SUPPLEMENTARY INFORMATION to Corley, *et al* Footprinting SHAPE-eCLIP reveals transcriptome-wide hydrogen bonds at RNA-protein interfaces

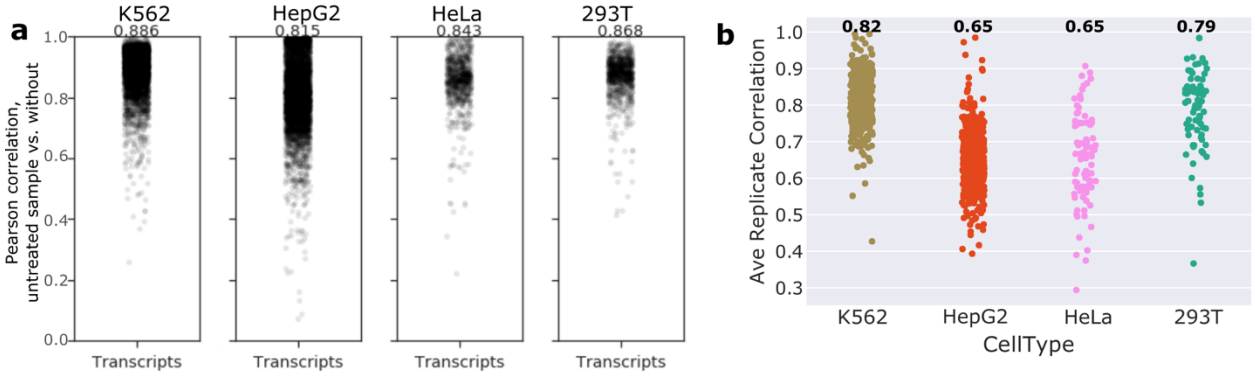


Figure S1. fSHAPE quality control metrics. Related to Figure 1. (a) Average Pearson correlations across transcripts comparing fSHAPE reactivities calculated by normalizing both -protein and +protein samples to an untreated sample versus fSHAPE reactivities calculated by simply normalizing the -protein sample to the +protein sample. Overall average correlation indicated at the top of each plot for data from each cell line. (b) Average fSHAPE replicate Pearson correlations across transcripts in four cell lines. Overall averages indicated above each cell line. Of note: correlations between both technical and biological replicates always increase with better read coverage (data not shown).

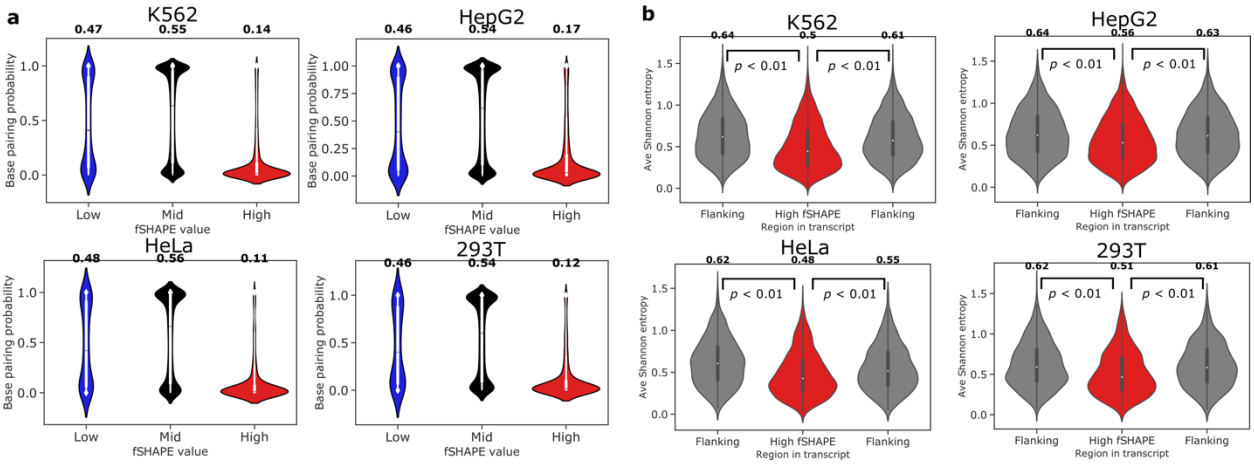


Figure S2. fSHAPE reactivities in the context of predicted RNA structures. Related to Figure 4. (a) Predicted base pairing probability densities for nucleotides grouped by low, medium, and high fSHAPE reactivities in four cell lines. Median and interquartile range displayed in white. Average base pairing probability indicated above each group. (b) Shannon entropy values predicted for 50-nucleotide regions containing high fSHAPE reactivities compared to 50-nucleotide flanking regions, for four cell lines. Median and interquartile range displayed in black. Average Shannon entropy indicated above each type of region.

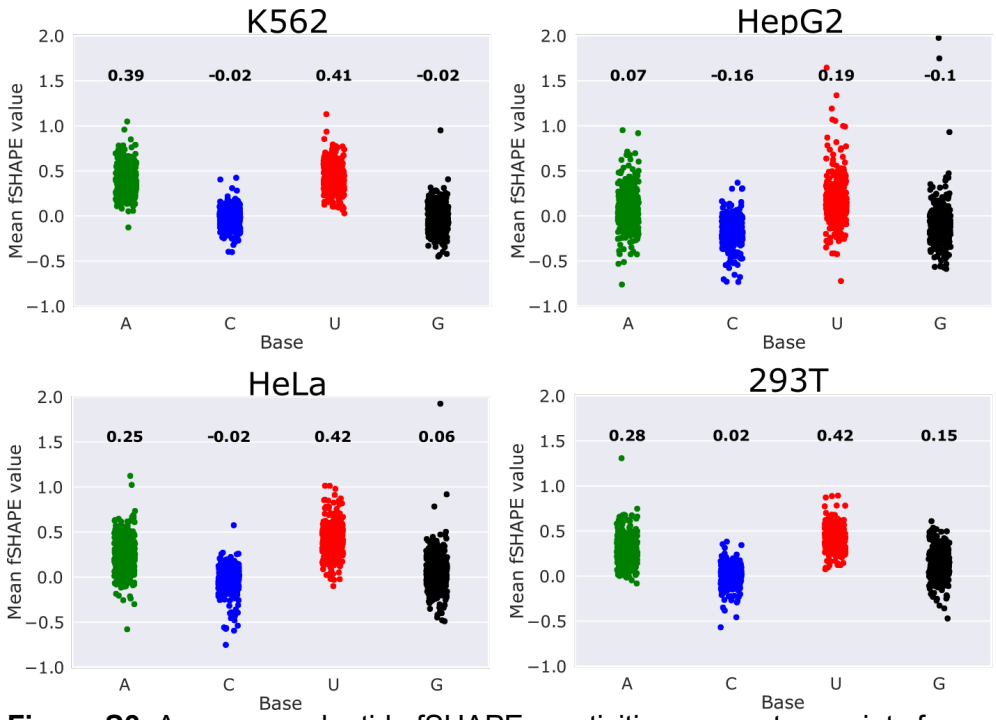


Figure S3. Average nucleotide fSHAPE reactivities across transcripts for each cell line. Related to Figure 4. Average indicated above each nucleotide.

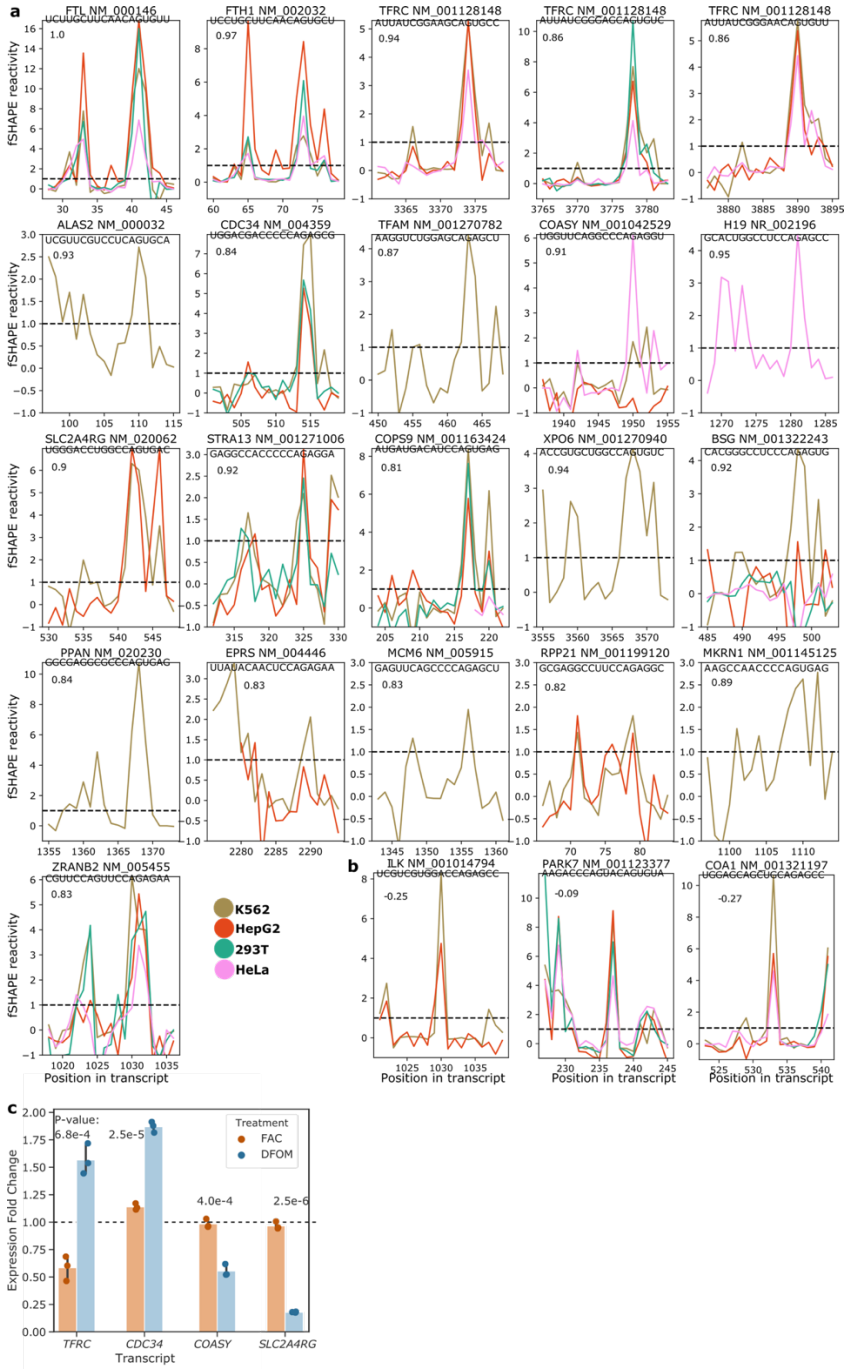


Figure S4. Predicting iron response elements based on fSHAPE reactivities. Related to Figure 5. (a) fSHAPE reactivity profiles in multiple cell lines of predicted iron response elements (IREs) in transcripts. Profiles are replicate averaged. Pearson correlation compared to *FTL* is indicated in top left corner; best value shown, if fSHAPE data available in multiple cell lines. Gene name, transcript ID (NCBI), and sequence indicated above each plot. IREs in (*FTL*), *FTH1*, *TFRC (*multiple), and *ALAS2* (top row) have been previously verified, the remainder are novel. (b) Example negative IRE matches. fSHAPE reactivity profiles of sequence matches to the iron response element whose fSHAPE reactivities do not match the *FTL* IRE. (c) Quantitative PCR results for candidate IRE-containing transcripts in response to high iron (FAC) and low iron (DFOM) conditions. *TFRC* is the positive control and is expected to increase in response to lower cellular iron levels. Expression fold change is relative to transcripts' expression in untreated samples.

