TT-TSS-seq and polyA-TSS-seq step-by-step protocol:

Day 1.

4SU labelling (Adapted from TT_{chem}-seq,(Gregersen et al. 2020)**)**

4SU: Dissolve 250 mg of 4SU in 1.92 mL of sterile tissue-culture grade DMSO (final concentration 0.5 M). Freeze in 100-500 µL aliquots and store at -20°C in the dark for up to 12 months.

1) Add 4SU to the tissue culture medium to a final concentration of 1 mM. Remove part of the media with a serological pipette and filter-sterilise it. Add the 4SU to the filtered media and then add the 4SU-media, ensuring the final concentration of 4SU is 1 mM. Incubate the cells with 4SU for 15 min.
2) Aspirate off the medium and stop the labelling by the addition of 1 mL of TRIzol per 10-cm dish (scale up as necessary if using a bigger dish). Scrape the cells off the plate with a cell lifter and collect the TRIzol–cell mixture into a microcentrifuge tube.
3) RNA extraction
4) Add 200 µL of chloroform to 1 mL of the TRIzol–cell mixture from Step 2) and shake well for 30 s. Let it stand 2-15 minutes at RT and wait until the phases separate.
5) Spin at 12,000g for 15 min at 4 °C.
6) Collect the aqueous phase (usually ~500 µL) and add 1.1 volumes of isopropanol (usually 550 µL) and 1 µL GlycoBlue coprecipitant. Mix well.
7) Leave O/N at -20C.
8) Spin at 12,000g for 20 min at 4 °C to pellet the RNA.
9) Wash the RNA pellet with 750 µL of 85% (v/v) ethanol without disrupting the pellet.
10) Spin at 7,500g for 5 min at 4 °C.
11) Remove and discard all ethanol and allow the RNA pellet to air-dry.
12) Resuspend the pellet in 50–100 µL of RNase-free water. Measure RNA concentration by NanoDrop to get a rough estimation of RNA quantity.

A. Day 2

(Optional) Assessing 4SU incorporation by dot or slot blot (Adapted from TT_{chem}-seq, ,(Gregersen et al. 2020)**)**

Buffers:

Biotin buffer: 833 mM Tris-HCl (pH 7.4) and 83.3 mM EDTA. Store at RT for up to 12 months.
Dot/slot blot blocking buffer: 10% (w/v) SDS and 1 mM EDTA in PBS. Store at RT for up to 12 months.
Dot/slot blot wash buffer I: 1% (w/v) SDS in PBS. Store at RT for up to 12 months.
Dot/slot blot wash buffer II: 0.1% w/v SDS in PBS. Store at RT for up to 12 months.
Dot/slot blot staining buffer: 0.5 M sodium acetate and 0.5% (w/v) methylene blue.

Prepare one tube with 5 µg of total RNA for each sample in a total volume of 197 µL of RNase-free water (before DNase digestion). Add 3 µL of biotin buffer and 50 µL of 0.1 mg/mL MTSEA biotin-XX linker (dissolved in DMF) and incubate at RT for 30 min in the dark

13) Purify biotinylated RNA with phenol/chloroform/isoamyl alcohol (25:24:1):
    1) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (v/v/v)) to the biotinylated RNA.
    2) Shake and spin at 12,000g for 5 min at 4 °C. Transfer the upper aqueous phase containing the RNA to a new tube.
    3) Precipitate the RNA from the aqueous phase by the addition of:
        - 1/10 volume of the aqueous phase (usually 25 µL) of 5 M NaCl
        - 1.1 volume of the aqueous phase (usually 275 µL) of isopropanol
        - 1 µL GlycoBlue per tube

4) Mix by inverting the tube a few times.
5) Incubate at RT for 10 min (or -20ºC overnight).
6) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
7) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.
8) Air-dry until the edges of the pellet become slightly transparent, before dissolving in the pellet in 10 µL RNase-free water, which usually takes ~2–3 min.

14) Cut Hybond-N membrane and Whatman paper to the correct size and soak in RNase-free water. Place the membrane on top of 2–3 sheets of Whatman paper in a dot/slot blot apparatus and seal it well. Turn opposite screws at the same time. Connect the apparatus to a vacuum pump and turn it on.
15) Add 10 µL of bromophenol blue (0.001% (wt/vol)) solution to each 10-µL sample containing 2–10 µg of biotinylated RNA and apply it to the membrane in a well.
16) After a couple of minutes, turn off the vacuum pump and disassemble the dot/slot blot apparatus. Cut the corners of the membrane to indicate the left/right and up/down orientations.
17) UV-crosslink the membrane at 0.2 J/cm2 (254 nm) in a Stratalinker or similar device.
18) Block the membrane by incubation in dot/slot blot blocking buffer for 20 min at RT.
19) Probe the membrane with a 1:50,000 dilution of 1 mg/mL HRP-conjugated streptavidin in dot/slot blot blocking buffer for 15 min at RT.
20) Wash the membrane 2x in dot/slot blot blocking buffer for 10 min.
21) Wash the membrane 2x in dot/slot blot wash buffer I for 10 min each.
22) Wash the membrane 2x in dot/slot blot wash buffer II for 10 min each.
23) Visualise the signal of the biotin-bound HRP-conjugated streptavidin by detection of ECL prime reagent. You may need to dilute the ECL reagent 1:5 in water as the signal can be strong.
24) Stain the membrane to assess RNA loading with dot/slot blot staining solution for 10 min at RT. De-stain with several washes in water (the last wash can be done O/N).

