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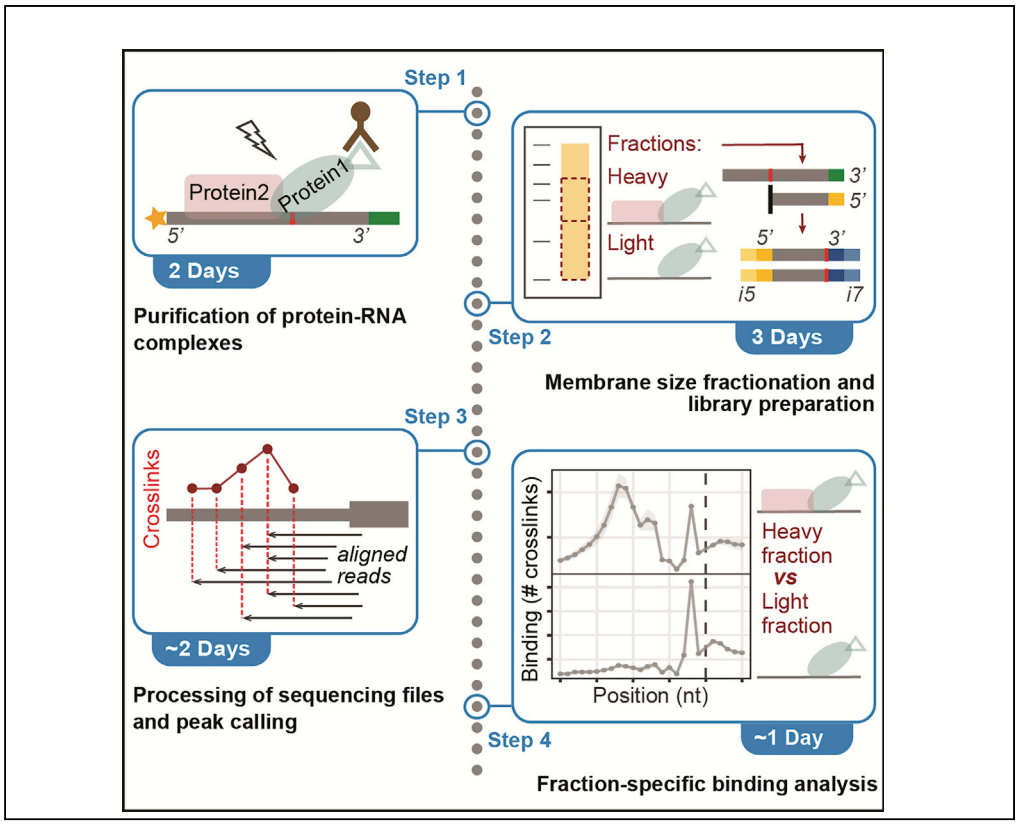
Deconvolution of *in vivo* protein-RNA contacts using fractionated eCLIP-seq



Thousands of RNA-binding proteins orchestrate RNA processing and altered protein-RNA interactions frequently lead to disease. Here, we present experimental and computational analysis pipelines of fractionated eCLIP-seq (freCLIP-seq), a modification of enhanced UV-crosslinking and RNA immunoprecipitation followed by sequencing. FreCLIP-seq allows transcriptome-wide analysis of protein-RNA interactions at single-nucleotide level and provides an additional level of resolution by isolating binding signals of individual RNA-binding proteins within a multicomponent complex. Binding occupancy can be inferred from read counts and crosslinking events.

Giulia Biancon, Emma Busarello, Poorval Joshi, Bluma J. Lesch, Stephanie Halene, Toma Tebaldi

giulia.biancon@yale.edu (G.B.)
stephanie.halene@yale.edu (S.H.)
toma.tebaldi@unitn.it (T.T.)

Highlights

Generation of crosslinked cell pellets and immunoprecipitation

Isolation of distinct RBP-RNA contacts within a complex by membrane size fractionation

Identification of binding peaks by a transcriptome-wide or region-specific approach

Annotation and differential analysis of binding occupancies

Biancon et al., STAR Protocols 3, 101823
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101823

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Deconvolution of *in vivo* protein-RNA contacts using fractionated eCLIP-seq

Giulia Biancon,[1,8,*] Emma Busarello,[2] Poorval Joshi,[1] Bluma J. Lesch,[3] Stephanie Halene,[1,4,5,6,7,9,*] and Toma Tebaldi[1,2,7,8,*]

[1]Section of Hematology, Department of Internal Medicine, Yale Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT, USA

[2]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

[3]Department of Genetics, Yale University School of Medicine, New Haven, CT, USA

[4]Yale Stem Cell Center, Yale University School of Medicine, New Haven, CT, USA

[5]Yale Center for RNA Science and Medicine, Yale University School of Medicine, New Haven, CT, USA

[6]Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

[7]These authors contributed equally

[8]Technical contact

[9]Lead contact

*Correspondence: giulia.biancon@yale.edu (G.B.), stephanie.halene@yale.edu (S.H.), toma.tebaldi@unitn.it (T.T.)
https://doi.org/10.1016/j.xpro.2022.101823

SUMMARY

Thousands of RNA-binding proteins orchestrate RNA processing and altered protein-RNA interactions frequently lead to disease. Here, we present experimental and computational analysis pipelines of fractionated eCLIP-seq (freCLIP-seq), a modification of enhanced UV-crosslinking and RNA immunoprecipitation followed by sequencing. FreCLIP-seq allows transcriptome-wide analysis of protein-RNA interactions at single-nucleotide level and provides an additional level of resolution by isolating binding signals of individual RNA-binding proteins within a multicomponent complex. Binding occupancy can be inferred from read counts and crosslinking events.

For complete details on the use and execution of this protocol, please refer to Biancon et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the specific steps for performing freCLIP-seq on inducible human erythroleukemia (HEL) cell lines expressing FLAG-tagged wild-type or mutant U2AF1 splicing factor. Together, U2AF1 and U2AF2 form the U2AF complex that recognizes the 3′ splice site (3′SS) and recruits U2 small nuclear ribonucleoproteins (snRNPs) for spliceosome activation. U2AF1 hotspot mutations affecting the S34 or Q157 residues alter RNA binding and are associated with myeloid malignancies and solid cancers. The application of freCLIP-seq allowed us to distinguish U2AF1 from U2AF2 binding signals and to identify distinct targets of the two U2AF subunits. Moreover, we were able to dissect changes in binding occupancy and targets under mutant (S34F or Q157R) *vs* wildtype conditions.

FreCLIP-seq is based on previously published iCLIP-seq and eCLIP-seq protocols (Hafner et al., 2021; König et al., 2010; Van Nostrand et al., 2016), with the introduction of the membrane size fractionation step that allows to separate RNA binding signals of individual proteins within a complex. The experimental protocol described here has been successfully applied with and without membrane size fractionation in several cellular models, including inducible and standard cell lines for

U2AF1 and U2AF2 binding analysis (Biancon et al., 2022; Glasser et al., 2022), and cytoplasmic fractions derived from primary mouse cells for AGO2 binding analysis (Griffin et al., 2022).

We also provide two possible computational approaches for the analysis of freCLIP-seq and eCLIP-seq data to address different research questions. Both approaches are based on read counts (coverage) and crosslinking-induced termination events to identify and quantify protein-RNA interactions. The first approach focuses on the transcriptome-wide identification of protein-RNA interactions, using the PureCLIP peak calling pipeline (Krakau et al., 2017). The second approach focuses on the comparison of binding occupancies on specific transcript regions, i.e., intron-exon junctions.

Our comprehensive protocol is highly adaptable at both experimental and computational levels. It provides suggestions and alternatives to handle different experimental conditions and to easily process obtained sequencing data. Critical steps are sufficiently detailed to allow researchers with different backgrounds and expertise to successfully follow the protocol.

Institutional permissions

Any experiments on live vertebrates or higher invertebrates must be performed in accordance with relevant institutional and national guidelines and regulations.

Cell preparation

Timing: ~2 weeks

Note: Make sure to grow sufficient cells for at least **2 crosslinked biological replicates** and 1 non-crosslinked control sample (Van Nostrand et al., 2016). To validate antibody specificity, we suggest to initially run the immunoprecipitation (IP) with 2 additional control samples: IP with anti-target antibody in cells lacking that target (in our case study, mouse anti-FLAG antibody in non-induced HEL cells lacking FLAG-tagged proteins) and IP with IgG control according to the antibody source (in our case study, mouse IgG).

1. Grow HEL suspension cells in T175 flasks up to 50% confluence in 100 mL RPMI 1640 supplemented with 9% tetracycline-negative FBS, 1% L-glutamine and 1% penicillin-streptomycin. Cells are cultured in 5% CO_2 at 37°C.
2. Add 1 μg/mL doxycycline to the cells for 48 h to induce the expression of FLAG-tagged U2AF1 proteins.

Note: The concentration of doxycycline may have to be titrated and optimized for each new model to avoid excessive protein expression in comparison to endogenous levels.

Note: Perform a comparative analysis between two induced conditions (in our case study, FLAG-tagged U2AF1 wildtype *vs* FLAG-tagged U2AF1 S34F or Q157R) to remove any potential induction-related bias.

UV-crosslinking

Timing: 2 h

3. Count cells and spin down 30 × 10^6 cells per pellet at 300 × *g*, 4°C, 5 min (consider preparing: biological replicates, n ≥ 2; technical replicates, n ≥ 2).
4. Each 30 × 10^6 cell pellet will be resuspended in 25 mL ice-cold PBS. Using a 10 mL serological pipet gently pipet up and down to generate a single cell suspension.

⚠ CRITICAL: Do not use a 25 mL pipet as it does not allow for single cell suspension. Optimal crosslinking occurs when HEL cells are at a density of 1.2 × 10^6 per mL hence the volumes are kept as mentioned above.

5. Add 10 mL of the cells in ice cold PBS per 10 cm Petri dish. For 30 × 10^6 cell pellet in 25 mL ice-cold PBS, use 3 Petri dishes (10 mL + 10 mL + 5 mL). Ideally, process 60 × 10^6 cells and plate 50 mL in 5 Petri dishes (10 mL in each).
6. Crosslink 5 Petri dishes at a time on ice. Remove the lid and make sure the layer of PBS covers the surface evenly.

⚠ CRITICAL: Check the UV crosslinker before starting (if using UV Stratalinker 2400 by Stratagene, press AUTOCROSSLINK → Start. The crosslinker should count down from 1,200 to 0 in 1 min. Do not proceed with crosslinking if this condition is not met).

7. Irradiate for 200 mJ/cm^2 at 254 nm (if using UV Stratalinker 2400 by Stratagene, press: Energy → 2000 → Start). Swirl the cells and irradiate again for 200 mJ/cm^2.
8. Collect the crosslinked HEL cells from the 5 Petri dishes into two 50 mL conical tubes. Use 6 mL ice-cold PBS to wash all 5 Petri dishes and distribute equally into the two 50 mL conical tubes. Spin down at 300 × *g*, 4°C, 5 min.
9. Prepare dry ice-absolute ethanol bath.
10. Aspirate the supernatant and snap-freeze the pellet in the dry ice-absolute ethanol bath. Pellets can be stored at −80°C until further use.

