Buffers

iCLIP lysis buffer
50 mM Tris-HCl pH 7.4
100 mM NaCl
1% NP-40 (Igepal CA630)
0.1% SDS
0.5% sodium deoxycholate (protect from light)
1:200 Protease Inhibitor Cocktail III (add fresh)

High salt wash buffer
50 mM Tris-HCl pH 7.4
1 M NaCl
1 mM EDTA
1% NP-40
0.1% SDS
0.5% sodium deoxycholate (protect from light)

Wash buffer
20 mM Tris-HCl pH 7.4
10 mM $MgCl_2$
0.2% Tween-20

5X PNK pH 6.5 buffer
350mM Tris-HCl pH 6.5
50mM $MgCl_2$

1X FastAP Buffer
10mM Tris pH 7.5
5mM $MgCl_2$
100mM KCl
0.02% Triton X-100

1x RNA Ligase Buffer
50mM Tris-HCl pH 7.5
10mM $MgCl_2$

PK Buffer
100mM Tris-HCl pH 7.4
50mM NaCl
10mM EDTA

RLT Buffer
Qiagen cat # 79216

Enzymes

Turbo DNase	2 U/µl	LifeTech	AM2239
RNase I	100 U/µl	LifeTech	AM2295
FastAP	1 U/µl	LifeTech	EF0652
Murine RNase Inhibitor	40 U/µl	NEB	M0314L
T4 PNK	10 U/µl	NEB	M0201L
T4 RNA ligase 1 high conc	30 U/µl	NEB	M0437M
Proteinase K	0.8 U/µl	NEB	P8107S
Q5 PCR Master Mix		NEB	M0494L
Protease Inhibitor Cocktail III		EMD Millipore	
AffinityScript reverse transcriptase		Agilent	600107
Exo-SAP-IT		Affymetrix	78201

Beads

Dynabeads M-280 sheep anti-rabbit	10 mg/ml	LifeTech	
Dynabeads Protein G	30 mg/ml	LifeTech	37002D
Dynabeads MyOne Silane	40 mg/ml	LifeTech	

Agencourt AMPure XP beads Beckman Coulter A63881

Primers

RNA oligos:

Original RNA adapters:

(RNA_A01 & RNA_B06 create a colorbalanced pair)

RNA_A01 /5phos/rArUrUrGrCrUrUrArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

RNA_B06 /5phos/rArCrArArGrCrCrArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

(RNA_C01 & RNA_D08 create a colorbalanced pair)

RNA_C01 /5phos/rArArCrUrUrGrUrArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

RNA_D08 /5phos/rArGrGrArCrCrArArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

RiL19 /5phos/rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrG/3SpC3/

New RNA adapters (avoids low-complexity issues in HiSeq 2500 cluster identification)

(RNA_X1A & RNA_X1B create a colorbalanced pair)

RNA_X1A /5Phos/rArUrArUrArGrG rNrNrNrNrN rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

RNA_X1B /5Phos/rArArUrArGrCrA rNrNrNrNrN rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

(RNA_X2A & RNA_X2B create a colorbalanced pair)

RNA_X2A /5Phos/rArArGrUrArUrA rNrNrNrNrN rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

RNA_X2B /5Phos/rArGrArArGrArU rNrNrNrNrN rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrGrUrArG/3SpC3/

---> All RNA barcode adapters: 200 uM stock concentration (store at -80C), 20 uM working concentration

RiL19 /5phos/rArGrArUrCrGrGrArArGrArGrCrCrGrUrCrGrUrG/3SpC3/

(stock 200 uM; working 40 uM)

DNA oligos:

AR17 ACACGACGCTCTTCCGA (stock 200 uM; working 20 uM)

rand103Tr3 /5Phos/NNNNNNNNNNAGATCGGAAGAGCACACGTCTG/3SpC3/ (stock 200 uM; working 80 uM)

(Below we order page-purified)

PCR_F_D501 AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

PCR_F_D502 AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCT

PCR_R_D701 CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

PCR_R_D702 CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

(See Illumina customer service letter for D503-508, D703-712; any standard Illumina HT primers work fine)

(stock 100 uM; working 20 uM)

Notes

For this protocol (& for ENCODE eCLIP-seq experiments), one 'experiment' is defined as 4 libraries: 2 eCLIP experiments on UV crosslinked biological replicate samples, 1 eCLIP experiment on a non-UV crosslinked sample, and 1 size-matched input control (taken from one of the two UV crosslinked samples). Additionally, an IgG-only IP is run on the Western gel to validate antibody specificity.

