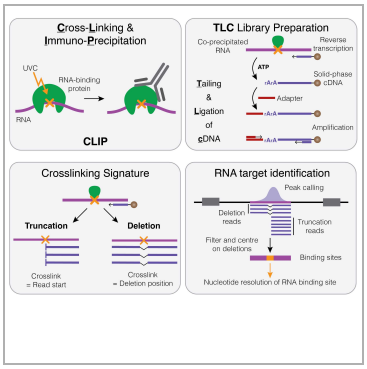

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We use this protocol and it's working

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TLC-CLIP

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ABSTRACT

RNA-binding proteins are instrumental for post-transcriptional gene regulation, controlling all aspects throughout the lifecycle of RNA molecules. However, transcriptome-wide methods to profile RNA-protein interactions *in vivo* remain technically challenging and require large amounts of starting material. Herein, we present an improved library preparation strategy for crosslinking and immunoprecipitation (CLIP) that is based on tailing and ligation of cDNA molecules (TLC). TLC involves the generation of solid-phase cDNA, followed by ribotailing to increase the efficiency of subsequent adapter ligation. These modifications result in a streamlined, fully bead-based library preparation strategy, which eliminates time-consuming purification procedures and drastically reduces sample loss, allowing the profiling of RNA-protein interactions from as few as 1000 cells.
In the accompanying manuscript, we have applied TLC-CLIP to four endogenous RNA-binding proteins, demonstrating its reproducibility and improved precision due to a higher number of crosslinking-induced deletions that serve as an intrinsic quality metric and increase both specificity and nucleotide-resolution.

GUIDELINES

While working with RNA during the inital parts of the protocol, keep samples cold (on ice) and in RNase-free environment. Use RNase-free water for reactions and buffers.

MATERIALS

Equipment

Equipment

UVP Crosslinker CL-3000

NAME

Analytic Jena

BRAND

UVPA849-95-0615-02

SKU

https://www.uvp.com/products/lab-equipment/uvp-crosslinker/

LINK

Equipment

Branson Tip Sonicator

NAME

Sonicator

TYPE

Branson

BRAND

LPe 40:0.50:4T

SKU

Equipment

ThermoMixer® C

NAME

Eppendorf

BRAND

Catalog No. 2231000680

SKU

https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html

LINK

Equipment

SureLock™ Tandem Midi Gel Tank	NAME
Electrophoresis System	TYPE
Invitrogen	BRAND
STM1001	SKU
https://www.thermofisher.com/order/catalog/product/STM1001	LINK

Equipment

Criterion Blotter with Plate Electrodes	NAME
Wet-transfer system	TYPE
Bio-Rad	BRAND
1704070	SKU
https://www.bio-rad.com/en-ch/product/criterion-blotter?ID=351faa8e-680e-4892-9778-c8c722e9b3b2	LINK

Equipment

Odyssey CLx

NAME

Imaging System

TYPE

LI-COR

BRAND

Odyssey CLx

SKU

https://www.licor.com/bio/odyssey-clx/

LINK

Equipment

Bioanalyzer

NAME

Bioanalyzer

TYPE

Agilent

BRAND

G2991AA

SKU

https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250

LINK

Any bioanalyzer will suffice.

SPECIFICATIONS

Equipment

Qubit Fluorometer	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238	LINK

Buffer and Stock Solutions

- 1M Tris-HCl pH=7.5 **Invitrogen - Thermo Fisher Catalog #15567-027**
- 5M NaCl **Ambion Catalog #AM9760G**
- Igepal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I8896**
- SDS, 10% Solution **Life Technologies Catalog #AM9822**
- Magnesium Chloride Solution BioUltra **Merck MilliporeSigma (Sigma-Aldrich) Catalog #68475-100ML-F**
- Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1379-500ml**
- PEG400 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #91893**
- Lithium chloride (8M) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L7026-100ML**
- EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #AM9260G**
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977049**

Chemicals

- Sodium deoxycholate (SDC) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #30970**
- DTT, 1M **Thermo Fisher Catalog #P2325**
- Methanol **Fisher Scientific Catalog #code356T**

Lithium dodecyl sulfate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L4632**

Commercial Kits

Pierce™ Rapid Gold BCA Protein Assay Kit **Thermo Fisher Catalog #A53225**

ProNex® Size-Selective Purification System **Promega Catalog #NG2002**

BioAnalyzer High Sensitivity Chip **Agilent Technologies Catalog #5067-4626**

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Reagents

Dynabeads™ Protein G for Immunoprecipitation **Thermo Fisher Catalog #10004D**

cOmplete™, Mini, EDTA-free (Protease Inhibitor) **Roche Catalog ##11836170001)**

Oligo(dT)25 Dynabeads **Thermo Fisher Scientific Catalog #61005**

Phusion HF Buffer Pack **Thermo Fisher Catalog #F518L**

Phusion High-Fidelity PCR Master Mix with HF Buffer - 500 rxns (50 ul vol)**New England Biolabs Catalog #M0531L**

Enzymes

RNase I (10 U/µL) **Thermo Fisher Catalog #EN0602**

TURBO™ DNase (2 U/µL) **Thermo Fisher Scientific Catalog #AM2238**

SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #AM2696**

T4 Polynucleotide Kinase - 2,500 units **New England Biolabs Catalog #M0201L**

T4 RNA Ligase 1 (ssRNA Ligase) - 5,000 units **New England Biolabs Catalog #M0204L**

Proteinase K Solution (20 mg/mL) **Thermo Fisher Scientific Catalog #AM2546**

SuperScript™ IV Reverse Transcriptase **Thermo Fisher Scientific Catalog #18090050**

Terminal Deoxynucleotidyl Transferase **Takara Bio Inc. Catalog #2230B**

T4 RNA Ligase High Concentration **New England Biolabs Catalog #M0437**

SDS-PAGE

NuPAGE™ LDS Sample Buffer (4X) **Invitrogen - Thermo Fisher Catalog #NP0008**

2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M6250**

NuPAGE™ 4-12% Bis-Tris Midi Protein Gels, 20-well, w/adapters **Thermo Fisher Catalog #WG1402A**

NuPAGE™ MOPS SDS Running Buffer (20X) **Invitrogen - Thermo Fisher Catalog #NP000102**