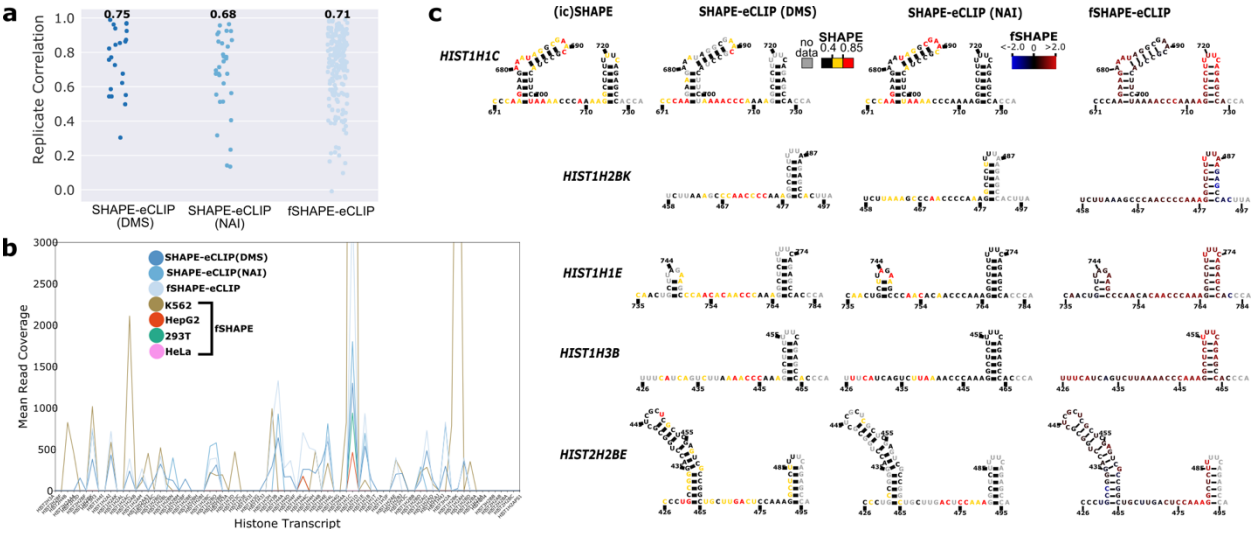


Figure S5. SHAPE-eCLIP and fSHAPE-eCLIP results. Related to Figure 6. (a) The average Pearson correlations between transcripts in replicate SHAPE-CLIP (DMS or NAI) or fSHAPE-CLIP experiments. Overall averages indicated above each group. (b) Mean read coverage at the 3' ends of histone trascripts in all fSHAPE datasets (K562 predominates) compared to SHAPE-CLIP and fSHAPE-CLIP experiments. (c) Predicted stem loop structures in histone transcripts overlaid with their SHAPE-eCLIP (DMS or NAI datasets) or fSHAPE-eCLIP reactivities, or icSHAPE reactivities where available. Bases are numbered by relative position in each transcript.

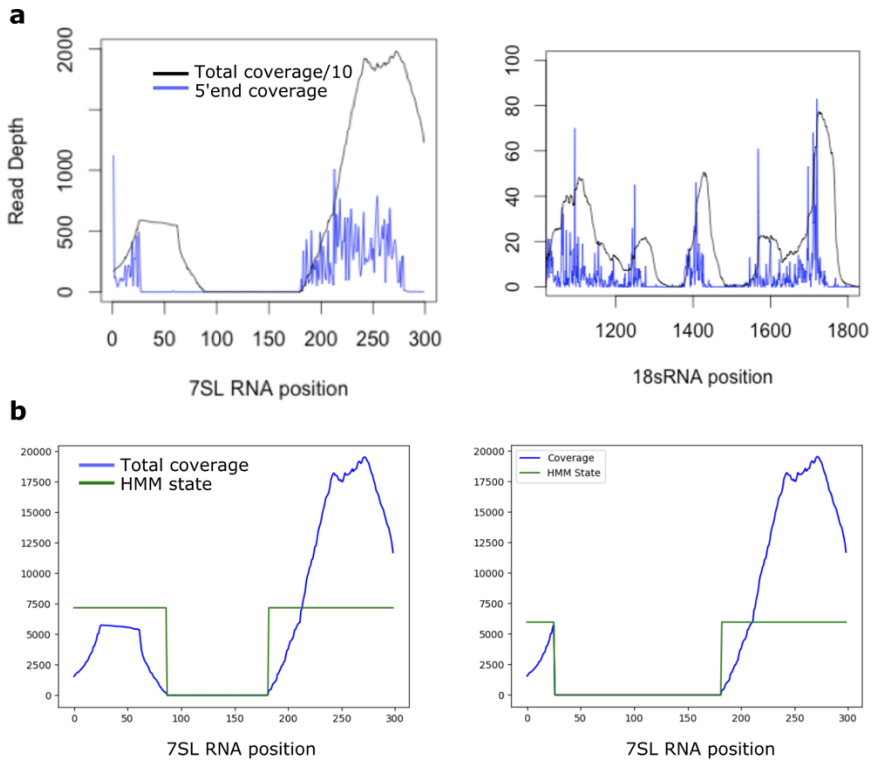


Figure S6. Reasoning for using a hidden Markov model to process read coverages in samples produced with RT-stop SHAPE methods (icSHAPE and fSHAPE). Related to Figure 1. (a) Read coverage is often uneven across transcripts, where coverages occasionally drop to zero or negligible reads. RT-stop based SHAPE methods rely on total coverage counts compared to the 5' end coverage counts to calculate RT-stop frequencies (5'end counts / total counts) at each nucleotide. In regions where total coverage drops to negligible densities, the 5'end coverage will always drop to zero counts before the drop in total coverage. These zero-valued 5'end coverage counts will be calculated as RT-stop frequencies of zero (0/total counts = 0), when in fact they should be calculated as "no data." (b) We used a two-state HMM to define regions of "gapped" total coverage" in our sequencing data, which were then extended to include the upstream positions where the 5'end coverage gap begins, and these regions were set to "no data" RT-stop frequencies.

Method	Determines RBP binding sites	Detects RNA Hydrogen bonds with RBPs	Probes RNA secondary structure	Transcripts covered	Reference
eCLIP	Yes	No	No	Transcripts bound by RBP of interest	(Van Nostrand et al., 2016)
SHAPE	No	No	Yes	All transcripts	(Flynn et al., 2016; Siegfried et al., 2014; Zubradt et al., 2017)
fSHAPE	No	Yes	No	All transcripts	This manuscript
SHAPE-eCLIP	Yes	No	Yes	Transcripts bound by RBP of interest	This manuscript
fSHAPE-eCLIP	Yes	Yes	No	Transcripts bound by RBP of interest	This manuscript
SHAPE-eCLIP + fSHAPE-eCLIP	Yes	Yes	Yes	Transcripts bound by RBP of interest	This manuscript

Table S1. Summary of methods discussed in this manuscript. Related to Figure 1.

Sample	Cell Line	Total Reads	Mapped Reads	%Mapped
fSHAPE -protein rep1	K562	488205440	220041090	45.07
fSHAPE -protein rep2	K562	449509340	193939211	43.14
fSHAPE +protein rep1	K562	474675397	177632371	37.42
fSHAPE +protein rep2	K562	399546049	214380454	53.66
untreated rep1	K562	169246638	121580081	71.84
untreated rep2	K562	248274587	174750357	70.39
fSHAPE -protein rep1	HepG2	381254232	147773034	38.76
fSHAPE -protein rep2	HepG2	400402773	217351947	54.28
fSHAPE +protein rep1	HepG2	341084582	199235899	58.41
fSHAPE +protein rep2	HepG2	262490678	128472705	48.94
untreated rep1	HepG2	179816667	132000895	73.41
untreated rep2	HepG2	206441539	143018701	69.28
fSHAPE -protein rep1	293T	396720043	58625034	14.78
fSHAPE -protein rep2	293T	261373987	16127640	6.17
fSHAPE +protein rep1	293T	224873324	62305628	27.71
fSHAPE +protein rep2	293T	223620844	71664269	32.05
untreated rep1	293T	117514555	69811752	59.41
untreated rep2	293T	122471399	69239719	56.54
fSHAPE -protein rep1	HeLa	385247373	41945298	10.89
fSHAPE -protein rep2	HeLa	139823501	15152703	10.84
fSHAPE +protein rep1	HeLa	390941420	91473409	23.40
fSHAPE +protein rep2	HeLa	390322340	77649074	19.89
untreated rep1	HeLa	208221981	133657309	64.19
untreated rep2	HeLa	152499397	91462156	59.98
SHAPE-eCLIP -DMS rep1 IP	K562	38507575	3267763	8.49
SHAPE-eCLIP -DMS rep1 Input	K562	38236715	3970591	10.38
SHAPE-eCLIP -DMS rep2 IP	K562	30031772	3896617	12.97
SHAPE-eCLIP -DMS rep2 Input	K562	26662691	3833100	14.38
SHAPE-eCLIP +DMS rep1 IP	K562	30202972	3243709	10.74
SHAPE-eCLIP +DMS rep1 Input	K562	27796355	1599309	5.75
SHAPE-eCLIP +DMS rep2 IP	K562	34204439	3541417	10.35
SHAPE-eCLIP +DMS rep2 Input	K562	35586786	1806514	5.08
SHAPE-eCLIP -NAI rep1 IP	K562	52038752	5023370	9.65
SHAPE-eCLIP -NAI rep1 Input	K562	45911093	8701095	18.95
SHAPE-eCLIP -NAI rep2 IP	K562	32422846	3112251	9.60
SHAPE-eCLIP -NAI rep2 Input	K562	44343542	8791660	19.83
SHAPE-eCLIP +NAI rep1 IP	K562	41002116	4221630	10.30
SHAPE-eCLIP +NAI rep2 IP	K562	42134793	5155322	12.24
fSHAPE-eCLIP -protein rep1 IP	K562	30248776	1783173	5.90
fSHAPE-eCLIP -protein rep1 Input	K562	61788254	5199523	8.42
fSHAPE-eCLIP -protein rep2 IP	K562	49112391	3862742	7.87
fSHAPE-eCLIP -protein rep2 Input	K562	35011020	2815761	8.04
fSHAPE-eCLIP +protein rep1 IP	K562	43370132	2140988	4.94
fSHAPE-eCLIP +protein rep1 Input	K562	55674156	4559061	8.19
fSHAPE-eCLIP +protein rep2 IP	K562	34489586	1974941	5.73
fSHAPE-eCLIP +protein rep2 Input	K562	42154910	3358947	7.97

Table S2. Sequencing statistics for fSHAPE, SHAPE-eCLIP, and fSHAPE-eCLIP data sets presented. Related to Figure 1. Mapped reads are defined as non-duplicate, uniquely mapped reads.

