>Day 0, prepare RNA or DNA marker for size selection.

Index	RNA or DNA
S0	Decade Marker (AM7778 from Life Technologies)
S1	130 nt long RNA or single strand DNA, 5'-OH
S2	190 nt long RNA or single strand DNA, 5'-OH
S3	~760 nt long RNA or single strand DNA, 5'-OH
S4	~820 nt long RNA or single strand DNA, 5'-OH

RNA or DNA (24 μM)	1
T4 PNK buffer	1
T4 PNK	1
ATP (γ-^{32}P, 6000 Ci/mmol, 7 μl/1 mCi, 24 μM)	1
RNase-free H_2O	6
TOTAL	10

Incubate at 37 °C for 1 h.
Add 50 μl water and purify through Micro Bio-Spin P-30 columns (Bio-Rad, 7326250).
Run samples on an 8% Urea-PAGE gel to verify the integrity.

> Day 1, 3' splint ligation.

20x annealing buffer:

HEPES pH 7.5	200 mM
NaCl	1 M
EDTA	20 mM

Mix-1, "Splint Mix":

	1x
KXS009 (100 μM, A-splint)	0.55
KXS010 (5 μM, U-splint)	0.55
KXS018 (50 μM, 3' adapter)	1
20x annealing buffer	1
TOTAL (per ligation reaction)	3.1

Mix-2, "Standard Mix":

	1x
Poly(A) Std Alex Set 6 (1 ng/µl)	0.1
Poly(A) Std Alex Set 7 (1 ng/µl)	0.1
ZF4 mRNA RNA (poly(A)-selected total RNA on 20190621, 18.4 ng/µl, only use this if the sample doesn't have zebrafish RNAs)	0.1
RNase-free H_2O	1.7
TOTAL (per ligation reaction)	2

Note: use at 0.1 ng of each spike-in RNA per 1 µg total RNA in the ligation mix. Scale accordingly when using different amount of total RNA.

Mix the following to a total of 20 µl:

	Volume (µl)
Sample RNA	x
Mix-1	3.1
Mix-2	2
RNase-free H_2O	14.9–x
TOTAL	20

Note: ideally use 0.5~8 µg sample RNA.

Heat the mixture at 65°C for 5 min and slowly cool to 18°C at 0.1°C/sec.

"Master Mix"

	1x
ATP (10 mM)	0.6
10x RNA ligase 2 buffer	3
1 M $MgCl_2$	0.3
RNasin Plus (Promega, N2611)	0.75
RNase-free H_2O	4.85
T4 RNA Ligase 2	1.5
TOTAL	10

Add 10 µl "Master Mix" to each reaction:
Incubate reactions at 18°C for ~16 h.

>Day 2, RNase T1 digestion and gel purification

Add the following the each reaction:

RNase-free H_2O	157
NaCl (5 M)	12
GlycoBlue (Invitrogen, AM9515)	1
Phenol/Chloroform	200

Note: Use phenol/chloroform instead of Trizol because the adaptor is DNA.

Phenol/chloroform extraction and ethanol precipitation on dry ice for 30 min.
Wash the pellet with 70% ethanol once and re-suspend in 10 µl water.

RNase T1 digestion
Note: The optimal RNase T1 concentration is 0.008 U/µl for 1~8 µg input total RNA.
RNase T1 buffer

Sodium Citrate pH 5.0	20 mM
EDTA	1 mM
Urea	7 M

Add 90 µl RNase T1 buffer and re-suspended RNA.
Incubate at 50 °C for 5 min and quick chill on ice for 5 min.
Add 0.8 µl RNase T1 (1 U/µl, Thermo Fisher, AM2283) to each reaction, and incubate at 23 °C for 30 min.
When finished, add 100 µl phenol/chloroform to each reaction for extraction.
Add 250 µl Inactivation/precipitation buffer from RNase T1 kit (make sure the ethanol has been added).
Leave on dry ice for 30 min.
Wash the pellet once with 70% ethanol.
Re-suspend in 12 µl 1x Gel loading buffer II (from the RNase T1 kit).
Resolve a 8% Urea-PAGE gel (500V for 25 min) alongside labelled size markers S1 (130 nt) and S3 (~760 nt).