Nitrocellulose Membrane 0.45 um **Bio-Rad Laboratories Catalog #1620115**

NuPAGE™ Transfer Buffer (20X) **Thermo Fisher Catalog #NP00061**

Precision Plus Protein All Blue Prestained Protein Standards **Bio-Rad Laboratories Catalog #1610373**

Thick Blot Filter Paper Precut 9.5 x 15.2 cm **Bio-Rad Laboratories Catalog #1704085**

Consumables

1.5 mL LoBind tubes **Eppendorf Catalog #022431021**

BRAND(TM) PCR TUBE STRIPS OF 8 ATTACHED SINGLE CAPS STANDARD PROFILE 0.2 ML **Merck MilliporeSigma (Sigma-Aldrich) Catalog #BR781332-120EA**

RNaseZap® **Thermo Scientific Catalog #AM9780**

PCR Seals **Thermo Scientific Catalog #AB0558**

Swann-Morton™ Sterile Disposable Stainless Steel Scalpels **Fisher Scientific Catalog #11798343**

Oligonucleotides

	Name	Sequence	Scale	Purification	Index
	3' Adapter				
	TLC-L3	/5Phos/AGATCGGAAGAGCACACGTCTGAAAAAAAAAAAAAAAAAAAAAAAA/3IR800CWN/	250nm	RNASE	
	5' Adapter				

	Name	Sequence	Scale	Purification	Index
	TLC_L01	/5Phos/NNNNATCACGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	CGTGAT
	TLC_L02	/5Phos/NNNNCGATGTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	ACATCG
	TLC_L03	/5Phos/NNNNTTAGGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	GCCTAA
	TLC_L04	/5Phos/NNNNTGACCANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	TGGTCA
	TLC_L05	/5Phos/NNNNACAGTGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	CACTGT
	TLC_L06	/5Phos/NNNNGCCAATNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	ATTGGC
	TLC_L07	/5Phos/NNNNCAGATCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	GATCTG
	TLC_L08	/5Phos/NNNNACTTGANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	TCAAGT
	TLC_L09	/5Phos/NNNNGATCAGNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	CTGATC
	TLC_L10	/5Phos/NNNNTAGCTTNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	AAGCTA
	TLC_L11	/5Phos/NNNNATGAGCNNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	GCTCAT
	TLC_L12	/5Phos/NNNNCTTGTANNNNNAGATCGGAAGAGCGTCGTG/3ddC/	100nm	STD	TACAAG
	cDNA Amplification				
	P5short_TLC-CLIP	ACACGACGCTCTTCCGATCT	100nm	PAGE	
	P7short_TLC-CLIP	TGACGTGTGCTCTTCCGATCT	100nm	PAGE	
	PCR amplification				
	P5_Universal_adapter	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	1um	PAGE	
	P7-1_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	ATCACG
	P7-2_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	CGATGTAT
	P7-3_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	TTAGGCAT
	P7-4_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	TGACCAAT
	P7-5_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	ACAGTGAT

	Name	Sequence	Scale	Purification	Index
	P7-6_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	GCCAATAT
	P7-7_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	CAGATCAT
	P7-8_TLC-CLIP	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T	250nm	PAGE	ACTTGAAT

SAFETY WARNINGS

Prolonged exposure to UVC light can cause skin and eye damage. Ensure that the UV-crosslinker functions properly and follow all safety precautions specified in the manufacturer's instructions.
Some of the chemicals and reagents used in this protocol can be hazardous if not handled properly. Always follow the safety precautions outlined in the Safety Data Sheets (SDS) provided by the manufacturer.

BEFORE START INSTRUCTIONS

Prepare Buffers

iCLIP Lysis Buffer

	Final Concentration	Stock Solution	For 500 ml
	50 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.5	25 ml
	100 mM NaCl	5 M NaCl	10 ml
	1% Igepal-CA-630	100% Igepal CA-630	5 ml
	0.1% SDS	10% SDS	5 ml
	0.5% sodium deoxycholate		2.5 g
	Add H2O		455 ml

High Salt Wash Buffer

	Final Concentration	Stock Solution	For 500 ml

	Final Concentration	Stock Solution	For 500 ml
	50 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.5	25 ml
	1 M NaCl	5 M NaCl	100 ml
	1 mM EDTA	0.5M EDTA, pH 8.0	1 ml
	1% Igepal-CA-630	100% Igepal CA-630	5 ml
	0.1% SDS	10% SDS	5 ml
	0.5% sodium deoxycholate		2.5 g
	Add H2O		364 ml

PNK Wash Buffer

	Final Concentration	Stock Solution	For 500 ml
	20 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.5	10 ml
	10 mM MgCl2	2 M MgCl2	2.5 ml
	0.2% Tween-20	100% Tween-20	1 ml
	Add H2O		486.5 ml

5X PNK Buffer

	Final Concentration	Stock Solution	For 10 ml
	350 mM Tris-HCl, pH 6.5	1 M Tris-HCl, pH 6.5	3.5 ml
	50 mM MgCl2	2 M MgCl2	0.25 ml
	5 mM DTT	1M DTT	0.05 ml
	Add H2O		6.2 ml

Freeze individual use aliquots to avoid freeze-thaw cycles.

4X Ligation Buffer

	Final Concentration	Stock Solution	For 10 ml
	200 mM Tris-HCl, pH 7.8	1 M Tris-HCl, pH 7.8	2 ml
	40 mM MgCl2	2 M MgCl2	0.2 ml

	Final Concentration	Stock Solution	For 10 ml
	4 mM DTT	1M DTT	0.04 ml
	Add H2O		7.76 ml

Freeze individual use aliquots to avoid freeze-thaw cycles.

Proteinase K Buffer

	Final Concentration	Stock Solution	For 50 ml
	100 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.4	5 ml
	50 mM LiCl	8 M LiCl	0.3125 ml
	1 mM EDTA	0.5 M EDTA	0.1 ml
	0.2% LiDS	1% LiDS	10 ml
	Add H2O		34.5875 ml

Oligo(dT) Binding Buffer

	Final Concentration	Stock Solution	For 50 ml
	20 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.4	1 ml
	1 M LiCl	8 M LiCl	6.25 ml
	2 mM EDTA	0.5 M EDTA	0.2 ml
	Add H2O		42.55 ml

Oligo(dT) Wash Buffer

	Final Concentration	Stock Solution	For 50 ml
	10 mM Tris-HCl, pH 7.4	1 M Tris-HCl, pH 7.4	0.5 ml
	150 mM LiCl	8 M LiCl	0.9375 ml
	0.1 mM EDTA	0.5 M EDTA	0.01 ml
	Add H2O		48.5525 ml

First-Strand (FS) Buffer (5X)

	Final Concentration	Stock Solution	For 10 ml
	250 mM Tris-HCl, pH 8.3	1 M Tris-HCl, pH 8.3	2.5 ml
	375 mM KCl	1 M KCl	3.75 ml
	15 mM MgCl2	1 M MgCl2	0.15 ml
	Add H2O		3.6 ml

Generation of preadenylated TLC-L3 adapter

TLC-L3 oligo was ordered from IDT at 250 nmole scale, carrying a 5' phosphorylation and 3' IRDye® 800CW (NHS Ester) (v3) modification and purified using RNase-free HPLC with a total yield of 21.1 nmoles.