PDB ID	Protein	Gene	Transcript	Chrom	Start	End
6HTU	STAU1	ARF1	NM_001658	chr1+	764	782
6DU4	METTL16	MAT2A	NM_005911	chr2+	1525	1553
5M73	SRP72	RN7SL1	NR_002715	chr14+	106	249
5M73	SRP72	RN7SL2	NR_027260	chr14-	106	249
4QOZ	SLBP/ERI1	HIST1H2BG	NM_003518	chr6-	407	432
4L8R	SLBP/ERI1	HIST1H2BG	NM_003518	chr6-	407	432
1MFQ	SRP19/SRP54	RN7SL1	NR_002715	chr14+	112	238
1E8O	SRP9/SRP14	RN7SL1	NR_002715	chr14+	1	48
5AOX	SRP6/SRP14	RN7SL1	NR_002715	chr14+	1	117
3SN2	IRP1	TFRC	NM_001313966	chr3-	2962	2990
3SNP	IRP1	FTL	NM_000146	chr19+	26	55
3SNP	IRP1	FTH1	NM_002032	chr11-	58	87

Table S3. 'Ground truth' Protein Data Bank (PDB) structures for modeling the RNA-protein hydrogen bonds that fSHAPE detects. Related to Figure 3. Protein and transcript represented in each structure is listed, as well as the relative position in the transcript of the RNA in the PDB structure.

Model	AUC	t (Å)
baseP<t	0.724	4.1
sugarP<t	0.558	2.9
backP<t	0.681	4.1
baseR>=t	0.672	3.0
backP<t AND sugarP<t	0.700	3.0
backP<t OR sugarP<t	0.604	3.0
backP<t AND baseP<t	0.796	3.0
backP<t OR baseP<t	0.640	3.0
baseP<t AND sugarP<t	0.648	2.9
baseP<t OR sugarP<t	0.681	2.9
sugarP<t AND sugarR>=t	0.544	3.0
sugarP<t AND baseR>=t	0.663	3.0
backP<t AND backR>=t	0.630	3.0
backP<t AND baseR>=t	0.773	4.1
baseP<t AND baseR>=t	**0.823**	**3.0**
(baseP<t OR backP<t) AND baseR>=t	0.792	3.0
baseP<t AND backP<t AND baseR>=t	**0.843**	**4.1**
(sugarP<t AND backP<t) AND baseR>t	0.655	2.7
(sugarP<t OR backP<t) AND baseR>=t	0.765	4.0
(sugarP<t OR baseP<t) AND baseR>=t	0.768	3.0
(sugarP<t AND baseP<t) AND baseR>=t	0.675	3.7
baseP<t OR backP<t OR sugarP<t	0.627	3.0
(baseP<t OR backP<t OR sugarP<t) AND (baseR>=t)	0.779	3.0
baseP<t AND sugarP<t AND sugarP<t AND baseR>=t	0.688	3.2
(baseP<t AND base>=t) OR (sugarP<t AND sugarR>=t) OR (backP<t AND backR>=t)	0.643	3.0
(baseP<t AND (backP<t OR sugarP<t)) AND baseR>=t	**0.820**	**3.5**

Table S4. High fSHAPE reactivities' agreement with various sets of RNA-protein hydrogen bonds from the structures in Table S2. Related to Figure 3. backP, sugarP, and baseP are hbonds between protein and RNA backbone, sugar, and base moieties, respectively. backR, sugarR, and baseR are hbonds between (other) RNA and RNA backbone, sugar, and base moieties, respectively. t is hydrogen bond length and angstroms.

Gene Target	Forward Primer (5’ – 3’)	Reverse Primer (5’ – 3’)
TFRC (positive ctrl)	AAAATCCGGTGTAGGCACAG	TTAAATGCAGGGACGAAAGG
RPL4 (negative ctrl)	GCTCTGGCCAGGGTGCTTTTG	ATGGCGTATCGTTTTTGGGTTGT
CDC34	GGGGACGTGTGTATCTCCAT	TGTGTACTCCCGATCCTTCC
COASY	GGCATCATCAACAGGAAGGT	GCCTCAGTCTCTGGGATGAC
SLC2A4RG	GTCCAGCCTGACTCCAGTGT	CCCTGGTAGACACGGTCACT

Table S5. Primers for quantification of transcripts under varying cellular iron conditions. Related to Figure 5.

Methods S1. Experimental protocols for fSHAPE, SHAPE-eCLIP, fSHAPE-eCLIP. Related to Figure 1.

fSHAPE Experimental Procedures

RNA footprinting by way of SHAPE structure probing

In this paper fSHAPE is based on the combination of "in vitro" ("-protein") and "in vivo" ("+protein") *in vivo* click SHAPE structure probing experiments performed in parallel. Untreated controls are not necessary for fSHAPE; the "in vitro" sample is simply normalized to the "in vivo" sample during data analysis. Detailed bench protocols for "in vitro" and "in vivo" icSHAPE structure probing are available in:
Flynn, R. A., Zhang, Q. C., Spitale, R. C., Lee, B., Mumbach, M. R., & Chang, H. Y. (2016). Transcriptome-wide interrogation of RNA secondary structure in living cells with icSHAPE. ***Nature Protocols***, 11(2), 273-290.

The "in vitro" and "in vivo" samples differ on when the RNA is probed with the structure probing reagent (NAI-N_3 in the case of icSHAPE). "in vivo" samples are treated at the cell stage, when protein is present, while "in vitro" samples are mock treated at this stage (typically with DMSO). This will look something like the following:

- Reduce each cell sample volume to 2 mL in plain media (no FBS) in 10 cm plates.
- Per 40 million cell sample: If probing "in vivo" add 170 uL 2 M NAI-N_3 structure probing reagent to the cells. If probing "in vitro" add 170 uL DMSO. Swirl to mix.
- Incubate plates at 37 C for 10 minutes.
- Gather cells, moving to 15 mL conicals. Spin down at 500 g for 3 minute.
- Remove supernatant (NAI-N_3 treated supernatant should go into its own waste container).
- Resuspend cells in 5mL cold PBS. Spin down and remove supernatant as before, washing again with cold PBS.

Following RNA extraction and some library preparation, "in vitro" RNA samples are refolded and treated with the structure probing reagent, now in the absence of protein. In vitro refolding and treating RNA typically looks something like the following:

- Heat ~10 µL "in vitro" RNA samples to 95 C for 2 minutes, then place on ice. Next add:
  - 6.6 µL 3.3X **SHAPE Folding Buffer**
  - 2.4 µL water
  - 1.0 µL RNase inhibitor
- Heat to 37 C for 5 minutes to re-fold the RNA.
- Add 1 µL 2 M NAI-N_3 and continue heating at 37 C for 10 minutes.
- Column clean-up "in vitro" samples to stop NAI reaction.

We should note that while the published icSHAPE protocol originally used truncation events during reverse transcription to indicate sites of NAI-N_3 modification, it is possible to instead use the mutational profiling method of probing reagent detection. This requires the simple addition of manganese chloride to the reverse transcription reaction as follows:

- **Anneal primer**:
  - To ~9µl of RNA, add:
    - 1µl RT primer (=5 pmoles, 1µl of 5µM)
    - 1µl of 10 mM dNTPs
  - Heat 65 C for 2 min in pre-heated PCR block, place immediately on ice (do <u>not</u> cool down in PCR block)

Errors are induced in the cDNA by manganese when Superscript II encounters NAI-N_3 adducts in the RNA. This "mutational profiling" records the location of NAI-N_3 reactions with RNA bases and is a crucial step of RNA secondary structure probing.

- **Prepare structure probing RT master mix** on ice; 10 µl per sample:
  - 10X SHAPE FS Buffer 2.0 µl
  - Murine RNase Inhibitor 0.2 µl
  - 0.1 M DTT 1.0 µl
  - Water 5.36 µl
  - 500 mM $MnCl_2$ 0.24 µl
  - Superscript II Enzyme 1.0 µl

- Add 10 µl to each sample, mix, incubate 45 C, 3 hours min in pre-heated PCR block
- *Really needs all 3 hours, since manganese-induced errors slow down the RT*

fSHAPE-eCLIP Experimental Procedures

eCLIP modified to footprint RNA

Buffers & Solutions

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)

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
5 mM NaCl

RLTW Buffer
1x Qiagen cat # 79216
0.025% Tween-20

PCR Elution Buffer
10 mM Tris-HCl pH7.5
20 mM NaCl
0.1 mM EDTA

Input RNA Adapter Mix (per sample)

TT Elution Buffer	0.2 µl
H_2O	0.8 µl
DMSO	0.8 µl
Inv5-8 Adapter, 200 µM	0.2 µl

80% Ethanol

1% Tween-20

1 M HCl

1 M NaOH

2 M 2-methylnicotinic acid imidazolide (NAI)

Dimethyl sulfoxide (DMSO), anhydrous

PKS Buffer
100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA
0.2% SDS

SHAPE Folding Buffer (3.3X)
333 mM HEPES, pH 8.0
20 mM $MgCl_2$
333 mM NaCl

SHAPE FS (10X)
500 mM Tris-HCl, pH 8.0
750 mM KCl
750 mM KCl

TT Elution Buffer
10 mM Tris-HCl pH 7.5
0.1 mM EDTA
0.01% Tween-20

IP RNA Adapter Mix (per sample)

TT Elution Buffer	0.3 µl
H_2O	2.085 µl
InvRNA1-4 Adapter, 200 µM	0.615 µl

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	M0492L
Protease Inhibitor Cocktail III		EMD Millipore	539134-1SET
Superscript II Reverse Transcriptase		Invitrogen	18064014
Exo-SAP-IT		Affymetrix	78201

Beads

Dynabeads M-280 sheep anti-rabbit	10 mg/ml	LifeTech	11204D
(or Dynabeads Protein G)	30 mg/ml	LifeTech	
Dynabeads MyOne Silane	40 mg/ml	LifeTech	37002D
Agencourt AMPure XP beads		Beckman Coμlter	A63881

Primers

RNA oligos:

Original RNA adapters:

InvRiL19: /5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrC/3SpC3/

(Order 100 nmole RNA oligo, standard desalting; storage stock 200 μM; working stock 40 μM; final concentration 1 μM (input), 4 μM (CLIP)).