⚠ CRITICAL: We advise to not exceed one-month storage to limit RNA degradation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-FLAG (clone M2)	Sigma-Aldrich	Cat#F1804; RRID: AB_262044
Chemicals, peptides, and recombinant proteins		
RNAse Away Reagent	Thermo Fisher Scientific	Cat#10328011 Note: For removal of RNAse contamination from surfaces, tools and gloves!
H_2O, RNAse and Endotoxin Tested	AmericanBio	Cat#AB02128-00500; CAS: 7732-18-5 Note: For buffer preparation.
EtOH, pure alcohol, 200 proof	AmericanBio	Cat#AB00515-00500; CAS: 64-17-5 Note: For buffer preparation and bead washing steps.
RPMI 1640	Thermo Fisher Scientific	Cat#11875093
Tetracycline-negative FBS	Gemini Bio-Products	Cat#100-800
L-glutamine	Thermo Fisher Scientific	Cat#25030081
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat#15140122; CAS: 3810-74-0
Doxycycline	Sigma-Aldrich	Cat#D3447; CAS: 10592-13-9
Tris HCl, 1 M solution, pH 7.4	AmericanBio	Cat#AB14044-01000; CAS: 7732-18-5, 77-86-1
Sodium chloride, 5 M solution	AmericanBio	Cat#AB13198-01000; CAS: 7732-18-5, 7647-14-5
NP-40 Surfact-Amps™ Detergent Solution	Thermo Fisher Scientific	Cat#85124; CAS: 9016-45-9
SDS, 10% Solution, RNase-free	Thermo Fisher Scientific	Cat#AM9822; CAS: 151-21-3
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750-10G; CAS: 302-95-4
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#11836170001; CAS: 25322-68-3, 30827-99-7
SUPERase•In™ RNase Inhibitor (20 U/μL)	Thermo Fisher Scientific	Cat#AM2696; CAS: 56-81-5
EDTA, 0.5 M Solution, pH 8.0	AmericanBio	Cat#AB00502-01000; CAS: 7732-18-5, 6381-92-6

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Magnesium chloride, 1 M solution	AmericanBio	Cat#AB09006-00100; CAS: 7732-18-5, 7791-18-6
TWEEN 20	AmericanBio	Cat#AB02038-00500; CAS: 9005-64-5
Tris, 2 M solution, pH 7.5	AmericanBio	Cat#AB14116-01000; CAS: 7732-18-5, 77-86-1
Potassium chloride solution	Sigma-Aldrich	Cat#60135; CAS: 7732-18-5, 7447-40-7
Triton X-100 Detergent	Bio-Rad	Cat#1610407; CAS: 9002-93-1
Tris-HCl 1 M, pH 6.5	Teknova	Cat#T1065; CAS: 77-86-1
DTT (5 g)	DOT Scientific	Cat#DSD11000-5; CAS: 3483-12-3
UltraPure™ 1 M Tris-HCl Buffer, pH 7.5	Thermo Fisher Scientific	Cat#15567027
UltraPure™ Urea	Thermo Fisher Scientific	Cat#15505035
NuPAGE™ MOPS SDS Running Buffer (20×)	Thermo Fisher Scientific	Cat#NP0001; CAS: 151-21-3, 68-12-2
NuPAGE™ Transfer Buffer (20×)	Thermo Fisher Scientific	Cat#NP00061
Methanol ACS reagent, ≥99.8%	Sigma-Aldrich	Cat#179337; CAS: 67-56-1
Novex™ TBE Running Buffer (5×)	Thermo Fisher Scientific	Cat#LC6675
Dynabeads™ Protein G for Immunoprecipitation	Thermo Fisher Scientific	Cat#10004D; CAS: 26628-22-8
Ambion™ RNase I (100 U/μL)	Thermo Fisher Scientific	Cat#AM2295; CAS: 56-81-5
TURBO™ DNase (2 U/μL)	Thermo Fisher Scientific	Cat#AM2239; CAS: 56-81-5
FastAP Thermosensitive Alkaline Phosphatase (1 U/μL)	Thermo Fisher Scientific	Cat#EF0651; CAS: 56-81-5, 9002-93-1
AffinityScript Multiple Temperature Reverse Transcriptase	Agilent Technologies	Cat#600107; CAS: 56-81-5, 7447-40-7
T4 Polynucleotide Kinase	New England Biolabs	Cat#M0201L; CAS: 56-81-5, 60-00-4
100% DMSO	New England Biolabs	Cat#B0515AVIAL; CAS: 67-68-5
T4 RNA Ligase 1 (ssRNA Ligase), High Concentration	New England Biolabs	Cat#M0437M; CAS: 60-00-4
NuPAGE™ LDS Sample Buffer (4×)	Thermo Fisher Scientific	Cat#NP0007; CAS: 56-81-5
ATP, [γ-^{32}P]	PerkinElmer	Cat#BLU002Z250UC
NuPAGE™ 4%–12%, Bis-Tris, 1.0–1.5 mm 10 W, Mini Protein Gels	Thermo Fisher Scientific	Cat#NP0321BOX
Precision Plus Protein Dual Color Standards	Bio-Rad	Cat#1610374
Nitrocellulose Membrane, Roll, 0.45 μm, 30 cm × 3.5 m	Bio-Rad	Cat#1620115; CAS: 9004-70-0
Mini Trans-Blot® Filter Paper, Pkg of 50	Bio-Rad	Cat#1703932
HyBlot CL® Autoradiography Film	Thomas Scientific	Cat#1141J51
Proteinase K Solution (20 mg/mL), RNA grade	Thermo Fisher Scientific	Cat#25530049; CAS: 39450-01-6
Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1)	Thermo Fisher Scientific	Cat#AM9722; CAS: 108-95-2, 67-66-3, 123-51-3
Chloroform	J.T.Baker	Cat#9180-01; CAS: 67-66-3
Dynabeads™ MyOne™ Silane	Thermo Fisher Scientific	Cat#37002D
Buffer RLT	QIAGEN	Cat#79216; CAS: 593-84-0
ATP (10 mM)	New England Biolabs	Cat#P0756S; CAS: 1310-73-2
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs	Cat#N0447S
ExoSAP-IT™ PCR Product Cleanup Reagent	Thermo Fisher Scientific	Cat#78200.200.UL
Sodium hydroxide solution (1.0 N, BioReagent, suitable for cell culture)	Sigma-Aldrich	Cat#S2770-100ML; CAS: 1310-73-2
Hydrochloric acid solution (1.0 N, BioReagent, suitable for cell culture)	Sigma-Aldrich	Cat#H9892-100ML; CAS: 7647-01-0
iQ™ SYBR® Green Supermix	Bio-Rad	Cat#1708880; CAS: 56-81-5, 67-68-5
Q5® High-Fidelity 2× Master Mix	New England Biolabs	Cat#M0492S
AMPure XP Reagent	Beckman Coulter	Cat#A63880
Novex™ TBE-Urea Gels, 6%, 12 well	Thermo Fisher Scientific	Cat#EC68652BOX
GelStar™ Nucleic Acid Gel Stain, 10,000×	Lonza	Cat#50535; CAS: 67-68-5
Critical commercial assays		
RNA Clean & Concentrator-5 kit	Zymo Research	Cat#R1016
Deposited data		
Sequencing data	Biancon et al. (2022) Griffin et al. (2022)	GEO: GSE195620; GSE181264

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
freCLIP-seq and eCLIP-seq analysis code	Biancon et al. (2022) This paper	GitHub: https://github.com/TebaldiLab/eCLIP_seq https://doi.org/10.5281/zenodo.7121228
Original Images	This paper	Mendeley Data: https://doi.org/10.17632/m7g92hkwmh.1
Experimental models: Cell lines		
Human: HEL	ATCC	TIB-180
Experimental models: Organisms/strains		
Mouse: Ago2 conditional knockout males (Ddx4-Cre/+;Ago2$^{flox/\Delta}$)	Griffin et al. (2022)	N/A
Oligonucleotides		
RNA oligos (X1A/B, X2A/B, RiL19) and DNA oligos (AR17, rand3Tr3)	Van Nostrand et al. (2016)	eCLIP Standard Operating Procedure v1.P 20151108
Illumina Adapters (D501-508, D701-D712)	Illumina Adapter Sequences	https://support-docs.illumina.com/SHARE/AdapterSeq/illumina-adapter-sequences.pdf Pages 50–51. Remember to order the reverse complement of the sequence reported for D701–D712.
Recombinant DNA		
CS-TRE-U2AF1-PRE-Ubc-tTA-I2G plasmids	Yamaguchi et al. (2012); Yoshida et al. (2011)	N/A
psPAX2	Addgene	Cat#12260
VSV.G	Addgene	Cat#14888
Software and algorithms		
FastQC	N/A	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
FastUniq	Xu et al. (2012)	https://sourceforge.net/projects/fastuniq/
Cutadapt	Martin (2011)	https://cutadapt.readthedocs.io/en/stable/
STAR v2.7.0f	Dobin et al. (2013)	https://github.com/alexdobin/STAR/releases
PureCLIP	Krakau et al. (2017)	https://github.com/skrakau/PureCLIP
Bedtools	Quinlan and Hall (2010)	https://github.com/arq5x/bedtools2
edgeR	Robinson et al. (2010)	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Tidyverse	CRAN	https://www.tidyverse.org/
data.table	CRAN	https://cran.r-project.org/web/packages/data.table/index.html
Ggseqlogo	CRAN	https://cran.r-project.org/web/packages/ggseqlogo/index.html
GenomicAlignments	Bioconductor	https://bioconductor.org/packages/release/bioc/html/GenomicAlignments.html
GenomicRanges	Bioconductor	https://bioconductor.org/packages/release/bioc/html/GenomicRanges.html
Bamsignals	Bioconductor	https://bioconductor.org/packages/release/bioc/html/bamsignals.html
BSgenome	Bioconductor	https://bioconductor.org/packages/release/bioc/html/BSgenome.html
Other		
RNAse-free filter tips	USA Scientific	Cat#1121-3810, 1120-1810, 1120-8810, 1126-7810
Low-binding microcentrifuge tubes	Sorenson BioScience	1.7 mL tubes, Cat#11700
Phase Lock Gel Heavy Tubes	Quantabio	2 mL tubes, Cat#2302830
UV crosslinker	Stratagene	UV Stratalinker 2400
Tube rotator	Any brand/model available in the lab	N/A
Sonicator	Branson	Digital Sonifier 450
Thermomixer	Benchmark Scientific	H5000-HC
Magnetic separation rack	Any brand/model available in the lab	N/A
Laboratory cold room	Yale University	Note: For IP and washing steps.
Radioactivity room (only if using γ-^{32}P-ATP, not mandatory)	Yale University	Note: Specific training courses are required. Refer to the safety guidelines of your institution.

