PAR clip

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PROTOCOL OUTLINE

1. Protocol Title

PAR-CLIP (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) - a step-by-step protocol to the transcriptome-wide identification of binding sites of RNA-binding proteins

2. Author Names

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4. Purpose

Post-transcriptional regulation (PTR) of messenger RNAs (mRNAs) plays important roles in diverse cellular processes (Ambros 2004; Halbeisen, Galgano et al. 2008). The fates of mRNAs are determined predominantly by their interactions with RNA-binding proteins (RBPs) and non-coding, guide-RNA-containing ribonucleoprotein complexes (RNPs). Taken together, they form mRNA-containing ribonucleoprotein complexes (mRNPs). The RBPs influence the structure and interactions of the RNAs and play critical roles in their biogenesis, stability, function, transport and cellular localization (Moore 2005; Keene 2007; Glisovic, Bachorik et al. 2008).

Given that hundreds of RBPs and RNPs and their networks remain to be studied and evaluated in a cell-type-dependent manner, the development of powerful tools to determine their binding sites or RNA recognition elements (RREs) is critical to enhance our understanding of PTR. It offers new opportunities for understanding both gene regulation and consequences of genetic variation in transcript regions aside from the open reading frame.

We recently developed a protocol for the transcriptome-wide isolation of RREs readily applicable to any protein or RNP directly contacting RNA (including RNA helicases, polymerases, or nucleases) expressed in cell culture models either naturally or ectopically (Hafner, Landthaler et al. 2010).

Briefly, immunoprecipitation of the RBP of interest is followed by isolation of the crosslinked and coimmunoprecipitated RNA. In the course of lysate preparation and immunoprecipitation, the mRNAs are partially degraded using Ribonuclease T1. The isolated crosslinked RNA fragments are converted into a cDNA library and deep-sequenced using Solexa

technology. By introducing photoreactive nucleosides that generate characteristic sequence changes upon crosslinking (see below), our protocol allows one to separate RNA segments bound by the protein of interest from the background un-crosslinked RNAs.

5. Theory

Typically, a combination of genetic, biochemical and computational approaches has been applied to identify mRNA-RBP or mRNA-RNP interactions. However, each of these methods has limitations. Microarray profiling of mRNA associated with immunopurified RBPs (RIP-ChIP) (Tenenbaum, Carson et al. 2000) is limited by incomplete enrichment of bound mRNAs and the difficulty of locating the RRE in the hundreds to thousands of nucleotide (nt) long target mRNA (Gerber, Luschnig et al. 2006; Landthaler, Gaidatzis et al. 2008).

Some of these problems were addressed by an *in vivo* UV 254 nm crosslinking and immunoprecipitation (CLIP) protocol (Ule, Jensen et al. 2003) that better defines the interaction site by isolating and sequencing small RNA segments crosslinked to RBPs. However, UV 254 nm crosslinking is not efficient, and the site of crosslinking is not revealed after sequencing of the isolated RNA fragment. To separate crosslinked sites from background noise, additional control crosslinking experiments are needed, e.g. including the use of knockout cells of the protein of interest.

To overcome these limitations, we developed a new protocol referred to as PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) (Hafner, Landthaler et al. 2010).

4-Thiouridine (4SU) and 6-thioguanosine (6SG) are readily incorporated into nascent RNAs by simply supplementing the media of cultured cells with the modified nucleoside (Favre, Moreno et al. 1986; Bezerra and Favre 1990). At the concentrations used in the presented protocol, neither of the tested photoreactive nucleosides showed any detectable toxic effects based on mRNA profiling or cell count. Irradiation of the cells by UV light of 365 nm leads to crosslinking of photoreactive nucleoside-labelled cellular RNAs to interacting RBPs. Using similar irradiation protocols, 4SU incorporation substantially enhances RNA recovery compared to UV 254 nm crosslinking, 6SG performs in between these two methods.

Most importantly, the sites of crosslinking can be easily identified by mapping characteristic T to C mutations (G to A in the case of 6SG, though less pronounced) in the sequenced cDNA libraries obtained from the recovered RNA initiated by the photocrosslinking itself. We presume that the structural change upon crosslinking of the modified nucleosides to aromatic amino acid side chains directs the incorporation of a non-cognate deoxynucleoside during reverse transcription of crosslinked RNAs. The presence of the mutations in sequence reads, together with the observation that multiple positions within a cluster of sequence reads can be altered, facilitates the separation from clusters of unaltered background sequences typically derived from abundant cellular RNAs.

For details on the bioinformatic analyses please refer to our recent publication (Hafner, Landthaler et al. 2010).

6. Equipment

Major equipment	365 nm UV-transilluminator

	Agarose gel chambers
	Balances (e.g. 0.1 mg - 64 g and 0.1 g - 4.2 kg)
	Block heater at 90°C
	CO_2 incubator for mammalian cell culture
	D-Tube Dialyzer Midi rack (EMD Biosciences, 71511-3)
	Equipment to cast and run 15 cm x 17 cm x 0.8 mm (or similar) polyacrylamide gels
	High speed floor centrifuge (capable of at least 13,000 x g)
	Magnetic rack for 1.5 ml microcentrifuge tubes and 15 ml conical tubes
	Multichannel pipet
	pH-meter
	Phosphorimager & imaging plates (or regular X-ray film and developer)
	Power supply
	Protein electrophoresis apparatus
	Radioisotope laboratory
	Refrigerated bench top microcentrifuge
	Rotating wheel
	Thermocycler
	Thermometer
	Thermomixer
	UV Stratalinker 2400 equipped with 365 nm light bulbs for crosslinking (Stratagene)
	Vortex mixer
	Water bath
	Water filter; MilliQBiocel water purification system
	X-ray exposure cassette