DNA oligos:

InvRand3Tr3: /5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/

(Order 100 nmole DNA oligo, standard desalting; storage stock 200 μM; working stock 80 μM; final concentration 3 μM).

InvAR17: CAGACGTGTGCTCTTCCGA (25 nmole DNA oligo, standard desalting; storage stock 200 μM; working stock 5 μM; final concentration 0.5 μM).

(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 RNA-seq primers work fine)

(stock 100 μM; working 20 μM)

Notes

- For this protocol one ‘experiment’ produces 6 libraries: 4 fSHAPE-eCLIP experiments on UV crosslinked biological replicate samples and 2 size-matched input control (taken from two UV crosslinked samples). Additionally, an IgG-only IP is run on the Western gel to validate antibody specificity.
    - ENCODE3 eCLIP experiments contained only one size-matched input, taken from one of the two biosamples
    - For other experiments, one can modify this design to add the paired size-matched input control from the other replicate, add a 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 as a shortcut. COLD = an analytical gel run on 10% of sample run as a standard Western blot; HOT = a preparative gel run on 80% of sample run for membrane cutting & RNA isolation
- When working with Protein G or other antibody-attached beads, be sure beads are never heated or dried (make sure master mixes are ready prior to removing supernatant from final washes)
- Bead washes should be done in the following order:
    1. Remove sample from magnet and buffer to beads
    2. Close tubes well and invert or flick tubes (do not vortex) to mix beads with buffer until solution is homogenous
    3. Transfer tubes to magnet and magnetically separate for 1-2 minutes. During this time it is recommended to slowly invert magnet with tubes a few times (this helps to collect any beads from tube caps).
    4. When the solution is transparent, remove most solution while ensuring the pipette tip does not touch the beads. Next, close the tubes and spin briefly for 1-3 seconds (desktop minicentrifuge), and place back on magnet. The remaining solution can then be removed using 200 µL (or smaller) pipette tips.
- Enzymes should be kept at -20C, preferentially in chilled enzyme coolers, keep them in cooler the whole time.
- We recommend the use of low-retention tips and tubes to ensure minimal sample loss
- Ensure proper hygiene for working with RNA samples (including separation from bacteria or other RNase-containing samples) as well as high-throughput sequencing libraries (we recommend physical separation of work performed on pre-amplified and post-PCR amplified material).

DAY -1

Grow cells to ~60-80 million cells per replicate, typically two replicates, referred to here at "Sample 1" and "Sample 2". Each replicate requires a pair of 30-40 million cell sub-samples: An "in vivo" sub-sample and an "in vitro" sub-sample. Note: A density of greater than 20 million cells/mL does not crosslink well and should be dispersed across plates.

Setup:

- Pre-chill 1X PBS (needs to be cold for washes).
- Set incubator to 37 C.
- Pre-chill metal plate for crosslinker. Set crosslinker to "4000" (400 mJ/cm^2) and 2 minutes.
- Fetch some dry ice.

Treat cells with NAI:

- Reduce each cell sample volume to 2 mL in plain media (no FBS) in 10 cm plates.
- Per 40 million cell sample pair: add 170 uL 2 M NAI structure probing reagent to one sample. This is the "in vivo" sub-sample. Add 170 uL DMSO to the other sample in the pair. This is the "in vitro" sub-sample. Swirl to mix.
- Incubate plates at 37 C for 10 minutes.

Crosslink cells and wash:

- Take lids off and crosslink plates.
- Gather cells, moving to 15 mL conicals. Spin down at 500 g for 3 minute.
- Remove supernatant (NAI treated supernatant is supposed to go into its own waste container).
- Resuspend cells in 5mL cold PBS. Spin down and remove supernatant as before, washing again with cold PBS.
- Spin down cells one more time, remove PBS supernatant, and flash freeze cells in dry ice.

DAY 0

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). ***I personally always use RNase inhibitor at this step.***

Couple antibody to magnetic beads (do this first)

Note: Process IgG identically to antibodies

- **Beads and antibodies:**
    - Use **125 µl beads** per sample
        - rabbit antibodies: use sheep anti-rabbit Dynabeads
        - mouse antibodies: use sheep anti-mouse Dynabeads
    - 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 cold **iCLIP Lysis Buffer,** 100 µl per sample
- **Bind antibody:**
    - Add antibody (10 µg per sample) to washed beads
    - Rotate at room temp, 45 min

Lyse cells (while ab and beads are binding)

- **Lyse cells:**

- Retrieve cell pellets from -80°C freezer, immediately add 1 mL cold **iCLIP Lysis Buffer + Protease Inhibitor Mix** to each pellet, pipette to resuspend

  2 Pellets per experiment:

  - Sample 1: IP-A (UV-crosslinked batch #1)
  - Sample 2: IP-B (UV-crosslinked batch #2)

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 3

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

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

- Lyse 5 mins on ice

RNase treat lysate (while ab+bead binding**):**

- Sonicate in Bioruptor on 'low' setting, 4°C, 5 min, 30sec on / 30 sec off
- Dilute RNase I in PBS at 1:50 on ice; use 10 µl diluted RNase I per sample
- To lysed sample(s), add 10 µl **Turbo Dnase** (mix immediately before use)
- To lysed sample(s), add 10 µl **diluted RNase I**, mix & immediately proceed to next step
- Incubate in Thermomixer at 1200 rpm, 37°C, 5 mins (exactly), place on ice
- Immediately add 11 µl **Murine RNase Inhibitor**, mix (if added earlier, ignore this step)
- Centrifuge 15,000g, 4°C, 3min
- Transfer cleared lysate to a new tube

Capture RBP-RNA complexes on beads

- Wash antibody beads 2x in 500 µl cold **iCLIP Lysis Buffer**
- Add cleared lysates to washed antibody beads
- Rotate 4°C, 2 h or overnight (in cold room) *Rotate 2h and proceed to "Day1"

DAY 1

Step: SAVE INPUT SAMPLES

- Mix samples well
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'HOT' (RNA) gel, store at 4°C
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'COLD' (western) gel; store at 4°C
- *Inputs are not necessary for both "in vitro"/"in vivo" samples, just pick one to make Inputs for. So at the very least you will have three samples per replicate: IPs for each "in vivo" and "in vitro", and an Input for one of these.*

Wash beads

- Wash 2x with 500 µL cold **High Salt Wash Buffer**
- Transition wash: add 500 µL cold **High Salt Wash Buffer**, mix, then add 500 µL cold **Wash Buffer**
- Wash 3x with 500 µL 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,** add **100** µl cold **Wash Buffer**, resuspend beads well
  - Move 20 µl to new tube #1 = **COLD IP Bead samples**

- For **HOT IP Bead samples,** remove remaining 80 µl Wash Buffer and add 20 µl Wash Buffer

Prepare input and IP samples for SDS-PAGE corresponding to following table:

Final Sample Composition for Gel Loading						
Buffer	**Cold Inputs**	**Cold IP Beads, "in vitro"**	**Cold IP Beads, "in vivo"**	**HOT Inputs**	**HOT IP Beads, "in vitro"**	**HOT IP Beads, "in vivo"**
Wash Buffer	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
4x NuPAGE LDS Buffer	7.5 µl	7.5 µl	7.5 µl	7.5 µl	7.5 µl	7.5 µl
1M DTT	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl

- Denature all samples in Thermomixer, 1200 rpm, 70°C, 10 min
- Cool on ice 1 min, spin briefly in minifuge
- For **all samples**, magnetically separate prior to loading (IP AND Inputs have beads)

Potential (not recommended) -20 C stopping point

Load and run gels **your loading scheme may vary depending on conditions**

- **Load HOT gel (4-12% Bis-Tris, 12-well, 1.5 mm). M = protein marker**

HOT GEL	1	2	3	4	5	6	7	8	9	10	11	12
Sample	M	**A Input**	m	**A-IP, "in vitro"**	m	**A-IP, "in vivo"**	m	**B Input**	M	**B-IP, "in vitro"**	m	**B-IP, "in vivo"**
Volume to Load	5 µl	30 µl	1 µl	30 µl	1 µl	30 µl	1 µl	30 µl	5 µl	30 µl	1 µl	30 µl
% of Sample Represented		2%		80%		80%		2%		80%		80%

- **Load COLD gel (4-12% Bis-Tris, 10-well, 1.5 mm)**

COLD GEL	1	2	3	4	5	6	7	8
Sample	M	**"in vitro" A-IP**	**"in vivo" A-IP**	**A-INPUT**	M	**"in vitro" B-IP**	**"in vivo" B-IP**	**B-INPUT**
Volume to Load	5 µl	15 µl	15 µl	15 µl	5 µl	15 µl	15 µl	15 µl
% of Sample Represented		10%	10%	1%		10%	10%	1%

- Save remaining 15 µl of COLD samples at -20 C for backup
- Run at 150V in 1X MOPS 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: 1x NuPAGE transfer buffer, 10% methanol
  - **COLD gel**: Prepare PVDF membrane(s): pre-wet 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 or iBlot transfer

  HOT gel: Nitrocellulose membrane from iBlot stack

- **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: 0.2-0.5 µg/ml in 5% milk in TBST, room temp, 1 hr.
- Wash 3x with TBST, 5 min each
- Probe with secondary antibody: 1:4000 Rabbit or Mouse TrueBlot HRP 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 each
- Mix equal volumes of ECL Reagent 1 + Reagent 2 (or 40:1 of ECL Plus Substrate A to Substrate B), 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 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 and centrifuge – place tube on ice if doing many samples

Release RNA from membrane

- Prepare **Proteinase K SDS mix** on ice, 150 µl per sample:
  - PKS Buffer 130 µl
  - Proteinase K 20 µl
- Mix, add **150 µl** Proteinase K SDS mix to membrane slices, incubate in Thermomixer at 1200 rpm, 37 C, 20 min (make sure all membrane slices are submerged)
- Further incubate in Thermomixer at 1200 rpm, 50 C, for an additional 20 min
- Transfer all solution to a fresh 1.5 mL DNA loBind tube
- Rinse membrane with 55 µl of water, and add to supernatant above (giving 200 µL total)

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

- Add 400 µl (2x volumes) RNA binding buffer, pipette mix well
- Add 700 µl (3.5x starting volume) of 100% ethanol & pipette mix well (take care to avoid spilling of sample)
- Transfer 650 µl of mixed sample to Zymo-Spin column
- Centrifuge 30 sec on benchtop minifuge or 5,000g
- Repeat column binding for sample: carefully pipette flow-through back onto column and centrifuge again, discard flow-through
- Repeat by reloading an additional 650 µl volume until all sample has been spun through column
- Add 400 µl RNA Prep Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through

- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 200 µl RNA Wash Buffer, centrifuge for 1 minute at 9,000g, discard flow through
- Centrifuge additional 2 mins
- Transfer column to new 1.5 mL tube (avoid getting Wash Buffer on column)
- **Elute: Add 10 µl H2O to column, let sit for 1 min, centrifuge for 30 sec at 9,000g**
- **Repeat elution in same eluate: take the flow-through and pipette it onto the column again, sit for 1 minute, and centrifigue 30 sec at 9,000g**
- **Place IP samples in -80 C until reverse transcription**

Potential -80 C stopping point

"In vitro" structure probing

- Set " in vivo" IPInput samples aside on ice.
- Heat ~10 µL "in vitro" samples to 95 C for 2 minutes, then place on ice. Next add:
    - 6.6 µL 3.3X **SHAPE Folding Buffer**
    - 2.4 µL water
    - 1.0 µL RNase inhibitor
- Heat to 37 C for 5 minutes to re-fold the RNA.
- Add 1 µL 2 M NAI and continue heating at 37 C for 10 minutes.
- Repeat Zymo clean-up on "in vitro" samples to stop NAI reaction.

