol, an extra wash may be needed to better remove adapter-dimer cDNA duplexes. Size-selection after PCR could be considered if additional washing does not sufficiently reduce adapter-dimer cDNAs.
[15] The water, 10X RNase H Buffer, and RNase H can be combined as a master-mix.

cDNA PCR for Illumina sequencing

Step 4: PCR for Illumina P5/P7 addition and indexing

1. Perform qPCR quantification of the library cDNA before library multiplexing PCR amplification[16].
2. For 50 µL PCR reactions, make a master mix combining for each reaction:

    16.5 µL sterile water

    10 µL 5X Q5 buffer

    1 µL 10 mM dNTP mix

    1 µL 10 µM P5(i5 index)R1 primer

    1 µL 10 µM P7(i7 index)R2 primer

    0.5 µL Q5 polymerase

2. Add 30 µL master mix to 20 µL cDNA + sterile water.
3. Perform the PCR reaction using a thermocycler with 105°C heated lid: 98°C, 1 min.; 6X or more cycles [98°C, 20 sec.; 65°C, 20 sec.; 72°C, 10 sec.]; 72°C, 5 min.; cool to room temperature.
    - cDNA over amplification will result in "daisy chain" PCR products due to insufficient amounts of primers at later amplification cycles. Use qPCR quantification of the cDNA to precises control both the cDNA input amount and PCR cycle number[13]. Alternatively, empirically determine cycle number by titrating PCR cycle numbers in scaled-down reactions to identify the number of cycles needed to sufficiently amplify cDNA without the formation of "daisy chain" PCR products.

[16] qPCR quantification of purified cDNA using Read1 and Read2 primers can be used to inform how much cDNA to include and how many cycles of PCR amplification to perform. See "cDNA quantification by qPCR" section in the ribosome profiling protocol introduced in Ferguson *et al.,* 2023 (*Nature Methods*) or Section 5.8 in McGlincy *et al.*, 2017 (*Methods*).