Consumables	1.5 ml polypropylene tubes
	1.5 ml siliconized tubes (BIO PLAS Inc., 4165SL)
	15 and 50 ml conical tubes (e.g. Falcon) as well as tubes withstanding high speed centrifugation (e.g. Sarsted, 13 ml centrifuge tube, 55.518)
	15-cm culture dishes
	5 µm Supor membrane syringe filter (Pall Acrodisc)
	Antibody (e.g. for FLAG-tagged RBPs: mouse monoclonal anti-FLAG M2 (Sigma, F1804))
	Cell scraper (Corning)
	D-Tube Dialyzer Midi, MWCO 3.5 kDa (EMD Biosciences, 71506-3)
	NuPAGE Novex 4-12 % BT Midi 1.0 gel (Invitrogen)
	pH paper (covering the range between pH 6.5 - 10)
	Plastic wrap

	QIAquick gel purification kit (Qiagen)
	Scalpels or razor blades
	Strips of 0.2 ml tubes (Thermo Scientific, AB-0264)
	Syringes (10 ml)

7. Materials

Reagents & Chemicals	2-Mercaptoethanol (14.3 M; Sigma, M6250)
	Agarose, electrophoresis grade (SeaKem LE Agarose, Lonza, 50004)
	Agarose, low melting (NuSieve GTG Agarose, Lonza, 50080)
	Appropriate cell culture medium and selection antibiotics
	APS (Ammonium persulfate)
	ATP (Adenosine triphosphate)
	Bromophenol blue
	BSA, acetylated (Ambion, AM2614)
	$CaCl_2 \cdot 2H_2O$
	Calf intestinal alkaline phosphatase (CIP)
	Chloroform
	Citric acid monohydrate
	Complete EDTA-free protease inhibitor cocktail (Roche)
	DMSO
	DNA ladder (25 bp)
	dNTPs: dATP, dCTP, dGTP, dTTP (0.1 M each; Fermentas, R0182)
	DTT
	Dynabeads Protein G (Invitrogen, 100-03D)
	EDTA disodium salt dihydrate (Sigma, E5134)
	EGTA, $C_{14}H_{20}N_2O_{10}Na_4$ (Sigma, E8145-10G)
	Ethanol (100 %)
	Ethidium bromide
	Ficoll type 400
	Formamide
	gamma-^{32}P-ATP, 10 mCi/ml, 6000Ci(222TBq)/mmol (Perkin Elmer, NEG002Z500UC)
	Glycerol
	Glycoblue or glycogen
	HCl, concentrated (Fisher Scientific, A144S)
	HEPES
	Isoamyl alcohol
	Isopropyl alcohol
	KCl
	KOH

	$MgCl_2 \cdot 6H_2O$
	MOPS SDS running buffer (20x; Invitrogen)
	$Na_2HPO_4 \cdot 7\ H_2O$ (Sigma, S9390-100G)
	NaCl
	NaF
	NaOH
	NP40 substitute (100 %; Sigma [74385])
	PBS (10x, commercially available)
	Phenol (saturated with 0.1 M citrate buffer, pH 4.3 ± 0.2, Sigma, P4682)
	Photoreactive nucleoside (Sigma; 4-thiouridine [T4509] / 6-thioguanosine [858412])
	Protein ladder (e.g. Biorad, 161-0374; 10 - 250 kDa)
	Proteinase K (lyophilizate; Roche, 03115801001)
	RNase T1 (Fermentas, EN0541); concentration 1,000 U/µl
	SDS (Fisher Scientific, BP166-500)
	SuperScript III Reverse Transcriptase (Invitrogen, 18080-044); includes 5x first-strand buffer
	T4 PNK (T4 Polynucleotide Kinase, NEB, M0201)
	T4 RNA Ligase 1 (NEB, M0204L)
	T4 RNA Ligase 2, truncated (e.g. NEB, M0242L); or: Rnl2(1-249)K227Q (our plasmid for expression of the his-tagged mutant is available at www.addgene.com, plasmid 14072; however, the purified enzyme will shortly also be available from NEB)
	Taq DNA polymerase (5 U/µl)
	TBE (Tris-Borate - EDTA buffer solution)
	TEMED (Tetramethylethylenediamine)
	Tris base (Fisher Scientific, BP152-1)
	Tris-HCl (Promega, PR-H5121)
	Triton X-100
	UreaGel - SequaGel - System, National Diagnostics, EC-833

RNA & DNA oligonucleotides	3’ adapter (DNA except for the 5' riboadenylate (rApp) residue): 5’ rAppTCGTATGCCGTCTTCTGCTTGT
	5’ adapter (RNA): 5’ GUUCAGAGUUCUACAGUCCGACGAUC
	3’ PCR primer (DNA): 5’ CAAGCAGAAGACGGCATACGA
	5’ PCR primer (DNA): 5’ AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA
	19-nt size marker (RNA):

	5’ CGUACGCGGGUUUAAACGA
	24-nt size marker (RNA): 5’ CGUACGCGGAAUAGUUUAAACUGU
	33-nt size marker (RNA): 5’ CAUCUUGGUCGUACGCGGAAUAGUUUAAACUGU
	35-nt size marker (RNA): 5’CUCAUCUUGGUCGUACGCGGAAUAGUUUAAACUGU

Solutions & buffers

<u>Step 1</u>

4-Thiouridine stock solution

Dissolve 250 mg 4-thiouridine in 960.5 µl DMSO.
(For a 1 M 6-thioguanosine solution first dehydrate the powder supplied by Sigma in a vacuum oven at room temperature overnight. Then dissolve 299.3 mg in 1 ml DMSO).