DAY 3

Inputs and IPs

FastAP treat input and IP RNA

- To 10 µL sample, add:
    - 6 µl H_2O
    - 2 µl 10X FastAP buffer
    - 1 µl RNase Inhibitor
    - 2 µl FastAP enzyme

- Mix, add **11 µl** to samples, mix, incubate in Thermomixer at 1200 rpm, 37 C, 10 min

PNK treat input and IP RNA

- Make **PNK master mix**; 75 µl per sample:
    - H_2O 45 µl
    - 5X PNK 6.5 buffer 20 µl
    - 1 M DTT 0.5 µl
    - Turbo DNase 1 µl
    - PNK enzyme 4 µl

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

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

- Add 200 µl (2x volumes) RNA binding buffer directly to 95 µL repaired RNA sample, pipette mix well
- Add 300 µl (3x starting volume) of 100% ethanol & pipette mix well (avoid spilling of sample)
- Transfer all sample to Zymo-Spin column, centrifuge 30 sec at 5,000g
- Repeat column binding for sample: carefully pipette flow-through back onto column and centrifuge again, discard flow-through
- Add 400 µl RNA Prep Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 200 µl RNA Wash Buffer, centrifuge for 1 minute at 9,000g, discard flow through

- Centrifuge additional 2 mins
- Transfer column to new 1.5 mL tube (avoid getting Wash Buffer on column)
- **Elute: Add 10 µl H2O to column, let sit for 1 min, centrifuge for 30 sec at 9,000g**
- **Repeat elution in same eluate: take the flow-through and pipette it onto the column again, sit for 1 minute, and centrifuge 30 sec at 9,000g**

Potential -80 C stopping point

3' linker ligate input RNA

- **Anneal adapter:**
  - **INPUTS:**
    - Take 5 µl of RNA (from above) – remainder of input is kept at -80 C as backup
    - Add 2 µl **RNA adapter (input) mix (InvRNA5-8)** **(9 µl aliquots)**
    - Incubate 65 C, 2 min → place on ice >1 min
  - **IPs:**
    - Take all of RNA from above (~10 µl)
    - Add 4 µl **IP RNA adapter mix (InvRNA1-4)** **(7.5 µl aliquots)**
    - Incubate 65 C, 2 min → place on ice >1 min

- **Prepare ligation master mix; 13.5/27 µl per Input/IP sample** at room temperature (not on ice)**:**
  - H_2O 2.8/5.6 µl
  - 10X NEB Ligase Buffer (with DTT) 2.0/4.0 µl
  - 0.1 M ATP 0.2/0.4 µl
  - 100% DMSO 0.6/1.2 µl
  - 1% Tween-20 0.4/0.8 µl
  - 50% PEG-8000 6.0/12.0 µl
  - Murine RNase Inhibitor 0.3/0.6 µl
  - RNA Ligase High Conc 1.2/2.4 µl
- Flick/pipette mix, add **13.5/27 µl** to each sample, flick/pipette-mix, incubate at room temp for 60 min
- Flick to mix every ~15 min

Silane cleanup input/IP RNA

- **Prepare beads:**
  - To 10 µl **MyONE Silane Beads** per sample, add 5x volume RLT (e.g. for 4 samples, use 40 µl of beads and add 200 µl of RLT) *Each IP sample counts as two samples because in twice the volume*
  - Pipette mix, magnetically separate, and remove supernatant
  - Resuspend beads in 63 µl RLTW buffer (RLT + 0.025% Tween-20) **per sample (**e.g. for 4 samples, use 250 µl RLTW buffer). Mix well. [25 uL 1% tween + 975 uL RLT]
- **Bind RNA:**
  - Add 61/122 µl of bead/RLTW mixture above to each Input/IP RNA sample, mix
  - Add 65/130 µl **100% EtOH** to each sample
  - Pipette mix 10 times, leave pipette tip in tube, pipette mix every ~3-5 min for 10 min
- **Wash beads:**
  - Magnetically separate and discard supernatant
  - Add 300 µl (PCR strip tubes) or 1 mL (1.5 mL tubes) **80% EtOH**, pipette resuspend
  - After 30 s, magnetically separate, remove supernatant
  - Repeat wash with 300 µl (PCR strip tubes) or 1 mL (1.5 mL tubes) **80% EtOH**
  - After 30 s, magnetically separate, remove supernatant
  - Wash 3rd time with 100 µl (PCR strip tubes) or 750 µl (1.5 mL tubes) **80% EtOH.**
  - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
  - Dry beads well, i.e. until they stop "shining - no ethanol should be left on the bottom of strip. Beads are over-dry when they change color from brown to orange/rusty color.
- **Elute RNA:**
  - Resuspend in **9.5 µl Silane Elution Buffer**, let sit for 5 min

(300 µl aliquots – save remainder for Silane cleanup after reverse transcription)

- Magnetically separate, transfer supernatants to strip tube(s) (will be **~9 µl**)

Potential -80 C stopping point

Reverse transcribe RNA (ALL IP and INPUTS)

- **Anneal primer** in 8-well strip tubes:
  - To ~9µl of RNA, add:
    - 1µl InvAR17 diluted primer (=5 pmoles, 1µl of 5µM)
    - 1µl of 10 mM dNTPs
  - Heat 65 C for 2 min in pre-heated PCR block, place immediately on ice (do not cool down in PCR block)

Errors are induced in the cDNA by manganese when superscript II encounters NAI adducts in the RNA. This "mutational profiling" records the location of NAI reactions with RNA bases and is a crucial step of RNA secondary structure probing.

- **Prepare structure probing RT master mix** on ice; 10 µl per sample:
  - 10X SHAPE FS Buffer 2.0 µl
  - Murine RNase Inhibitor 0.2 µl
  - 0.1 M DTT 1.0 µl
  - Water 5.36 µl
  - 500 mM $MnCl_2$ 0.24 µl
  - Superscript II Enzyme 1.0 µl
- Add 10 µl to each sample, mix, incubate 45 C, 3 hours min in pre-heated PCR block
- *Really needs all 3 hours, since manganese-induced errors slow down the RT*

Cleanup cDNA

- **ExoSAP Treatment**
  - Add **2.5µl ExoSAP-IT** to each sample, vortex, spin down
  - Incubate 37°C for 15 mins on PCR block
  - Add 1 µl **0.5 M EDTA**, pipette-mix
- **RNA removal**
  - Add 3 µl of **1 M NaOH**, pipette-mix
  - Incubate 70°C, 10 min on PCR block
  - Add 3 µl of **1 M HCl**, pipette-mix (to fix pH)

Silane cleanup cDNA

- **Prepare beads:**
  - Take **5 µl MyONE Silane beads** per sample and add 5x volume RLT buffer, mix well
  - Magnetically separate and remove supernatant
  - Resuspend beads in 93 µL **RLTW buffer per sample**
- **Bind cDNA:**
  - Add **90 µl beads+RLTW** to each sample
  - Add **108 µl 100% EtOH**
  - Pipette mix (10+ times), leave pipette tip in tube, pipette mix twice (every 5min) **for total incubation of 10 minutes at room temp**
- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Wash 2× with 300 µl 80% EtOH (add 80% ethanol, move back and forth on magnet, magnetically separate, remove supernatant)
  - Wash 1x with 150 µl 80% EtOH (spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip)
  - Air-dry 5 min

5’ linker ligate cDNA (on-bead, in 10 µl)

- **Add cDNA adapter mix**
  - Add 1.1 µl of TT Elution Buffer
  - Add 0.6 µl InvRand10_3Tr3 adapter
  - Add 0.8 µl 100% **DMSO**
  - Heat at 70°C, 2 min, place immediately on ice for >1 min

- **Prepare ligation master mix** on bench:
  - H_2O 1.4 µl
  - 10x NEB RNA Ligase Buffer (with DTT) 1 µl
  - 0.1M DTT 0.2 µl
  - 0.1M ATP 0.1 µl
  - 1% Tween-20 0.2 µl
  - 50% PEG 8000 3.6 µl
  - RNA Ligase high conc 1 µl
  - Deadenylase enzyme 0.3 µl
- Flick to mix twice, spin down briefly, add **7.8 µl** to each sample: stir sample with pipette tip, then add master mix slowly with stirring; needs to be homogeneous
- Incubate at room temp overnight on rotator

DAY 4

Silane cleanup linker-ligated cDNA

- To each sample **add 5 µl of Silane Elution Buffer,** making 15 µl total.
- **Prepare beads:**
  - Take **2.5 µl MyONE Silane beads** per sample, add 5x volume RLT
  - Magnetically separate and remove supernatant
  - Resuspend beads in 47 µL RLTW buffer per sample
- **Bind RNA:**
  - Add **45 µl** beads+**RLTW buffer mix** to each sample
  - Add **45 µl 100% EtOH**
  - Pipette mix, leave pipette tip in tube, pipette mix twice, for 10 min total
- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Wash 2× with 300 µl 80% EtOH (add 80% ethanol, move back and forth on magnet, magnetically separate, remove supernatant)
  - Wash 1x with 150 µl 80% EtOH (spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip)
  - Air-dry 5 min
- **Elute ligated cDNA:**
  - Resuspend in 25 µl **Silane Elution Buffer**, let sit for 5 min
  - Magnetically separate, transfer **25 µl** sample to new tube

Potential -80 C stopping point

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

- **qPCR conditions (preset protocol: 30 cycle, PowerSYBR, no melt)**
    - 95 C for 10 min
    - 95 C for 15 sec ⟵ 30x
    - 60 C for 1 min -> take image
    - **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

- 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

- Prepare **PCR** on ice; 40 µl total per sample:
    - Ligated cDNA 16 µl (save remainder at -80 C as backup)
    - 20 µM right primer (D50x) 2 µl
    - 20 µM left primer (D70x) 2 µl
    - 2x Q5 PCR master mix 20 µl

- 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

SPRI cleanup library

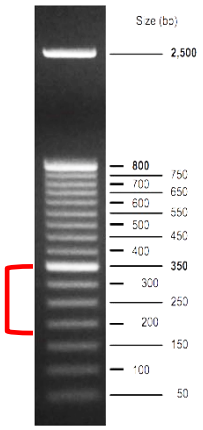
- Resuspend **AmpureXP beads** well by vortexing
    a. (Note: beads should be incubated at room temp for 15 min prior to use)
- Add 72 µl bead suspension (do not separate) per 40 µl PCR reaction and pipette mix well
- Incubate at room temp for 10 min (pipette mix 2-3x during incubation)
- Magnetically separate, wash beads 3x with **80% EtOH**
- Dry beads for 5 min on magnet (do not over-dry, i.e. the pellet will 'crack')
- Resuspend in **20 µl of PCR Elution Buffer** and incubate for 5 minutes at room temperature
- Magnetically separate and transfer supernatant to new tubes