1. Set up 50 µl of 100 µM TLC-L3 adapter (5 nmoles) with 25 µl 10X 5' DNA Adenylation Reaction Buffer, 25 µl 1 mM ATP and 50 µl Mth RNA Ligase (1nmol) in a total volume of 200 µl using the 5' DNA Adenylation Kit - 50 rxns **New England Biolabs Catalog #E2610L**.
2. Incubate at 65ºC for 2 hours followed by inactivation at 85ºC for 10 minutes (reaction turns cloudy).
3. Clean up using the Nucleotide Removal Kit **Qiagen Catalog #28304** by mixing the 200 µl preadenylation reaction with 4.8 ml PNI buffer and distributing over 10 columns of Nucleotide Removal Kit.
4. Spin down at 6000 rpm for 30 seconds.
5. Wash once in 750 µl PE and spin for 1 minute at 6000 rpm, followed by an empty spin at full speed.
6. Transfer to a new collection tube and add 50 µl H_2O per column and incubate at RT for 2 minutes
7. Spin at 6000 rpm for 1 minute to elute
8. Combine eluates at an approximate final concentration of 10 µM and prepare 1 µM working stocks to be stored at -20ºC.

UV crosslinking and generation of cell lysates

45m

1 UV crosslinking and generation of cell lysates

Grow desired cell line to ~80% confluency in appropriate culture conditions.

1.1 Remove media, wash once in ice-cold PBS and drain cells of all fluid. 5m

1.2 Transfer the plate onto ice, remove lid of the culture dish, and crosslink at 254 nm with 300 mJ/cm^2. 5m

On ice

Equipment

UVP Crosslinker CL-3000	NAME
Analytic Jena	BRAND
UVPA849-95-0615-02	SKU
https://www.uvp.com/products/lab-equipment/uvp-crosslinker/	LINK

1.3 Scrape cells into 5 ml PBS, count then aliquot desired number of cells and spin down. 10m

1.4 Resuspend cell pellet in iCLIP Lysis Buffer - 1 ml buffer for 1 million cells - scale accordingly. 5m

1.5 Place lysates on ice for 5 minutes. 5m

On ice 00:05:00

1.6 Sonicate lysates for 10-20 seconds at 0.5sec ON and 0.5sec OFF at 10% amplitude to reduce viscosity. 5m

-80 °C Safe Stopping Point

1.7 Quantify cell lysates with Pierce BCA Quantification Assay or other method of choice to determine protein concentration. 10m

Expected result

We aim for lysate concentrations of 0.5 µg/µl for CLIP experiments.

Concentrations will differ between samples and cell types - adjust the amount of iCLIP Lysis Buffer used in step 1.4 to your samples to reach the desired concentration.

Preparation of bead-antibody mixture 45m

2

Preparation of bead-antibody mixture

We routinely use 1-2 µg of antibody per IP in 25-50 µg of cell lysate.

Note

100 µl of protein-G beads bind 20-30 µg of antibody - calculate total volume of beads necessary based on amount of antibody used for experiment.

2.1 Wash appropriate amount of beads twice in 1 ml iCLIP Lysis Buffer, then resuspend in 100 µl per antibody 5m

2.2 Add antibody to beads and incubate at room temperature for 30-60 minutes rotating. 30m

Room temperature

00:30:00

Note

Continue with section 3 'RNase and DNase Treatment of Lysates' during this incubation step.

2.3 Wash beads twice in 1 ml iCLIP Lysis buffer then resuspend in 10 µl 6X Protease Inhibitor per IP. 5m

Note

Add the appropriate amount of antibody-bead mixture to cell lysate to achieve 1X Protease Inhibitor concentration.

RNase and DNase Treatment of Lysates 30m

3 **RNase and DNase Treatment of Lysates**

Perform these steps while beads are coupling to antibodies.

Note

Ensure to use the same concentration and the same volume of lysate for a given RBP after optimisation of RNase concentrations.

When optimising RNase concentrations, starting points can be a final amount of 0.25U, 0.025U and 0.005U of RNaseI (EN0602) for 50.000 293T cells (50 µl of lysate at ~0.5 µg/µl).

Note

We aim for a lysate concentration of ~0.5 µg/µl, more concentrated lysates can lead to higher background signal, but can be necessary for lowly expressed RBPs.

3.1 Make serial dilutions to achieve desired RNaseI dilution then add 10 µl of RNaseI and 2 µl of Turbo DNase to cell lysates 5m

3.2 Digest RNA for exactly 3 minutes at 37°C shaking at 1100 rpm then immediately transfer to ice and incubate for another 3 minutes. 10m

Note

Keep digestion time consistent between experiments to avoid over-digestion of RNA.

3.3 Spin lysates for 10 minutes at 4°C at 16,000g then transfer lysates to a fresh tube. 15m

4 °C 00:10:00

Immunoprecipitation and Washes 2h 30m

4 **Immunoprecipitation and Washes** 5m

Keep the timing of IP consistent between experiments as RNaseI remains in samples and has residual activity even at 4°C.

4.1 Set up 50 µl of RNase-treated lysate with 10 µl of antibody-bead mixture in 6X Protease Inhibitor and incubate for 2 hours at 4°C. 2h

4 °C 02:00:00

4.2 Magnetically attract beads and remove supernatant. 5m

> Note
>
> Unbound fraction of IP can be kept to test IP efficiency via Western Blot when optimising conditions.