4-Thiouridine-containing growth medium

Component	**Stock**	**Final conc.**	**Amount/liter medium**
4-thiouridine (in DMSO)	1 M	100 µM	100 µl

1x PBS

Component	**Stock**	**Final conc.**	**Amount/liter**
PBS	10 x	1 x	100 ml
H_2O to 1 liter	n/a	n/a	900 ml

<u>Step 2</u>

NP40 lysis buffer

Component	**Stock**	**Final conc.**	**Amount/liter**
HEPES-KOH, pH 7.5	1 M	50 mM	50 ml
KCl	1 M	150 mM	150 ml
EDTA-NaOH, pH 8.0	0.5 M	2 mM	4 ml
NaF	0.5 M	1 mM	2 ml
NP40 substitute	100 %	0.5 % (v/v)	5 ml
H_2O to 1 liter	n/a	n/a	788.5 ml
DTT (fresh)	1 M	0.5 mM	0.5 ml
Complete EDTA-free protease inhibitor cocktail (fresh)	n/a	n/a	1 tablet / 50 ml

<u>Step 3</u>

Citrate-phosphate buffer pH 5.0

Component	Stock	Final conc.	Amount/liter
Citric acid monohydrate	n/a	n/a	4.7 g/l
$Na_2HPO_4 \cdot 7\ H_2O$	n/a	n/a	9.2 g/l
H_2O to 1 liter	n/a	n/a	

Step 4

IP-wash buffer

Component	Stock	Final conc.	Amount/liter
HEPES-KOH, pH 7.5	1 M	50 mM	50 ml
KCl	1 M	300 mM	300 ml
NP40 substitute	100 %	0.05 % (v/v)	0.5 ml
H_2O to 1 liter	n/a	n/a	649 ml
DTT (fresh)	1 M	0.5 mM	0.5 ml
Complete EDTA-free protease inhibitor cocktail (fresh)	n/a	n/a	1 tablet / 50 ml

High-salt wash buffer

Component	Stock	Final conc.	Amount/liter
HEPES-KOH, pH 7.5	1 M	50 mM	50 ml
KCl	1 M	500 mM	500 ml
NP40 substitute	100 %	0.05 % (v/v)	0.5 ml
H_2O to 1 liter	n/a	n/a	449 ml
DTT (fresh)	1 M	0.5 mM	0.5 ml
Complete EDTA-free protease inhibitor cocktail (fresh)	n/a	n/a	1 tablet / 50 ml

Dephosphorylation buffer (10x)

Component	Stock	Final conc.	Amount/liter
Tris-HCl, pH 7.9	1 M	50 mM	50 ml
NaCl	3 M	100 mM	33.3 ml
$MgCl_2 \cdot 6H_2O$	1 M	10 mM	10 ml
H_2O to 10 ml	n/a	n/a	906.2 ml
DTT (fresh)	1 M	1 mM	0.5 ml

Step 5

Phosphatase wash buffer

Component	Stock	Final conc.	Amount/liter
Tris-HCl, pH 7.5	1 M	50 mM	50 ml
EGTA-NaOH, pH 7.5	0.5 M	20 mM	40 ml
NP40 substitute	100 %	0.5 % (v/v)	5 ml

H_2O to 1 liter	n/a	n/a	905 ml

Polynucleotide kinase (PNK) buffer without DTT

Component	Stock	Final conc.	Amount/liter
Tris-HCl, pH 7.5	1 M	50 mM	50 ml
NaCl	3 M	50 mM	16.7 ml
$MgCl_2 \cdot 6H_2O$	1 M	10 mM	10 ml
H_2O to 1 liter	n/a	n/a	923.3 ml

PNK buffer with DTT

Component	Stock	Final conc.	Amount/liter
Tris-HCl, pH 7.5	1 M	50 mM	50 ml
NaCl	3 M	50 mM	16.7 ml
$MgCl_2 \cdot 6H_2O$	1 M	10 mM	10 ml
H_2O to 1 liter	n/a	n/a	918.3 ml
DTT (fresh)	1 M	5 mM	5 ml

1x SDS PAGE loading buffer

Component	Stock	Final conc.	Amount/10 ml
Tris-HCl, pH 6.8	1 M	50 mM	0.5 ml
EDTA-NaOH, pH 8.0	0.5 M	2 mM	0.04 ml
Glycerol	50 %	10 % (v/v)	2 ml
SDS	20 %	2 % (v/v)	1 ml
DTT	1 M	100 mM	1 ml
Bromophenol blue	n/a	0.1 % (w/v)	10 mg
H_2O to 10 ml	n/a	n/a	

Step 6

1x MOPS running buffer

Dilute 1:20 from commercially available 20x buffer (Invitrogen).

Step 7

Proteinase K storage buffer

Component	Stock	Final conc.	Amount/10 ml
Proteinase K powder	n/a	20 mg / ml	200 mg
Tris-HCl, pH 8	1 M	50 mM	0.5 ml
$CaCl_2 \cdot 2H_2O$	1 M	30 mM	30 µl
Glycerol	100 %	50 %	5 ml
H_2O to 5 ml	n/a	n/a	

2x Proteinase K buffer

Component	Stock	Final conc.	Amount/10 ml
Tris-HCl, pH 7.5	1 M	100 mM	1 ml

EDTA-NaOH, pH 8.0	0.5 M	12.5 mM	0.25 ml
NaCl	3 M	150 mM	0.5 ml
SDS	20 %	2 % (v/v)	1 ml
H_2O to 10 ml	n/a	n/a	7.25f ml

Acidic Phenol/Chloroform/IAA (25:24:1)
Combine 25 ml acidic phenol, 24 ml chloroform and 1 ml isoamyl alcohol (overlay with with 0.1 M citrate buffer, pH 4.3 ± 0.2 which you can take from the acidic phenol bottle).

Step 8

50% DMSO
Mix 1 ml DMSO with 1 ml H_2O.