Gel-purify library

- Prepare **3% low melting temp agarose gel (**NuSieve GTG, cat# 50080) in 1% TBE
    - 50 mL for 7 x 10 cm tray, 80 mL for 15 x 7 cm tray, 120 mL for 15 x 10 cm tray
    - Add agarose gradually to pre-heated TBE while mixing with stir bar
    - Melt agarose by microwaving and stirring in ~30 sec intervals (careful not to boil over)
    - Cool down, add **1:10,000 SybrSafe**, mix, pour gel

- **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 µl Orange G + 7.5 µl H2O)
    - Load on gel → if needed, 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

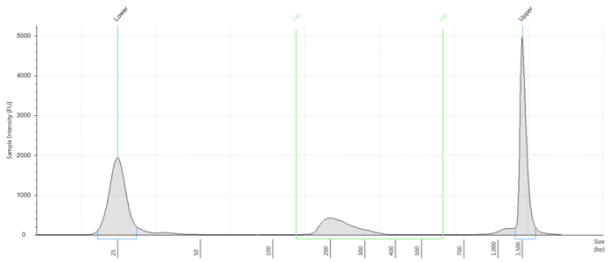


- **Cut & elute gel** using Qiagen MinElute gel extraction kit:
  - Weigh 15 mL conical with gel slice (blank with empty conical tube)
  - Calculate gel weight, add 6x volumes of **Buffer QG** to melt gel (e.g. for 100 mg gel, add 600 µl QG)
  - Melt gel at room temp (do <u>not</u> heat) on benchtop (can shake to help melt, but don't vortex)
  - After gel is melted, add 1x volume of original gel of **isopropanol** & <u>mix</u> <u>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 µl 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 from 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 DNA tapestation)

- 3 µl D1000 loading buffer, 1 µl sample
- Vortex to mix, spin down in microfuge
- Correctly quantify by adding a region to each sample and dragging boundaries to include the <u>entire</u> library peak (usually ~150-600bp).

Example of a good trace:



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total	Region Comment	Color
140	600	233	2.79	19.2	65.55		

SHAPE-eCLIP Experimental Procedures

eCLIP modified to probe RNA structure, on either *in vivo* or *in vitro* RNA

Buffers & Solutions

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)

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
5 mM NaCl

RLTW Buffer
1x Qiagen cat # 79216
0.025% Tween-20

PCR Elution Buffer
10 mM Tris-HCl pH7.5
20 mM NaCl
0.1 mM EDTA

Input RNA Adapter Mix (per sample)

TT Elution Buffer	0.2 µl
H_2O	0.8 µl
DMSO	0.8 µl
Inv5-8 Adapter, 200 µM	0.2 µl

80% Ethanol

1% Tween-20

1 M HCl

1 M NaOH

2 M 2-methylnicotinic acid imidazolide (NAI)

Dimethyl sulfoxide (DMSO), anhydrous

PKS Buffer
100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA
0.2% SDS

SHAPE Folding Buffer (3.3X)
333 mM HEPES, pH 8.0
20 mM $MgCl_2$
333 mM NaCl

SHAPE FS (10X)
500 mM Tris-HCl, pH 8.0
750 mM KCl
750 mM KCl

TT Elution Buffer
10 mM Tris-HCl pH 7.5
0.1 mM EDTA
0.01% Tween-20

IP RNA Adapter Mix (per sample)

TT Elution Buffer	0.3 µl
H_2O	2.085 µl
InvRNA1-4 Adapter, 200 µM	0.615 µl

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	M0492L
Protease Inhibitor Cocktail III		EMD Millipore	539134-1SET
Superscript II Reverse Transcriptase		Invitrogen	18064014
Exo-SAP-IT		Affymetrix	78201

Beads

Dynabeads M-280 sheep anti-rabbit	10 mg/ml	LifeTech	11204D
(or Dynabeads Protein G)	30 mg/ml	LifeTech	
Dynabeads MyOne Silane	40 mg/ml	LifeTech	37002D
Agencourt AMPure XP beads		Beckman Coµlter	A63881

Primers

RNA oligos:

Original RNA adapters:

InvRiL19: /5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrC/3SpC3/

(Order 100 nmole RNA oligo, standard desalting; storage stock 200 µM; working stock 40 µM; final concentration 1 µM (input), 4 µM (CLIP)).

DNA oligos:

InvRand3Tr3: /5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/

(Order 100 nmole DNA oligo, standard desalting; storage stock 200 µM; working stock 80 µM; final concentration 3 µM).

InvAR17: CAGACGTGTGCTCTTCCGA (25 nmole DNA oligo, standard desalting; storage stock 200 µM; working stock 5 µM; final concentration 0.5 µM).

(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 RNA-seq primers work fine)

(stock 100 µM; working 20 µM)

Notes

- For this protocol one ‘experiment’ is defined as 6 libraries: 4 SHAPE-eCLIP experiments on UV crosslinked biological replicate samples and 2 size-matched input control (taken from two UV crosslinked samples). Additionally, an IgG-only IP is run on the Western gel to validate antibody specificity.
    - ENCODE3 eCLIP experiments contained only one size-matched input, taken from one of the two biosamples
    - For other experiments, one can modify this design to add the paired size-matched input control from the other replicate, add a 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 as a shortcut. COLD = an analytical gel run on 10% of sample run as a standard Western blot; HOT = a preparative gel run on 80% of sample run for membrane cutting & RNA isolation
- When working with Protein G or other antibody-attached beads, be sure beads are never heated or dried (make sure master mixes are ready prior to removing supernatant from final washes)
- Bead washes should be done in the following order:
    5. Remove sample from magnet and buffer to beads
    6. Close tubes well and invert or flick tubes (do not vortex) to mix beads with buffer until solution is homogenous
    7. Transfer tubes to magnet and magnetically separate for 1-2 minutes. During this time it is recommended to slowly invert magnet with tubes a few times (this helps to collect any beads from tube caps).
    8. When the solution is transparent, remove most solution while ensuring the pipette tip does not touch the beads. Next, close the tubes and spin briefly for 1-3 seconds (desktop minicentrifuge), and place back on magnet. The remaining solution can then be removed using 200 µL (or smaller) pipette tips.
- Enzymes should be kept at -20C, preferentially in chilled enzyme coolers, keep them in cooler the whole time.
- We recommend the use of low-retention tips and tubes to ensure minimal sample loss
- Ensure proper hygiene for working with RNA samples (including separation from bacteria or other RNase-containing samples) as well as high-throughput sequencing libraries (we recommend physical separation of work performed on pre-amplified and post-PCR amplified material).

DAY -1

Grow cells to ~60-80 million cells per replicate, typically two replicates, referred to here at "Sample 1" and "Sample 2". Each replicate requires a pair of 30-40 million cell sub-samples: A sub-sample treated with the structure probing reagent (NAI) and an untreated control. Treated samples may either be treated "in vivo", or once RNA is removed from the cell and purified, i.e. "in vitro". Note: A density of greater than 20 million cells/mL does not crosslink well and should be dispersed across plates.

Setup:

- Pre-chill 1X PBS (needs to be cold for washes).
- Set incubator to 37 C.
- Pre-chill metal plate for crosslinker. Set crosslinker to "4000" (400 mJ/cm^2) and 2 minutes.
- Fetch some dry ice.

Treat cells with NAI:

- Reduce each cell sample volume to 2 mL in plain media (no FBS) in 10 cm plates.
- Per 40 million cell sample pair: If probing "in vivo" add 170 uL 2 M NAI structure probing reagent to the sub-sample. If probing "in vitro" add 170 uL DMSO. Add 170 uL DMSO to the untreated sample in the pair. Swirl to mix.
- Incubate plates at 37 C for 10 minutes.

Crosslink cells and wash:

- Take lids off and crosslink plates.
- Gather cells, moving to 15 mL conicals. Spin down at 500 g for 3 minute.
- Remove supernatant (NAI treated supernatant is supposed to go into its own waste container).
- Resuspend cells in 5mL cold PBS. Spin down and remove supernatant as before, washing again with cold PBS.
- Spin down cells one more time, remove PBS supernatant, and flash freeze cells in dry ice.

DAY 0

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). ***I personally always use RNase inhibitor at this step.***

Couple antibody to magnetic beads (do this first)

Note: Process IgG identically to antibodies

- **Beads and antibodies:**
    - Use **125 µl beads** per sample
        - rabbit antibodies: use sheep anti-rabbit Dynabeads
        - mouse antibodies: use sheep anti-mouse Dynabeads
    - 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 cold **iCLIP Lysis Buffer,** 100 µl per sample
- **Bind antibody:**
    - Add antibody (10 µg per sample) to washed beads
    - Rotate at room temp, 45 min

Lyse cells (while ab and beads are binding)

- **Lyse cells:**
  - Retrieve cell pellets from -80°C freezer, immediately add 1 mL cold **iCLIP Lysis Buffer + Protease Inhibitor Mix** to each pellet, pipette to resuspend

    2 Pellets per experiment:
    - Sample 1: IP-A (UV-crosslinked batch #1)
    - Sample 2: IP-B (UV-crosslinked batch #2)

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

  (One 20M cell IgG IP is good for 10 IP experiments & can be stored after IP and denaturation in NuPage buffer + DTT)
  - Lyse 5 mins on ice

RNase treat lysate (while ab+bead binding**):**

- Sonicate in Bioruptor on 'low' setting, 4°C, 5 min, 30sec on / 30 sec off
- Dilute RNase I in PBS at 1:50 on ice; use 10 µl diluted RNase I per sample
- To lysed sample(s), add 10 µl **Turbo Dnase** (mix immediately before use)
- To lysed sample(s), add 10 µl **diluted RNase I**, mix & immediately proceed to next step
- Incubate in Thermomixer at 1200 rpm, 37°C, 5 mins (exactly), place on ice
- Immediately add 11 µl **Murine RNase Inhibitor**, mix (if added earlier, ignore this step)
- Centrifuge 15,000g, 4°C, 3min
- Transfer cleared lysate to a new tube

Capture RBP-RNA complexes on beads

- Wash antibody beads 2x in 500 µl cold **iCLIP Lysis Buffer**
- Add cleared lysates to washed antibody beads
- Rotate 4°C, 2 h or overnight (in cold room) *Rotate 2h and proceed to "Day1.5" if probing "in vitro" RNA

DAY 1

Step: SAVE INPUT SAMPLES (If using "in vivo" probed RNA)