B. DNase treatment

1) Prepare DNase I mix:

RDD 10x buffer	10 µL
DNase I (40X) (Qiagen)	2.5 µL
Sample	
Water	To 100 µL

2) Incubate at 37 degrees for 1 h
3) Clean up either using the RNeasy MinElute Cleanup kit or by phenol/chloroform/isoamyl alcohol:

If clean up is performed using the RNeasy MinElute Cleanup kit, add 1.5× (v/v) 100% ethanol relative to the RLT buffer to retain <200-nt RNA fragments. To a 100 µL sample, add 350 µL of buffer RLT and 525 µL of 100% ethanol. Add 700 µL of the mixture to the column and spin for 15 s at ≥8000 x g and discard the flow-through, then repeat with the remaining mixture. Follow the remaining protocol as recommended by Qiagen.

a) Bring the solution to 250 µL with RNase-free water.
b) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (vol/vol/vol)) to the RNA.
c) Shake and spin at 12,000g for 5 min at 4 °C.
d) Transfer the upper aqueous phase containing the RNA to a new tube. If samples were split in different reactions, re-merge them at this point.

e) Precipitate the RNA from the aqueous phase by addition of: 1/10 volume of the aqueous phase of 5 M NaCl,1.1 volume of the aqueous phase of isopropanol 1 µL GlycoBlue per tube
f) Mix by inverting the tube a few times.
g) Incubate at -20ºC overnight (at very least 2h)
h) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
i) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.

J) air-dry until the edges of the pellet become slightly transparent, before dissolving in the pellet in RNase-free water

4) Measure the RNA concentration using a Qubit RNA BR Assay Kit (should be >1 µg/µL) and check the RNA integrity on a Bioanalyzer.

C. Alkaline phosphatase treatment

Quick CIP treatment removes 5'-phosphate from uncapped RNA molecules, preventing T4 RNA ligase 1 activity.

25) Set up the following 100 µL reaction in a 1.5 mL tube. If needed, split samples in multiple reactions. Do not use more than 7ul of CIP per reaction. For 100 µg, split into 4x 30 U CIP reactions (6 µL CIP). Use 1.2 U of CIP per µg of RNA.

Component	Amount (µL)	Final amount / conc.
RNA sample		Maximum 29 µg
CutSmart buffer (10x)	10	1x
Quick CIP (NEB), 5 µ/µL	No more than 7 µL	1.2 U per ug of RNA
RNasin Plus (Promega)	1	
RNase-free water		
Total	**100**	

26) Incubate samples for 2 h at 37°C.
27) Heat-inactivate at 80°C for 2 min.
28) Purify RNA:
    1) Bring the solution to 250 µL with RNAse-free water.
    2) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (vol/vol/vol)) to the RNA.
    3) Shake and spin at 12,000g for 5 min at 4 °C.
    4) Transfer the upper aqueous phase containing the RNA to a new tube.
       If samples were split in different reactions, re-merge them at this point.
    5) Precipitate the RNA from the aqueous phase by the addition of:
       - 1/10 volume of the aqueous phase of 5 M NaCl
       - 1.1 volume of the aqueous phase of isopropanol
       - 1 µL GlycoBlue per tube
    6) Mix by inverting the tube a few times.
    7) Incubate at -20ºC overnight (at very least 2h).
    8) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
    9) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.

    Air-dry until the edges of the pellet become slightly transparent, before dissolving in the pellet in RNase-free water

D. Day 3

Decapping of mRNAs

29) Set up the following 20 µL reaction. Use 7 U of mRNA decapping enzyme (100 U/µL) per µg of RNA, e.g. for 100 µg RNA, use 7 µL of mRNA-decapping enzyme in 1 reaction.

Component	Amount (µL)	Final amount / conc.
RNA		
MDE buffer (10x)	2	1x
mRNA decapping enzyme (NEB), 100 U/µL		7 U per µg RNA
Water		
Total	**20**	

30) Incubate samples for 2 hours at 37 °C.