10x RNA ligase buffer without ATP

Component	**Stock**	**Final conc.**	**Amount/10 ml**
Tris-HCl, pH 7.6	1 M	0.5 M	5 ml
$MgCl_2 \cdot 6H_2O$	1 M	0.1 M	1 ml
2-Mercaptoethanol	14.3 M	0.1 M	0.07 ml
Acetylated BSA	20 mg/ml	1 mg/ml	0.5 ml
H_2O to 10 ml	n/a	n/a	3.43 ml

2x Formamide loading dye

Component	**Stock**	**Final conc.**	**Amount/10 ml**
EDTA-NaOH, pH 8.0	0.5 M	50 mM	2 ml
Bromophenol blue	n/a	0.05 % (w/v)	5 mg
Add formamide to 10 ml	n/a	n/a	

10x TBE

Component	**Stock**	**Final conc.**	**Amount/liter**
Tris base	n/a	445 mM	53.9 g
Boric acid	n/a	445 mM	27.5 g
EDTA-NaOH, pH 8.0	0.5 M	10 mM	20 ml
H_2O to 1 l	n/a	n/a	

0.4 M NaCl

Component	**Stock**	**Final conc.**	**Amount/500 ml**
NaCl	3 M	0.4 M	66.7 ml
H_2O to 500 ml	n/a	n/a	433.3 ml

Step 9

10x RNA ligase buffer with ATP

Component	Stock	Final conc.	Amount/10 ml
Tris-HCl, pH 7.6	1 M	0.5 M	5 ml
$MgCl_2 \cdot 6H_2O$	1 M	0.1 M	1 ml
2-Mercaptoethanol	14.3 M	0.1 M	0.07 ml
Acetylated BSA	20 mg/ml	1 mg/ml	0.5 ml
ATP	100 mM	2 mM	0.2 ml
H_2O to 10 ml	n/a	n/a	3.23 ml

<u>Step 10</u>

10x dNTP solution

Component	Stock	Final conc.	Amount/10 ml
dATP	0.1 M	2 mM	0.2 ml
dCTP	0.1 M	2 mM	0.2 ml
dGTP	0.1 M	2 mM	0.2 ml
dTTP	0.1 M	2 mM	0.2 ml
H_2O to 10 ml	n/a	n/a	9.2 ml

150 mM KOH/20 mM Tris base

Component	Stock	Final conc.	Amount/1 ml
KOH	5 M	150 mM	30 µl
Tris base	1 M	20 mM	20 µl
H_2O	n/a	n/a	950 µl

150 mM HCl

Component	Stock	Final conc.	Amount/1 ml
HCl, concentrated	12.1 M	150 mM	12.4 µl
H_2O to 1 ml	n/a	n/a	987.6 µl

<u>Step 11</u>

10x PCR buffer

Component	Stock	Final conc.	Amount/10 ml
Tris-HCl, pH 8.0	1 M	100 mM	1 ml
KCl	1 M	500 mM	5 ml
2-Mercaptoethanol	14.3 M	10 mM	7 µl
Triton X-100	100 %	1 % (v/v)	0.1 ml
$MgCl_2 \cdot 6H_2O$	1 M	20 mM	2 ml
H_2O to 10 ml	n/a	n/a	1.9 ml

DNA loading dye (5x)

Component	Stock	Final conc.	Amount/10 ml
EDTA-NaOH, pH 8.0	0.5 M	50 mM	1 ml

Bromophenol blue	n/a	0.2 % (w/v)	20 mg
Ficoll type 400	n/a	20 % (w/v)	2 g
H_2O to 10 ml	n/a	n/a	

8. Protocol

Duration

Preparation	Expanding cell line(s)	Approximately two weeks depending on the desired number of cells
	Antibody testing	Variable
	Buffers etc.	1 day
	Radiolabelling of RNA size markers	1.5 days
Protocol	Total	7 days
	Day 1	3 - 4 h
	Day 2	10 - 12 h
	Day 3	5 - 6 h
	Day 4	3 - 4 h
	Day 5	5 - 6 h
	Day 6	6 - 7 h
	Day 7	4 - 5 h

Preparation Expand cells in appropriate growth medium containing selection antibiotics as appropriate to maintain your stable cell line. We usually prepare lysates from 3 - 5 ml wet cell pellet from crosslinked cells per experiment, which corresponds to 20 - 50 15-cm cell culture plates (for HEK293). However, if material is limiting, we have performed successful PAR-CLIPs experiments from less than 0.5 ml wet cell pellet (200 x 10^6 HEK293 cells (10 15-cm plates) will yield approximately 1 ml of wet cell pellet).

Grow cells to approximately 80% confluence. 14 h before crosslinking add 4SU to a final concentration of 100 µM directly to the cell culture medium. 6SG (100 µM) can also be used as the photoactivatable ribonucleoside. Induce expression of protein, if necessary.

Tip *If you want to add 4SU to e.g. 50 15-cm cell culture plates containing 20 ml of growth medium each, prepare 5 x 53 ml (taking the pipetting error into account) of growth medium into a sterile, empty bottle (e.g. an empty medium bottle from your last expansion to 50 plates). Add 132.5µl 1 M 4SU and mix. Additional reagents such as doxycycline (e.g. 1 µg/ml) to induce protein expression may be added. Aliquot 5 ml of prepared growth medium containing 4SU per 15-cm plate.*

Step 0 Buffer preparations, antibody testing and radiolabelling of RNA size markers

Buffers Buffer recipes and required reagents are listed above. Allow approximately one day for general preparations including buffer preparation. All pH measurements and adjustments are performed at room temperature. Buffers and all perishable reagents should be refrigerated for storage. We use water purified by a Millipore water purification system.

On the day before you start the PAR-CLIP procedure fill the required amounts of the individual buffers into e.g. 50 ml conical tubes and refrigerate them. The table below gives a rough guide to the required amounts of the individual buffers (but only of those that will be used in quantities above 1 ml on the first two days). Add DTT and protease inhibitors on the day of the experiment.