- Mix samples well
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'HOT' (RNA) gel, store at 4°C
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'COLD' (western) gel; store at 4°C
- *Input samples are not necessary for both treated and untreated SHAPE samples, just pick one to make Inputs for. So at the very least you will have three samples per replicate: IPs for each untreated and treated sub-sample, and an Input for one of these.*

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 back and forth on magnet, magnetically separate beads, add 500 µl **1xFastAP** buffer, mix, remove supernatant
- Wash 1x with 500 µl **1xFastAP buffer** (keep tubes on ice, remove supernatant only when FastAP mix is ready for next step)

FastAP treat beads

- **Prepare FastAP master mix** on ice; 50 µl per sample:
  - H_2O 38 µl
  - 10x FastAP buffer 5 µl

- Murine RNase Inhibitor 2 µl
- Turbo DNase 2 µl
- FastAP enzyme 3 µl

- Mix, add **50 µl** to each sample, incubate in Thermomixer at 1200 rpm, 37°C, 10 min

PNK treat beads

- While beads are incubating, **prepare PNK master mix** on ice; 150 µl per sample:
  - H_2O 116 µl
  - 5X PNK pH 6.5 buffer 30 µl
  - T4 PNK enzyme 4 µl

- Mix, add **150 µl** to each sample, incubate in Thermomixer at 1200 rpm, 37°C, 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 back and forth on magnet, magnetically separate beads, add 500 µl **High salt wash buffer**, move back and forth on magnet, remove supernatant
- Transition to Wash buffer: add 500 µl cold **High salt wash buffer**, move back and forth on magnet, magnetically separate beads, add 500 µl **Wash buffer**, move back and forth on magnet, remove supernatant
- Wash 1x with 500µL cold **Wash buffer**
- Transition to 1xLigase buffer (no DTT): add 500 µl **Wash buffer**, move back and forth on magnet, magnetically separate beads, add 300 µl **1xLigase buffer (no DTT)**, move back and forth on 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 8.4 µl
  - 10x Ligase buffer (**no DTT**) 3 µl
  - 0.1 M ATP 0.3 µl
  - 100% DMSO 0.9 µl
  - 1% Tween-20 0.6 µl
  - 50% PEG 8000 9 µl
  - Murine RNase Inhibitor 0.4 µl
  - RNA Ligase high conc. 2.4 µl

- Mix carefµlly by pipetting or flicking (do not vortex)
- Add **25 µl** to each sample
- To each sample, add 2.5 µl of **InvRiL19 (40 µM stock)** to each sample
- Incubate at room temperature for 75 min; flick to mix every ~10 min

Wash beads (resume IgG sample here)

- Wash 2x with 500µL cold **Wash buffer**, magnetically separate, remove supernatant

DAY 1.5

Step: SAVE INPUT SAMPLES (If using "in vitro" probed RNA, otherwise Inputs have already been reserved and should proceed to Wash beads.)

- Mix samples well
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'HOT' (RNA) gel, store at 4°C
- To new tube, take 20 µL (2%) of Sample 1 (A-Input), and Sample 2 (B-Input) for 'COLD' (western) gel; store at 4°C
- *Input samples are not necessary for both treated and untreated SHAPE samples, just pick one to make Inputs for. So at the very least you will have three samples per replicate: IPs for each untreated and treated sub-sample, and an Input for one of these.*

Wash beads

- Wash 2x with 500 µL cold **High Salt Wash Buffer**
- Transition wash: add 500 µL cold **High Salt Wash Buffer**, mix, then add 500 µL cold **Wash Buffer**
- Wash 3x with 500 µL 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,** add **100** µl cold **Wash Buffer**, resuspend beads well
  - Move 20 µl to new tube #1 = **COLD IP Bead samples**
  - For **HOT IP Bead samples,** remove remaining 80 µl Wash Buffer and add 20 µl Wash Buffer

Prepare input and IP samples for SDS-PAGE corresponding to following table:

Final Sample Composition for Gel Loading						
Buffer	**Cold Inputs**	**Cold IP Beads, NAI treated**	**Cold IP Beads, untreated**	**HOT Inputs**	**HOT IP Beads, NAI treated**	**HOT IP Beads, untreated**
Wash Buffer	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl
4x NuPAGE LDS Buffer	7.5 µl	7.5 µl	7.5 µl	7.5 µl	7.5 µl	7.5 µl
1M DTT	3 µl	3 µl	3 µl	3 µl	3 µl	3 µl

- Denature all samples in Thermomixer, 1200 rpm, 70°C, 10 min
- Cool on ice 1 min, spin briefly in minifuge
- For **all samples**, magnetically separate prior to loading (IP AND Inputs have beads)

Potential (not recommended) -20 C stopping point

Load and run gels **your loading scheme may vary depending on conditions**

- **Load HOT gel (4-12% Bis-Tris, 12-well, 1.5 mm). M = protein marker**

HOT GEL	1	2	3	4	5	6	7	8	9	10	11	12
Sample	M	**A Input**	m	**A-IP, treated**	m	**A-IP, untreated**	m	**B Input**	M	**B-IP, treated**	m	**B-IP, untreated**
Volume to Load	5 µl	30 µl	1 µl	30 µl	1 µl	30 µl	1 µl	30 µl	5 µl	30 µl	1 µl	30 µl
% of Sample Represented		2%		80%		80%		2%		80%		80%

- **Load COLD gel (4-12% Bis-Tris, 10-well, 1.5 mm)**

COLD GEL	1	2	3	4	5	6	7	8
Sample	M	**Treated A-IP**	**Untreated A-IP**	**A-INPUT**	M	**Treated B-IP**	**Untreated B-IP**	**B-INPUT**
Volume to Load	5 µl	15 µl	15 µl	15 µl	5 µl	15 µl	15 µl	15 µl
% of Sample Represented		10%	10%	1%		10%	10%	1%

- Save remaining 15 µl of COLD samples at -20 C for backup
- Run at 150V in 1X MOPS 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: 1x NuPAGE transfer buffer, 10% methanol
  - **COLD gel**: Prepare PVDF membrane(s): pre-wet 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 or iBlot transfer

  HOT gel: Nitrocellulose membrane from iBlot stack
- **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: 0.2-0.5 µg/ml in 5% milk in TBST, room temp, 1 hr.
- Wash 3x with TBST, 5 min each
- Probe with secondary antibody: 1:4000 Rabbit or Mouse TrueBlot HRP 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 each
- Mix equal volumes of ECL Reagent 1 + Reagent 2 (or 40:1 of ECL Plus Substrate A to Substrate B), 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 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 and centrifuge – place tube on ice if doing many samples

Release RNA from membrane

- Prepare **Proteinase K SDS mix** on ice, 150 µl per sample:
    - PKS Buffer 130 µl
    - Proteinase K 20 µl
- Mix, add **150 µl** Proteinase K SDS mix to membrane slices, incubate in Thermomixer at 1200 rpm, 37 C, 20 min (make sure all membrane slices are submerged)
- Further incubate in Thermomixer at 1200 rpm, 50 C, for an additional 20 min
- Transfer all solution to a fresh 1.5 mL DNA loBind tube
- Rinse membrane with 55 µl of water, and add to supernatant above (giving 200 µL total)

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

- Add 400 µl (2x volumes) RNA binding buffer, pipette mix well
- Add 700 µl (3.5x starting volume) of 100% ethanol & pipette mix well (take care to avoid spilling of sample)
- Transfer 650 µl of mixed sample to Zymo-Spin column
- Centrifuge 30 sec on benchtop minifuge or 5,000g
- Repeat column binding for sample: carefully pipette flow-through back onto column and centrifuge again, discard flow-through
- Repeat by reloading an additional 650 µl volume until all sample has been spun through column
- Add 400 µl RNA Prep Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 200 µl RNA Wash Buffer, centrifuge for 1 minute at 9,000g, discard flow through
- Centrifuge additional 2 mins
- Transfer column to new 1.5 mL tube (avoid getting Wash Buffer on column)
- **Elute: Add 10 µl H2O to column, let sit for 1 min, centrifuge for 30 sec at 9,000g**
- **Repeat elution in same eluate: take the flow-through and pipette it onto the column again, sit for 1 minute, and centrifigue 30 sec at 9,000g**
- **Place IP samples in -80 C until reverse transcription**

Potential -80 C stopping point

"In vitro" structure probing

(only if using "in vitro" probing, otherwise proceed with "in vivo" probed samples' Inputs to Day 3)

- Set Input and untreated samples aside on ice. Up until this point the "in vitro" treated and untreated IP samples are identical.
- Heat ~10 µL "in vitro" IP samples to 95 C for 2 minutes, then place on ice. Next add:
    - 6.6 µL 3.3X **SHAPE Folding Buffer**
    - 2.4 µL water
    - 1.0 µL RNase inhibitor
- Heat to 37 C for 5 minutes to re-fold the RNA.
- Add 1 µL 2 M NAI and continue heating at 37 C for 10 minutes.
- Repeat Zymo clean-up on "in vitro" samples to stop NAI reaction.

Carry all "in vitro" IP and Input samples into Day 3

Potential -80 C stopping point

DAY 3

Input samples only - if using "in vivo" probing.
Input and IP samples together - if using "in vitro" probing.

FastAP treat RNA

- To 10 µL sample, add:
  - 6 µl H_2O
  - 2 µl 10X FastAP buffer
  - 1 µl RNase Inhibitor
  - 2 µl FastAP enzyme

- Mix, add **11 µl** to samples, mix, incubate in Thermomixer at 1200 rpm, 37 C, 10 min

PNK treat RNA

- Make **PNK master mix**; 75 µl per sample:
  - H_2O 45 µl
  - 5X PNK 6.5 buffer 20 µl
  - 1 M DTT 0.5 µl
  - Turbo DNase 1 µl
  - PNK enzyme 4 µl

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

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

- Add 200 µl (2x volumes) RNA binding buffer directly to 95 µL repaired RNA sample, pipette mix well
- Add 300 µl (3x starting volume) of 100% ethanol & pipette mix well (avoid spilling of sample)
- Transfer all sample to Zymo-Spin column, centrifuge 30 sec at 5,000g
- Repeat column binding for sample: carefully pipette flow-through back onto column and centrifuge again, discard flow-through
- Add 400 µl RNA Prep Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 500 µl RNA Wash Buffer, centrifuge for 30 sec, discard flow through
- Add 200 µl RNA Wash Buffer, centrifuge for 1 minute at 9,000g, discard flow through
- Centrifuge additional 2 mins
- Transfer column to new 1.5 mL tube (avoid getting Wash Buffer on column)
- **Elute: Add 10 µl H2O to column, let sit for 1 min, centrifuge for 30 sec at 9,000g**
- **Repeat elution in same eluate: take the flow-through and pipette it onto the column again, sit for 1 minute, and centrifuge 30 sec at 9,000g**