31) Purify RNA:
   1) Bring the solution to 250 µL with RNAse-free water.
   2) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (vol/vol/vol)) to the RNA.
   3) Shake and spin at 12,000g for 5 min at 4 °C.
   4) Transfer the upper aqueous phase containing the RNA to a new tube.
   5) Precipitate the RNA from the aqueous phase by the addition of:
      - 1/10 volume of the aqueous phase (usually 25 µL) of 5 M NaCl
      - 1.1 volume of the aqueous phase (usually 275 µL) of isopropanol
      - 1 µL GlycoBlue per tube
   6) Mix by inverting the tube a few times.
   7) Incubate at -20ºC overnight (at least 2h).
   8) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
   9) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.
   10) Air-dry until the edges of the pellet become slightly transparent, before dissolving in the pellet in **13 µL** RNase-free water, which usually takes ~2–3 min.

E. 5’ adapter ligation

32) Set up the following 20 µL reaction in a PCR tube.

Component	Amount (µL)	Final amount / conc.
RNA	10 µL	
5’ oligo (DNA:RNA hybrid) (100 µM)	2	10 µM
Mix RNA and oligo first, then denature for 2 mins at 70 degrees, then leave for 2 mins on ice		
10x T4 RNA ligase 1 Buffer	2	1x
10mM ATP	2	1 mM
T4 RNA ligase 1, **High Conc** (30K U/mL)	1	30 U
RNasin Plus (Promega)	1	
DMSO	2	
Total	**20**	

33) Incubate at 25 °C for 2 h, then 16 °C for 16 hours, and **turn the heated lid off**.

F. Clean up RNA to remove free 5' adapters

RNAClean XP kit. Follow the single-tube format protocol.

G. (Optional) Pool samples

Measure cleaned-up RNA samples using a Qubit RNA BR Assay Kit.

Pool samples which have different sample barcodes for early multiplexing. We tend to pool all of the biological replicates for a certain sample type here. Make sure all RNAs are included in equal amounts.

H. Day 4.

Biotinylation of 4SU-RNA (Adapted from TT_{chem}-seq)

Biotin buffer: 833 mM Tris-HCl (pH 7.4) and 83.3 mM EDTA. Store at RT for up to 12 months. ***MTSEA*** *biotin-XX linker: Make a 10x stock of 1 mg/mL MTSEA biotin-XX linker in DMF and store at -80°C for up to 6 months.*

34) Bring all RNA samples to 200 µL each. A maximum of 100 µg of total RNA per reaction here.
35) Add 3 µL of biotin buffer and 50 µL of 0.1 mg/mL MTSEA biotin-XX linker (dissolved in DMF) to the RNA samples and incubate at RT for 30 min in the dark.
36) Purify biotinylated RNA with phenol/chloroform/isoamyl alcohol (25:24:1):
    1) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (v/v/v)) to the biotinylated RNA.
    2) Shake and spin at 12,000g for 5 min at 4 °C.
    3) Transfer the upper aqueous phase containing the RNA to a new tube.
    4) Precipitate the RNA from the aqueous phase by the addition of:
        - 1/10 volume of the aqueous phase (usually 25 µL) of 5 M NaCl
        - 1.1 volume of the aqueous phase (usually 275 µL) of isopropanol
        - 1 µL GlycoBlue per tube
    5) Mix by inverting the tube a few times.
    6) Incubate at -20ºC overnight (at very least 2h). Maybe better to do this one for 2 h.
    7) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
    8) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.
    9) Air-dry until the edges of the pellet become slightly transparent, before dissolving in the pellet in **50 µL** RNase-free water, which usually takes ~2–3 min.

I. Streptavidin pull-down (Adapted from TT_{chem}-seq)

Pull-down wash buffer: 100 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1 M NaCl and 0.1% (vol/vol) Tween-20. Store at RT for up to 12 months and pre-warm to 55°C before use.
Elution buffer: 100 mM DTT freshly dissolved in RNase-free water. Should be prepared fresh each time.
The µMACs Streptavidin Kit contains µMACS Streptavidin MicroBeads, 20 µColumns and equilibration buffers for both protein and nucleic acid applications.

37) Denature the biotinylated RNA at 65ºC for 10 min, followed by rapid cooling on ice for 5 min.
38) Add 200 µL of µMACS Streptavidin MicroBeads to the RNA and incubate for 15 minutes at RT on a rotating wheel.

39) Place a µColumn in the magnetic field of a µMACS magnetic separator placed on a MACS multistand. Prepare the column by rinsing it with 100 µL of nucleic acid equilibration buffer. Initiate flow and remove air bubbles from the column by very gently pressing the top of the column with the plunger of a syringe of the appropriate size (**5 mL**).
40) Apply the µMACS streptavidin MicroBeads and RNA mix to the top of the column, keeping the µColumn on the magnetic separator. Optionally, collect the flow-through as 'non-4SU-labeled, preexisting RNA'. Collect in 2 mL tubes.
41) Wash the column twice with 500 µL of **pre-warmed (55 °C)** pull-down wash buffer. Can collect wash buffer.
42) Prepare fresh elution buffer. 0.0154g of DTT in 1 mL or 0.0077g in 0.5 mL.
43) Add 100 µL of elution buffer (RT) to elute the 4SU+ RNA and collect the eluate. Repeat the elution with an additional 100 µL of elution buffer 5 min later and pool the two eluates. Collect in 2 mL tubes.
44) Clean up and concentrate the 4SU+ RNA eluates (and non-4SU-labeled, preexisting RNA, if collected at step 49), using the **RNeasy MinElute Cleanup Kit**.
    - To efficiently capture <200-nt fragments from the MinElute spin columns, add a 1.5x volume of 100% ethanol relative to RLT buffer. For a 200 µL sample, add 700 µL of RLT buffer and 1050 µL of 100% ethanol. Add 700 µL of the mixture to the column and spin for 15 s at ≥8000 g and discard the flow-through. Repeat for 2 more rounds until all the mixture has been added to the column. Follow the remaining protocol as recommended by Qiagen.
    - Elute the RNA in **18 µL** of RNase-free water.