MATERIALS AND EQUIPMENT

Oligonucleotide	Stock concentration	Working concentration
X1A/B, X2A/B	200 μM	20 μM
RiL19	200 μM	40 μM
AR17	200 μM	20 μM
rand3Tr3	200 μM	80 μM
D501-508, D701-D712	100 μM	20 μM

Lysis Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (1 M)	50 mM	5 mL
NaCl (5 M)	100 mM	2 mL
NP-40 (10% solution)	1%	10 mL
SDS (10% solution)	0.1%	1 mL
Sodium deoxycholate (10% solution)	0.5%	5 mL
H_2O	N/A	77 mL
Total	**N/A**	**100 mL**

High-salt Wash Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (1 M)	50 mM	5 mL
NaCl (5 M)	1 M	20 mL
EDTA (0.5 M)	1 mM	0.2 mL
NP-40 (10% solution)	1%	10 mL
SDS (10% solution)	0.1%	1 mL
Sodium deoxycholate (10% solution)	0.5%	5 mL
H_2O	N/A	58.8 mL
Total	**N/A**	**100 mL**

PNK Wash Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (1 M)	20 mM	2 mL
$MgCl_2$ (1 M)	10 mM	1 mL
Tween 20 (20% solution)	0.2%	1 mL
H_2O	N/A	96 mL
Total	**N/A**	**100 mL**

1× FastAP Buffer		
Reagent	Final concentration	Amount
Tris, pH 7.5 (2 M)	10 mM	0.25 mL
$MgCl_2$ (1 M)	5 mM	0.25 mL
KCl (3 M solution)	100 mM	1.67 mL
Triton X-100	0.02%	10 μL
H_2O	N/A	47.82 mL
Total	**N/A**	**50 mL**

5× PNK pH 6.5 Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 6.5 (1 M)	350 mM	17.5 mL
$MgCl_2$ (1 M)	50 mM	2.5 mL
DTT (100 mM)	5 mM	2.5 mL
H_2O	N/A	27.5 mL
Total	**N/A**	**50 mL**

10× RNA Ligase Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.5 (1 M)	500 mM	25 mL
$MgCl_2$ (1 M)	100 mM	5 mL
H_2O	N/A	20 mL
Total	**N/A**	**50 mL**
Dilute 1:10 in H_2O for **1× RNA Ligase Buffer**.		

Proteinase K Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.5 (1 M)	100 mM	10 mL
NaCl (5 M)	50 mM	1 mL
EDTA (0.5 M)	10 mM	2 mL
H_2O	N/A	87 mL
Total	**N/A**	**100 mL**

Proteinase K/Urea Buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.5 (1 M)	100 mM	10 mL
NaCl (5 M)	50 mM	1 mL
EDTA (0.5 M)	10 mM	2 mL
Urea	7 M	42 g
H_2O	N/A	87 mL
Total	**N/A**	**100 mL**

⚠ CRITICAL: Above listed buffers can be stored at 4°C for up to one month. Use them cold.

All buffers contain harmful or toxic reagents. Wear protective gloves/eye protection/face protection.

1× SDS Running Buffer		
Reagent	Final concentration	Amount
NuPAGE™ MOPS SDS Running Buffer (20×)	1×	50 mL
deionized H_2O	N/A	950 mL
Total	N/A	1,000 mL
We suggest to prepare it fresh for each experiment. It can be stored at 23°C.		

1× Transfer Buffer		
Reagent	Final concentration	Amount
NuPAGE™ Transfer Buffer (20×)	1×	50 mL
Methanol (100%)	10%	100 mL
deionized H_2O	N/A	850 mL
Total	**N/A**	**1,000 mL**
We suggest to prepare it fresh for each experiment.		

⚠ CRITICAL: Store it at 4°C and use it cold.

1× TBE Running Buffer		
Reagent	Final concentration	Amount
Novex™ TBE Running Buffer (5×)	1×	200 mL
deionized H_2O	N/A	800 mL
Total	N/A	1,000 mL
We suggest to prepare it fresh for each experiment. It can be stored at 23°C.		

STEP-BY-STEP METHOD DETAILS

Purification of protein-RNA complexes

Timing: 2 days

The experimental pipeline has two major steps. This first step allows to purify protein-RNA complexes, constituted by the protein(s) of interest and bound fragmented RNAs from crosslinked cell lysates.

⚠ CRITICAL: Always keep samples, buffers and reagents on ice. Frequently spray surfaces/tools/gloves with 70% ethanol and RNAse Away Reagent for decontamination.

Day 1

1. Cell lysis.
   a. Prepare **Lysis Mix**: to 10 mL Lysis Buffer, add 400 µL Proteinase Inhibitor Cocktail (1 tablet dissolved in 400 µL H_2O) and 20 µL SUPERase•In™ RNase Inhibitor.
   b. Thaw crosslinked cell pellets on ice. Resuspend each pellet in 1.2 mL Lysis Mix.
2. Bead preparation (**Dynabeads™ Protein G**).
   a. Vortex Protein G beads, supplied in a solution containing sodium azide as preservative, for 30 s to obtain a homogeneous suspension.
   b. Per each sample, transfer 50 µL of bead suspension to a labeled Low Binding tube.

   Note: The volume of Protein G beads can be scaled up or down according to the experimental conditions. Standard iCLIP-seq and eCLIP-seq suggest to use 100–125 µL beads/sample (König et al., 2010; Van Nostrand et al., 2016).

   c. Place Low Binding tubes on the magnetic rack, which will collect beads on the side of the tubes.
   d. Wait 30 s until the solution is clear.
   e. Keeping the tubes on the magnet, remove the supernatant without disturbing beads.
   f. Wash Protein G beads 2× with 500 µL Lysis Mix.
      i. Remove the tubes from the magnetic rack.
      ii. Resuspend beads in 500 µL Lysis Mix.
      iii. Replace tubes onto magnetic rack.
      iv. Wait 30 s until the solution is clear and discard supernatant.
      v. Repeat for a second time.
   g. Resuspend beads in 500 µL Lysis Mix.
   h. Add 12 µg of anti-FLAG antibody (12 µL of 1 mg/mL stock stored at −20°C) to the bead slurry.

   Note: The amount of antibody depends on the antibody quality, purity, binding strength, and specificity. This should be optimized in preliminary experiments following manufacturer's instructions for IP application.

i. Rotate tubes at 23°C for 60 min. In the meantime, go to **3. Partial RNA digestion.**
j. When cell lysates are ready at the end of step 2, wash Protein G beads with bound antibody 2× with 500 µL Lysis Mix.
k. Resuspend beads in 500 µL Lysis Mix and keep on ice.

3. Partial RNA digestion.

Optional: Sonicate samples (not required for cytoplasmic fraction as indicated in Brugiolo et al., 2017):

Clean sonicator tip with RNAse Away Reagent before using it.
Transfer each lysate to a 15 mL tube and place the tube in an ice beaker.

⚠ CRITICAL: Avoid foaming. Keep the probe approximately 0.5 cm from the bottom of the tube without touching tube sides.

Sonicate with 6 pulses each 10 s at 30% amplitude, 20 s pause.

Note: We use the Branson Digital Sonifier 450. Sonication conditions may need to be converted to suit another instrument.

Transfer 1.2 mL lysate to Low Binding tube.
Spin down at 16,000 × *g*, 4°C, 10 min.
Transfer the supernatant into a fresh Low Binding tube (leave some to prevent carryover).

a. Prepare a dilution of RNAse I at 1:30 in H_2O [Low RNase].

Note: Several RNAse dilutions should be initially tested with the help of P32-labeling in step 9 (Huppertz et al., 2014). With a 1:50 High RNase dilution, immunoprecipitated proteins are bound to short RNAs migrating as a less diffuse band ~5 kD above the expected molecular weight of the protein (this also helps in determining the actual protein size). With Low RNase conditions the band shifts up and becomes more diffuse.

b. Add 10 µL of diluted RNAse I and 5 µL of TURBO™ DNase to each lysate.
c. Incubate at 37°C exactly for 3 min while shaking at 1,100 rpm.
d. Place immediately on ice for at least 3 min.

4. Immunoprecipitation.
a. For each cell lysate, save 24 µL (2%) as **pre-IP input sample for library preparation**, and other 24 µL as **pre-IP control for western blot.** Store at 4°C.

Note: Paired pre-IP input samples are sequenced to allow normalization for background signal and potential differences in transcript abundances between conditions.

b. Magnetically remove the Lysis Mix from the Protein G beads with bound antibody.
c. Add the remaining cell lysate to the beads.
d. Rotate beads/lysate mix for 1 h at 4°C in the cold room.
e. Wash beads with bound protein-RNA complexes 3× with 800 µL High-salt Wash Buffer.
   i. Short spin and magnetic separation (let sit for 2 min in the cold room).
   ii. Discard supernatant.
   iii. Add 800 µL High-salt Wash Buffer, vortex for few seconds until the beads are fully resuspended, 2 min rotation in the cold room.
   iv. Repeat for other 2 times.
f. Wash 1× with 800 µL PNK Wash Buffer for 2 min rotation in the cold room as in **4.e.**

g. Wash 1× with 800 µL 1× FastAP Buffer for 2 min rotation in the cold room as in **4.e.** At the end, remove residual buffer with fine tip.

5. 3′ end dephosphorylation.
   a. FastAP treatment. Prepare **FastAP Master Mix** for *n*+1 samples as follows:

Reagent	Amount
10× FastAP Buffer	10 µL
SUPERase·In™ RNase Inhibitor	2 µL
TURBO™ DNase	1 µL
FastAP enzyme	8 µL
H_2O	79 µL
Mix, add 100 µL/sample, incubate in Thermomixer at 1,200 rpm, 37°C, 15 min. Then, short spin and magnetic separation to remove Master Mix.	

   b. PNK treatment. Prepare **PNK Master Mix** for *n*+1 samples as follows:

Reagent	Amount
5× PNK pH 6.5 Buffer	60 µL
100 mM DTT	3 µL
SUPERase·In™ RNase Inhibitor	5 µL
TURBO™ DNase	1 µL
T4 PNK enzyme	7 µL
H_2O	224 µL
Mix, add 300 µL/sample, incubate in Thermomixer at 1,200 rpm, 37°C, 20 min. Then, short spin and magnetic separation to remove Master Mix.	

6. Wash beads with bound protein-RNA complexes as in **4.e**.
   a. Wash 1× with 800 µL PNK Wash Buffer for 2 min rotation in the cold room.
   b. Wash 1× with 800 µL High-salt Wash Buffer for 2 min rotation in the cold room.
   c. Wash 2× with 800 µL PNK Wash Buffer for 2 min rotation in the cold room.
   d. Wash 2× with 300 µL 1× RNA Ligase Buffer for 2 min rotation in the cold room. At the end, remove residual buffer with fine tip.
7. 3′ linker ligation.
   a. Prepare **3′ Ligation Master Mix** for *n*+1 samples as follows:

Reagent	Amount
10× RNA Ligase Buffer	3 µL
100 mM ATP	0.3 µL
100% DMSO	0.8 µL
SUPERase•In™ RNase Inhibitor	0.4 µL
T4 RNA Ligase High Concentration	2.5 µL
50% PEG 8000	9 µL
H_2O	9 µL
Mix carefully, add 25 µL/sample, flick.	

   b. To each sample, add 5 µL of a different barcoded RNA adapter at 20 µM: X1A, X1B, X2A, X2B.
   c. Incubate at 23°C for 75 min flicking every 10 min.

Pause point: Keep at 4°C for 12–16 h.