Potential -80 C stopping point

3' linker ligate input RNA

- **Anneal adapter:**
  - **INPUTS:**
    - Take 5 µl of RNA (from above) – remainder of input is kept at -80 C as backup
    - Add 2 µl **RNA adapter (input) mix (InvRNA5-8)** **(9 µl aliquots)**
    - Incubate 65 C, 2 min → place on ice >1 min
  - **IPs (if following "in vitro" probing):**
    - Take all of RNA from above (~10 µl)
    - Add 4 µl **IP RNA adapter mix (InvRNA1-4)** **(7.5 µl aliquots)**
    - Incubate 65 C, 2 min → place on ice >1 min

- **Prepare ligation master mix; 13.5/27 µl per Input/IP sample** at room temperature (not on ice)**:**

- H_2O 2.8/5.6 µl
- 10X NEB Ligase Buffer (with DTT) 2.0/4.0 µl
- 0.1 M ATP 0.2/0.4 µl
- 100% DMSO 0.6/1.2 µl
- 1% Tween-20 0.4/0.8 µl
- 50% PEG-8000 6.0/12.0 µl
- Murine RNase Inhibitor 0.3/0.6 µl
- RNA Ligase High Conc 1.2/2.4 µl

- Flick/pipette mix, add **13.5/27 µl** to each sample, flick/pipette-mix, incubate at room temp for 60 min
- Flick to mix every ~15 min

Silane cleanup input/IP RNA

- **Prepare beads:**
  - To 10 µl **MyONE Silane Beads** per sample, add 5x volume RLT (e.g. for 4 samples, use 40 µl of beads and add 200 µl of RLT) *Each IP sample, if following "in vitro" probing, counts as two samples because in twice the volume*
  - Pipette mix, magnetically separate, and remove supernatant
  - Resuspend beads in 63 µl RLTW buffer (RLT + 0.025% Tween-20) **per sample (**e.g. for 4 samples, use 250 µl RLTW buffer). Mix well. [25 uL 1% tween + 975 uL RLT]

- **Bind RNA:**
  - Add 61/122 µl of bead/RLTW mixture above to each Input/IP RNA sample, mix
  - Add 65/130 µl **100% EtOH** to each sample
  - Pipette mix 10 times, leave pipette tip in tube, pipette mix every ~3-5 min for 10 min

- **Wash beads:**
  - Magnetically separate and discard supernatant
  - Add 300 µl (PCR strip tubes) or 1 mL (1.5 mL tubes) **80% EtOH**, pipette resuspend
  - After 30 s, magnetically separate, remove supernatant
  - Repeat wash with 300 µl (PCR strip tubes) or 1 mL (1.5 mL tubes) **80% EtOH**
  - After 30 s, magnetically separate, remove supernatant
  - Wash 3rd time with 100 µl (PCR strip tubes) or 750 µl (1.5 mL tubes) **80% EtOH.**
  - Spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip
  - Dry beads well, i.e. until they stop "shining - no ethanol should be left on the bottom of strip. Beads are over-dry when they change color from brown to orange/rusty color.

- **Elute RNA:**
  - Resuspend in **9.5 µl Silane Elution Buffer**, let sit for 5 min **(300 µl aliquots – save remainder for Silane cleanup after reverse transcription)**
  - Magnetically separate, transfer supernatants to strip tube(s) (will be **~9 µl**)

Potential -80 C stopping point

Reverse transcribe RNA (ALL IP and INPUTS, applies to both "in vitro" and "in vivo" probing)

- **Anneal primer** in 8-well strip tubes:
  - To ~9µl of RNA, add:
    - 1µl InvAR17 diluted primer (=5 pmoles, 1µl of 5µM)
    - 1µl of 10 mM dNTPs
  - Heat 65 C for 2 min in pre-heated PCR block, place immediately on ice (do not cool down in PCR block)

Errors are induced in the cDNA by manganese when superscript II encounters NAI adducts in the RNA. This "mutational profiling" records the location of NAI reactions with RNA bases and is a crucial step of RNA secondary structure probing.

- **Prepare structure probing RT master mix** on ice; 10 µl per sample:
  - 10X SHAPE FS Buffer 2.0 µl
  - Murine RNase Inhibitor 0.2 µl
  - 0.1 M DTT 1.0 µl

- Water 5.36 µl
- 500 mM $MnCl_2$ 0.24 µl
- Superscript II Enzyme 1.0 µl

- Add 10 µl to each sample, mix, incubate 45 C, 3 hours min in pre-heated PCR block
- *Really needs all 3 hours, since manganese-induced errors slow down the RT*

Cleanup cDNA

- **ExoSAP Treatment**
  - Add **2.5µl ExoSAP-IT** to each sample, vortex, spin down
  - Incubate 37°C for 15 mins on PCR block
  - Add 1 µl **0.5 M EDTA**, pipette-mix
- **RNA removal**
  - Add 3 µl of **1 M NaOH**, pipette-mix
  - Incubate 70°C, 10 min on PCR block
  - Add 3 µl of **1 M HCl**, pipette-mix (to fix pH)

Silane cleanup cDNA

- **Prepare beads:**
  - Take **5 µl MyONE Silane beads** per sample and add 5x volume RLT buffer, mix well
  - Magnetically separate and remove supernatant
  - Resuspend beads in 93 µL **RLTW buffer per sample**
- **Bind cDNA:**
  - Add **90 µl beads+RLTW** to each sample
  - Add **108 µl 100% EtOH**
  - Pipette mix (10+ times), leave pipette tip in tube, pipette mix twice (every 5min) **for total incubation of 10 minutes at room temp**
- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Wash 2× with 300 µl 80% EtOH (add 80% ethanol, move back and forth on magnet, magnetically separate, remove supernatant)
  - Wash 1x with 150 µl 80% EtOH (spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip)
  - Air-dry 5 min

5' linker ligate cDNA (on-bead, in 10 µl)

- **Add cDNA adapter mix**
  - Add 1.1 µl of TT Elution Buffer
  - Add 0.6 µl InvRand10_3Tr3 adapter
  - Add 0.8 µl 100% **DMSO**
  - Heat at 70°C, 2 min, place immediately on ice for >1 min
- **Prepare ligation master mix** on bench:
  - H_2O 1.4 µl
  - 10x NEB RNA Ligase Buffer (with DTT) 1 µl
  - 0.1M DTT 0.2 µl
  - 0.1M ATP 0.1 µl
  - 1% Tween-20 0.2 µl
  - 50% PEG 8000 3.6 µl
  - RNA Ligase high conc 1 µl
  - Deadenylase enzyme 0.3 µl
- Flick to mix twice, spin down briefly, add **7.8 µl** to each sample: stir sample with pipette tip, then add master mix slowly with stirring; needs to be homogeneous
- Incubate at room temp overnight on rotator

DAY 4

Silane cleanup linker-ligated cDNA

- To each sample **add 5 µl of Silane Elution Buffer,** making 15 µl total.
- **Prepare beads:**
  - Take **2.5 µl MyONE Silane beads** per sample, add 5x volume RLT
  - Magnetically separate and remove supernatant
  - Resuspend beads in 47 µL RLTW buffer per sample
- **Bind RNA:**
  - Add **45 µl** beads+**RLTW buffer mix** to each sample
  - Add **45 µl 100% EtOH**
  - Pipette mix, leave pipette tip in tube, pipette mix twice, for 10 min total
- **Wash beads:**
  - Magnetically separate, remove supernatant
  - Wash 2× with 300 µl 80% EtOH (add 80% ethanol, move back and forth on magnet, magnetically separate, remove supernatant)
  - Wash 1x with 150 µl 80% EtOH (spin briefly in picoFuge, magnetically separate, remove residual liquid with fine tip)
  - Air-dry 5 min
- **Elute ligated cDNA:**
  - Resuspend in 25 µl **Silane Elution Buffer**, let sit for 5 min
  - Magnetically separate, transfer **25 µl** sample to new tube

Potential -80 C stopping point

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
- **qPCR conditions (preset protocol: 30 cycle, PowerSYBR, no melt)**
  - 95 C for 10 min
  - 95 C for 15 sec ⟵ 30x
  - 60 C for 1 min -> take image
  - **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

- 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
- Prepare **PCR** on ice; 40 µl total per sample:
  - Ligated cDNA 16 µl (save remainder at -80 C as backup)
  - 20 µM right primer (D50x) 2 µl
  - 20 µM left primer (D70x) 2 µl
  - 2x Q5 PCR master mix 20 µl
- 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

SPRI cleanup library

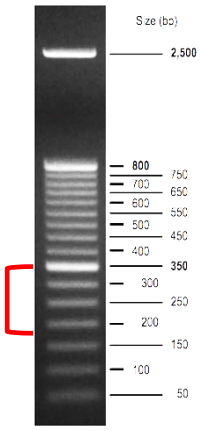
- Resuspend **AmpureXP beads** well by vortexing
    a. (Note: beads should be incubated at room temp for 15 min prior to use)
- Add 72 µl bead suspension (do not separate) per 40 µl PCR reaction and pipette mix well
- Incubate at room temp for 10 min (pipette mix 2-3x during incubation)
- Magnetically separate, wash beads 3x with **80% EtOH**
- Dry beads for 5 min on magnet (do not over-dry, i.e. the pellet will 'crack')
- Resuspend in **20 µl of PCR Elution Buffer** and incubate for 5 minutes at room temperature
- Magnetically separate and transfer supernatant to new tubes

Gel-purify library

- Prepare **3% low melting temp agarose gel (**NuSieve GTG, cat# 50080) in 1% TBE
    - 50 mL for 7 x 10 cm tray, 80 mL for 15 x 7 cm tray, 120 mL for 15 x 10 cm tray
    - Add agarose gradually to pre-heated TBE while mixing with stir bar
    - Melt agarose by microwaving and stirring in ~30 sec intervals (careful not to boil over)
    - Cool down, add **1:10,000 SybrSafe**, mix, pour gel

- **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 µl Orange G + 7.5 µl H2O)
    - Load on gel → if needed, 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



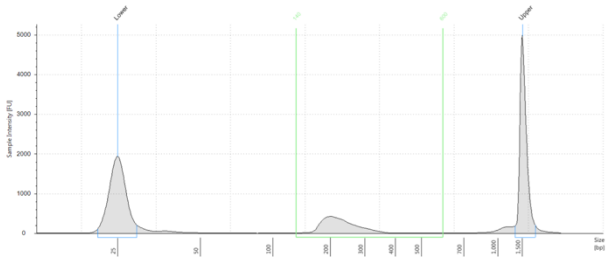
- **Cut & elute gel** using Qiagen MinElute gel extraction kit:
    - Weigh 15 mL conical with gel slice (blank with empty conical tube)
    - Calculate gel weight, add 6x volumes of **Buffer QG** to melt gel (e.g. for 100 mg gel, add 600 µl QG)
    - Melt gel at room temp (do not heat) on benchtop (can shake to help melt, but don't vortex)
    - After gel is melted, add 1x volume of original gel of **isopropanol** & mix well (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 µl 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 from 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 DNA tapestation)

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

- Correctly quantify by adding a region to each sample and dragging boundaries to include the <u>entire</u> library peak (usually ~150-600bp).

Example of a good trace:



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	%of Total	Region Comment	Color
140	600	233	2.79	19.2	65.55		