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PAL-seq V2 protocol

Starting material:

1. **Total RNA** – 25 µg is typical for non-limited samples, we don't have a good sense of the lower limit but have successfully made libraries from what we believe to be relatively small amount of starting RNA from 5-EU timecourse samples.
2. **Poly(A)-tail length standards** – These are necessary for monitoring the sequencing and computational pipelines to call tail lengths. The standards are RNA molecules with defined poly(A) lengths, prepared as in Subtelny et al., 2014 and Eisen et al., 2020. The optimal concentrations for addition to a library are ~0.1 ng/µg.
3. **Gel visualization markers** – Any radiolabeled RNA that has a 3′ A tract of at least 8 As. If performing the splint ligation with a splint designed to ligate to terminal Us, a non-A standard and a standard that contains an A tract and a terminal uridine is recommended. We generally use three markers, all 5'-phosphate labeled: (1) 75 nt RNA with 60 terminal adenosines, (2) 40 nt RNA ending in 29 adenosines and a terminal uridine, and (3) 32 nt RNA of heterogeneous sequence. These markers are used as internal standards to monitor ligation to the 3′ adaptor. They then get removed from the sample during the first gel purification.

Step 1. Splint ligation
This step is identical to the splint ligation of PAL-seq in Subtelny et al., 2014 (in the protocols section of the Bartel lab website), with 2 exceptions. First, we include a splint that can capture terminally uridylated tails, and second, we add a second biotin to the splint adapter because of reports that this increases capture efficiency.

Oligos:

Split adapter: 5'-pAGATCGGAAGAGCACACGTCT-dT•Biotin-dT•Biotin-ddC-3
Poly(A) splint: 5'-TTCCGATCTTTTTTTTT-3'
Poly(A) terminal U splint: 5'-TTCCGATCTATTTTTTT-3'

Protocol:
1. Prepare the ligation mix:

Reagent	Vol (µL)
Water	5.1
Splint adapter (50 µM)	2.3
Poly(A) splint (50 µM)	1.6
Poly(A) terminal U splint (50 µM)	0.32

ATP (10 mM)	1.28
10x RNA ligase 2 buffer	6.4
MgCl2 (100 mM)	6.4
Rnasin Plus	1.6
Total	25

2. Incubate at 22°C for 5 min.
3. While incubation is happening, prepare a standard master mix of the poly(A)-tail length standards and a marker master mix of the hot markers. Add poly(A)-tail length standards and radiolabeled spikes (marker mix), as detailed above. Store these mixes on ice. Make enough marker mix that you can set aside a small amount to run on a gel later with your post-ligation samples
4. Add standard/marker mix to RNA samples and bring the final volume of each sample to 35.8 µL (example below):

Reagent	Vol (µL)
Water	19.8
RNA	10
Standard mix	3
Marker mix	3
Total	35.8

5. Add RNA mixture to ligation mixture, incubate at 22°C for 5 min
6. Add 3.2 µL T4 RNL 2, incubate at 18°C overnight (typically ~12-16 hours)
7. Following ligation, bring the volume up to 400 µL with water and phenol-chloroform extract (with basic phenol), precipitate with 4 µL linear acrylamide as carrier, and then proceed to T1 digestion.

Step 2. T1 digestion

We perform this digestion with more RNase than Subtelny et al., 2014 in PAL-seq because we found that 0.006 U/µL was the lowest concentration we could use to cut almost all molecules of a 720 nt transcript at least once.

Protocol:

1. Spin down ligated RNA and resuspend with 9.6 µL water. Your final volume will be 10 µL, you can set aside 0.5 µL of this to run on a 10% gel to check the ligation.
2. Denature the RNA in 1x RNAse T1 sequence buffer:

Reagent	Vol (µL)
RNA	9.5

RNase T1 sequence buffer	89.5
Total	99

3. Incubate at 50°C for 5 min, then put onto ice.
4. Prepare a 0.6 U/µL dilution of RNAse T1 by diluting RNAse T1 (stock concentration of 1 U/µL) in 1x RNAse T1 sequence buffer.
5. Add 1 µL of 0.6 U/µL RNase T1, mix each sample by inversion immediately after adding RNAse T1. Final concentration of RNAse T1 in reactions is 0.006 U/µL.
6. Briefly spin down samples, then mix gently by flicking tubes or lightly vortexing.
7. Incubate at room temperature for 30 min.
8. Add 300 µL phenol-chloroform pH 7, then add 200 µL water.
9. Complete phenol extraction as normal, ending up with approximately 270 µL of aqueous phase after chloroform extraction.
10. Add 880 µL precipitation/inactivation reagent to aqueous phase along with 5 µL linear acrylamide.
11. Precipitate at -20 C for at least 2 h.