Day 2

8. Wash beads with bound protein-RNA complexes as in **4.e.**
   a. Magnetically separate (let sit for 5 min) and remove supernatant.

b. Wash beads 1× with 800 μL PNK Wash Buffer for 2 min rotation in the cold room.
c. Wash 2× with 800 μL High-salt Wash Buffer for 2 min rotation in the cold room.
d. Wash 2× with 800 μL PNK Wash Buffer for 2 min rotation in the cold room.
e. Resuspend beads in 1 mL PNK Wash Buffer and keep on ice.
f. Dilute 4× NuPAGE™ Loading Buffer to 1.5× in H_2O.
g. Add 20 μL 1.5× NuPAGE™ Buffer to **pre-IP controls for western blot** saved on Day 1.
h. Take out 100 μL of the 1 mL beads in PNK Wash Buffer, perform magnetic separation, discard supernatant and resuspend beads in 20 μL 1.5× NuPAGE™ Buffer as **post-IP controls for western blot.** Keep on ice.

9. Optional: P32-labeling.

Note: We usually perform radiolabeling because it helps with visualization and purification of cross-linked protein-RNA complexes after SDS-PAGE and membrane transfer, in the optimization of RNA digestion conditions and in the identification of potential non-specific signal. However, as reported in Van Nostrand et al. (2016), this is not a mandatory step.

a. Take out another 100 μL beads of the remaining 900 μL beads in PNK Wash Buffer. Prepare **PNK Master Mix** for *n*+1 samples as follows:

Reagent	Amount
T4 PNK enzyme	0.2 μL
10× PNK buffer	0.4 μL
H_2O	3 μL

Discard the buffer from the beads, add 3.6 μL/sample.
b. Add 0.4 μL γ-32P-ATP (corresponding to 4 μCi).

⚠ CRITICAL: The half-life of γ-32P-ATP is 14.3 days. Adjust γ-32P-ATP and H_2O volumes accordingly (e.g., if the use date is 2 weeks after the calibration date, double γ-32P-ATP volume using 0.8 μL and reduce H_2O to 2.6 μL https://www.perkinelmer.com/tools/calculatorrad/product).

c. Mix well by pipetting up and down and incubate in Thermomixer at 1,100 rpm, 37°C, 5 min.
d. Perform short spin and magnetic separation.
e. Discard the buffer after magnetic separation and resuspend radioactive beads in 20 μL 1.5× NuPAGE™ Buffer.
f. Remove the buffer from the remaining cold beads (800 μL).
g. Collect NuPAGE™ Buffer + radioactive beads and combine with corresponding cold beads.

10. Prepare samples for gel loading.
a. Denature experimental samples and pre-/post-IP controls for western blot in Thermomixer at 70°C, 10 min, without shaking.
b. Collect eluates and keep on ice. Store controls for western blot at −80°C.

11. SDS-PAGE and membrane transfer.
a. Have ready the prepared 1× SDS Running Buffer and 1× Transfer Buffer.
b. Use 10-well NuPAGE™ 4%–12% Bis-Tris gels. Peel off the tape and place the gel cassette into the electrophoresis tank with the lower cassette side facing you.

Note: We use the Thermo Fisher Scientific Mini Gel Tank that can allocate up to 2 gels per run. Remove the cassette clamp from the unused chamber to run only one gel. See manufacturer's instructions available here: https://www.thermofisher.com/order/catalog/product/A25977.

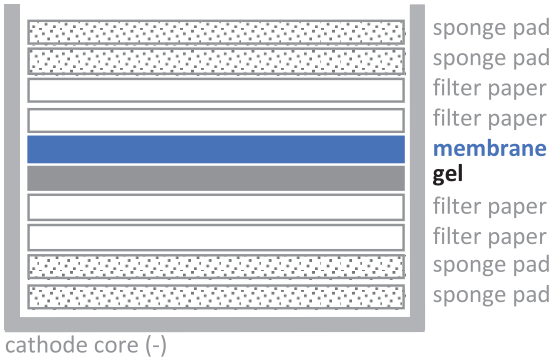
c. Fill the tank with 1× SDS Running Buffer.
d. Load 20 μL eluted samples and 6 μL pre-stained protein ladder.

⚠ CRITICAL: Leave one lane free for cutting (max 4 samples/gel). Immediately before loading, use a pipette or syringe to rinse the wells 3 times with 1× SDS Running Buffer.

e. Run at 180 V, 55 min.
f. After the gel run, cut off wells and dye front (after P32-labeling, it contains free radioactive ATP).
g. Soak sponge pads and filter papers in pre-cold 1× Transfer Buffer.
h. Use 0.45 μm nitrocellulose membranes.

Note: Nitrocellulose membranes can represent a source of bacterial contamination (Van Nostrand et al., 2017; Rosenberg et al., 2021). Using Bio-Rad membranes (Cat#1620115) we consistently obtained mapping rates between 80%–90%. If low mapping rates are encountered, consider changing membranes.

i. Assemble the transfer stack as follows:



⚠ CRITICAL: Use a roller to remove air bubbles trapped in the stack!

Note: We use the Thermo Fisher Scientific XCell II™ Blot Module in the XCell SureLock system. See manufacturer's instructions available here: https://www.thermofisher.com/order/catalog/product/EI9051.

j. Fill the blot module with 1× Transfer Buffer.
k. Transfer at 30 V, 90 min.
l. After the transfer, rinse the membrane in 1× PBS and wrap it in plastic wrap.

Pause point: Store the membrane at −80°C for 12–16 h.

Optional: If you performed P32-labeling: Tape the wrapped membrane to the cassette with intensifying screen (we use Kodak BioMax Cassette with BioMax MS Intensifying Screen). Use luminescent stickers to help aligning the developed film after exposure to the membrane.

Pause point: Expose the membrane to X-ray film for 12–16 h.

Fractionation and library preparation

Timing: 3 days

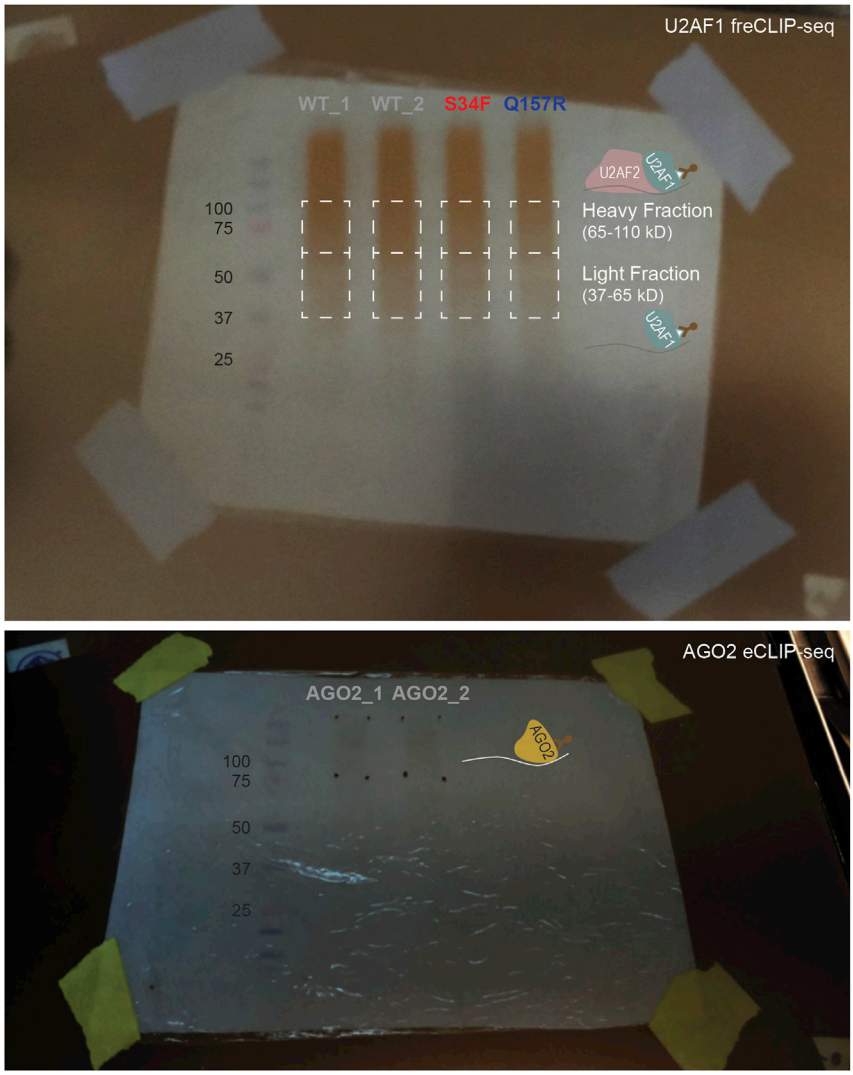


Figure 1. Autoradiographic visualization and membrane size-selection

Top: representative U2AF1 freCLIP-seq membrane cutting to obtain U2AF1 light and heavy fractions in U2AF1 wildtype and mutant (S34F or Q157R) conditions. Figure adapted and reprinted with permission from (Biancon et al., 2022). Bottom: representative AGO2 eCLIP-seq membrane cutting to obtain AGO2-RNA complexes. See also Figure S1.

This second major step of the experimental pipeline extracts RNAs from protein-RNA complexes and converts isolated RNAs into cDNA high-throughput sequencing libraries.

Day 3

12. Membrane cutting (with or without fractionation).
    a. Optional if you performed P32-labeling: after exposure for 12–16 h, proceed with autoradiographic visualization. Use the signal on the developed film as a mask for cutting out regions of interest. Remember that you are working with radioactive RNA!

Note: In case of experimental samples with absent or faint signal, see troubleshooting 1.

    b. Cut each sample (and each fraction) with a fresh sterile scalpel blade.

Note: The average molecular weight of 70 nt long RNA is ~20 kD. The 3′ RNA linker is 34 nt long, ~10 kD. Cut the membrane considering the size of the protein with the crosslinked fragmented RNA (Huppertz et al., 2014; Van Nostrand et al., 2016). For U2AF1 freCLIP-seq (Biancon et al., 2022), we cut a "light fraction" between 37 kD (U2AF1 size) and 65 kD, containing U2AF1 monomer plus bound RNA, and a "heavy fraction" between 65 kD (U2AF2 size) and 110 kD, containing U2AF2 and U2AF heterodimer plus bound RNA (Figure 1 top panel). To apply our pipeline without membrane size fractionation, cut a lane from the protein size to approximately 80 kD above it. For AGO2 eCLIP-seq (Griffin et al., 2022), according to our western blot results and to the signal on the developed film, we cut a lane starting between 75-100 kD (AGO2 size is 97 kD) and ending approximately at 250 kD (Figure 1 bottom panel).

Note: Control samples should show absent or significantly reduced signal in comparison to experimental samples (Figure S1), resulting in insufficient material for library preparation and sequencing and therefore confirming insignificant non-specific binding.

c. Place each membrane region on a fresh glass slide and slice it into 1–2 mm pieces. Transfer all the pieces to a Low Binding 1.7 mL tube.