Cut out gel pieces between the size markers S1 and S3.
Apply each gel piece through a Gel Breaker Tube (IST Engineering, 3388110).
Elute macerated gel pieces in 0.8 ml Elution buffer (300 mM NaCl, 10 mM HEPES pH 7.5), optionally supplemented with 8 µl SUPERase•In (Invitrogen, AM2696).
Leave tubes on a thermal mixer, shaking at 1000~1400 rpm, with 15 sec shaking and 1 min 45 sec on hold, at 23°C overnight (16h).

> Day 3, beads capture, 5' dephosphorylation and 5' ligation
Apply each gel eluate to two Corning Spin-X columns (Corning, 8160). Then combine them.
Add 3 µl Glycoblue and 700 µl isopropanol for precipitation. Precipitate on dry ice for 30 min.

Wash the pellet with 70% ethanol once.
Re-suspend each RNA sample in 100 µl water.

Capture the ligated RNA
2xB&W buffer

Tris-HCl or HEPES, pH 7.5	10 mM
EDTA	1 mM
NaCl	2 M

Prepare Dynabeads M-280 (Invitrogen, 60210) based following the manufacture's protocol (use a proper magnetic stand for handling with the beads):
Use 50 µl beads per reaction.
Wash with 0.5 ml Buffer A (0.1 M NaOH, 50 mM NaCl) twice.
Wash with 0.5 ml Buffer B (0.1M NaCl) twice.
Wash with 0.5 ml 2xB&W buffer twice.
Re-suspend in 2xB&W buffer (twice the original volume, 100 µl per reaction)

Add RNA to the beads (final 200 µl for each sample).
Incubate at 23 °C for 20 min, using a thermal mixer, with 15 sec shaking and 1 min 15 sec on hold.
Wash the beads once with 200 µl 1xB&W buffer.
Wash the beads twice with 200 µl pre-warmed (50 °C 1xB&W buffer).
Wash the beads twice with 200 µl 1xT4 PNK buffer (New England Biolabs, M0201S).

5' phosphorylation
Using a magnetic stand, remove the supernatant from the beads and add the T4 PNK mix:

	1x
T4 PNK buffer (10x)	5
T4 PNK (New England Biolabs, M0201S)	2.5
SUPERase•In (Invitrogen, AM2696)	1.25
ATP (10 mM)	5
RNase-free water	36.25
TOTAL	50

Incubate at 37 °C for 60 min, using a thermal mixer, with 15 sec shaking and 1 min15 sec on hold.
Wash the beads 3 times with 100 µl 1x T4 RNA ligase 1 buffer (New England Biolabs, M0204S).

5' ligation

Using a magnetic stand, remove the supernatant from the beads, and add the ligation mix:

	1x
T4 RNA ligase 1 buffer (10x)	2.5
T4 RNA ligase 1 (New England Biolabs, M0204S)	2.5
ATP (10 mM)	1.2
SUPERase•In (Invitrogen, AM2696)	1.25
5' adapter (200 µM, KXRNA016)	3.3
RNase-free water	14.25
TOTAL	25

Incubate at 23 °C overnight ~16 h, using a thermal mixer, with 15 sec shaking and 1 min 15 sec on hold.

>Day 4, reverse transcription, base hydrolysis and cDNA purification

Reverse transcription

Using a magnetic stand, remove the supernatant from the beads and wash the beads with 200 µl RNase-free water, 3 times.

Remove the supernatant from the beads and add the following mix to the beads:

RT primer (barcoded for each sample, 100 µM)	1
RNase-free water	17
TOTAL	18

Incubate at 65 °C for 5 min and chill on ice.

Add the following mix:

	1x
5x First strand buffer	6
0.1M DTT	1.5
10mM dNTP	1.5
SUPERase•In	1.5
SuperScript III (Invitrogen, 18080044)	1.5
TOTAL	12

Incubate at 50 °C for 60 min, using a thermal mixer, with 15 sec shaking and 1 min 15 sec on hold.

Base hydrolysis

Add 5 µl 1 M NaOH to the beads of each reaction (per 30 µl reaction).

Incubate on a thermal mixer or a heat block (with heated top) at 90 °C for 10 min. (Use foil to wrap the cap to avoid cap from popping when taken out from the thermal mixer)

When finished, place samples on ice and add the following:

1 M HEPES (pH 7.5)	59
NaCl (5 M)	6

Using a magnetic stand, separate the supernatant from the beads and transfer it to a new tube.