It is important to add 1.5× (vol/vol) ethanol relative to the RLT buffer to retain <200-nt RNA fragments. This differs from the recommended RNeasy MinElute protocol, which selects for RNA fragments >200 nt and discards the smaller fragments.

45) Measure the RNA concentration using the Qubit RNA HS Assay Kit. This is important to see whether the 4SU% was as expected. Purified 4SU+ RNA can be stored for a few weeks at -80°C before library preparation.

I. (Optional) PolyA selection

Buffers

Binding Buffer: 20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA.
Washing Buffer B: 10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA.
10 mM Tris-HCl, pH 7.5

1) Calculate volume of Dynabeads Oligod(T) (Invitrogen) to use (1 mg beads per 75µg RNA):

RNA (750 ng/µL)	75 µg	250 µg	___ µg
RNA volume	100 µL	333 µL	___ µL
Beads volume (~5 mg/mL)	200 µL (1mg)	666 µL (3.33 mg)	___ µL (__ mg)

2) Resuspend the Dynabeads in the bottle by vortexing for >30 sec, or tilting and rotating for 5 min.
3) Transfer 2 volumes of Dynabeads to a tube (where 1 volume is the volume of RNA, see table).
4) Add 2 volumes of Binding Buffer (up to at least 1 mL) and resuspend.
5) Place the tube in a magnetic stand for 1 min and discard the supernatant.
6) Remove the tube from the magnet and resuspend the Dynabeads in 2 volumes of Binding Buffer (as the initial volume of Dynabeads collected in step c)).
7) Adjust the concentration of the total RNA sample to 750ng/µL with RNase-free water or with 10 mM Tris-HCl pH 7.5 (bringing the RNA to 1 volume as calculated above).
8) Add 1 volume (same as RNA) of Binding Buffer.

9) Heat to 65°C for 2 min to disrupt secondary structures. Immediately place on ice.
10) Add the total RNA + Binding Buffer (ex. 200 µL) to the washed beads (obtained in step 5, ex. 200 µL).
11) Mix thoroughly and allow binding by rotating continuously on a mixer for 5 min at RT.
12) Place the tube on the magnet for 1-2 min and carefully remove all the supernatant.
13) Remove the tube from the magnet and add 2 volumes of Washing Buffer B. Mix by pipetting carefully a couple of times.
14) Apply to the magnet for 1 min and remove the supernatant.
15) Repeat wash (steps 12-13) once more.
16) Perform one of the following:

    To elute mRNA from the beads, remove the Washing Buffer B and add 9 µL 10 mM Tris-HCl. Incubate at 75°C (up to 80°C) for 2 min, then place the tube on the magnet and quickly transfer the supernatant containing the mRNA to a new RNase-free tube. Repeat the elution a second time (9 µL) to increase the yield. Total volume 18 µL.

J. Alkaline fragmentation

46) Make aliquots of 10 µg of RNA (or more, but max 10-20 µL). From polyA selection 18 µL.
47) Add a corresponding volume of 10x Fragmentation Reagent (Ambion RNA Fragmentation Reagents) on the side of the tube.
48) Spin tubes down and incubate at 70ºC for (3.5 minutes for yeast, 5.5 minutes for mammalian RNA). Fragment 2 tubes at a time.
49) Add the corresponding volume of 10x Stop Solution, and place tubes on ice.
50) Verify size using Agilent Bioanalyzer/Tapestation if no clean-up step is done.
    Migration of RNA in the fragmentation buffer looks ~100nt lower than cleaned-up RNA.

K. RNA fragments clean-up

51) Bring the RNA volume up to 100 µL.
52) Clean up RNA from using RNeasy MinElute Clean-up columns.
    Use 1.5x volume of 100% EtOH as recommended in step 48 (525 µL EtOH for 100 µL RNA).
53) Elute 2 times in 14 µL water
54) Verify size using Agilent Bioanalyzer/Tapestation.

L. End repair with alkaline phosphatase

Quick CIP treatment repairs the 3' end of alkaline-fragmented RNA to 3'OH, thereby making it ligation competent.

55) Set up the following 100 µL reaction in 1.5 mL tube.