⚠ CRITICAL: Use tweezers to remove the plastic wrap and handle the membrane but don't forget to clean them with 70% ethanol and RNAse Away Reagent between each sample (and each fraction).

13. RNA extraction.
    a. Submerge the membrane pieces in 200 µL Proteinase K Buffer. Add 10 µL Proteinase K. Incubate in a Thermomixer at 1,100 rpm, 37°C, 20 min.
    b. Add 200 µL Proteinase K/Urea Buffer. Incubate in Thermomixer at 1,100 rpm, 37°C, 20 min.
    c. Spin 2 mL Phase Lock Gel tubes at 16,000 × *g*, 23°C, 1 min before use.
    d. Transfer the supernatant (without membrane slices) to a Phase Lock Gel tube.
    e. Add 400 µL phenol/chloroform. Incubate in Thermomixer at 1,100 rpm, 30°C, 5 min.
    f. Spin down at 16,000 × *g*, 23°C, 5 min.
    g. Add 400 µL chloroform. Incubate in Thermomixer at 1,100 rpm, 30°C, 5 min.
    h. Spin down at 16,000 × *g*, 23°C, 5 min.
    i. Transfer the upper aqueous layer (350 µL, avoid the gel barrier) to 15 mL conical tube.
    j. RNA Cleanup (RNA Clean & Concentrator-5 kit. All centrifugation steps are carried out at 13,000 × *g*, 23°C, 30 s).
        i. Add 700 µL RNA Binding Buffer.
        ii. Add 1,050 µL ethanol 100% and mix.
        iii. Transfer 750 µL sample to Zymo-Spin IC Column. Spin down and discard flow-through. Repeat 2× to transfer all sample volume.
        iv. Add 400 µL RNA Prep Buffer. Spin down and discard flow-through.
        v. Add 700 µL RNA Wash Buffer. Spin down and discard flow-through.
        vi. Add 400 µL RNA Wash Buffer. Spin down and discard flow-through. Centrifuge for additional 2 min.
        vii. Transfer column to a Low Binding tube. Add 12 µL DNase/RNase-Free Water to column, let sit for 1 min and spin down.

⏸ **Pause point:** Store the extracted CLIP RNA at −80°C.

14. Processing of pre-IP input samples.
    a. Take out 10 µL from the pre-IP input samples stored at 4°C on Day 1.
    b. FastAP treatment. Prepare **FastAP Master Mix** for *n*+1 samples as follows:

Reagent	Amount
10× FastAP Buffer	2.5 µL
SUPERase•In™ RNase Inhibitor	0.5 µL
FastAP enzyme	2.5 µL
H_2O	10 µL
Mix, add 15.5 µL/sample, incubate in Thermomixer at 1,200 rpm, 37°C, 15 min.	

c. PNK treatment. Prepare **PNK Master Mix** for *n*+1 samples as follows:

Reagent	Amount
5× PNK pH 6.5 Buffer	20 µL
100 mM DTT	1 µL
SUPERase•In™ RNase Inhibitor	1 µL
TURBO™ DNase	1 µL
T4 PNK enzyme	7 µL
H_2O	45 µL
Mix, add 75 µL/sample, incubate in Thermomixer at 1,200 rpm, 37°C, 20 min. Then, short spin and magnetic separation.	

d. Silane cleanup of input RNA (**Dynabeads™ MyOne™ Silane**).
   i. Vortex Silane beads for 30 s. Magnetically separate 20 µL beads per sample in Low Binding tubes, remove supernatant.
   ii. Wash beads with 900 µL Buffer RLT (let sit 30 s).
   iii. Resuspend beads in 300 µL Buffer RLT and add them to sample, mix.
   iv. Add 10 µL 5 M NaCl.
   v. Add 615 µL 100% EtOH.
   vi. Mix and rotate at 23°C for 15 min.
   vii. Magnetically separate and remove supernatant.
   viii. Resuspend beads in 1 mL 75% EtOH and move suspension to a new Low Binding tube.
   ix. After 30 s, magnetically separate and remove supernatant.
   x. Wash beads 2× with 75% EtOH (let sit 30 s).
   xi. Quick spin and perform magnetic separation to remove residual liquid.
   xii. Air-dry 5 min (do not over-dry!).
   xiii. Resuspend beads in 10 µL H_2O, let sit 5 min.
   xiv. Magnetically separate and transfer supernatant to new Low Binding tubes: 5 µL for proceeding with library preparation (keep on ice), 5 µL as backup sample (store at −80°C).

e. 3′ linker ligation to input RNA.
   i. Add 1.5 µL 100% DMSO.
   ii. Add 0.5 µL RiL19 barcoded RNA adapter at 40 µM.
   iii. Incubate in Thermomixer at 65°C, 2 min, no shaking. Place immediately on ice for at least 1 min.
   iv. Prepare **3′ Ligation Master Mix** for *n*+1 samples as follows:

Reagent	Amount
10× T4 RNA Ligase Reaction Buffer	2 µL
10 mM ATP	2 µL
100% DMSO	0.3 µL
SUPERase·In™ RNase Inhibitor	0.2 µL
T4 RNA Ligase High Concentration	1.3 µL
50% PEG 8000	8 µL
Mix carefully, add 13.8 µL/sample, flick, incubate at 23°C for 75 min (flick to mix every 15 min).	

f. Silane cleanup of linker-ligated input RNA (**Dynabeads™ MyOne™ Silane**).
   i. Vortex Silane beads for 30 s. Magnetically separate 20 µL beads per sample in Low Binding tubes, remove supernatant.
   ii. Wash beads with 900 µL Buffer RLT (let sit 30 s).
   iii. Resuspend beads in 61.6 µL Buffer RLT and add them to sample, mix.
   iv. Add 61.6 µL 100% EtOH. Mix every 5 min for 15 min.
   v. Magnetically separate and remove supernatant.
   vi. Resuspend beads in 1 mL 75% EtOH and move suspension to a new Low Binding tube.
   vii. After 30 s, magnetically separate and remove supernatant.
   viii. Wash beads 2× with 75% EtOH (let sit 30 s).
   ix. Quick spin and perform magnetic separation to remove residual liquid.
   x. Air-dry 5 min (do not over-dry!).
   xi. Resuspend beads in 10 µL H_2O, let sit 5 min.
   xii. Magnetically separate and transfer supernatant to a new Low Binding tube.

Pause point: Store the extracted INPUT RNA at −80°C. CLIP and INPUT RNA samples are now all synchronized.

Day 4

15. Reverse transcription.
    a. Primer annealing.
       i. Thaw stored 10 µL CLIP and INPUT RNA samples.

Note: If you performed P32-labeling, CLIP samples are still radioactive.

       ii. Transfer all the samples to 8-tube PCR strips. Make a RTneg tube with 10 µL H_2O.
       iii. Add 0.5 µL AR17 primer at 20 µM.
       iv. Place at 65°C for 2 min in pre-heated PCR block. Place immediately on ice for at least 1 min.
    b. Prepare **Reverse Transcription Mix** for *n*+1 samples as follows:

Reagent	Amount
10× AffinityScript RT buffer	2 µL
100 mM DTT	2 µL
dNTPs (10 mM each)	2 µL
SUPERase•In™ RNase Inhibitor	0.3 µL
AffinityScript Multi-Temp RT	0.9 µL
H_2O	2.8 µL
Mix, add 10 µL/sample, incubate in pre-heated PCR block at 55°C, 45 min.	

16. RT cleanup.
    a. Add 3.5 µL ExoSAP-IT™ to each sample, pipette-mix.
    b. Incubate in pre-heated PCR block at 37°C, 15 min.
    c. Add 1 µL 0.5 M EDTA.
    d. Add 3 µL 1 M NaOH, pipette-mix.
    e. Incubate in pre-heated PCR block at 70°C, 12 min.
    f. Add 3 µL 1 M HCl, pipette-mix.
17. Silane cleanup of cDNA (**Dynabeads™ MyOne™ Silane**).
    a. Vortex Silane beads for 30 s. Magnetically separate 10 µL beads per sample in Low Binding tubes, remove supernatant.
    b. Wash beads with 500 µL Buffer RLT (let sit 30 s).

c. Resuspend beads in 93 µL Buffer RLT and add them to sample, mix.
d. Add 111.6 µL 100% EtOH. Mix, let sit for 5 min, mix, let sit for other 5 min. Then, magnetically separate and remove supernatant.
e. Resuspend beads in 1 mL 80% EtOH and move suspension to a new Low Binding tube.

Note: If performed P32-labeling, at this point samples are not radioactive anymore.

f. After 30 s, magnetically separate and remove supernatant.
g. Wash beads 2× with 80% EtOH (let sit 30 s). Quick spin and perform magnetic separation to remove residual liquid.
h. Air-dry 5 min (do not over-dry!).
i. Resuspend beads in 5 µL 5 mM Tris-HCl pH 7.5, let sit 5 min (do not remove from beads).

18. 3′ linker ligation to cDNA (5′ ligation considering the original RNA fragment).
a. Add 0.8 µL rand3Tr3 ssDNA adapter at 80 µM.

Note: Added to single strand cDNA fragments, this adapter works as a randomer-UMI because it contains an inline randomer of 10 nt that allows to recognize and remove PCR duplicates.

b. Add 1 µL 100% DMSO.
c. Incubate in Thermomixer at 75°C, 2 min, no shaking. Place immediately on ice for at least 1 min.
d. Prepare 5′ **Ligation Master Mix** for *n*+1 samples as follows:

Reagent	Amount
10× T4 RNA Ligase Reaction Buffer	2 µL
10 mM ATP	2 µL
T4 RNA Ligase High Concentration	0.5 µL
50% PEG 8000	9 µL
Mix carefully, add 13.5 µL/sample, mix by stirring contents.	

e. Add another 1 µL T4 RNA Ligase High Concentration to each sample, flick to mix.
f. Incubate in Thermomixer at 1,200 rpm, 23°C, 30 s. Place tubes in a rack on your bench.

Pause point: Flick ideally every hour, at least twice, before incubating at 23°C for 12–16 h.

Day 5

19. Silane cleanup of linker-ligated cDNA (**Dynabeads™ MyOne™ Silane**).
a. Vortex Silane beads for 30 s. Magnetically separate 5 µL beads per sample in Low Binding tubes, remove supernatant.
b. Wash beads with 500 µL Buffer RLT (let sit 30 s).
c. Resuspend beads in 60 µL Buffer RLT and add them to sample, mix.
d. Add 60 µL 100% EtOH. Mix, let sit 5 min, mix, let sit other 5 min. Then, magnetically separate and remove supernatant.
e. Resuspend beads in 1 mL 75% EtOH and move suspension to a new Low Binding tube.
f. After 30 s, magnetically separate and remove supernatant.
g. Wash beads 2× with 75% EtOH (let sit 30 s). Quick spin and perform magnetic separation to remove residual liquid.
h. Air-dry 5 min (do not over-dry!).
i. Resuspend beads in 27 µL 10 mM Tris-HCl pH 7.5, let sit 5 min.

j. Magnetically separate, transfer 25 µL supernatant to a new Low Binding tube and place on ice.