For other experiments, one can modify this design to add the paired size-matched input control from the other replicate, remove the non-UV crosslinked sample, or add additional library controls (IgG, FLAG or V5 pulldown on wild-type cells, etc.) if desired.

Although we do not standardly do P32 labeling, we still use the "HOT" and "COLD" membranes nomenclature from iCLIP & other CLIP protocols. COLD = 10% of sample run as a standard Western blot; HOT = 80% of sample run for membrane cutting & RNA isolation

DAY 1

Prepare iClip lysis mix

- Pre-chill iCLIP lysis buffer
- Per sample (20 million cells): add **5.5 µl 200x Protease Inhibitor Cocktail III** to **1 mL iCLIP lysis buffer**, mix
    - ** Note: For tissues or cell-types with high endogenous RNAse, add 11 µl **Murine RNase Inhibitor per 1 mL lysis buffer** at this step (works for ES, Neuronal Stem Cell, many tissues). This may need to be further increased for particularly difficult samples (e.g. Pancreas).

Lyse cells (Do this first)

- **Lyse cells:**
    - Retrieve cell pellets from -80 degC freezer, immediately add 1 mL cold **iCLIP lysis mix** to each pellet, pipette to resuspend

      3 Pellets per experiment:

        - Sample 1: IP-A (UV-crosslinked batch #1)
        - Sample 2: IP-B (UV-crosslinked batch #2)
        - Sample 3: nonX-UV (non-UV crosslinked, batch #3)

IMPORTANT: for ENCODE, Sample 1 and Sample 2 MUST be different biological replicates. The simplest way to do this is to have different culture start date and culture end dates. If dates are similar, you must make sure before starting that the samples actually meet ENCODE criteria for being distinct biological replicate samples.

Potential sample 4

- Sample 4: IgG (non-UV-crosslinked batch #3)

(One 20 M IgG IP is good for 10 IP experiments & can be stored after IP and denaturation in NuPage buffer + DTT)

- Lyse 15 mins on ice

Couple antibody to magnetic beads (start while lysate on ice)

Note: Process IgG identically to antibodies

- **Beads and antibodies:**
  - Use **125 µl beads** per sample
    - rabbit antibodies: use sheep anti-rabbit beads
    - mouse antibodies: use sheep anti-mouse beads
  - Use **10 µg antibody** per sample

- **Prepare beads:**
  - Magnetically separate beads, remove supernatant
  - Wash beads 2x in 500 µl cold **iCLIP lysis buffer**
  - Resuspend beads in 100 µl cold **iCLIP lysis buffer**

- **Bind antibody:**
  - Add antibody (10 µg) to 100 µl washed beads
  - Rotate, room temp, 45 min

RNase treat lysate (while ab+bead binding**):**
- Sonicate in Bioruptor at 'low' setting, 4 degC, 5 min, 30sec on / 30 sec off
- Dilute RNase I in PBS at 1:25 on ice; use 10 µl diluted RNase I per sample
- Add 2 µl **Turbo DNase**, mix Immediately before use,
- Add 10 µl **diluted RNase I**, mix & immediately proceed to next step
- Incubate in Thermomixer at 1200 rpm, 37 degC, 5 mins (exactly), place on ice
- Immediately add 11 µl **Murine RNase Inhibitor**, mix (If added earlier, ignore this step)
- Centrifuge 15,000g, 4 degC, 15min
- Transfer supernatant to a new tube

Capture RBP-RNA complexes on beads

- Wash antibody beads 2x in 500 µl cold **iCLIP lysis buffer**
- Remove 20 µL (2%) of Sample 1, 2, 3 as BACKUP inputs for western; store at 4 degC
- Add remainder to washed antibody beads
- Rotate 4 degC, 2 h or overnight (in cold room)

Step: SAVE INPUT SAMPLES: Remove Input Samples
- Mix samples well
- To new tube, take 20 µL (2%) of Sample 1 (A-Input) for 'HOT' gel, store at 4 degC
- To new tube, take 20 uL (2%) of Sample 1 (A-Input), 2 (B-Input), 3 (NX-Input), 4 (IgG-Input) for COLD gel; store at 4 degC
- To new tube, take 2 uL (0.2%) of Sample 3 (NX-Input) for COLD gel 0.1% input lane; store at 4 degC
- To new tubes, take 5 tubes of 20ul each of sample 4 (IgG-Input) as COLD IgG Input samples.