Component	Amount (µL)	Final amount / conc.
RNA sample		
CutSmart buffer (10x)	10	1x
Quick CIP (NEB)	2	30 U
RNasin Plus (Promega)	1	
RNase-free water		-
Total	**100**	

56) Incubate samples for 1 h at 37°C.
57) Heat-inactivate at 80°C for 2 min.

58) Purify biotinylated RNA with phenol/chloroform/isoamyl alcohol (25:24:1):
    1) Add 250 µL of phenol/chloroform/isoamyl alcohol (25:24:1 (vol/vol/vol)) to the biotinylated RNA.
    2) Shake and spin at 12,000g for 5 min at 4 °C.
    3) Transfer the upper aqueous phase containing the RNA to a new tube.
    4) Precipitate the RNA from the aqueous phase by the addition of:
        - 1/10 volume of the aqueous phase (usually 25 µL) of 5 M NaCl
        - 1.1 volume of the aqueous phase (usually 275 µL) of isopropanol
        - 1 µL GlycoBlue per tube
    5) Mix by inverting the tube a few times.
    6) Incubate at -20ºC overnight (at very least 2h).
    7) Spin at 20,000g for 20 min at 4 °C to pellet the RNA. Discard the supernatant.
    8) Wash the RNA pellet with 500 µL of 85% (vol/vol) ethanol without disrupting the pellet and spin at 20,000g for 5 min at 4 °C. Be sure to remove as much residual ethanol as possible.
    9) Air-dry until the edges of the pellet become slightly transparent, before dissolving the pellet in **10 µL** RNase-free water, which usually takes ~2–3 min.

M. Day 5.

3' adapter ligation (Adapted from iCLIP2, (Buchbender et al. 2020))

T4 RNA Ligase 2, truncated KQ (T4 Rnl2tr R55K, K227Q) specifically ligates the preadenylated 5´ end of DNA or RNA to the 3´ OH end of RNA. The enzyme does not use ATP for ligation but requires pre-adenylated linkers.

59) Set up the following 20 µL reaction.

Component	Amount (µL)	Final amount / conc.
Purified fragmented RNA	10 µL	
50% PEG8000	4 µL	10%
Pre-adenylated L3-App (20 µM)	2 µL	2 µM
Mix RNA, adapter & PEG, heat denature at 70 degrees for 2 mins, 3 mins on ice		
10x T4 RNA Ligase Reaction Buffer	2 µL	1x
T4 RNA Ligase 2, truncated KQ (200K U/mL) (NEB)	1 µL	200 U
RNAsin Plus (Promega)	1 µL	
Total	**20 µL**	

PCR Block – Folder: A1 – Program: "22"

60) Incubate 2 hours at 25°C, and 16ºC 16 hours (turn the heated lid off here).
61) Heat inactivation 65°C for 20 min (turn the heated lid back on here).

N. Day 6

RNA Clean-up

Remove template RNA and adapters using **RNAClean XP kit.** Follow the single-tube format protocol. Output: fragmented RNA of size 200-300 bp with 5' and 3' adapter

O. Reverse transcription

62) Prepare mix 1

Component	Amount (µL)	Final amount / conc.
RNA (? ng)	24 µL	
Primer *NEBNext_ver2RT* (0.5pmol/µL)	1 µL	0.5 pmol
dNTP mix (10mM **each**)	2 µL	
Total	**27 µL**	

NEBNext_ver2RT: GGATC**GACGTGTGCT**

63) Incubate mix 1 for 5 minutes at 65°C.

64) In the meantime, prepare mix 2:

Component	Amount (µL)	Final amount / conc.
SSIII FS buffer	8 µL	
DTT (0.1M)	2 µL	
SuperScript III (Thermo Fisher)	2 µL	
RNasin Plus (Promega)	1 µL	
Total	**13 µL**	

65) Place RNA on ice for 1 min and add 13 µL mix 2 to each tube.

66) Continue RT thermal programme:

25ºC 5min
42ºC 20min
50 ºC 40 min
80ºC 5min
4ºC ∞

67) Remove the template RNA by adding 1 µL of the following mix directly to the PCR tube and incubating at 37 degrees for 30 mins.

Component	Amount (µL)	Final amount
RNase H (5U/µL) (NEB)	2	5 U
RNase cocktail (10X) (Thermo Fisher)	2.22	-
Total	**4.22**	

P. cDNA Clean-up with AMPure XP beads:

1) Add X µL Agencourt AMPure XP beads (3x the volume of the RT reaction) directly to the RT reaction and mix well by pipetting (15 times)
2) Add X µL isopropanol (1.7x volume of the RT reaction). Mix by extensive pipetting (15 times). For samples that are 46.44 ul add 139.32 µL of AMPure and 78.95 µL of isopropanol. For samples that are 48 µL add 144 µL of AMPure and 81.9 µL of isopropanol.