20. cDNA quantification by qPCR.
    a. Dilute cDNA samples 1:10 in H_2O. As controls, use RT H_2O (RTneg) and qPCR H_2O. Samples and controls are quantified in triplicate.
    b. Prepare **qPCR Master Mix** for *n*+3 samples as follows:

Reagent	Amount
iQ™ SYBR® Green Supermix	5 µL
qPCR primer mix (10 µM each D502/D702)	0.4 µL
H_2O	3.6 µL
Mix, add 9 µL/sample in 96 well qPCR plate, add 1 µL diluted cDNA or controls, mix, seal.	

    c. Run qPCR ("Quick Plate_96 wells_SYBR Only" 2-step protocol in the BIO-RAD CFX96 Touch Real-Time PCR Detection System) with the following cycling conditions:

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	15 s	39 cycles
Annealing/Extension	60°C	30 s	
Melt/Dissociation	55°C	5 s	1
	95°C	50 s	

    d. Use automatically calculated Cq values. The number of cycles for final PCR amplification will be 3 cycles less than the Cq of the diluted CLIP sample.

Note: The "3 cycles less" rule may be adjusted considering the type of sample (CLIP or INPUT), final library concentration and possible adapter bands.

21. cDNA amplification.
    a. Organize 8-tube PCR strips according to the Cq values of each sample.
    b. Transfer 12.5 µL CLIP samples or 10 µL INPUT samples in the assigned strips. For each PCR strip, make a PCRneg tube with 10 µL H_2O. Store the remaining 12.5 µL CLIP samples and 15 µL INPUT samples at −80°C for troubleshooting 2 (**pre-AMP libraries**).
    c. Dispense **PCR Master Mix** in 8-tube PCR strips as follows:

Reagent	Amount
Q5® High-Fidelity 2× Master Mix	25 µL
H_2O	7.5 µL CLIP or 10 µL INPUT
20 µM right primer D50×	2.5 µL
20 µM left primer D70×	2.5 µL

Note: For adapter pairs and sample pooling, we follow Illumina guidelines reported in Table S1 (and previously available as document #770-2017-004-B). Refer to Illumina website for current guidelines: https://support.illumina.com/downloads/index-adapters-pooling-guide-1000000041074.html. Illumina adapters were purchased from Integrated DNA Technologies, IDT (100 nmole DNA Oligos, PAGE purification).

For PCRneg tubes, use adapter pair of one of the library samples in the strip.

    d. Mix and run PCR with the following **cycling conditions**:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	6 cycles
Annealing	68°C	30 s	
Extension	72°C	40 s	
Denaturation	98°C	15 s	X cycles (-3 rule)
Annealing/Extension	72°C	1 min	
Final extension	72°C	1 min	1
Hold	4°C	forever	

Note: INPUT samples are typically amplified with 9 total cycles (6+3); CLIP samples, especially freCLIP fractions, typically required 16–18 total cycles (6+10–12). If Cq value=22, keep 18 total cycles; if Cq value ≥22 but lower than H_2O Cq, use 19 total cycles, do not increase further! As reported in Van Nostrand et al. (2016), 18 cycles will yield ~30%–50% PCR duplicate reads.

22. SPRI cleanup of amplified libraries (**AMPure XP beads**).
    a. Vortex AMPure XP beads for 30 s.
    b. Add 90 µL beads to 50 µL PCR products, mix well and incubate at 23°C, 10 min (mix 2× during incubation).
    c. Magnetically separate and remove supernatant.
    d. Wash beads 2× with 75% EtOH (let sit 30 s).
    e. Short spin and magnetic separation to remove residual liquid.
    f. Air-dry 5 min (do not over-dry!).
    g. Resuspend beads in 20 µL H_2O, let sit 5 min.
    h. Magnetically separate, transfer 18 µL supernatant to a new Low Binding tube (**post-AMP libraries**).

Pause point: Store at −80°C for up to one month before sequencing.

23. Optional: Library visualization by TBE-Urea gel.
    a. Have ready the prepared 1× TBE Running Buffer.
    b. Take out 6 µL purified post-AMP libraries and mix with 1 µL 6× loading dye.
    c. Run libraries on 6% TBE-Urea gel at 180 V, 40 min.
    d. Stain gel with GelStar™ (3 µL GelStar™ in 50 mL 1× TBE Running Buffer). Gentle shaking at 23°C, 10 min.
    e. Visualize gel (Figure 2A). Library size should be between 175–350 bp.

⚠ CRITICAL: Adapter dimers around 150 bp negatively affect sequencing. See troubleshooting 2.

Note: Primer excess (<100 bp) does not affect sequencing because these short fragments cannot bind to the flow cell. Consider buying new AMPure XP beads!

EXPECTED OUTCOMES

Steps 1–22 of the experimental pipeline will allow to obtain freCLIP-seq or eCLIP-seq libraries that are ready for sequencing.

Right after SPRI cleanup, purified post-AMP libraries can be visualized by TBE-Urea gel (Figure 2A). This step is useful to quickly verify the presence of libraries within the correct bp range and possible adapter contamination. However, we discourage to perform this step with low concentration libraries (high Cq values) to avoid wasting precious sample.

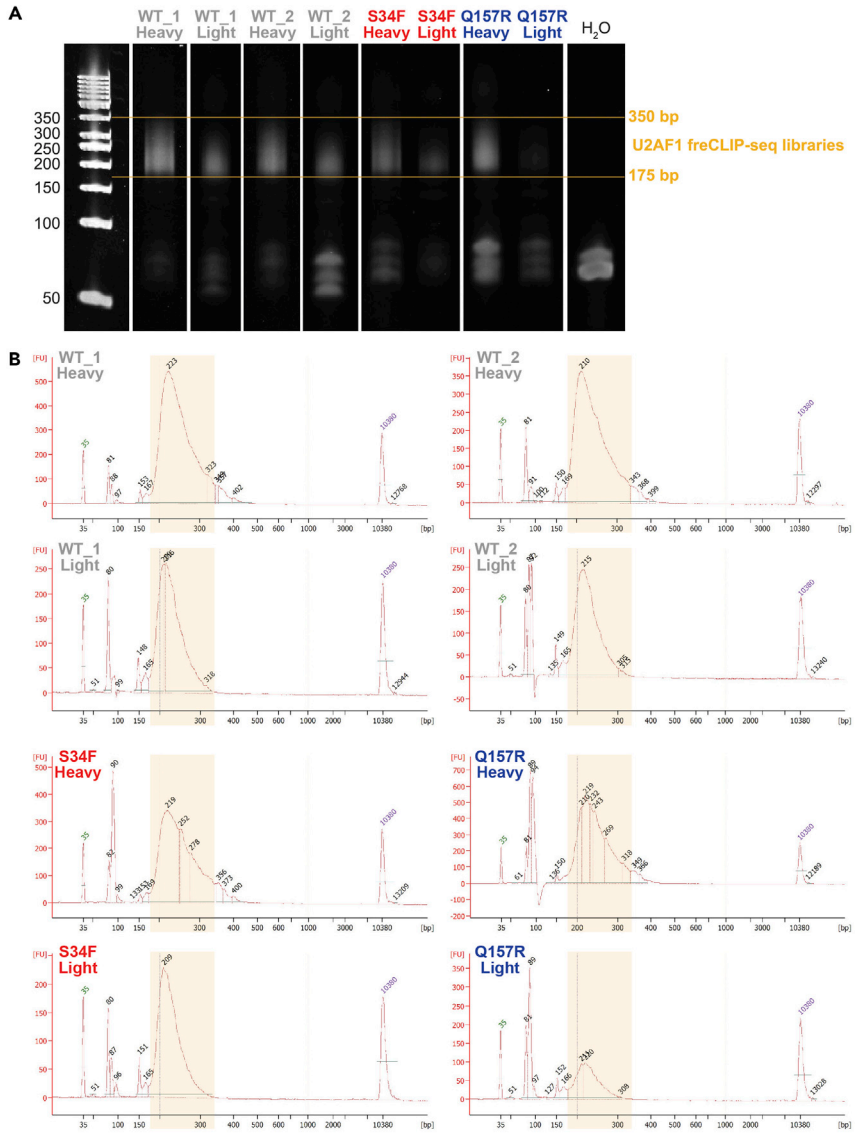


Figure 2. Quality control of representative U2AF1 freCLIP-seq libraries before sequencing

(A) Visualization by TBE-Urea gel. Yellow lines mark the expected library size.

(B) Visualization by Agilent 2100 Bioanalyzer. Peak size is reported. Lower and upper marker peaks are indicated, respectively, in green and purple. These libraries are ready for sequencing: 175–350 bp range (yellow box), good concentration, no adapter dimers (<10%), primer excess can be ignored.

Before sequencing, purified post-AMP libraries need to be subjected to quality control and quantification through the steps listed below. These steps were performed by the Yale Center for Genome Analysis (YCGA).

Quality control (QC) by automated electrophoresis. Run purified post-AMP libraries on Agilent 2100 Bioanalyzer using the High Sensitivity DNA Assay (Cat#5067-4626). Assess library size (175–350 bp), concentration, primer excess (80 bp peak) and adapter dimers (150 bp peak) (Figure 2B). For libraries with over 10% adapter dimers (troubleshooting 2), proceed to step 2.

Optional: **Size-selection by gel purification.** Run libraries on 2% Invitrogen™ E-Gel™ EX Agarose Gels (Cat#A42346), and gel-extract using QIAGEN QIAquick Gel Extraction Kit (Cat#28706). Repeat QC.

Library quantification. To quantify adapter-ligated fragments in the libraries, use Roche KAPA Library Quantification Kit optimized for Illumina/LightCycler 480 (Cat#07960298001). Required concentration: 1.15 nM (for NovaSeq).

Our freCLIP-seq and eCLIP-seq libraries were deep-sequenced on Illumina HiSeq 2500 system paired-end 75 bp, and on Illumina HiSeq 4000 or NovaSeq 6000 system paired-end 100 bp, at 80 million reads/sample.

Alternative strategy

Single-end CLIP (seCLIP). As reported in Van Nostrand et al. (2017), seCLIP exploits a new 3′ linker ligation strategy that allows for single-end sequencing thus reducing sequencing costs. In the paired-end eCLIP, the crosslinked binding site corresponds to the nucleotide right before the 5′ of the second paired-end read (R2). seCLIP protocol inverts this read structure removing the need of paired-end sequencing. Moreover, the new released seCLIP protocol (Blue et al., 2022) introduced biotin labeling for RNA visualization overcoming the absence of visualization methods or the use of radioactivity. Our pipeline can be easily adapted for seCLIP, at both the experimental and computational level.

QUANTIFICATION AND STATISTICAL ANALYSIS

We here describe our computational pipeline for the analysis of freCLIP-seq and eCLIP-seq data. Sample datasets to repeat our approach are available in GEO under the accession numbers GSE195620 (U2AF1 freCLIP-seq) and GSE181264 (AGO2 eCLIP-seq).

Pre-processing of next-generation sequencing data

This section lists all the computational steps necessary to obtain aligned eCLIP-seq reads (BAM files), starting from sequencing raw data (FASTQ files). The details are specified for paired-end eCLIP-seq libraries, but can be easily adapted to single-end libraries. An implementation of these steps is available as a bash script (https://github.com/TebaldiLab/eCLIP_seq). For any step, alternative computational tools can be used, with additional care optionally necessary to optimize the compatibility of input/output file formats.