Wash beads
- Wash 2x with 900µL cold **High salt wash buffer**
- Wash 1x with 500µL cold **Wash buffer**
- Transition to 1xFastAP buffer: add 500 µl cold **Wash buffer**, move through magnet, separate on magnet, add 500 µl **1xFastAP** buffer, mix, remove supernatant
- Wash 1x with 500 µl **1xFastAP buffer**

⇨ (If doing **IgG samples**: **pause** the IgG sample here and store on ice in Wash buffer)

FastAP treat beads (all samples except IgG)

- **Prepare FastAP master mix** on ice; 100 µl per sample:
  - H_2O 79 µl
  - 10x FastAP buffer 10 µl
  - Murine RNase Inhibitor 2 µl
  - Turbo DNase 1 µl
  - FastAP enzyme 8 µl

- Mix, add **100 µl** to each sample, incubate in Thermomixer at 1200 rpm, 37 degC, 15 min

PNK treat beads

- While beads are incubating, **prepare PNK master mix** on ice; 300 µl per sample:
  - H_2O 224 µl
  - 5X PNK pH 6.5 buffer 60 µl
  - 0.1 M DTT 3 µl
  - Murine RNase Inhibitor 5 µl
  - Turbo DNase 1 µl
  - T4 PNK enzyme 7 µl

- Mix, add **300 µl** to each sample, incubate in Thermomixer at 1200 rpm, 37 degC, 20 min

Wash beads

- Magnetically separate bead suspension, remove supernatant
- Wash 1x with 500µL cold **Wash buffer**
- Transition to High salt wash buffer: add 500 µl cold **Wash buffer**, move through magnet, separate on magnet, add 500 µl **High salt wash buffer**, move through magnet, remove supernatant
- Transition to Wash buffer: add 500 µl cold **High salt wash buffer**, move through magnet, separate on magnet, add 500 µl **Wash buffer**, move through magnet, remove supernatant
- Wash 1x with 500µL cold **Wash buffer**
- Transition to 1xLigase buffer (no DTT): add 500 µl **Wash buffer**, move through magnet, separate on magnet, add 300 µl **1xLigase buffer (no DTT)**, move through magnet, remove supernatant
- Wash 2X with 300 µl **1xLigase buffer (no DTT)**
- Prepare the 3' ligation master mix
- Just before adding the 3' ligation master mix, briefly spin tubes in minifuge, magnetically separate, remove residual liquid with fine tip

Ligate 3' RNA linker (on-bead)

- **Prepare 3' ligation master mix** on ice; 25 µl per sample:
  - H_2O 9 µl
  - 10x Ligase buffer (no DTT) 3 µl
  - 0.1 M ATP 0.3 µl
  - 100% DMSO 0.8 µl
  - 50% PEG 8000 9 µl
  - Murine RNase Inhibitor 0.4 µl
  - RNA Ligase high conc. 2.5 µl

- Mix carefµlly by pipetting or flicking (do not vortex)

- Add **25 µl** to each sample
- To each sample, add 2.5 µl of each of two different **barcoded RNA adapters** to each sample

  Acceptable RNA adapter pairs:
  - A01 + B06
  - C01 + D08
  - A03 + G07
  - A04 + F05
  - X1-A + X1-B
  - X2-A + X2-B
- Incubate at room temperature for 75 min; flick to mix every ~10 min

Wash beads (resume IgG sample here)

- Add 500µL cold **Wash buffer**, magnetically separate, remove supernatant
- Transition to High salt wash buffer: add 500 µl cold **Wash buffer**, move through magnet, separate on magnet, add 500 µl **High salt wash buffer**, move through magnet, remove supernatant
- Wash 1x with 500µL cold **High salt wash buffer**
- Transition to Wash buffer: add 500 µl cold **High salt wash buffer**, move through magnet, separate on magnet, add 500 µl **Wash buffer**, move through magnet, remove supernatant
- Wash 2x with 500µL cold **Wash buffer**

Prepare samples for gel loading

- **IP-Bead samples (HOT and COLD)**:
  **** Note: HOT & COLD are named relative to iCLIP gels; neither is radioactive in eCLIP**
  HOT = CLIP gel – for membrane transfer & RNA isolation
  COLD = WESTERN gel – for western imaging
  - **Remove s/n, a**dd **100** µl cold **Wash buffer**, resuspend beads well
  - Move 20 µl to new tube #1 = **COLD IP-WB samples**
  - Remaining 80 uL = **HOT IP samples**