3) Incubate for 5min at room temp. Check the isopropanol does not separate into an upper layer. Make sure the isopropanol isn't cold as this seems to increase the likelihood of separation.
4) Place on a magnetic rack and let beads collect for 2-3 minutes.
5) Remove the supernatant.
6) Without removing the beads from the magnet, add 200 µL 85% ethanol and incubate for 30 sec, then remove the supernatant.
7) Repeat 85% ethanol wash.
8) Spin down briefly, place back on magnets and remove the remaining ethanol (use an aspirator to remove any ethanol near the top and bottom of the tube. Hover the aspirator ~3 mm from the beads for 30 s to further dry the beads).
9) Leave the tube open to dry the beads. Wait until they are fully dry (no longer shiny).
10) To elute, add 2x 11.5 µL water, pipette 30 times to ensure a homogeneous solution and incubate for 3 mins.
11) Place back on magnets and transfer supernatant to a new tube.

Q. First PCR (cDNA pre-amplification) (Adapted from iCLIP2)

68) Prepare mix of 1:1 P5Solexa_s and NEBNext_i7_s primer (10µM each).
69) Prepare the following PCR mix:

Component	Amount (µL)	Final amount / conc.
cDNA	22.5 µL	
Primer mix of P5Solexa_s and NEBNext_i7_s (each 10µM)	1.5 µL	~300nM each
2x Phusion HF PCR Mastermix (NEB)	25 µL	1x
Total	**50 µL**	

Take ProNex beads out of the fridge so they have time to warm up whilst the PCR program is running.

70) Run PCR programme:

98ºC 30''
98ºC 10'' |
65ºC 30'' | x 6 cycles
72ºC 30'' |
72ºC 3min
16ºC ∞

S. First ProNex size selection to remove primer-dimers (From iCLIP2, (Buchbender et al. 2020))

71) Equilibrate the ProNex Chemistry to RT for 30 minutes. In the meantime, prepare the ULR Ladder for size selection and reference:

ULR Ladder reference		ULR Ladder for size selection	
1 µL	ULR ladder	1 µL	ULR ladder
49 µL	Water or ProNex Elution Buffer	24 µL	Water
		25 µL	2x Phusion PCR Master Mix

72) Resuspend the ProNex Chemistry beads by vortexing until the solution has a homogenous color.

73) Check the volume of your sample and add a 1:2.95 volume of ProNex Chemistry beads to the sample. For 50 µL of sample, add 147.5 µL of ProNex Chemistry beads and mix by pipetting up and down 30 times. If the sample is not 50 µL, either adjust the sample volume with nuclease-free water or use a different amount of beads to ensure the ratio is the same.
74) Incubate the ProNex Chemistry on the samples at RT for 10 min.
75) Place the samples on a magnetic stand for 2 min. Discard the supernatant.
76) Leave the beads on the magnetic stand and add 300 µL ProNex Wash Buffer to the samples.
77) If necessary, scale up the volume of ProNex Wash Buffer to cover all beads on the magnet (increase volume of wash buffer proportionally to volume of sample and beads). While the beads are magnetically attracted, incubate the ProNex Wash Buffer for 30–60 s before removal.
78) Repeat the last wash of the magnetically attracted beads with another 300 µL ProNex Wash Buffer for 40–60 s. Discard the supernatant.
79) Allow the samples to air-dry for ~8–10 min (<60 min) until cracking starts (hover aspirator near beads for 30 s to dry them, otherwise this takes much longer than 10 min)
80) Remove the beads from the magnetic stand and start eluting the samples.
81) Elute the the samples from the beads in 23 µL water (or ProNex Elution Buffer).
82) Elute the ULR Ladder for size selection in 50 µL water (or ProNex Elution Buffer).
83) Resuspend all samples by pipetting.
84) Let stand for 5 min at RT.
85) Return samples to magnetic stand for 1 min.
86) Carefully transfer eluted cDNA to a clean tube.
87) Check the selection efficiency of your samples: compare the ULR Ladder with and without ProNex size selection on a High Sensitivity D1000 TapeStation Kit (or Bioanalyzer or polyacrylamide gels).

R. Optimization of second PCR cycles (Adapted from iCLIP2,(Buchbender et al. 2020))

88) Prepare mix of 1:1 i5 and i7 primers (10µM each).
89) Prepare PCR mix:

Component	Amount (µL)	Final amount / conc.
cDNA	1 µL	
i5 and i7 primers mix (each 10µM)	0.5 µL	~500nM
2x Phusion HF PCR Mastermix (NEB)	5 µL	1x
Water	3.5 µL	
Total	**10 µL**	

90) Run PCR programme to test the number of cycles:

98ºC 30''
98ºC 10'' |
65ºC 30'' | x 6-11 cycles
72ºC 30'' |
72ºC 3min
16ºC ∞
(Usually 9 cycles)

We recommend a range of 6-11 cycles. 6-9 cycles yield good libraries, while libraries amplified with 10-13 cycles will contain higher PCR duplicate amounts. Consider that you will amplify a 2.5-times more concentrated cDNA (see PCR mix below), therefore, one cycle less is needed than in the optimisation PCR.