1. **Quality control** with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For multiple samples, QC can be visually inspected with MultiQC (https://multiqc.info/). Output: quality control reports.
2. **Deduplication** with FastUniq, a tool for removal of duplicates in paired short reads, not requiring complete genome sequences as prerequisites (Xu et al., 2012). Output: FASTQ files with unique reads that are used in the following step.
3. **Adapter removal** with Cutadapt (Martin, 2011). In case of paired-end libraries, this step is performed on R2, removing the first 10 nucleotides (randomer-UMI) and removing the sequence of the 3′ RNA linker (Table S2) from the end of the read. Only reads longer than 10 nucleotides after linker removal are kept. Output: FASTQ files with trimmed reads, to be used in the following step.
4. **Alignment** to a reference genome with a splice-aware aligner. In our case studies, we used the STAR aligner (Dobin et al., 2013) with default options, and we aligned reads to human and mouse reference genomes using annotations provided by GENCODE. Output: BAM files with aligned reads.

U2AF1 freCLIP-seq and AGO2 eCLIP-seq metrics are available in Table S3.

Identification of transcriptome-wide protein-RNA interactions with a peak calling algorithm

This section lists all the computational steps necessary to identify and compare protein-RNA interaction locations, starting from aligned reads (BAM files). In the simplest experimental design, CLIP and INPUT libraries with ideally 2 or more biological replicates are expected. An implementation of these steps is available as bash and R scripts (https://github.com/TebaldiLab/eCLIP_seq). The case study shown is based on a dataset where AGO2 binding to cytoplasmic RNAs was quantified in mouse

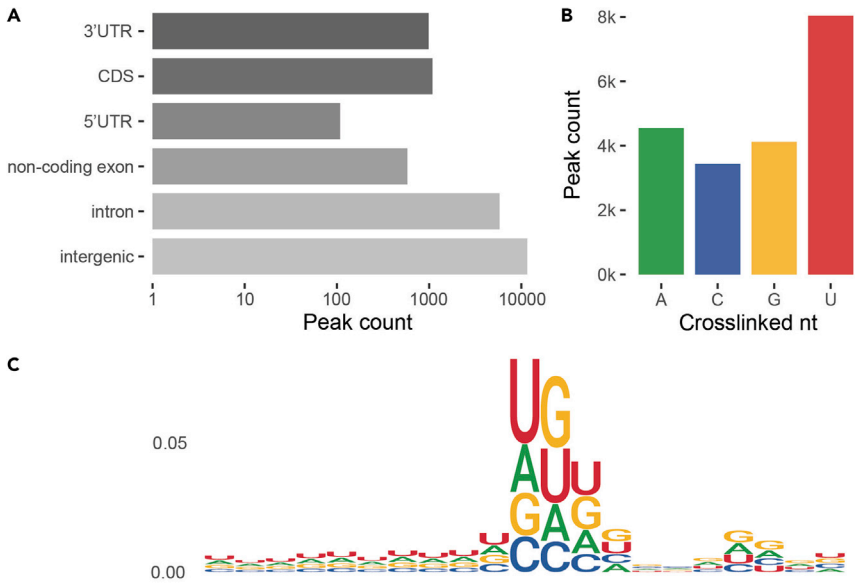


Figure 3. Characterization of transcriptome-wide protein-RNA interactions

(A) Distribution of AGO2 eCLIP-seq peaks in genome regions.

(B) Distribution of crosslinked nucleotides in AGO2 eCLIP-seq peaks.

(C) Sequence logo of AGO2 eCLIP-seq peak regions (10 nucleotide on each side from the crosslinked nucleotide).

whole testis cells (Griffin et al., 2022). For any step, alternative computational tools can be used, with additional care optionally necessary to optimize the compatibility of input/output file formats.

5. **Peak calling** with PureCLIP (Krakau et al., 2017). PureCLIP uses a Hidden Markov Model-based approach, and performs peak calling using both read coverage and crosslinking-induced termination events. Output: BED files with the locations of the predicted interaction peaks.
6. **Downstream analysis of PureCLIP peaks.** This analysis is performed within the R environment and includes the following steps:
   a. Comparison with controls (INPUT and/or IgG) and selection of CLIP specific peaks. This comparison requires to collect coverage for each peak in each CLIP and INPUT replicate, with bedtools (Quinlan and Hall, 2010) *coverage* function (alternatively, *featureCounts* or similar tools). The selection of CLIP specific peaks is performed using differential expression tools, *edgeR* (Robinson et al., 2010) in our example, by comparing CLIP *vs* INPUT (and optionally IgG) samples.
   b. Location analysis (distribution of CLIP specific peaks on transcript regions, Figure 3A).
   c. Sequence analysis (identification of consensus sequences in CLIP specific peaks, Figures 3B and 3C).
   d. Differential analysis (optional, if multiple CLIPs can be compared).
   e. Functional enrichment analysis (based on the genes with CLIP specific peaks).

Comparison of binding occupancy between different conditions in specific transcript regions

This section lists all the computational steps necessary to quantify and compare occupancy profiles of specific transcript regions, starting from aligned reads (BAM files) and an annotation listing the regions of interest. Binding profiles are based on the position and the number of crosslinking sites, calculated from R2 5′ end. The case study shown is based on a dataset where RNA binding of the splicing factor U2AF1 was quantified in HEL cells and a comparison between wildtype and mutant U2AF1 was performed, specifically looking at intron-exon junctions (Biancon et al., 2022). An implementation of these steps is available as bash and R scripts (https://github.com/TebaldiLab/eCLIP_seq). For any step, alternative computational tools can be used, with additional care optionally necessary to optimize the compatibility of input/output file formats.

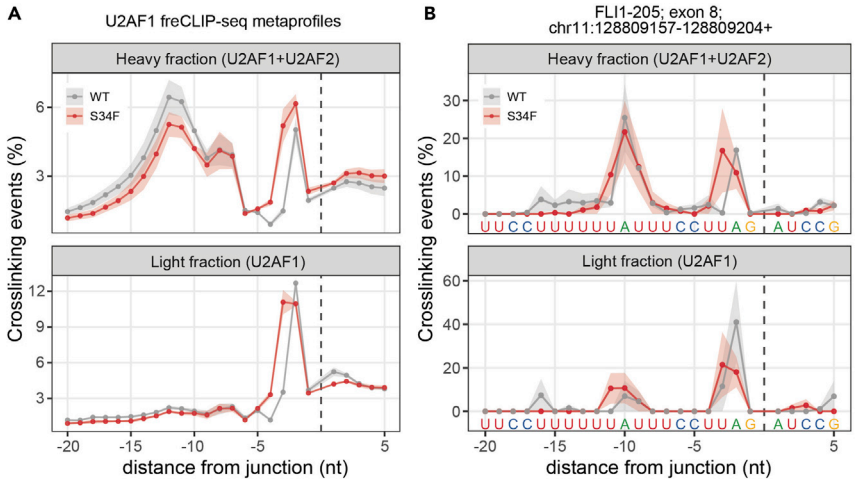


Figure 4. Comparison of freCLIP-seq occupancies between different conditions in specific transcript regions

(A) Metaprofile (mean ± SEM) of U2AF1 freCLIP-seq occupancies in all intron-exon junctions, comparing wildtype with the S34F mutant samples.

(B) Profile (mean ± SEM) of U2AF1 freCLIP-seq occupancies in a selected intron-exon junction, comparing wildtype with the S34F mutant samples.

7. **Conversion of reads into putative crosslinking sites.** This step converts a BAM file with aligned reads into a BAM file with mapped putative crosslinking sites. First the input BAM file is converted into a BED file, with *bedtools bamtobed* function. Next, the crosslinking site is calculated for each read as the upstream nucleotide with respect to the read 5′ end. Finally, the bed file with putative crosslinking sites can be converted back to a BAM file with the *bedToBam* function.
8. **Downstream analysis of crosslinking sites in specific regions.** This analysis is performed within the R environment and includes the following steps:
   a. Quantification of binding across each region of interest (in the case study, the genomic coordinates of each intron-exon junction are stored in a *GRanges* object). Coverage on each nucleotide of each region is collected from the crosslink BAM files generated in the previous step with the *bamProfile* function.
   b. Generation of metaprofiles (cumulative signal on all intron-exon junctions). Regions considered to generate the metaprofile can be selected according to a coverage filter. Normalization across different CLIP libraries can be achieved by representing the occupancy at each nucleotide as the fraction, or percentage, of signal with respect to the total signal across the regions. Metaprofile can be generated for specific subsets of junctions, i.e., considering only internal exons (Figure 4).
   c. Sequence analysis (performed on all the intron-exon junctions used to generate the metaprofile). It can be used to split metaprofiles according to sequence motifs (e.g., introns ending in CAG *vs* UAG).
   d. Differential analysis (identification of regions where binding occupancy is significantly different when comparing two conditions, e.g., wildtype *vs* mutant U2AF1).
   e. Functional enrichment analysis (based on the genes with differentially bound intron-exon junctions).

LIMITATIONS

FreCLIP-seq, as other CLIP-seq protocols, requires a significant number of cells limiting the application on primary patient-derived cells. We suggest to use at least 10×10^6 cells for standard eCLIP-seq, and $20–30 \times 10^6$ cells for freCLIP-seq.

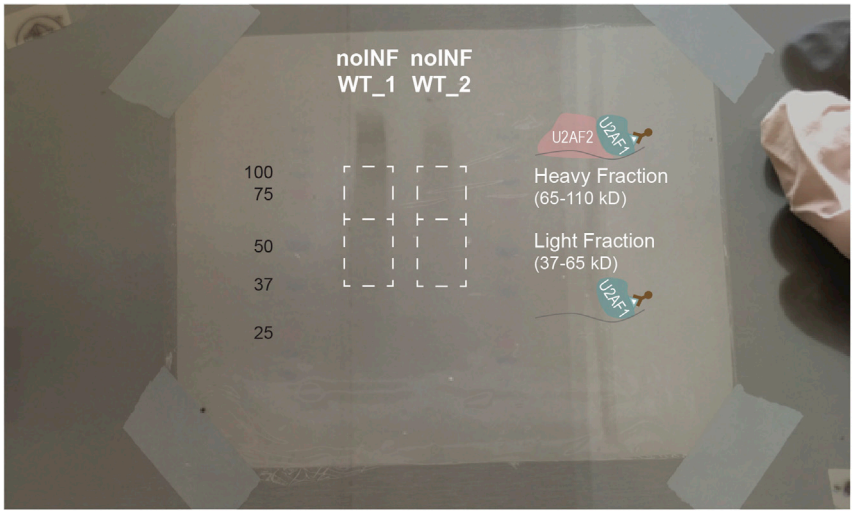


Figure 5. Issues during autoradiographic visualization

Representative U2AF1 freCLIP-seq samples obtained from not-infected HEL cells.

Please note that eCLIP-seq technologies do not directly measure RNA binding affinity. By mapping and counting the end of eCLIP-seq reads we can quantify the binding occupancy with single-nucleotide resolution.