- For **COLD IP-WB samples:**
  - **COLD IP-WB samples** 20.0 µl

  Add:
  - 4x NuPAGE buffer 7.5 µl
  - 1M DTT 3.0 µl

- For **HOT IP samples:**
  - Place sample on magnet, remove supernatant
  - Resuspend in **elution/loading master mix**; 30 µl per sample:
    - Wash buffer 20.0 µl
    - 4x NuPAGE buffer 7.5 µl
    - 1M DTT 3.0 µl

- For **HOT Input samples**, mix:
  - Input sample 20.0 µl (Saved in step x)
  - 4x NuPAGE buffer 7.5 µl
  - 1M DTT 3.0 µl

- For **COLD input samples**, mix:

Input sample:	1x	0.1x
Input sample	20.0 µl /	2.0 µl (Saved in Step: SAVE INPUT SAMPLES)
Wash buffer	0 µl /	18.0 µl
4x NuPAGE buffer	7.5 µl	
1M DTT	3.0 µl	

- For **IgG samples**:
  - Resuspend beads in 100 uL of wash buffer
  - 4x NuPAGE buffer 37.5 µl
  - 1M DTT 15.0 µl

  (Final volume 150 uL -> load 15 uL per well)

- Denature all samples in Thermomixer, 1200 rpm, 70 degC, 10 min
- Cool on ice 1 min, spin briefly in minifuge
- For **all samples**, transfer supernatant to new tube (IP AND Inputs have beads)

Load and run gels

- Load HOT gel (4-12% Bis-Tris, 10-well, 1.5 mm) with (M) pre-stained markers and (m) diluted pre-stained marker (2 uL marker, 2 uL 4x NuPAGE buffer, 6 uL Wash Buffer)

1	2	3	4	5	6	7	8	9	10
M	Input	(m)	A-IP	(m)	B-IP	(m)	NX-IP	M	(m)

Load:

HOT Input: 30 uL volume (30 uL denatured sample = 20 uL input lysate = 2% of input). HOT Input (for library prep) should come from crosslinked samples (either Sample A or Sample B).

IP-NX, IP-A, IP-B: 30 uL volume (80% of IP)

- Load COLD gel gel (4-12% Bis-Tris, -well, 1.5 mm)

1	2	3	4	5	6	7	8	9	10
NX-input (1:10 diluted)	IgG Input	IgG bead	NX-Input	NX-IP	M	A-IP	A-INPUT	B-IP	B-INPUT

Load:

Input & 1:10 input: Load 15 ul, save remaining 15 uL as backup (15ul denatured sample = 10ul lysate = 1% or 0.1% Input respectively)

IP: Load 15 ul, save remaining 15 uL as backup (15ul denatured sample = 10% of IP bead sample).

IgG: Load 15 uL, save remaining

- (All saved samples at -20C)
- Run at 150V in 1xMOPS running buffer, 75 min or until dye front is at the bottom

Transfer to membranes

- **Prepare transfer:**
  - (Have pre-prepared COLD (4 deg) transfer buffer with methanol: 1xNuPAGE transfer buffer, 10% methanol

- **COLD gel**: Prepare PVDF membrane(s): pre-flash 10 s in methanol, move to transfer buffer with methanol
- **HOT gel**: Prepare Nitrocellulose membrane(s): incubate in transfer buffer for > 1 min
- Wet sponges and Whatman papers in transfer buffer with methanol
- Assemble transfer stacks, from bottom to top (black side of stack holder on bottom):

  1x sponge – 2x Whatman paper – gel – membrane – 2x Whatman paper – 1x sponge

  Cold gel: PVDF membrane

  HOT gel: Nitrocellulose membrane

- **Transfer:**
  - overnight 30V (preferred) OR
  - 2 hr 200 mA (if doing this, only hook up one transfer box per power supply)

Day 2

- Remove HOT membrane, rinse quickly once with sterile 1X PBS, wrap in Saran wrap, store at -20C

Develop COLD membrane

- Block in 5% milk in TBST, room temp, 30 min
- Probe with primary antibody: 0.2 ug/ml (1:5000 for a 1 mg/ml stock; check antibody) in 5% milk in TBST, room temp, 1 hr.
- Wash 3x with TBST, 5 min
- Probe with secondary antibody: 1:4000 Rabbit TrueBlot HRP in in 5% milk in TBST, room temp, 1 – 3 h
    - (Note: if western fails or signal is low, 1:1000 gives higher signal)
- Wash 3x with TBST, 5 min
- Mix equal volumes of ECL Buffer A + Buffer B (or 40:1 of ECL Plus Buffer 1 to Buffer 2), add to membrane and incubate (mix/rotate) for 1-5 min. (1ml final volume per membrane)
- Develop 30 sec & 5 min, then judge signal (15 min maximum; if 15 sec is still too bright, expose two films