4.3 Wash 2 x in 200 µl High Salt Buffer and keep the second wash for at least 1 minute at 4°C. 10m

4.4 Wash 2 x in 200 µl PNK Wash Buffer and keep in PNK Wash Buffer until ready to proceed. 10m

Dephosphorylation and first adapter ligation 45m

5 **Dephosphorylation and first adapter ligation**

5.1 Prepare PNK reaction and add 20 µl per sample. 10m

	PNK Reaction	x 1	x #
	5X PNK Buffer	4 µl	
	SuperaseIN	0.5 µl	
	T4 PNK	0.5 µl	
	H2O	15 µl	

5.2 Incubate at 37°C for 20 minutes with interval mixing in ThermoMixer. 20m

1800 rpm, 37°C 15 seconds shaking every 2 minutes

Equipment

Eppendorf Thermomixer C Model 5382 NAME

Thermomixer C TYPE

Eppendorf BRAND

5382000023 SKU

5.3 Remove PNK reaction and wash once in 200 µl PNK Wash Buffer. 5m

5.4 Prepare Ligation mix and add 20 µl per sample. 10m

	Ligation Mix	x 1	x #
	4X Ligation Buffer	5 µl	
	T4 RNA Ligase	1 µl	

	Ligation Mix	x 1	x #
	1 µM L3 Adapter	1 µl	
	SuperaseIN	0.5 µl	
	H2O	8.5 µl	
	PEG400	4 µl	

Prepare Ligation Reaction without PEG, mix then add PEG400 and mix by pipetting 10X with P1000.

5.5 Incubate at 16°C overnight or at 25°C for 75 minutes with interval mixing in ThermoMixer.

1800 rpm, 16°C 15 seconds shaking every 2 minutes

Overnight

Purification of RNA-protein complexes

4h 30m

6 Purification of RNA-protein complexes

Purification via SDS-PAGE can be omitted to enable a 2-day workflow (see "Omission of PAGE purification"). Please refer to the critical discussion of this step in Ernst et al. (2023) before choosing this workflow.

6.1 Remove ligation reaction and wash twice in 200 µl High Salt Buffer and twice in 200 µl PNK Wash Buffer. 20m

> **Note**
>
> When omitting PAGE purification, see step case "noPAGE" for how to proceed.

6.2 Resuspend in 20 µl 1X LDS sample buffer containing 5% ß-mercapto-ethanol. 5m

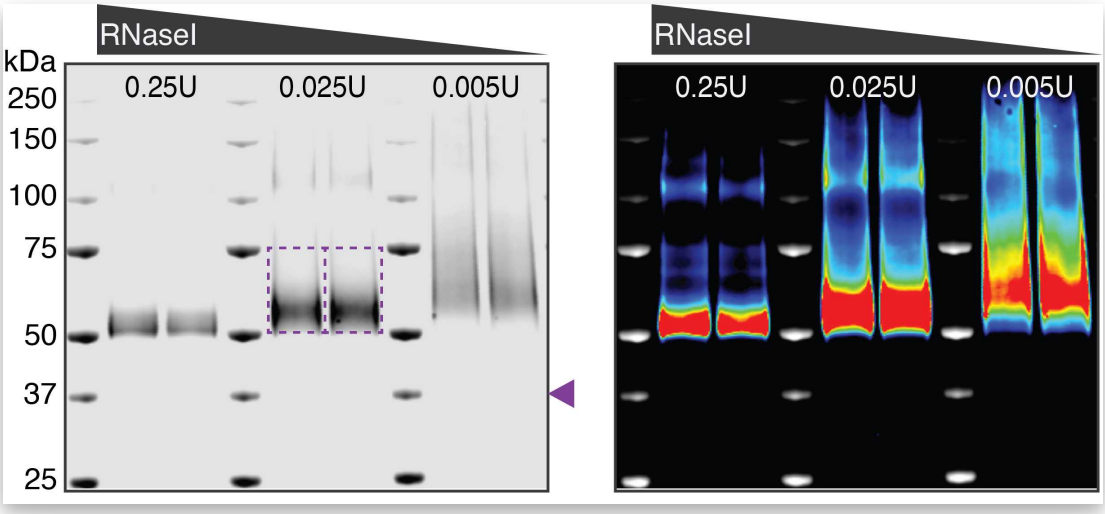
6.3 Denature at 70°C for 1 minute then proceed to PAGE purification. 5m

6.4 Resolve RNA-protein complexes on NuPAGE 4-12% Bis-Tris Gel at 180V for 60 minutes. 1h 15m

6.5 Transfer onto nitrocellulose in 1X NuPAGE transfer buffer with 10% methanol at 30V for 2 hours at RT. 2h 15m

6.6 Scan nitrocellulose membrane on Licor infrared scanner with 169 µm resolution. 15m

Expected result



Representative image for hnRNPA1 at three different RNaseI concentrations.

The image shows a representative example of the membrane scan for hnRNPA1 (indicated by a purple triangle), testing three different concentrations for RNaseI (0.25, 0.025 and 0.005U). The image is shown in greyscale (left) and pseudo colouring with higher contrast (right) to show different signal intensities. Purple rectangles indicate the region processed for library preparation.

6.7 Place nitrocellulose membrane on filter paper soaked in PBS to cut out region of interest and place nitrocellulose pieces in 1.5 ml LoBind Tubes. 15m

Note

Region of interest usually corresponds to ~20-60 kDa above the molecular weight of the RBP of interest due to the ligation of TLC-L3 adapter (~15.9 kDa) and additional weight depending on the length of associated RNA molecule (70 nt of RNA are on average 20kDa).

Step 6.7 includes a Step case.
noPAGE

RNA Purification

1h 30m

step case

noPAGE

1. When omitting PAGE purification, magnetic beads bound to RNA-protein complexes in step 6.2 can be directly resuspended in 100 µl Proteinase K buffer containing 100 µg of Proteinase K and incubated at 50°C at 800 rpm for 45 minutes (see Step 7.1).
2. Attract beads and transfer supernatant to fresh PCR tubes containing oligo(dT) beads (see Step 7.4).

7 RNA Purification

Upon capture on oligo(dT) beads (Step 7.5), resuspend beads throughout all steps by vortexing unless otherwise stated. Depending on the reaction, oligo(dT) beads can be sticky and mixing by pipetting can cause unnecessary loss of material through retention in pipette tips.

7.1 Add 200 µl of Proteinase K buffer containing 100 µg of Proteinase K to LoBind tube containing nitrocellulose pieces and incubate at 50°C at 800 rpm for 45 minutes. 1h

800 rpm, 50°C, 00:45:00

Note

1 µl of Proteinase K reaction can be dot blotted on nitrocellulose to visualise RNA release.