Buffer	**Amount per sample**
PBS	About 1 l for 20 - 30 15-cm cell culture plates
Citrate-phosphate buffer	5 ml
NP40 lysis buffer	3 ml per ml cell pellet volume
IP wash buffer	3 ml + 1/10 cell pellet volume
High-salt wash buffer	3 ml
Phosphatase wash buffer	2 ml
PNK buffer without DTT	7 ml

Antibodies This protocol was originally developed using anti-FLAG antibodies; use of a different antibody will likely require the optimization of the appropriate IP and wash conditions prior to starting a large scale experiment. Please ensure that the optimal salt concentration for antibody binding is maintained throughout the protocol; washes of the immunoprecipitate with high salt may disrupt antibody/antigen interactions.

If in doubt, you can use NP40 lysis buffer instead of the IP and the high-salt wash buffers, however, removal of non-specifically interacting RNAs might be less efficient.

The table below shows the protocol modifications for an anti-AGO2 antibody (Millipore, 04-642) that we have also used successfully:

Step	**Anti-FLAG antibody**	**Anti-AGO2 antibody**
Wash after IP	3x IP wash buffer	3x NP40 lysis buffer
KCl concentration	*300 mM*	*150 mM*
High-salt wash	3x high-salt wash buffer	3x NP40 lysis buffer
KCl concentration	*500 mM*	*150 mM*

Except for these two buffers, all the other washing steps are performed as described.

Radiolabelling On day 3 you will need 5'-^{32}P-radiolabelled RNA size markers. It is advisable to prepare them before starting the PAR-CLIP experiment. Perform a standard radiolabelling procedure with T4 PNK and gamma-^{32}P-ATP according to the manufacturer's guidelines and gel purify the markers (e.g. phosphorylate 1 µM RNA size marker in a 10 µl reaction volume using 1 µl of conventional gamma-^{32}P-ATP). Keep radioactive gel pieces from the running front of this gel as markers to implant into gels for alignment of phosphorimager printouts to exposed gels later on.

!! Consult your institute's Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.

Step 1 UV-Crosslinking of 4-thiouridine-labelled cells (Day 1)

Overview In this first step the RNA-binding protein of interest is crosslinked to its bound mRNAs targets which incorporated the photoactivatable ribonucleoside into nascent transcripts during the labelling step. The cells are then collected and the resulting cell pellet will be used as the input for the following PAR-CLIP procedure.

Duration *for 50 15-cm cell culture plates about 2 - 3 h, for cells grown in suspension culture about 1 h depending on the culture volume.*

For adherent cells:

1.1 Decant growth medium.
1.2 Wash cells once with 5 ml ice-cold PBS per plate and remove PBS completely by decanting and inverting the cell culture dish.
1.3 Place plates on a tray filled with ice to keep cells cold and irradiate uncovered with 0.15 J/cm^2 total energy of 365 nm UV light in a Stratalinker 2400 or similar device.
1.4 Add 3 ml PBS per plate and dislodge cells with a cell scraper. Transfer to pre-chilled 50 ml centrifugation tubes on ice. After the cells from the last have plate been collected, centrifuge at 500 x g for 5 min at 4ºC and discard the supernatant. Expect to obtain about 5 ml of wet cell pellet from 50 15-cm plates.
1.5 (**Optional: Pause point**) Unless you want to continue directly with cell lysis, snap freeze the cell pellet in liquid nitrogen and store at -80ºC. Cell pellets can be stored for at least 12 months.

Tip *Keep cell suspensions on ice until centrifugation.*

For cells grown in suspension culture:

1.1 Collect cells by centrifugation at 500 x g for 5 min at 4ºC.

1.2 Wash cells by resuspending in 20 ml ice-cold PBS and spin again at 500 x g for 5 min at 4ºC.
1.3 Resuspend cells in 20 ml ice-cold PBS and transfer into one 15-cm cell culture plate.
1.4 Place plate on a tray with ice and irradiate uncovered with 0.2 J/cm^2 of 365 nm UV light in a Stratalinker 2400 or similar device.
1.5 Transfer cells into a 50 ml centrifugation tube and collect by centrifugation at 500 x g for 5 min at 4ºC and discard the supernatant.
1.6 (**Optional: Pause point**) Unless you want to continue directly with cell lysis, snap freeze the cell pellet in liquid nitrogen and store at -80ºC. Cell pellets can be stored for at least 12 months.

Step 2 Preparation of cell lysate for immunoprecipitation (Day 2)

Overview The cell pellet obtained on day 1 will be lysed in preparation for immunoprecipitation. Partial RNase T1 digestion of mRNAs facilitates the recovery of crosslinked mRNPs.

Duration *2 h*

2.1 Thaw the crosslinked cell pellet on ice. Prepare the magnetic beads (see step 3) while the pellet thaws. Then resuspend the cell pellet in 3 cell pellet volumes of NP40 lysis buffer and incubate on ice for 10 min.
2.2 Clear the cell lysate by centrifugation at 13,000 x g for 15 min at 4ºC.
2.3 Clear the lysate further by filtering it through a 5 µm membrane syringe filter. Attach the syringe filter to a 20 ml syringe, remove the plunger and transfer the supernatant into the syringe. Be careful to hold the syringe above the 50 ml conical tube since the lysate will start to drip through the filter by gravitation only at first. Then insert the plunger and gently apply pressure until all of the lysate is filtered. Depending on the initial viscosity of the lysate, it might be necessary to exchange a clogged filter for a fresh one.
2.4 Add RNase T1 to a final concentration of 1 U/µl and incubate in a water bath for 15 min at 22ºC. Mix by inversion from time to time. Cool reaction for 5 min on ice before proceeding.
2.5 Remove a 10 µl aliquot for immunoblotting as a control for the protein levels used as input and freeze at -20°C.