Cut HOT membrane

- Note RBP band on film with respect to prestained protein markers
- Place HOT membrane on clean glass/metal surface
- Using a fresh razor blade, cut lane from HOT membrane from the RBP band to 75 kDa above it
- Slice membrane pieces into ~1-2 mm slices, use a fresh razor blade for each sample
- Transfer slices to Eppendorf tube – place tube on ice if doing many samples
- Collect slices at the bottom of tube (centrifuge if necessary)

Release RNA from membrane

- Prepare **Proteinase K mix** on ice, 200 µl per sample:
    - PK buffer 160 µl
    - Proteinase K 40 µl
- Mix, add **200 µl** Proteinase K mix to membrane slices, incubate in Thermomixer at 1200 rpm, 37 C, 20 min (make sure all membrane slices are submerged)
- Prepare Urea/PK buffer: Dissolve 420 mg Urea in 500 µL PK buffer, then add PK buffer to final volume of 1 mL
- Add **200 µl** Urea/PK buffer to samples, mix, incubate in Thermomixer at 1200 rpm, 37 C, for an additional 20 min

Purify RNA

- Add 400 µL **acid phenol/chloroform/isoamyl alcohol** (pH 6.5), mix well by shaking, incubate in Thermomixer at 1200 rpm, 37 C, 5 min
- Spin briefly in picoFuge, transfer all except membrane slices to Phaselock gel HEAVY tube, incubate in Thermomixer at 1200 rpm, 37 C, 5 min
- Centrifuge at 13000g, 15 min, room temp (gel should have separated phenol and aqueous phases)
- Transfer aqueous layer to new 15 mL conical tube (at least 3 mL volume tube)

Zymo column cleanup (replaces precipitation) – RNA Clean & Concentrator-5 columns (Cat R1016)

*** Note: we replaced precipitation with the column preps to avoid phenol/chloroform carryover, which SIGNIFICANTLY inhibits Exo-SAP and can cause adapter dimer libraries. Either can work, but column cleanup is significantly safer for large-scale experiments**

- Add 2 volumes RNA binding buffer (usually 2 x ~400 = 800 uL)
- Add equal volume 100% ethanol & mix (usually ~1200 uL)
- Transfer 750 uL of mixed sample to Zymo-Spin column
- Centrifuge 30 sec & discard flow-through
- Repeat spins by reloading additional 750 uL volume until all sample has been spun through column
- Add 400 uL RNA Prep Buffer, centrifuge for 30 sec, discard flow through
- Add 700 uL RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 400 uL RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Centrifuge additional 2 mins
- Transfer column to new 1.5 mL tube (avoid getting wash buffer on column)
- **INPUT: Add 10 uL H2O to column, let sit for 1 min, centrifuge for 30 sec**
- **CLIP: Add 10 uL H2O to column, let sit for 1 min, centrifuge for 30 sec**
- **Store at -80 C until RT**

~~(Struck through is previous precipitation version of the protocol)~~

- ~~Add 400 uL **chloroform**, mix well by shaking, centrifuge 13,000g, 1 min, room temp~~
- ~~Transfer aqueous (upper) phase to new tube – AVOID TRANSFERRING ORGANIC PHASE (leave some aqueous phase if necessary, organic phase inhibits later steps)~~
- ~~add 2 µL **GlycoBlue**, 30 µL **3M NaOAc (pH 5.5),** vortex, spin briefly in picoFuge~~
- ~~Add 1 mL cold **100% EtOH**, mix well by inverting, precipitate at -80 C (O/N, or for at least 1h)~~

Day 3

START Inputs only →

- ~~Store CLIP samples at -80 C until RT~~

~~Precipitate input RNA~~

- ~~Centrifuge samples at 13,000g, 15 min, 4 degC~~
- ~~Locate pellet, carefully remove supernatant~~
- ~~Carefully add 750 µL **75% ice-cold EtOH**~~
- ~~Centrifuge at max speed, 5 min, 4 degC~~
- ~~Locate pellet, remove supernatant~~
- ~~Spin briefly in picoFuge, remove residual liquid with fine tip~~
- ~~Air-dry until dry (~10 mins).~~
- ~~Resuspend in **20 µL H_2O**~~

FastAP treat input RNA

- To 10 µL sample, add:
  - 10 uL H_2O
  - 2.5 µl 10X FastAP buffer
  - 0.5 µl RNase Inhibitor
  - 2.5 µl FastAP enzyme