91) Run 2 µL of the amplified library on capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system (or Bioanalyzer or polyacrylamide gels).

S. Second PCR amplification (Adapted from iCLIP2, (Buchbender et al. 2020))

From the results, estimate the minimum number of PCR cycles required to amplify half of the library. Consider that you will amplify a 2.5-times more concentrated cDNA. Therefore, one cycle less is needed than in the optimisation PCR, usually 8 cycles.

92) Prepare PCR mix.

Component	Amount (µL)	Final amount / conc.
cDNA	10 µL	
i5 and i7 primers mix (each 10µM)	2 µL	~500nM
2x Phusion HF PCR Mastermix (NEB)	20 µL	1x
Water	8 µL	
Total	**40 µL**	

93) Run PCR programme with the adjusted cycle number:

98ºC	30''	
98ºC	10''	|
65ºC	30''	| XX cycles
72ºC	30''	|
72ºC	3min	
16ºC	∞	

94) Test 2 µL of the amplified library with capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system (or Bioanalyzer or polyacrylamide gels).

95) If everything looks fine, also amplify the second half of the library and combine it with the first half.

96) This is a good point to multiplex different samples or replicates that should be sequenced together (they must have different barcodes). Determine the concentration of the library, for example, using the concentration under the peak with TapeStation software, and combine the different samples in equal molarities.

T. Second ProNex size selection to remove residual primers, (Buchbender et al. 2020)

The second cDNA size selection step aims to discard excess primer that would negatively impact sequencing results (fragments of 58/61 nt) and retain the amplified library (original inserts >20 nt without adapters = 155 nt with adapters). Use the 150 nt/75 nt fragment ratios to determine size selection efficiency and quantify recovery of 150 nt fragments from the input ULR ladder to monitor for absolute sample loss.

97) Estimate the size selection efficiency of this second size selection by comparing the ULR Ladder with and without size selection on the High Sensitivity D1000 TapeStation Kit (or Bioanalyzer) to determine the optimal sample-to-bead ratio.
98) Equilibrate the ProNex Chemistry beads to RT for 30 min and resuspend the beads by vigorous vortexing.
99) Based on the optimal sample-to-ProNex (v/v) ratio of **1:2.3 (or whichever ratio you have determined)**, calculate and add the required volume of beads to your samples (e.g. for a 40 µL sample, add 92 µL beads). Mix by pipetting up and down 30 times.
100) Incubate the ProNex Chemistry on the samples at RT for 10 min.
101) Place the samples on a magnetic stand for 2 min. Discard the supernatant.
102) Leaving the beads on the magnetic stand, add 300 µL ProNex Wash Buffer to the sample. Do not resuspend the bead in wash buffer as this causes up to 20% sample loss. For larger samples, increase the volume of wash buffer to the volume of sample and beads. While the beads are magnetically attracted, incubate the ProNex Wash Buffer for 30–60 s before removal. If necessary, scale up the volume of ProNex Wash Buffer to cover all beads on the magnet.
103) Repeat the last wash of the magnetically attracted beads with another 300 µL ProNex Wash Buffer for 40–60 s. Discard the supernatant.
104) Air dry samples for ~8–10 min (<60 min) until cracking starts (can hover aspirator near beads for ~30s to speed this up)
105) Repeat the washing and air-dry sample for ~3 min (<60 min).
106) Remove the beads from the magnetic stand.
107) Elute beads of the sample in 20 µL water (or ProNex Elution Buffer).
108) Elute the ULR Ladder sample (for size selection) in 50 µL water (or ProNex Elution Buffer).
109) Resuspend all samples by pipetting.
110) Let them stand for 5 min at RT.
111) Return the samples to the magnetic stand for 1 min, then carefully transfer the eluted cDNA to a clean tube or well.
112) To check for primer removal, test 2 µL of the purified library with capillary gel electrophoresis using the High Sensitivity D1000 Kit in a TapeStation system (or Bioanalyzer). Calculate the ratio of intensities of the 150 nt and 75 nt fragments, which should be around 15.

U. Library quantification

113) Measure the library with the Qubit High Sensitivity D1000 Kit to determine concentration (ng/µL) and determine the median fragment size from the TapeStation peak (nt).
114) Using concentration and the median fragment size, determine the molarity of the library with the help of the following calculation:
Library molecular weight = (median fragment peak [nt] ∗ 650 g/mol) / nt
Molar concentration = Qubit concentration [ng/µL] / molecular weight
115) Dilute the library to 10 nM and submit 20 µL for sequencing.
116) Store at −20 °C.