7.2 Meanwhile, prepare 10 µl of oligo(dT) beads per sample and wash once in 1 ml oligo(dT) Binding Buffer. 10m

7.3 Resuspend oligo(dT) beads in 50 µl Binding Buffer per sample and distribute in fresh PCR tubes. 5m

7.4 Transfer Proteinase K reaction to oligo(dT) beads and incubate at RT for 10 minutes rotating. 15m

7.5 Wash twice in 125 µl oligo(dT) Wash Buffer and once in 20 µl 1X First-Strand (FS) Buffer by vortexing. 15m

Reverse Transcription

50m

8 Reverse Transcription and RNA elution

8.1 Prepare RT reaction and add 10 µl to beads - vortex to mix. 10m

	Reverse Transcription	x1	x #
	5X FS Buffer	2 µl	
	10mM dNTPs	0.5 µl	
	0.1M DTT	0.1 µl	
	SuperaseIN	0.3 µl	
	Superscript IV	0.1 µl	
	H2O	7 µl	

8.2 Incubate at 50°C for 15 minutes with interval mixing then heat up to 96°C on thermomixer. 25m

Note

Seal tubes with PCR plates seal to avoid lids from opening at higher temperatures on heatblock.

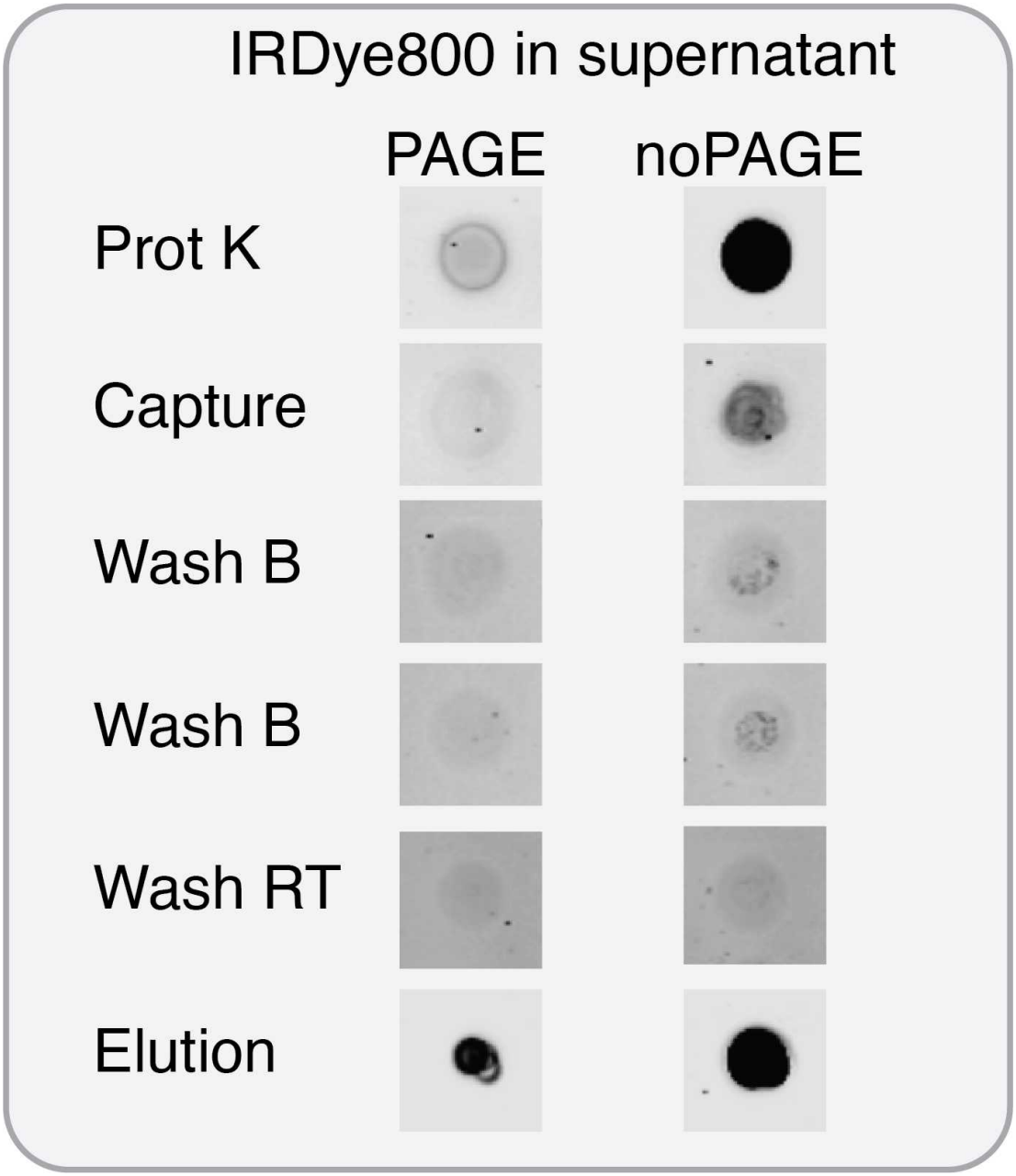
8.3 When 96°C is reached, vortex for 30 seconds on heatblock, spin down, and immediately place on magnet on ice. 5m

8.4 Remove supernatant and wash beads once in 60 µl oligo(dT) Wash buffer and once in 20 µl 1X T4 RNA Ligase buffer - vortex to mix. 10m

Note

Supernatant can be kept to visualise the elution of RNA-TLC-L3 hybrid through dot blotting on nitrocellulose.

Expected result



Example of dot blots at different steps during the protocol to visualise the amount of adapter-ligated RNA.

Second adapter ligation

50m

9 Second adapter ligation

9.1 Add 5 µl of 5' adapter mix to beads and vortex to mix. 5m

	5' Adapter Mix	x1	x #
	10X T4 RNA Ligase Buffer	2 µl	
	10 µM L## oligo	2 µl	
	DMSO	1 µl	

Note

Ensure balanced nucleotide composition of in-read barcodes that are used at this step, by using at least four different adapters that, when multiplexed, have a balanced 'per base sequence content' for the barcode sequence.