Tip *Take the cell pellet out of the -80°C freezer and put it on ice first thing in the morning since the thawing process takes a long time.*

Tip *Both incubation temperature and duration are critical at this step for a controlled partial RNase T1 digestion.*

Tip *Designate a set of pipetmans for working with RNases to avoid contamination at later RNA isolation and cDNA library preparation steps.*

Step 3 Preparation of the magnetic beads (Day 2)

Overview The antibody is conjugated to protein G magnetic beads to be used in the subsequent immunoprecipitation. Protein G is the optimal Ig-binding protein for anti-FLAG antibodies based on species and isotype. The choice of protein A vs. protein G should be considered depending on the antibody used.

Duration *1.5 h*

3.1 Transfer 10 µl of Protein G magnetic particles per ml cell lysate (typically approx. 100 - 150 µl of beads) to a 1.5 ml microtube. Put the magnetic rack on ice. Wash the beads twice with 1 ml of citrate-phosphate buffer.
3.2 Resuspend the beads in twice the volume of citrate-phosphate buffer relative to the original volume of bead suspension (i.e. 200 - 300 µl).
3.3 Add antibody to a final concentration of 0.25 µg/µl and inc ubate on a rotating wheel for 40 min at room temperature.
3.4 Collect the beads and wash twice in 1 ml of citrate-phosphate buffer to remove unbound antibody.
3.5 Resuspend beads in twice the volume of citrate-phosphate buffer relative to the original volume of bead suspension.

Tip *This step is performed while the cell pellet is thawing.*

Tip *Be careful to not let the magnetic beads dry out.*

Step 4 Immunoprecipitation and second RNase T1 treatment (Day 2)

Overview The mRNA-RBP complex of choice is isolated from the lysate by immunoprecipitation. A second RNase T1 digestion ensures that only the RNA-segment bound, crosslinked and protected by the RBP is recovered and sequenced. This enables the precise definition of the binding sites.

Duration *2 h*

4.1 Add 20 µl of freshly prepared antibody-conjugated magnetic beads per ml of partially RNase T1 treated cell lysate from step 2 and incubate in a 15 ml centrifugation tube on a rotating wheel for 1 h at 4ºC.
4.2 Collect magnetic beads on a magnetic particle collector for 15 ml centrifugation tubes (Invitrogen) and remove the supernatant. Keep an aliquot for immunoblotting.
4.3 Add 1 ml of IP wash buffer and transfer to 1.5 ml polypropylene tubes.
4.4 Wash beads 2 times in 1 ml of IP wash buffer.
4.5 Resuspend beads in 1x original bead volume IP wash buffer.

4.6 Add RNase T1 (Fermentas, 10,000 U/µl) to a final concentration of 100 U/µl and incubate the bead suspension in a water bath for 15 min at 22 ºC. Cool on ice for 5 min.
4.7 Wash beads 3 times with 1 ml high-salt wash buffer.
4.8 Resuspend beads in 1 volume of dephosphorylation buffer.

Tip *Both RNase T1 incubation temperature and time are crucial to avoid overdigestion of RNA that could result in RNA segments too short to be mapped uniquely to transcript or genomic sequences.*

Step 5 Dephosphorylation and radiolabelling of RNA segments crosslinked to immunoprecipitated proteins (Day 2)

Overview The RNAs crosslinked by the RBP of interest are radiolabelled using T4 PNK and gamma-^{32}P-ATP in order to visualize them by autoradiography after fractionation by SDS-PAGE (next step).

Duration *2 h*

5.1 Add calf intestinal alkaline phosphatase (CIP from NEB) to a final concentration of 0.5 U/µl, and incubate the suspension for 10 min at 37ºC and mixing at 800 rpm.
5.2 Wash beads twice in 1 ml of phosphatase wash buffer.
5.3 Wash beads twice in polynucleotide kinase (PNK) buffer without DTT.
5.4 Resuspend beads in one original bead volume of PNK buffer containing DTT.
5.5 Add gamma-^{32}P-ATP to a final concentration of 0.1 µCi/µl and T4 PNK (NEB) to 1 U/µl to the bead suspension. Incubate the suspension for 30 min at 37ºC and 800 rpm, mixing manually every 5 - 10 min.
5.6 Add 100 µM non-radioactive ATP and incubate for another 5 min at 37ºC. This ensures that all RNAs are fully 5' phosphorylated which is required for the 5' adapter ligation (step 9)
5.7 Wash the magnetic beads 5 times with 800 µl of PNK buffer without DTT; dispose of the radioactive buffer according to local guidelines.
5.8 Resuspend the beads in 65 µl of 1x SDS-PAGE loading buffer and incubate for 5 min in a heat block at 90ºC to denature and release the immunoprecipitated RBP with the crosslinked radiolabelled RNAs from the beads. Vortex.
5.9 Remove the magnetic beads on the separator and transfer the supernatant to a clean 1.5 ml microtube. (**Pause point**: you can freeze the supernatant and continue with the protocol at another time).

!! *Consult your institute's Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.*

Tip *Remove the gamma-^{32}P-ATP from the freezer and place it at room temperature during the dephosphorylation incubation time so that it is thawed by the time you need it.*

Step 6 SDS-PAGE and electroelution of crosslinked RNA-protein complexes from gel slices (Days 2 and 3)

Overview Size fractionation of the radiolabelled and crosslinked RNA protein complexes is achieved by SDS-PAGE. The band corresponding to the expected mass of the protein will be excised and the crosslinked RNA protein complexes electroeluted. This step ensures that only the band corresponding to the correct RBP is isolated and additionally prevents any unbound but labelled RNA from further processing. *See figure 1A.*

Duration *4.5 h*

6.1 Load 2 x 30 µl of the supernatant into two adjacent wells on a Novex Bis-Tris 4 - 12% (Invitrogen) precast SDS-PAGE gel and leave at least one lane empty between different samples / different proteins of interest. On both sides of the gel load a protein ladder. Keep the remaining 5 µl of the bead eluate for immunoblotting.