V. Reagents table

Step	Ingredient	Supplier	Catalogue number
4SU labeling	4SU	Glentham Life Sciences	GN6085
RNA extraction	TRIzol	Thermo Fisher	15596026
	Glycoblue Coprecipitant	Thermo Fisher	AM9515
Dot/slot blot	MTSEA biotin-XX linker	Biotium	BT90066
	Hybond-N membrane	Cytiva	RPN203B
	ECL reagent	Cytiva	RPN2232
	HRP-conjugated streptavidin	Thermo Fisher	N100
DNase digestion	RNase-Free DNase	Qiagen	79254
	RNeasy MinElute Cleanup kit	Qiagen	74204
	Qubit RNA BR assay kit	Thermo Fisher	Q10210
	Agilent RNA 6000 Nano reagents (Bioanalyzer)	Agilent	5067-1511
Alkaline phosphatase treatment	Quick CIP	NEB	M0525S
	RNasin PLUS	Promega	N2611
Decapping	mRNA decapping enzyme	NEB	M0608S
5' adapter ligation	T4 RNA ligase 1	NEB	M0437
Clean up to remove 5' adapters	RNAClean XP	Beckman Coulter	A63987
Streptavidin pull down	µMACS Streptavidin Starting Kit	Miltenyi Biotec	130-091-287
	µMACS Streptavidin Kit	Miltenyi Biotec	130-133-282
	Qubit RNA HS Assay kit	Thermo Fisher	Q32852
PolyA selection	Dynabeads Oligo(dT)	Thermo Fisher	61002
	Magnetic stand	Thermo Fisher	12321D
Alkaline fragmentation	10x Alkaline Fragmentation Buffer	Thermo Fisher	AM8740
	TapeStation High Sensitivity RNA ScreenTape	Agilent	5067-5579
	High Sensitivity RNA ScreenTape Ladder	Agilent	5067-5581
	High Sensitivity RNA ScreenTape Sample Buffer	Agilent	5067-5580
3' adapter ligation	T4 RNA ligase II, truncated KQ	NEB	M0242L
Reverse transcription	SuperScript III	Thermo Fisher	18080051
	RNase H	NEB	M0297L
	RNase cocktail	Thermo Fisher	AM2286
cDNA cleanup	AMPure XP	Beckman Coulter	A63880
Library PCR	Phusion HF PCR MM	NEB	M0531S
DNA size selection	ProNex Chemistry	Promega	NG2001
Library quantification	TapeStation HS D1000 Reagents	Agilent	5067-5585
	TapeStation HS D1000 ScreenTape	Agilent	5067-5584
	Qubit 1X dsDNA HS	Thermo Fisher	Q33230

W. Primers table

Name	Sequence 5'-3'
TSS_Adapter5_01	CACGACGCTCTTCCGATCTNNNNNCGrUrGrArUrNrNrNrN
TSS_Adapter5_02	CACGACGCTCTTCCGATCTNNNNNACrArUrCrGrNrNrNrN
TSS_Adapter5_03	CACGACGCTCTTCCGATCTNNNNNGCrCrUrArArNrNrNrN
TSS_Adapter5_04	CACGACGCTCTTCCGATCTNNNNNTGrGrUrCrArNrNrNrN
TSS_Adapter5_05	CACGACGCTCTTCCGATCTNNNNNCArCrUrGrUrNrNrNrN
TSS_Adapter5_06	CACGACGCTCTTCCGATCTNNNNNATrUrGrGrCrNrNrNrN
TSS_Adapter5_07	CACGACGCTCTTCCGATCTNNNNNGArUrCrUrGrNrNrNrN
TSS_Adapter5_08	CACGACGCTCTTCCGATCTNNNNNTCrArArGrUrNrNrNrN
TSS_Adapter5_09	CACGACGCTCTTCCGATCTNNNNNCTrGrArUrCrNrNrNrN
TSS_Adapter5_10	CACGACGCTCTTCCGATCTNNNNNAArGrCrUrArNrNrNrN
TSS_Adapter5_11	CACGACGCTCTTCCGATCTNNNNNGCrUrCrArUrNrNrNrN
TSS_Adapter5_12	CACGACGCTCTTCCGATCTNNNNNTArCrArArGrNrNrNrN
NEBNext_ver2L3-App	/5rApp/AGATCGGAAGAGCACACGTC/3ddC/ (5' adenylated and 3' dideoxy-C modified)
NEBNext_ver2RT	GGATCGACGTGTGCT
NEBNext i7_s	GACGTGTGCTCTTCCGATCT
TSS_P5Solexa_s	ACACGACGCTCTTCCGATCT
NEBNext i701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
NEBNext i501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i507	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTCTTCCGATCT
NEBNext i508	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCTTCCGATCT

Pricing:

Including poly(A) pulldown: approximately 120 USD per sample.

Excluding poly(A) pulldown: approximately 100 USD per sample.

References:

Buchbender A, Mutter H, Sutandy FXR, Kortel N, Hanel H, Busch A, Ebersberger S, Konig J. 2020. Improved library preparation with the new iCLIP2 protocol. *Methods* **178**: 33–48.

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