- Mix, incubate in Thermomixer at 1200 rpm, 37 C, 15 min

PNK treat input RNA

- Make **PNK master mix**; 75 µl per sample:
    - H_2O 45 µl
    - 5xPNK 6.5 buffer 20 µl
    - 0.1M DTT 1 µl
    - Turbo DNase 1 µl
    - Murine RNase Inhibitor 1 µl
    - PNK enzyme 7 µl

- Mix, add **75 µl** to samples, mix, incubate in Thermomixer at 1200 rpm, 37 C, 20 min

Silane cleanup input RNA

- **Prepare beads:**
    - Magnetically separate 20 µl **MyONE Silane beads** per sample, remove supernatant
    - Wash 1x with 900 µl **RLT buffer**
    - Resuspend beads in 300 µL **RLT buffer** per sample

- **Bind RNA:**
    - Add beads in 300 µl **RLT buffer** to sample, mix
    - Add 10 µL **5M NaCl**
    - Add 615 µL **100% EtOH**
    - Mix, rotate at room temp, 15 min

- **Wash beads:**
    - Magnetically separate, remove supernatant
    - Add 1 mL **75% EtOH**, pipette resuspend and move suspension to **new tube**
    - After 30 s, magnetically separate, remove supernatant
    - Wash 2x with **75% EtOH** (let sit 30 s)
    - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
    - Air-dry 5 min

- **Elute RNA:**
    - Resuspend in **10 µl H_2O**, let sit for 5 min
    - Magnetically separate
    - Transfer 5 uL of supernatant to new tube (for 3' linker ligation below)
    - Transfer remainder of supernatant to new tube & store at -20 (this is the backup input RNA sample)

3' linker ligate input RNA

- **Anneal adapter:**
    - Take 5 µl of RNA (from above)
    - Add 1.5 µl 100% DMSO
    - Add 0.5 µl **RiL19** adapter
    - Incubate 65 C, 2 min
    - Place on ice >1 min

- **Prepare ligation master mix; 13.5 µl per sample:**
    - 10x NEB Ligase Buffer (with DTT) 2.0 µl

- 0.1M ATP 0.2 µl
- Murine RNase Inhibitor 0.2 µl
- 100% DMSO 0.3 µl
- 50% PEG 8000 8.0 µl
- RNA Ligase high conc 1.3 µl
- H_2O 1.5 µl

- Flick/pipette mix, add **13.5 µl** to each sample, flick/pipette-mix, incubate at room temp for 75 min
- Flick to mix every ~15 min

Silane cleanup input RNA

Note: can start next CLIP sample precipitation spin in parallel

- **Prepare beads:**
  - Magnetically separate 20 µl **MyONE Silane beads** per sample, remove supernatant
  - Wash 1x with 900 µl **RLT buffer**
  - Resuspend beads in 61.6 µL **RLT buffer**

- **Bind RNA:**
  - Add beads in 61.6 µl **RLT buffer** to sample, mix
  - Add 61.6 µL **100% EtOH**
  - Pipette mix, leave pipette tip in tube, pipette mix every ~3-5 min for 15 min

- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Add 1 mL **75% EtOH**, pipette resuspend and move to **new tube**
  - After 30 s, magnetically separate, remove supernatant
  - Wash 2x with **75% EtOH** (30 s)
  - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
  - Air-dry 5 min

- **Elute RNA:**
  - Resuspend in **10 µl H_2O**, let sit for 5 min
  - Magnetically separate, transfer supernatant to new tube

- Possible stopping point (Can store input samples at -80 C until next day)

←END Inputs only

~~(Struck through is previous precipitation version of the protocol)~~

~~Precipitate CLIP RNA (this can be done simultaneously to Silane cleanup above)~~

- ~~Centrifuge samples at 13000g, 15 min, 4 C~~
- ~~Locate pellet, remove supernatant~~
- ~~Carefully add 750 µL 75% ice-cold EtOH~~
- ~~Centrifuge at max speed, 5 min, 4 C~~
- ~~Locate pellet, remove supernatant~~
- ~~Spin briefly in picoFuge, remove residual liquid with fine tip~~
- ~~Air-dry until dry (~10 mins)~~

~~• Resuspend in **10 µL H_2O**~~

All CLIP and INPUT samples are now synchronized.