TROUBLESHOOTING

Problem 1

Experimental samples with absent or faint signal on autoradiogram after IP (step 12). Absent signal may represent an antibody-related issue during IP. Faint signal may represent an issue during P32-labeling and visualization (e.g., P32 signal may not be proportional to the amount of RNA and film development issues may produce a dark film) (Figure 5).

Potential solution

In case of absent signal, perform western blot on saved pre-IP and post-IP samples to evaluate IP efficiency of the used antibody and adjust IP conditions. In case of faint signal, as in Figure 5, proceed with the experimental pipeline.

Problem 2

Adapter dimers (step 23). Post-AMP libraries can present adapter bands (Figure 6). Adapter dimers cause index hopping and demultiplexing issues and need to be removed.

Potential solution

We typically manage adapter troubleshooting starting from TBE-Urea gel visualization (Figure 6A). In case of a bright adapter band on the TBE-Urea gel, use the stored pre-AMP library and repeat final PCR with another adapter pair (always following pooling guidelines). In case of a visible but faint adapter band, use the stored pre-AMP library and repeat final PCR with the same adapter pair but 1 cycle less.

However, changing PCR conditions may be not sufficient to get rid of adapters, especially in low concentrated libraries usually containing high adapter levels. For libraries with over 10% adapter dimers after QC by Bioanalyzer (Figure 6B), perform gel extraction (QIAGEN QIAquick Gel Extraction Kit, Cat#28706) and repeat QC.

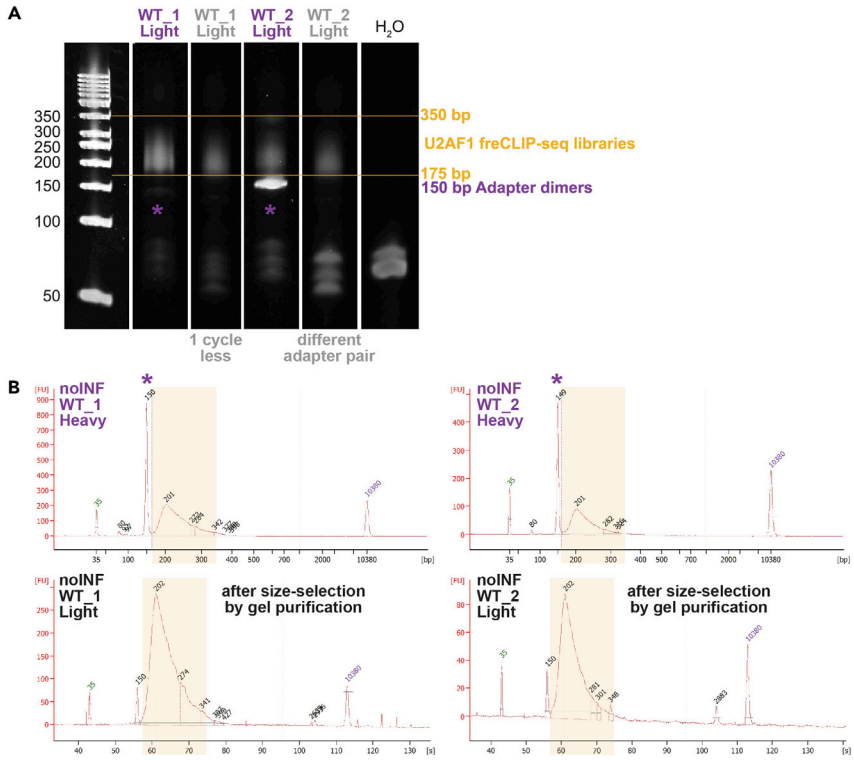


Figure 6. Presence and removal of adapter dimers

(A) Visualization by TBE-Urea gel of representative U2AF1 freCLIP-seq libraries. Yellow lines mark the expected library size. Purple stars indicate adapter dimers around 150 bp in post-AMP libraries. Gray-labeled samples are the purple-labeled samples after re-amplification changing PCR cycles or adapters.

(B) Visualization by Agilent 2100 Bioanalyzer of representative U2AF1 freCLIP-seq libraries obtained from not-infected HEL cells. Yellow boxes mark the expected library size. Peak size is reported. Purple stars indicate adapter dimers' peak around 150 bp in post-AMP libraries. Black-labeled samples are the respective purple-labeled samples after gel purification.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Stephanie Halene (stephanie.halene@yale.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets. Raw and analyzed U2AF1 freCLIP-seq sequencing data are available in GEO under the accession number GSE195620 (Biancon et al., 2022). Raw and analyzed AGO2 eCLIP-seq sequencing data are available in GEO under the accession number GSE181264 (Griffin et al., 2022). Original autoradiogram and gel images have been deposited in Mendeley Data at https://doi.org/10.17632/m7g92hkwmh.1.

Software used for the analyses are described and referenced in the quantification and statistical analysis subsections and are listed in the key resources table.

Analysis scripts in bash and R, with all the computational steps described in the quantification and statistical analysis subsections, are available on GitHub (https://github.com/TebaldiLab/eCLIP_seq https://doi.org/10.5281/zenodo.7121228).

Any additional information required to perform the experimental part or run the computational part of the here presented pipeline is available upon request from the lead contact.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101823.

ACKNOWLEDGMENTS

We thank Brooke Sullivan, Emma Sykes, Christopher Castaldi, and the Yale Center for Genome Analysis for library sequencing and troubleshooting, Benjamin Walters (Yale University) for the preparation of AGO2 lysates, and Valentina Botti, Karla Neugebauer, and Jun Lu (Yale University) for helpful suggestions. This study was funded in part by the Edward P. Evans Foundation, the Frederick A. DeLuca Foundation, the NIH/NIDDK R01DK102792, and the NIH/NIDDK R01DK124788 (to S.H.). T.T. was supported by AIRC under MFAG 2020 (ID. 24883 project). G.B. was supported by NIH/NIDDK Cooperative Centers of Excellence in Hematology U24DK126127. B.J.L. was supported by the NIH R01HD098128.

AUTHOR CONTRIBUTIONS

Conceptualization: G.B., T.T., and S.H.; methodology: G.B., P.J., and S.H.; investigation: G.B. and P.J.; formal analysis: G.B., E.B., and T.T.; resources: B.J.L. and S.H.; validation: E.B., P.J., and B.J.L.; writing: G.B., T.T., and S.H.; visualization: G.B. and T.T.; supervision: T.T. and S.H.; project administration: S.H.; funding acquisition: G.B., T.T., and S.H.

DECLARATION OF INTERESTS

S.H., consultancy, Forma Therapeutics.

REFERENCES

Biancon, G., Joshi, P., Zimmer, J.T., Hunck, T., Gao, Y., Lessard, M.D., Courchaine, E., Barentine, A.E.S., Machyna, M., Botti, V., et al. (2022). Precision analysis of mutant U2AF1 activity reveals deployment of stress granules in myeloid malignancies. Mol. Cell *82*, 1107–1122.e7.

Blue, S.M., Yee, B.A., Pratt, G.A., Mueller, J.R., Park, S.S., Shishkin, A.A., Starner, A.C., Van Nostrand, E.L., and Yeo, G.W. (2022). Transcriptome-wide identification of RNA-binding protein binding sites using seCLIP-seq. Nat. Protoc. *17*, 1223–1265.

Brugiolo, M., Botti, V., Liu, N., Müller-McNicoll, M., and Neugebauer, K.M. (2017). Fractionation iCLIP detects persistent SR protein binding to conserved, retained introns in chromatin, nucleoplasm and cytoplasm. Nucleic Acids Res. *45*, 10452–10465.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Glasser, E., Maji, D., Biancon, G., Puthenpeedikakkal, A.M.K., Cavender, C.E., Tebaldi, T., Jenkins, J.L., Mathews, D.H., Halene, S., and Kielkopf, C.L. (2022). Pre-mRNA splicing factor U2AF2 recognizes distinct conformations of nucleotide variants at the center of the pre-mRNA splice site signal. Nucleic Acids Res. *50*, 5299–5312.

Griffin, K.N., Walters, B.W., Li, H., Wang, H., Biancon, G., Tebaldi, T., Kaya, C.B., Kanyo, J., Lam, T.T., Cox, A.L., Halene, S., Chung, J.J., and Lesch, B.J. (2022). Widespread association of the Argonaute protein AGO2 with meiotic chromatin suggests a distinct nuclear function in mammalian male reproduction. Genome Res *32*, 1655–1668.

Hafner, M., Katsantoni, M., Köster, T., Marks, J., Mukherjee, J., Staiger, D., Ule, J., and Zavolan, M. (2021). CLIP and complementary methods. Nat. Rev. Methods Prim. *1*, 20.

Huppertz, I., Attig, J., D'Ambrogio, A., Easton, L.E., Sibley, C.R., Sugimoto, Y., Tajnik, M., König, J., and Ule, J. (2014). iCLIP: protein–RNA interactions at nucleotide resolution. Methods *65*, 274–287.

König, J., Zarnack, K., Rot, G., Curk, T., Kayikci, M., Zupan, B., Turner, D.J., Luscombe, N.M., and Ule, J. (2010). iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. Nat. Struct. Mol. Biol. *17*, 909–915.

Krakau, S., Richard, H., and Marsico, A. (2017). PureCLIP: capturing target-specific protein–RNA interaction footprints from single-nucleotide CLIP-seq data. Genome Biol. *18*, 240.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. *17*, 10.

Van Nostrand, E.L., Pratt, G.A., Shishkin, A.A., Gelboin-Burkhart, C., Fang, M.Y., Sundararaman, B., Blue, S.M., Nguyen, T.B., Surka, C., Elkins, K., et al. (2016). Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nat. Methods *13*, 508–514.

Van Nostrand, E.L., Nguyen, T.B., Gelboin-Burkhart, C., Wang, R., Blue, S.M., Pratt, G.A., Louie, A.L., and Yeo, G.W. (2017). Robust, cost-effective profiling of RNA binding protein targets with single-end enhanced crosslinking and immunoprecipitation (seCLIP). Methods Mol. Biol. *1648*, 177–200.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics *26*, 841–842.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140.

Rosenberg, M., Levy, V., Maier, V.K., Kesner, B., Blum, R., and Lee, J.T. (2021). Denaturing cross-linking immunoprecipitation to identify footprints for RNA-binding proteins. STAR Protoc. *2*, 100819.

Xu, H., Luo, X., Qian, J., Pang, X., Song, J., Qian, G., Chen, J., and Chen, S. (2012). FastUniq: a fast de novo duplicates removal tool for paired short reads. PLoS One *7*, e52249.

Yamaguchi, T., Hamanaka, S., Kamiya, A., Okabe, M., Kawarai, M., Wakiyama, Y., Umino, A., Hayama, T., Sato, H., Lee, Y.S., et al. (2012). Development of an all-in-one inducible lentiviral vector for gene specific analysis of reprogramming. PLoS One *7*, e41007.

Yoshida, K., Sanada, M., Shiraishi, Y., Nowak, D., Nagata, Y., Yamamoto, R., Sato, Y., Sato-Otsubo, A., Kon, A., Nagasaki, M., et al. (2011). Frequent pathway mutations of splicing machinery in myelodysplasia. Nature *478*, 64–69.