Option 1:
Samples can be multiplexed at this stage if the amounts of starting materials are similar. Doing so can reduce number of cDNA samples to purify on gels.
For samples to be sequenced on the same lane, take out half of each sample (50 µl) and combine them.
Add 3 µl Glycoblue. Add 2.5x volume ethanol or 1x volume isopropanol to the combined sample for precipitation.
For the other half of each sample, add 2 µl GlycoBlue and 150 µl ethanol. Keep them in –20°C indefinitely. They can serve as backups and be processed later.

Option 2:
Add 2 µl Glycoblue and 300 µl ethanol to each sample for precipitation.

Precipitate cDNA on dry ice for 30 min or at –20 °C overnight.
Wash the pellet once with 70% ethanol.
Re-suspend each pellet in 10 µl 1x Gel loading buffer II (from the RNase T1 kit).
Run samples on a 6% Urea-PAGE gel (500 V for 20 min) alongside labelled size markers S2 (190 nt) and S4 (~800 nt).

Cut out band between size markers S2 and S4.
Apply each gel piece through a Gel Breaker Tube (IST Engineering, 3388110).
Elute macerated gel pieces in 0.8 ml Elution buffer (300 mM NaCl, 10 mM HEPEs pH 7.5).
Leave tubes on a thermal mixer, shaking at 1000~1400 rpm, with 15 sec shaking and 1 min 45 sec on hold, at 23°C overnight (16h).

>Day 5, PCR test and PCR again for QC
Incubate each eluate sample at 50 °C for additional 60 min, using a thermal mixer, 1000 rpm.
Apply each gel eluate to two Corning Spin-X columns (Corning, 8160). Then combine them.
Add 2 µl Glycoblue and 700 µl isopropanol for precipitation on dry ice for 30 min.
Wash with 70% ethanol once and re-suspend in 30 µl buffer EB (QIAGEN) or 10 mM Tris pH 7.5 or 10 mM HEPES pH 7.5.

PCR amplification test (do not use Phusion)

	1x	10x
5x KAPA buffer	2	20
dNTPs (10 mM)	0.3	3
KAPA HiFi (Roche, 7958897001)	0.1	1
F-primer (KXS080, 10 μM)	0.3	3
R-primer (KXS081, 10 μM)	0.3	3
cDNA	0.2	2
H_2O	6.8	68
TOTAL	10	100

1	95 °C	3 min
2	98 °C	20 sec
3	60 °C	15 sec
4	72 °C	30 sec
5	Go to step 2	x more cycles
6	72 °C	3 min

For each sample, setup 100 μl reaction. Take out 10 μl for each aliquot, a total of 5 aliquots.
Each aliquot has different cycles for PCR, x=11, 13, 15, 17, 19.
Use the remaining part (50 μl) of each sample for another round PCR for QC, after determining the best cycles for PCR (to avoid over-cycling).

Purify second-round PCR products with AMPure XP beads (Beckman, A63881) at a ratio of beads : DNA = 1.2:1, following the manufacturer's protocol.
Repeat this step once more. Elute with 30 μl water or 10 mM Tris pH 7.5 or 10 mM HEPES pH 7.5.
Take 3 μl of cDNA, as well as 10 μl of PCR for QC.
For QC, perform Qubit and Fragment analysis for only the PCR products.
Perform qPCR for both cDNA and PCR products.

>Sample Mix for sequencing
Load 0.72 fmol per lane.
Multiplex samples and precipitate the mixture with ethanol for each lane if necessary.

Read1 primer (for the first 5 random nucleotides and the barcode): KXS078
Read2 and Read3 primer (for 3' UTR and poly(A) tails): KXS079

Buffers for Klenow extension mix

Note that the 3 final buffers have volumes that differ by 1 ml. This is for convenience and serves as a visual check that they are in the correct positions in the HiSeq reagent carousel and also to check that the liquid level has decreased after sequencing, in case there's an issue with the fluidics.