Reverse transcribe RNA (ALL CLIP and INPUTS)

- **Anneal primer** in 8-well strip tubes:
  - Mix 10µl of RNA with 0.5µl AR17 primer (using Rainin pipette + tips)
  - Heat 65 C for 2 min in pre-heated PCR block, place immediately on ice (do not cool down in PCR block)

- **Prepare reverse transcription master mix** on ice; 10 µl per sample:
  - H_2O 4.0 µl
  - 10x AffinityScript Buffer 2.0 µl
  - 0.1M DTT 2.0 µl
  - dNTPs (**25 mM each**) 0.8 µl
  - Murine RNase Inhibitor 0.3 µl
  - AffinityScript Enzyme 0.9 µl

- Add 10 µl to each sample, mix, incubate 55 C, 45 min in pre-heated PCR block

Cleanup cDNA

- **ExoSAP Treatment**
  - Add 3.5 µl **ExoSAP-IT** to each sample, vortex, spin down
  - Incubate 37 degC for 15 mins on PCR block
  - Add 1 µl **0.5M EDTA**, pipette-mix
- **RNA removal**
  - Add 3 µl of **1M NaOH**, pipette-mix
  - Incubate 70 degC, 12 min on PCR block
  - Add 3 µl of **1M HCl**, pipette-mix (to fix pH)

Silane cleanup cDNA

- **Prepare beads:**
  - Magnetically separate 10 µl **MyONE Silane beads** per sample, remove supernatant
  - Wash 1x with 500 µl **RLT buffer**
  - Resuspend beads in 93 µL **RLT buffer**

- **Bind RNA:**
  - Add beads in 93 µl **RLT buffer** to sample, mix
  - Add 111.6 µL **100% EtOH**
  - Pipette mix, leave pipette tip in tube, pipette mix twice, for 5 min

- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Add 1 mL **80% EtOH**, pipette resuspend and move to **new tube**
  - After 30 s, magnetically separate, remove supernatant
  - Wash 2x with **80% EtOH** (30 s)
  - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
  - Air-dry 5 min

- **Elute RNA:**
    - Resuspend in 5 µl 5 mM tris-Cl pH 7.5, let sit for 5 min (do not remove from beads)

5' linker ligate cDNA (on-bead)

- **Anneal linker:**
    - Add 0.8 µl **rand3Tr3** adapter
    - Add 1 µl 100% **DMSO**
    - Heat at 75 degC, 2 min, place immediately on ice for >1 min

- **Prepare ligation master mix** on ice; 12.8 µl per sample:

10x NEB RNA Ligase Buffer (with DTT)	2.0 µl
0.1M ATP	0.2 µl
50% PEG 8000	9.0 µl
RNA Ligase high conc	0.5 µl
H_2O	1.1 µl

- Flick to mix, spin down, add 12.8 µl to each sample: stir sample with pipette tip, then add master mix slowly with stirring; needs to be homogeneous
- Add another 1 µl **RNA Ligase high conc** to each sample, flick to mix
- Incubate on Thermomixer at 1200 rpm, room temp for 30 s, then put on bench
- Flick, ideally every hour, at least a few times before leaving overnight
- Incubate at room temp overnight

Day 4

Silane cleanup linker-ligated cDNA

- **Prepare beads:**
    - Magnetically separate 5 µl **MyONE Silane beads** per sample, remove supernatant
    - Wash 1x with 500 µl **RLT buffer**
    - Resuspend beads in 60 µL RLT buffer per sample

- **Bind RNA:**
    - Add beads in 60 µl **RLT buffer** to each sample, mix
    - Add 60 µL **100% EtOH**
    - Pipette mix, leave pipette tip in tube, pipette mix twice, for 5 min

- **Wash beads:**
    - Magnetically separate, remove supernatant
    - Add 1 mL **75% EtOH**, pipette resuspend and move to **new tube**
    - After 30 s, magnetically separate, remove supernatant
    - Wash 2x with **75% EtOH** (30 s)
    - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
    - Air-dry 5 min

- **Elute RNA:**
    - Resuspend in 27 µl **10mM Tris-HCl pH 7.5**, let sit for 5 min
    - Magnetically separate, transfer **25 µL** sample to new tube

qPCR quantify cDNA

- **Prepare qPCR master mix**; 9 µl per sample:
  - PowerSybr 2x master mix 5.0 µl
  - H_2O 3.6 µl
  - qPCR primer mix 0.4 µl (10 uM each qPCR-grade D5x/D7x mix)

- Mix, dispense into 384-well qPCR plate, add **1 µl 1:10 diluted (in H_2O) cDNA**, seal, mix
- Run qPCR, note Cq values
  - **Cycle # for final PCR will be 3 cycles less than the Ct of the 1:10 diluted sample**