Step 3. Size selection
This gel is cut between 100 nt and 750 nt. The Typically the 750 nt band of Century Plus marker (Thermo Fisher) is too faint to see with a short exposure, so we usually run decade and a radiolabeled 720 nt long RNA as the ladder. Note that the separation is run for only a short amount of time to decrease the amount of gel during the elution.

Protocol:
1. Spin down RNA following T1 digest.
2. Resuspend pellet in 10 µL 1x formamide loading dye. Boil at 85°C for 5 min.
3. Run out on a 6% urea-polyacrylamide gel at 5 W for 10 min then 12 W for 20 min.
4. Visualize then cut between 100 and 750 nt. Shred gel slice then elute overnight at room temperature in 840 µL 0.3 M NaCl.
5. The next day transfer the elution to 2 spin-x columns (Corning), approximately 450-500 µL in each column. Spin through at max speed in a microfuge for 3 min.
6. Combine the two fractions of each sample, then split into two tubes with approximately 400 µL each (often I have about 820 µL total, add ethanol assuming its 400 µL each).
7. Add 5 µL linear acrylamide to each precipitation, and precipitate with ethanol as usual at -20°C for at least 2 h.

Step 4. Capture ligated products on beads.
The ligated and unligated molecules cannot be resolved on a gel, so we use the biotinylated splint adapter to isolate the ligated products and proceed. Once going onto beads there are no pause points until after the reverse transcription. At this point we converge with the original Subtelny et al., 2014 PAL-seq protocol. This is reproduced below.

Protocol:
Resuspend each precipitation in 99.6 µL water and then combine the two precipitations. While resuspending the RNA, batch wash beads to prepare for capture:

1. Aliquot 100 µL M-280 streptavidin Dynabeads (Thermo Fisher) per reaction into a tube. (Note that we have also successfully used MyOne Streptavidin C1 beads from Thermo Fisher, which have a smaller bead diameter).
2. Wash 2x with an equal volume of Solution A.
3. Wash 2x with an equal volume of Solution B.
4. Wash 2x with an equal volume of 2X B&W buffer.
5. Resuspend the beads in twice the starting volume of 2X B&W buffer.
6. Distribute 201 µL of washed beads to a new tube for each reaction.

To the washed beads:

1. Add 200 µL RNA to the beads.
2. Incubate at room temperature for 15 minutes while rotating.
3. Briefly spin the beads down to collect anything that pooled in the lid into the bottom, then magnetize the beads
4. Wash 1x with 400 µL 1X B&W buffer.
5. Wash 2x with 400 µL Wash buffer warmed to 50°C, leaving in wash buffer for 1 min or so before magnetizing.
6. Wash 2x with 400 µL 1X PNK buffer, during the second wash transfer beads to a new tube.

Recipes:

Solution A:
0.1 M NaOH
50 mM NaCl

Solution B:
0.1 M NaCl

2X B&W buffer (Dilute 1:1 for 1X B&W buffer):
10 mM Tris-HCl, pH 7.5
1 mM EDTA
2 M NaCl
0.1% Tween-20

Wash buffer:
10 mM Tris-HCl, pH 7.5
1 mM EDTA

50 mM NaCl

Step 5: 5' kinase reaction

Protocol:
After removing the last wash from the beads in the prior step, resuspend in PNK reaction mixture:

Reagent	Volume (µL)
Water	70
ATP (10 mM)	10
10X PNK buffer	10
SUPERase·In	5
PNK	5
Total	100

Incubate at 37°C for 1 h while rotating.

After incubation:

1. Magnetize beads and remove supernatant.
2. Wash 3x in 100 µL 1X ligase buffer.

Step 6: 5' ligation:
For this ligation we used a mixture of 4 phased 5' adapters that would result in sequencing the cloned products out of phase from each other. We did this because initially during TAIL-seq library preps (Chang et al., 2014) we found that we cloned the 5.8 S rRNA very frequently, and its abundance could result in problems during the sequencing. By using the phased adapters we could diversify the sequencing at each cycle and fixed this issue. For PAL-seq V2 this is not a consideration since no sequencing from the 5' occurs. Both the phased version of the adapter and the unphased version, used for PAL-seq V2, are below. Note that the portion beginning with the random sequence is RNA (denoted using IDT's "r" convention), but the rest is DNA.