1. Prepare the 2x Klenow Buffer as below

	Volume
Water	4.8 ml
10x NEB Buffer 2 (from the Klenow kit, or New England Biolabs, B7002S)	1.2 ml
Tween 20 (10%, v/v)	12 µl
Total	6 ml

2. Make 1x Klenow Buffer

	Volume
2x Klenow Buffer	3 ml
Water	3 ml
Total	6 ml

3. Make Klenow Reaction Mixture

	Volume
2x Klenow Buffer	2.5 ml
Water	2.39 ml
dTTP (100 mM, Thermo Fisher, #10219012)	2.5 µl
Klenow polymerase (New England Biolabs, M0210L)	100 µl
Total	5 ml

4. Prepare the Wash buffer

	Volume
Tris pH 7.5 (1 M)	80 µL
500 mM EDTA (pH 8.0)	80 µL
Water	3.84 mL
Total	4 mL

>Sequencing

1. Use each 4 µl lane mix to make 125 µl hybridization mix. Then use that entire volume to cluster a flow cell on the cBot cluster generation system. This is not

standard for a sequencing core. To cluster onboard on a HiSeq, larger volumes (and thus more sample) are required, so we have preferred the cBot clustering.

2. Following cluster generation, the standard sequencing primer (KXS078) is hybridized. A total of 12 cycles of standard sequencing-by-synthesis is performed, which first sequenced the 5-nt random region (used to call clusters) and then sequenced the 6-nt barcode region (which required seven cycles).
3. The flow cell was stripped, a second sequencing primer (KX079) was annealed, and two dark cycles were performed in order to extend this primer past the two nucleotides corresponding to the RNA 3′ termini. The custom extension of the primer through the poly(A) tail region with Klenow was then performed with 50 μM dTTP as the only nucleotide present in the reaction.
4. Following the primer extension, 40 cycles of standard sequencing-by-synthesis are performed to complete the read 1, which was generated using two sequencing primers and had a total length of 52 nt (12 cycles before and 40 cycles after the Klenow reaction).
5. The flow cell was then stripped, and the second sequencing primer (KXS079) was used for 255 cycles of standard sequencing-by-synthesis to generate read 2.
6. The XML files used for configuring the HiSeq 2500 are available on GitHub (github.com/coffeebond/PAL-seq).

>Oligos and primers

Name	Sequence	Note
KXS009	GTACATGCTTTTTTTT	A splint for PAL-seq_v3,4
KXS010	GTACATGCATTTTTTT	U splint for PAL-seq_ v3,4
KXS018	/5Phos/GCATGTACATACGGCTGTCTCTTATACACATCTGACGCTGCCGACGA/iBiodT//3ddC/	3' adapter for PAL-seq_ v3,4
197420602/KXRNA016	CAAGCAGAAGACGGCArUrArCrGrArGrArUr(N:25252525)r(N)r(N)r(N)	5' adapter for PAL-seq_ v3,4
KXS068	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**ATCACG**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 1
KXS069	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**CGATGT**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 2
KXS070	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**TTAGGC**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 3
KXS071	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**TGACCA**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 4
KXS072	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**ACAGTG**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 5
KXS073	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**GCCAAT**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 6
KXS074	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**CAGATC**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 7
KXS075	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**ACTTGA**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 8

KXS076	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**GATCAG**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 9
KXS077	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**TAGCTT**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 10
KXS085	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**GGCTAC**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 11
KXS086	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**CTTGTA**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 12
KXS165	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**AGTCAA**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 13
KXS166	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**AGCGCT**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 14
KXS167	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**GCGTCA**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 15
KXS168	AATGATACGGCGACCACCGAGATCTACACTCNNNNN**CACGGT**GCGTCAGATGTGTATAAGAGACAGCC	PAL-seq v4 RT primer with barcode 16
KXS080	AATGATACGGCGACCACCGAGATC	P5 primer
KXS081	CAAGCAGAAGACGGCATACGAGAT	P7 primer
KXS078	AATGATACGGCGACCACCGAGATCTACACTC	PAL-seq v4 read 1 sequencing primer (random region + barcode + extra)
KXS079	GCGTCAGATGTGTATAAGAGACAGCCGTATGTACATGC	PAL-seq v4 read 2 & 3 sequencing primer (poly(A) region and 3'UTR)

Xiang & Bartel, 2021 (PMID: 34213414); Xiang, Ly, Bartel, 2024 (PMID: 38460509)