6.2 Run the gel in 1x MOPS SDS running buffer for 45 - 60 min at 200 V until the loading dye has reached the bottom of the gel.

6.3 Disassemble the gel chamber (the buffer will be radioactive!) and gently dismantle the gel, leaving it mounted on one plate. Cut the protruding bottom of the gel so that the gel lies flat on the phosphorimager screen.

6.4 To facilitate the alignment of the gel to the phosphorimager paper printout later on, place three tiny radioactive gel pieces (which you collected earlier when radiolabelling the size markers) asymmetrically into three of the four corners of the gel. Radioactive gel pieces could be collected earlier from the bottom of the gel from radiolabelling the size markers (see above).

6.5 After placing the gel pieces wrap the gel in plastic wrap and expose the gel to a blanked phosphorimager screen for 15 min. Visualize it on a phosphorimager. Have a second blanked screen ready and expose it during the scanning process should the first exposure indicate that a longer exposure is necessary.

6.6 Print the scanned image file at its original size (100%). Align the transparently wrapped gel on top of the printout guided by the implanted gel pieces for precise positioning. Cut out the bands that correspond to the expected size of the RBP (see figure 1A).

6.7 Add 800 µl of H_2O to a D-Tube Dialyzer Midi Tube used for electroelution and let stand at room temperature for 5 min. Remove the water. Take care not to pierce the membrane.

6.8 Transfer the excised bands to the dialyzer tube and add 800 µl 1x MOPS SDS running buffer.

6.9 Place the tubes into the electroelution rack in a standard and sufficiently-sized agarose gel chamber, such that the membrane is exposed to the flow of the current (for details see manufacturer’s instructions). Fill the chamber with 1 x MOPS SDS running buffer until the tubes are covered by buffer.

6.10 Electroelute the crosslinked RNA-RBP complex at 100 V for 1.5 h. Reverse the current for 2 min to release any protein attached on the dialysis membrane.

6.11 Transfer the solution to two siliconized tubes so that each contains around 350 µl (you will not be able to fully recover the original 800 µl). (**Pause point**: freeze the solution at -20°C and continue the next day).

!! *Consult your institute's Radiation Safety Officer for proper ordering, handling, and disposal of radioactive materials.*

Tip *To confirm that the correct band was excised from the gel run another small scale SDS page gel with 1 or 2 µl of the remaining 5 µl of your sample (see above). After transferring the SDS gel to a nitrocellulose membrane, first take another autoradiography exposure (after exposing for 1 - 2 h) and then use protein-specific antibodies to perform a standard immunoblot. After overlaying the resulting images you should be able to establish which band corresponds to your protein of interest and proceed with the protocol.*

Tip *In case you observe more than one band you can also cut all which correspond to other co-purifying RNA cross-linking proteins.*

Tip *Make sure that the membrane of the dialyzer tube is aligned correctly to allow flow of current.*

Tip *Use aerososol barrier tips and take general precautions to avoid any RNase contaminations since you will be working with RNA from now on until the reverse transcription on day 6. Clean your pipettes prior to starting to work with RNA.*

Tip *Use siliconized tubes until you have obtained your cDNA library; at low concentrations, nucleic acids have a tendency to stick to the tube walls.*

Step 7 Proteinase K digestion (Day 3)

Overview In this step the recovered RBP is proteolyzed and the crosslinked RNA is recovered so that it can serve as the input material for subsequent adapter ligations and Solexa sequencing.

Duration *3.5 h*

7.1 Add one volume of 2x Proteinase K Buffer, followed by the addition of Proteinase K (Roche) to a final concentration of 1.2 mg/ml. Incubate for 30 min at 55ºC. If the volume per tube exceeds 800 µl at this stage split the sample once more into two tubes per original tube.

7.2 Add one volume of acidic phenol/chloroform/isoamyl alcohol, vortex and spin at 20,000 g for 10 min at 4°C. Recover the upper aqueous phase without disturbing the interphase and pipet into two siliconized tubes.

7.3 Add an equal volume of chloroform, vortex and spin at 20,000 x g for 10 min at 4°C. Again recover the aqueous phase without disturbing the interphase.

7.4 Add 1/10 volume 3 M NaCl, 1 µl of glycogen (10 mg/ml stock) and 3 volumes of 100% ethanol.

7.5 Precipitate the RNA for 1 h on ice and spin at 20,000 g for 15 min at 4°C. (**Pause point**: precipitate the RNA overnight at -20°C).

7.6 Take off the supernatant, air dry the pellets and dissolve in a total of 10 µl of H_2O.

Tip *Monitor the radioactivity of the supernatant and the pellet to assess efficiency of the ethanol precipitation.*

Tip *Repeat the phenol/chloroform/IAA extraction until there is no precipitate visible in the interphase (usually once is sufficient but two or more times might be needed).*

Step 8 3' adapter ligation for cDNA library preparation (Day 3 overnight, day 4, beginning of day 5)

Overview The recovered 5'-^{32}P-phosphorylated RNA is now carried through a standard cDNA library preparation protocol which was originally described for the cloning of small regulatory RNA (Hafner, Landgraf et al. 2008). As a first step, a preadenylated 3' adapter is ligated by T4 Rnl2(1-249)K227Q. *See figure 1B.*

Duration *Day 3: 30 min (+ overnight incubation), Day 4: 4 - 5 h (but highly dependent on required exposure time), Day 5: 2 h*

8.1 Prepare the following reaction mixture for ligating the 3' adapter, multiplying the volumes by the number of ligation reactions (plus one for the size markers, see below) to be performed plus another extra volume to account for pipetting error:
2 µl of 10x RNA ligase buffer (without ATP),
6 µl 50% DMSO
1 µl of 100 µM preadenylated 3' adapter.
Add 9 µl of the reaction mixture to each sample (so that you have 19 µl per tube now).