Oligos (phasing nucleotides are bold):

Non-phased 5′ adapter (for PAL-seq V2):
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
Tr(N)r(N)r(N)r(N)r(N)r(N)r(N)r(N)

Phased 5′ adapters, mixed at an equal molar ratio prior to ligation (for TAIL-seq, Chang et al., 2014):
Phasing nucleotides are shown in bold.
Phase 1:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
Tr(N)r(N)**rA**r(N)r(N)r(N)r(N)r(N)r(N)

Phase 2:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
Tr(N)r(N)**rGrA**r(N)r(N)r(N)r(N)r(N)r(N)

Phase 3:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
Tr(N)r(N)**rCrGrA**r(N)r(N)r(N)r(N)r(N)r(N)

Phase 4:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC
Tr(N)r(N)**rUrCrGrA**r(N)r(N)r(N)r(N)r(N)r(N)

Protocol:
After removing the last wash from the beads in the prior step, resuspend in the 5'-ligation reaction mixture:

	Volume (µL)
Water	11.05
5' adapter (100 uM)	6.5
10x ligase buffer	2.5
ATP (10 mM)	1.2
Superase in	1.25
T4 RNA ligase 1	2.5
Total	25

Incubate at room temperature for 16 h while rotating.

After incubation:

1. Add 75 µL water to each reaction.
2. Magnetize beads and remove supernatant.
3. Wash 3x with 100 µL water, during last wash transfer beads to a new tube.

Step 7: Reverse transcription

In the RT/PCR that we developed in this protocol, the molecules are first constructed and then the cluster-generating sequences are flipped relative to the cloned insert during the PCR. This is because the libraries are compatible with either paired-end TAIL-seq sequencing or single-end PAL-seq sequencing by a simple change of PCR primers. If you are not interested in running TAIL-seq, the sequences of the 5′ and 3′ adapters can be modified to dispense with the requirement of changing the position of the cluster generating sequences.

Oligos:

RT primer (indexing portion in bold):
CAAGCAGAAGACGGCATACGAGAT**BBBBBB**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA

Indices listed at the end of the protocol.

Protocol:
After removing the last wash from the beads in the prior step, resuspend in the following mixture:

	Volume (µL)
Water	28
RT primer (100 µM)*	2
Total	30

*each sample is given a unique indexing primer based on the samples that will be sequenced in the same lane. This is for sequencing cDNA libraries using TAIL-seq. If you are performing PAL-seq, the molecules are re-barcoded during the PCR step, and so the barcodes introduced here will not be read. The molecules can be barcoded with the same sequence at this step.

Incubate at 65°C for 5 min, then put onto ice.

To the chilled mixture, add:

	Volume (µL)
5X First strand buffer	12.8
0.1 M DTT	6
10X dNTPs	14
Total	32.8

Incubate at 48°C for 3 min, then put onto ice.

Remove 6 µL of the reaction and transfer to a new tube for the no-RT control (I combine all my no-RT controls in a single tube). To the remaining 56.8 µL add 2 µL SuperScript III, mix well, then incubate at 50°C for 90 minutes while rotating.

Following the incubation:

1. Add 10 µL of 1 M NaOH to each reaction. Scale the amount of NaOH for the no-RT sample based on its relative volume.
2. Incubate at 90°C for 10 min.
3. Magnetize beads and then transfer the supernatant to a new tube.
4. Add 50 µL 1 M HEPES-NaOH, pH 7
5. Bring volume up to 350 µL with water, add 39 µL 3 M NaOAc, pH 5.2, 5 µL linear acrylamide and 1 mL 100% Ethanol.
6. Mix well then precipitate at -20°C for at least 2 h.

Step 8: cDNA purification

This gel is cut from ~160 to ~810 nt. Decade (Thermo Fisher; used to cut just above the 150mer) and a radiolabeled 720 nt long RNA are used to gauge these sizes. It's approximate, though, and it's important just to be consistent between libraries that will be compared.