9.2 Incubate at 75°C for 2 minutes then immediately place on ice. 5m

9.3 Add 4 µl of Ligation mix at the top of the tube, spin down and vortex to mix. 5m

	Ligation mix	x1	x #
	0.1 M ATP	0.5 µl	
	TdT (14U/µl)	0.5 µl	
	T4 RNA Ligase High Conc. (30U/µl)	0.5 µl	
	H20	2.5 µl	

Note

Beads can be sticky, avoid touching with pipette tip when adding ligation mix!

9.4 Add 10 µl PEG8000, spin down and vortex, then resuspend beads by pipetting up and down 10X at slow speed. 10m

9.5 Incubate at 37°C for 20 minutes, then cool down to 20°C. 20m

37 °C 00:20:00

9.6 Add 1 µl of T4 RNA Ligase (High Concentration) and pipette to mix before incubating overnight at 20°C. 5m

Overnight 20 °C

cDNA pre-amplification 1h

10 cDNA pre-amplification

10.1 Add 100 µl of oligo(dT) Wash buffer to ligation reaction and place on magnet. 5m

Note

Beads don't resuspend properly at this point - apply magnetic field from different sides of the tube until beads move swiftly from one side to the other in order to aid resuspension.

10.2 Discard supernatant and wash once more with 100 µl oligo(dT) supernatant and once in 20 µl 1X Phusion HF Buffer - vortex to mix. 10m

10.3 Prepare cDNA pre-amplification mix and resuspend beads in 25 µl - vortex to mix. 5m

	cDNA amplification	x1	x #
	2X Phusion HF PCR Mastermix	12.5 µl	
	10 µM P7&P5 short	1.25 µl	
	H2O	11.25 µl	

10.4 Amplify with the following programme: 20m

1. 98°C - 30 seconds
    2. 98°C - 10 seconds
    3. 65°C - 30 seconds
    4. 72°C - 30 seconds
        Go to Step #2 6 times
5. 72°C - 3 minutes
6. 16°C - HOLD

Note

When preparing libraries after omission of PAGE purification a total of 6 pre-amplification cycles are sufficient.

10.5 Wash 1-2 µl of oligo(dT) beads per sample in oligo(dT) Binding buffer and resuspend in 5 µl oligo(dT) binding buffer per sample. 5m

10.6 Add 5 µl of oligo(dT) beads to PCR reaction and rotate at RT for 5 minutes. 10m

10.7 Place on magnet and transfer amplified cDNA in supernatant to a fresh tube. 5m

Note

Pre-amplified cDNA can be stored at 4°C for short-term or -20°C for longer term.

cDNA size selection 50m

11 **cDNA size selection**

11.1 Perform ProNex Size Selection using 2.8X ProNex beads - add 84 µl of ProNEX beads and mix by pipetting up and down 10 times. 5m

> **Note**
>
> The exact ratio of ProNEX beads might have to be optimised for different batches of beads.

11.2 Incubate for 10 minutes at RT then place on magnet for 5 minutes. 15m

11.3 Discard supernatant and wash twice in 200 µl Wash Buffer with beads remaining on the magnet. 5m

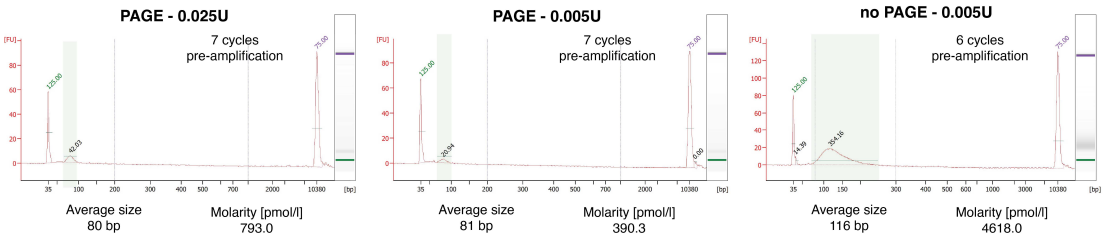
11.4 Remove Wash buffer and air-dry the beads on magnet until pellet starts to show cracks. 10m

11.5 Resuspend in 23 µl H_2O and incubate for 5 minutes at RT. 10m

11.6 Place on magnet and transfer 20 µl of supernatant to a new tube. 5m

Expected result

Pre-amplified cDNA can be run on High Sensitivity Bioanalyser Chip as anadditional quality control step, but will most likely only show signal for strong RBPs.



PCR amplification

1h 45m

12 PCR amplification

Estimate the necessary number of PCR cycles for ideal library amplification by running a test qPCR on 1 µl of pre-amplified cDNA and subtract 3-4 cycles from the obtained Ct value for the final library amplification. Overamplification of TLC-CLIP libraries should be avoided to minimise the number of PCR duplicates and increase the final yield of usable reads.

12.1

1h

Prepare qPCR Mastermix and add 9 µl to 1 µl of cDNA.

	Test qPCR	x 1	x #
	2X PowerUP SYBR Green Mastermix	5 µl	
	10 µM P5 + P7 primers	0.5 µl	
	H2O	3.5 µl	

Determine optimal cycle number by running the following programme:

1. 98°C - 30 seconds

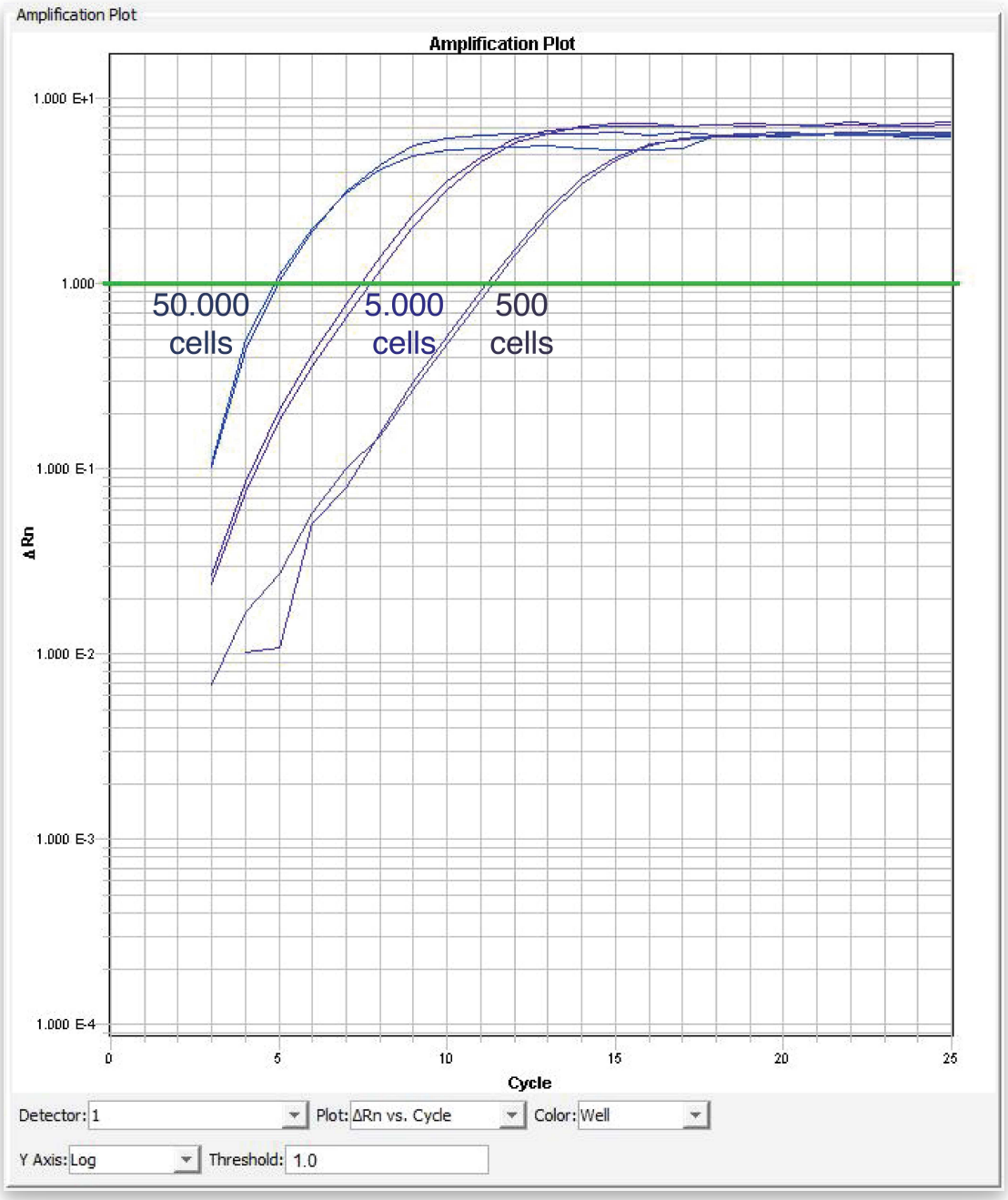
20 cycles of:

2. 98°C - 10 seconds
3. 68°C - 30 seconds
4. 72°C - 30 seconds

Note

Calculate required PCR cycles for amplification by removing 3-4 cycles from the determined Ct value.

Expected result



Amplification curves for duplicate libraries from different starting material.

12.2 Mix 10 µl of cDNA with 30 µl of PCR Mastermix and amplify with the following programme: 45m

	PCR	x 1	x #
	2X Phusion HF Mastermix	20 µl	
	10 µM P5 + P7 primers	1 µl	
	H2O	9 µl	

1. 98°C - 30 seconds
   # of cycles:
   2. 98°C - 10 seconds
   3. 68°C - 30 seconds
   4. 72°C - 30 seconds
5. 72°C - 3 minutes
6. 16°C - HOLD

Note

Add different i7 indexes at this point to allow greater multiplexing.

Note

10 µl of cDNA is used for PCR amplification to allow repetition in case of substantial over-amplification or unexpected size profile.

Size-selection of libraries 50m

13 **Size-selection of libraries**

13.1 Perform ProNex Size Selection using 1.8X ProNex beads - add 72 µl of ProNEX beads and mix by pipetting up and down 10 times. 5m

13.2 Incubate for 10 minutes at RT then place on magnet for 5 minutes. 15m

13.3 Discard supernatant and wash twice in 200 µl Wash Buffer while on magnet. 5m

13.4 Air-dry the beads on magnet until pellet starts to show cracks 10m

13.5 Resuspend in 17.5 µl H2O and incubate for 5 minutes at RT. 10m

13.6 Place on magnet and transfer 15 µl of supernatant to a new tube. 5m

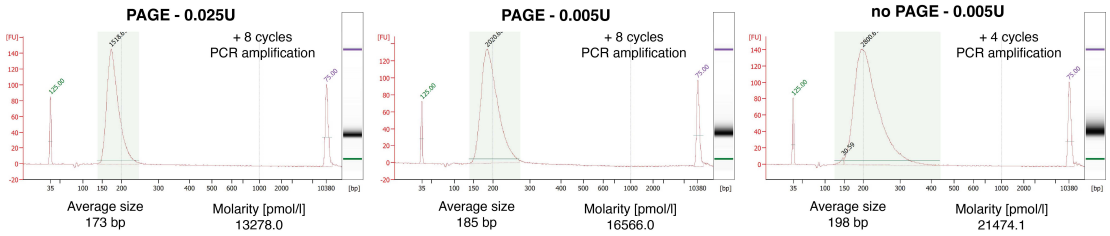
Quality Control and Quantification 1h

14 **Quality Control and Quantification**

Prepare 1:3 or 1:5 dilution for QC and quantification.

14.1 Run 1 µl on Agilent High Sensitivity DNA Chip. 1h

Expected result



14.2 Quantify libraries using Qubit High Sensitivity dsDNA kit. 20m

Multiplexing and sequencing

15 Multiplexing and sequencing

Multiplex samples in the final pool at the desired ratios and sequence on Illumina NextSeq500 using the High Output Kit for 75 cycles with the addition of 5% PhiX.

Demultiplexing and Trimming

16 Demultiplexing and Trimming of Reads using Flexbar

This is done in a two-step approach for the following reason:
The easiest way to trim 3' adapter contamination is to specifcy the entire adapter sequence (see Flexbar_2 below) with 'N' being used for the i7 index positions so that this step does not have to be run individually for different i7 indeces.

However, if this is specified together with --umi-tags, any bases on the 3' end that might correspond to the i7 index will be added to the read header and appended to the UMI which interferes with umi-tools as some UMIs will have more characters than others.

As such it is easiest to handle the demultiplexing based on In-read barcodes as well as the UMIs in the first step, and then trim remaining homopolymers at the 5' end as well as adapter contamination at the 3' end in a second step.