    **** Note: we use the automatically calculated Ct for this; this '3 cycle less' rule may change based on your lab setup, so for the first couple CLIPs it is best to err on the side of 1 or 2 extra PCR cycles. If final libraries are > 50 nM (especially if > 100 nM), you should back off a couple cycles.**

PCR amplify cDNA

- **Prepare PCR** on ice; 50 uL total per sample:
  - 2x Q5 PCR master mix 25.0 µl
  - H_2O 5.0 µl
  - 20 µM right primer (D50x) 2.5 µl
  - 20 µM left primer (D70x) 2.5 µl

- Dispense into 8-well strips, add **12.5 µl CLIP sample + 2.5 H_2O**; for **inputs**, use **10 µl + 5 µl H_2O**; mix

- PCR conditions (cycle # depending on library):
  - 98 C for 30 s
  - 98 C for 15 sec -> 68 C for 30 sec -> 72 C for 40 sec (x6 cycles)
  - 98 C for 15 sec -> 72 C for 60 sec (x ? cycles)
  - 72 C 1 min
  - 4 C hold
- Typical: Input 9 total cycles (6 + 3), CLIP 16 (6 + 10) total cycles
  - Note that 18 cycles will yield ~30-50% PCR duplicated libraries (further increasing above 18 cycles), which can be ok for RBPs with few specific targets but will be challenging for broad binders.
- **Cycle # for final PCR: 3 cycles less than the qPCR Ct of the 1:10 diluted sample**

SPRI cleanup library

- Resuspend **AmpureXP beads** well
- Add 90 µl bead suspension (do not separate) per 50 µl PCR reaction, pipette mix well, incubate room temp 10 min (pipette mix 2-3x during incubation)
- Magnetically separate, wash beads 2x with **75% EtOH**
- Dry beads for 5 min on magnet
- Move from magnet, resuspend in **20 µl H_2O**, let sit for 5 min
- Magnetically separate, transfer **18 µl** to new tubes

Gel-purify library

- Prepare **3% low melting temp agarose gel (**Seakem GTG LMP) in 1% TBE
    - 120 ml for larger gel tray
    - mix often while microwaving (low melting temp gel tends to boil over rapidly)
    - cool down, add **1:10,000 SybrSafe**, mix, pour

- **Prepare samples and run gel:**
    - Add 6 µl **6x OrangeG** buffer to each sample (18 µl of sample), mix
    - Prepare two **50 bp ladder** samples in Orange G buffer (Per well: 0.5 µl ladder + 2 uL Orange G + 7.5 uL H2O)
    - Load on gel, leave 1 empty well between samples, ladder on both sides of the gel
    - Run ~95V for 50 mins (longer gives better resolution but larger cut sizes)

- **Gel-extract library from gel:**
    - Under blue light illumination, cut gel slices 175-350 bp and place into 15 mL conical tubes, using fresh razor blades for each sample; keep cross-contamination to minimum
        - Keep in mind: adapter-dimer (including RNA adapter) is 142 bp, so anything below 175 will cluster & create reads on the HiSeq, but is too short to map and will be wasted

- **Cut & elute gel** using Qiagen MinElute gel extraction kit:
    - Weigh 15 mL conical with gel slice (blank with empty conical tube)
    - Calculate gel weight, add 6x volumes of **Buffer QG** to melt gel (e.g. for 100 mg gel, add 600 µL QG)
    - Melt gel at room temp (do <u>not</u> heat) on benchtop (can shake to help melt, but don't vortex)
    - After gel is melted, add 1x volume of original gel of **isopropanol** & <u>mix well</u> (100 mg gel = 100 µl isopropanol)
    - Load on column (750 µl per spin, can do multiple spins, all spins max speed 1 min)
        - **NOTE:** if gel weight is >400 mg, wash 1x with 500 uL Buffer QG after every 4 spins)
    - After all sample has been spun through, wash 1x with 500 µl **Buffer QG**
    - Add 1X with 750 µl **Buffer PE**, spin 1 min, pour out flow-through, spin again 2 min max speed
    - Carefully move column to new 1.5 mL tube (avoid any carryover of PE – if any liquid is visible on the outside of the column redo 2 min max speed spin)
    - Using a fine tip, pipette all remaining PE buffer off of the plastic purple rim of the MinElute column
    - Air dry 2 mins
    - Carefully add 12.5 µl **Buffer EB** directly to the center of the column, incubate 2 min room temp, spin max speed
    - For improved yield – repeat the elution (take the flow-through and add it to the column again)

Quantitate library (D1000 tapestation)

- 3 µl D1000 loading buffer, 1 µl sample
- Vortex to mix, spin down in microfuge