Protocol:

1. Spin down RNA from prior step.
2. Resuspend in 6 µL 1x formamide loading dye.
3. Run out sample on a thin 6% urea-polyacrylamide gel at 2 W for 10 min then 6 W for 20 min.
4. Excise from 160 to 810 nt and shred the gel slice.
5. Elute in 420 µL 0.3 M NaCl overnight at room temperature while rotating.
6. The following day, transfer elutions to a 50°C thermomixer and shake for 1 h
7. Transfer elutions to a spin-x column (Corning) and spin through at max speed for 4 min.
8. Add 5 µL linear acrylamide and 1 mL of ethanol to each elution, precipitate at -20°C for 2 h.
9. Resuspend the precipitated cDNA in 20 µL of water and store at -20°C. These libraries cannot be sequenced as cDNA using PAL-seq V2 but can be sequenced as TAIL-seq libraries. You have to swap the cluster generating sequences using PCR in the next step if you want to run PAL-seq V2.

Step 9: PCR amplification

This is to swap the cluster generating sequences. We generally do 6–15 cycles but I'd suggest doing at least 6 cycles so that the molecules with the correct cluster sequences account for the vast majority of what gets loaded onto the flow cell.

Oligos (indexing portion in bold):

Flipped p7 primer
AATGATACGGCGACCACCGAGATCTACACGTGACTGGAGTTCAGACGTGTGCTCTTC
CGA

Flipped p5 primers:
CAAGCAGAAGACGGCATACGAGAT**BBBBBB**CGTCTTTCCCTACACGACGCTCTTCC

Indices listed at the end of the protocol.

Protocol:
To perform the PCR, combine the following:

	Volume (µL)
10X Titanium Taq PCR buffer	5
10X dNTPs	5
Flipped P7 primer (20 uM)	0.5
Flipped P5 primer (20 uM)	0.5
Water	37
Titanium Taq	1
cDNA	2
Total	50

Cycle as follows:
1. 98°C for 1 min
2. 94°C for 30 sec
3. 57°C for 1.5 min
4. Repeat steps 2-3 7 more times
5. 57°C for 10 min

Clean up with AMPure XP beads (Beckman Coulter) using 40 µL of beads for 50 µL of PCR according to the manufacturer's instructions, below.

1. Add 40 µL well-mixed AMPure XP beads to each sample and mix thoroughly
2. Incubate for 5 min at RT
3. Place sample onto a magnet with magnet to pellet the beads for 5 min
4. Remove supernatant and discard
5. Without moving the tube from magnet, add 200 µL freshly prepared 80% ethanol and let stand for at least 30 seconds
6. Remove ethanol, repeat ethanol wash once more.

7. After removing wash, remove tubes from magnet and let air dry for 2 minutes or until beads are visibly dry (For me, this is always 2 min, but for others in lab they waited longer).
8. Resuspend beads with 51 µL water, incubate at RT for 2 min
9. Pellet beads with magnet for 5 min and transfer supernatant to a new tube.
8. Repeat steps 1-9 with 40 µL fresh Ampure XP beads, except elute with 21 µL water
9. Store at -20 C until ready for sequencing.

Step 10: Preparing lane mixes and sequencing

Because we often want to add several µL of each sample in a lane to get the correct concentration, we mix the samples that will be sequenced together, precipitate them, and resuspend them in 3.6 µL water (4 µL total) for sequencing on a Hiseq 2500. We have had tremendous difficulty getting the loading concentrations correct because the qPCR systematically underestimates the concentration of our libraries (due to poor qPCR performance with the poly(A)-rich samples). This issue is compounded by the fact that samples from different species have different average tail lengths and this needs to be compensated for in the loading because the qPCR is more accurate when tails are shorter. For mammalian samples, the following loading should work well, but for other samples optimization may be needed. It is better to err on the side of underloading the flow cell initially and scale up in a subsequent sequencing round because the quality scores are often better (as compared to overloading) and the concentration can often be more accurately assessed.

Protocol for sequencing of PAL-seq V2 libraries is performed on a HiSeq 2500 operating in Rapid mode:

1. Mix together samples that will be run in the same lane based on qPCR concentrations so that you have a final total library amount of 0.375 pmol. (if you're combining 10 libraries then you'd use 0.0375 pmol of each). Note that if your samples are sufficiently concentrated, you don't need to precipitate them to combine, you can just make a master mix with 0.375 pmol in 4 µL.
2. To precipitate the samples, bring the volume up to 350 µL, add 39 µL sodium acetate, add 5 µL linear acrylamide, add 1 mL 100% ethanol and precipitate at -20 C for at least 2 h. Spin down and resuspend in 3.6 µL (4 µL final volume).
3. Note that the amounts described above are for making 125 µL hybridization mix and using 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.
4. Following cluster generation, the standard sequencing primer is hybridized (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'). Sequencing is performed by running two dark cycles (two rounds of standard sequencing-by-synthesis where imaging is skipped, enabling sequencing of poly(A) tails terminating in non-adenosine bases in the next step) and then a custom primer extension reaction (Subtelny et al., 2014) using

50 µM dTTP as the only nucleotide present in the reaction. Following the primer extension, 50 cycles of standard sequencing-by-synthesis are performed.