16.1 Demultiplexing based on In-read barcodes

Flexbar.v3.4 was used.
This command demultiplexes based on in-read barcodes and moves UMIs in the read header.

-r sample.fastq.gz # Fastq file demultiplexed based on i7 index but not trimmed
-b TLC_barcodes.fasta # Fasta file specifying in-read barcodes and UMIs
--barcode-unassigned # Generates file containing all unassigned barcodes
--barcode-trim-end LTAIL # Defines barcode position within the read
--barcode-error-rate 0 # Determines number of mismatches and indels allowed in barcode
--umi-tags # Wildcard character 'N' specified within barcode fasta file will be appended to read name separated by underscode
-n 4 # Number of threads
-t path/sample_demult # Prefix of output files
-z GZ # Output files are compressed using gzip

Command

Flexbar_1

```
flexbar -r sample.fastq.gz -b TLC_barcodes.fasta --barcode-unassigned --barcode-trim-end LTAIL --barcode-error-rate 0 --umi-tags -n 4 -t path/sample_demult -z GZ
```

Example of TLC_barcodes.fasta file

Command

Fasta file containing in-read barcodes for demultiplexing with Flexbar.

```
>TLC_L01
NNNNNCGTGATNNNN
>TLC_L02
NNNNNACATCGNNNN
>TLC_L03
NNNNNGCCTAANNNN
>TLC_L04
NNNNNTGGTCANNNN
>TLC_L05
NNNNNCACTGTNNNN
>TLC_L06
NNNNNATTGGCNNNN
>TLC_L07
NNNNNGATCTGNNNN
>TLC_L08
NNNNNTCAAGTNNNN
>TLC_L09
NNNNNCTGATCNNNN
>TLC_L10
NNNNNAAGCTANNNN
>TLC_L11
NNNNNGCTCATNNNN
>TLC_L12
NNNNNTACAAGNNNN
```

16.2 Trimming of adapter and homopolymers

flexbar.v3.4 was used
This command trims remaining homopolymers at the 5' end of reads as well as adapter contamination at the 3' end.

-r sample_demult.fastq.gz # Demultiplexed fastq files from previous trimming step
--adapter-seq
'AGATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTT

G'
Seqeunce of the Illumina Multiplexing Index Read Sequencing Primer with 'N' designating the position of different i7 indices.
--adapter-trim-end RIGHT # Defines which end of the read to trim the adapter sequence off
--adapter-error-rate 0.1 # Determines how many mismatches and indels are allowed for the adapter sequence to be removed
--adapter-min-overlap 1 # Minimum required overlap for adapter to be removed
--min-read-length 18 # Discards reads shorter than 18 nucleotides after trimming
-n 2 # Number of threads
--htrim-left T # Trims poly(T) from the left side of the read
--htrim-max-length 2 # Defines maximum length of poly(T) stretch to be trimmed as 2
--htrim-min-length 1 # Defines minimum length of poly(T) stretch as 1
-t path/sample_tr1-2 # Prefix of output files
-z GZ # Output files are compressed using gzip

Command

Flexbar_2

```
flexbar -r sample_demult.fastq.gz --adapter-seq 'AGATCGGAAGAGCACACGTCTGAACTCCAGTCACNNNNNNATCTCGTATGCCGTCTTCTGCTTG' --adapter-trim-end RIGHT --adapter-error-rate 0.1 --adapter-min-overlap 1 --min-read-length 18 --length-dist -n 2 --htrim-left T --htrim-max-length 2 --htrim-min-length 1 -t path/sample_tr1-2 -z GZ
```

Remove space from read header before mapping to keep UMI

Command

```
zcat $sample_tr1-2.fastq.gz | tr ' ' ':' | gzip -c > path/mapready/sample_tr1-2.fastq.gz
```

Mapping and Deduplication

17 Mapping is performed with STAR, keeping only uniquely mapping reads and removing the penalty for opening deletions or insertions.

17.1 STAR mapping

STAR v.2.7.3a was used

```
--runThreadN 8                          # Number of threads
-genomeDir /genome_index/STAR/    # Path to genome directory
--readFilesIn sample_tr1-2.fastq.gz    # Path to fastq file
--readFilesCommand zcat                # Uncompession Command for compressed fastq
--outSAMtype BAM SortedByCoordinate # Output sorted by coordinate
--limitBAMsortRAM 20000000000     # Maximum available RAM for sorting BAM
--outSAMattributes All                 # Include all attributes
--outSAMunmapped Within              # Output unmapped reads within the main SAM file
--runRNGseed 42                        # random number generator seed
--outTmpDir {localdir}                 # path to temporary directory
--outFilterMultimapNmax 1              # maximum number of multiple alignments allowed for a read
--outSJfilterReads Unique              # Only uniquely mapping reads are considered for splice junction output
--alignEndsType Extend5pOfRead1    # Fully extend only the 5' end of the read, local alignment for 3' end
--scoreDelOpen 0                       # Remove deletion open penalty
--scoreInsOpen 0                       # Remove insertion open penalty
--outFileNamePrefix path/sample      # Prefix for output files
```

Command

STAR_mapping

```
STAR --runThreadN 8 -genomeDir /genome_index/STAR/ --readFilesIn sample_tr1-2.fastq.gz  --readFilesCommand zcat --outSAMtype BAM SortedByCoordinate --limitBAMsortRAM 20000000000 --outSAMattributes All --outSAMunmapped Within --runRNGseed 42 --outTmpDir {localdir} --outFilterMultimapNmax 1 --outSJfilterReads Unique --alignEndsType Extend5pOfRead1 --scoreDelOpen 0 --scoreInsOpen 0 --outFileNamePrefix mapping/star/sample
```

17.2 Deduplication with umi-tools

UMI-tools v.1.0.1 was used in dedup mode:

-I mapping/star/sample_tr1-2.bam # mapped bam file to read
-L logs/umi_tools/umi_tools_sample.log # file with logging information
-S mapping/star_dd/sample_dd.bam # output file
--extract-umi-method read_id # Barcodes are contained at the end of the read header
--method unique # Reads group share the exact same UMI
--spliced-is-unique # Two reads that start in the same position on the same strand and having the same UMI are considered unique if one is spliced and the other is not.

Command

umitools_dedup

```
umi_tools dedup -I mapping/star/sample_tr1-2.bam -L logs/umi_tools/umi_tools_sample.log -S mapping/star_dd/sample_dd.bam --extract-umi-method read_id --method unique --spliced-is-unique
```