5. The flow cell is then stripped. A barcode sequencing primer (5'-AGATCGGAAGAGCGTCGTGTAGGGAAAGACG-3') is annealed and 7 cycles of standard sequencing-by-synthesis are performed to read the barcode.
6. The flow cell is stripped and the same primer as used in the first sequencing read is hybridized again and used for 250 cycles of standard sequencing-by-synthesis. For cases where a read corresponds to a polyadenylated mRNA, the first read (Read 1) is the reverse complement of the 3′ end of the mRNA immediately preceding the poly(A) tail and is used to identify the mRNA, the second read (index read) is the sample barcode, and the third read (Read 2) sequences through the poly(A) tail and is used to quantify poly(A)-tail length. The intensity files of these runs are saved and used for poly(A)-tail length determination, along with the Illumina FastQ files. The XML files used for configuring the HiSeq 2500 are available on our GitHub (github.com/kslin/PAL-seq).

Klenow extension recipes (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).

2x Klenow Buffer	**Volume**
Water	4.8mL
10x NEB Buffer 2	1.2mL
Tween 20 (10%)	12µL
Total	*6mL*

Klenow Reaction Mix	
2x Klenow Buffer	2.5 mL
Water	2.39 mL
100mM dTTP	2.5µL
Klenow polymerase	100 µL
Total	*5 mL*

1x Klenow Buffer	
2x Klenow Buffer	3 mL
Water	3 mL
Total	*6 mL*

HT2 Wash Buffer	

HT2	4 mL
500mM EDTA	80µL
Total	*4mL*

Full sequence for indexing primers:

For PCR (index is bold, sequencing primer binding site underlined):
Flipped p5 primer 1:
CAAGCAGAAGACGGCATACGAGAT**GCACTA**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 2:
CAAGCAGAAGACGGCATACGAGAT**TGTAGC**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 3:
CAAGCAGAAGACGGCATACGAGAT**CGGATT**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 4:
CAAGCAGAAGACGGCATACGAGAT**ACCAGT**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 5:
CAAGCAGAAGACGGCATACGAGAT**GTGACA**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 6:
CAAGCAGAAGACGGCATACGAGAT**TAACCG**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 7:
CAAGCAGAAGACGGCATACGAGAT**CTAGAC**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 8:
CAAGCAGAAGACGGCATACGAGAT**AGTTCA**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 9:
CAAGCAGAAGACGGCATACGAGAT**GACTAG**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 10:
CAAGCAGAAGACGGCATACGAGAT**TTCGAT**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 11:
CAAGCAGAAGACGGCATACGAGAT**AATTGC**CGTCTTTCCCTACACGACGCTCTTCC
Flipped p5 primer 12:
CAAGCAGAAGACGGCATACGAGAT**ACGCTG**CGTCTTTCCCTACACGACGCTCTTCC

For RT (Note that having different indices in the RT step is only for sequencing cDNA libraries using TAIL-seq. To prepare PAL-seq V2 libraries, only one of these indices is needed, as the libraries are barcoded in the PCR step.):

Primer 1:

CAAGCAGAAGACGGCATACGAGAT**GCACTA**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 2:
CAAGCAGAAGACGGCATACGAGAT**TGTAGC**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 3:
CAAGCAGAAGACGGCATACGAGAT**CGGATT**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 4:
CAAGCAGAAGACGGCATACGAGAT**ACCAGT**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 5:
CAAGCAGAAGACGGCATACGAGAT**GTGACA**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 6:
CAAGCAGAAGACGGCATACGAGAT**TAACCG**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 7:
CAAGCAGAAGACGGCATACGAGAT**CTAGAC**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 8:
CAAGCAGAAGACGGCATACGAGAT**AGTTCA**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 9:
CAAGCAGAAGACGGCATACGAGAT**GACTAG**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA
Primer 10:
CAAGCAGAAGACGGCATACGAGAT**TTCGAT**GTGACTGGAGTTCAGACGTGTGCTCT
TCCGA