8.2 Prepare ~40 fmol of a 1:100 dilution of 5'-^{32}P-labelled RNA size markers (19-nt and 24-nt size marker at equimolar concentrations, see above). This controls for successful ligation and indicates the length of the bands that will later on be cut out from the gel.

8.3 Denature the RNA to disrupt secondary structures by incubating for 30 s at 90°C. Place the tubes on ice immediately for 30 s.

8.4 Add 1 µl of Rnl2(1-249)K227Q ligase (1 µg/µl) to the ligation reactions, mix gently and incubate overnight on ice in the cold room or in an insulated ice bucket covered with a lid.

8.5 The next morning, cast a 15% denaturing 8 M urea polyacrylamide gel (we use the UreaGel system from National Diagnostics) and wait until the polymerization process is complete. Our gels measure 15 cm x 17 cm x 0.8 mm and contain about 25 ml gel volume with a 20 well comb.

8.6 Pre-run the gel for 30 min at 30 W using 1x TBE buffer. After the pre-run, flush the wells with 1x TBE.

8.7 Add 20 µl of formamide gel loading solution to the samples to stop the ligation reactions.

8.8 Denature the RNA for 30 s at 90ºC.

8.9 Load each sample into one well (or two) of the gel. Load the size markers symmetrically on both sides of the gel to allow for approximation of the length of the ligated samples between them. Use the center of the gel to guarantee even running of the gel. Make sure to space different samples appropriately, typically at a two-well distance, to avoid cross contamination. Ensure that the overall loading of the gel is asymmetrical.

8.10 Run the gel for 45 min at 30 W in 1x TBE buffer until the bromophenol blue dye is close to the bottom of the gel.

8.11 Dismantle the gel, leaving it mounted on one glass plate. To facilitate the alignment of the gel to the phosphorimager paper printout, again implant three tiny radioactive gel pieces asymmetrically at three of the four corners of the gel. Cover the gel in plastic wrap.

8.12 Expose the gel for at least 1 h to a phosphorimager screen. If the radioactivity of the recovered RNA is weak, you can expose the gel overnight, placing the exposure cassette in a -20ºC freezer. Allow the cassette to return to room temperature before opening it.

8.13 Align the gel on top of a printout scaled to 100 % according to the position of the three radioactive gel pieces. Cut out the bands in between the ligated products of the 19-nt and above the 24-nt marker (**NOTE**: We do not recommend cutting of RNA that is running below the 19-nt marker line. For our bioinformatic analyses, all sequences shorter than 20nt are discarded due to the increased probability of mapping to multiple locations and the uncertainty defining its genetic location. Our bioinformatic analysis pipeline discards reads under 20nt lengths for that reason. In case you would like to cut a larger size range, two longer size markers [33-nt and 35-nt], which we also have successfully used, are also included in the materials and methods section). Also, cut the ligated 19- and 24-nt size markers, which will serve once more as a ligation control in the next step (see figure 1B).

8.14 Place the cut gel pieces in siliconized tubes and add 350 µl 0.4 M NaCl (ensure that the gel pieces are covered by NaCl). Elute the ligation products overnight at 4°C shaking at 800 rpm.

8.15 Transfer the supernatant into a new siliconized tube and add 1 ml 100 % ethanol. Precipitate the RNA for 1 h on ice and spin at 20,000 g for 15 min at 4°C.

8.16 Take off the supernatant, air dry the pellets and dissolve in a total of 9 µl H_2O. Dissolve the ligated markers in 12 µl H_2O.

Tip *Keep the supernatant from the ethanol precipitation. In case no pellet should form after the precipitation you can add 1 µl of glycogen to it and precipitate again. We do not routinely add glycogen at this stage since the relatively high amount of glycogen might interfere with the subsequent reaction which is performed in a low volume. The linear acrylamide eluted from the gel usually is a sufficient carrier.*

Step 9 5’ adapter ligation for cDNA library preparation (Day 5, beginning of day 6)

Overview In this step the 5’ adapter is joined to the 3’ ligated RNA to enable the cDNA synthesis in the next step. *See figure 1C.*

Duration *Day 5: about 5 h but again highly dependent on required exposure time; day 6: 2 h*

9.1 Prepare the following reaction mixture for the ligation of the 5’ adapter, multiplying the volumes by the number of ligation reactions to be performed (again also include the positive control) plus one extra volume to account for pipetting error:
1 µl of 100 µM 5’ adapter
2 µl of 10x RNA ligase buffer with ATP
6 µl 50% aqueous DMSO
Combine 9 µl of this mixture with 9 µl of sample.
Remember to also process the ligated markers from the last step. Ligate 9 µl out of the 12 µl and keep 3 µl as an unligated control for the next gel.

9.2 Denature the RNA by incubation for 30 s at 90°C. Place the tube immediately on ice for 30 s.

9.3 Add 2 µl of T4 RNA ligase 1 (10 U/µl), mix gently, and incubate for 1 h at 37°C.

9.4 In the meantime, cast a 12% denaturing 8 M urea polyacrylamide gel and wait until the polymerization process is complete. We again use 0.8 mm spacers and a 20 well comb.

9.5 Pre-run the gel for 30 min at 30 W in 1x TBE buffer. After the pre-run, gently flush the wells with 1x TBE.

9.6 Add 20 µl of formamide gel loading solution, incubate the samples at 90°C for 30 s and load them on the gel. Make sure to space different samples appropriately, typically at a two-well distance, to avoid cross contamination.
Load 50% of the ligated markers on the left side and 50% on the right side. Load the remaining 3 µl unligated marker on either side but remember which (see figure 1C).

9.7 Run the gel for 45 min at 30 W using 1x TBE buffer until the bromophenol blue dye is close to the bottom of the gel. Disassemble and image the gel as described above for the 3’ ligation (start with an exposure roughly twice as long as for the 3’ ligation) and excise the new ligation product (again include the ligated markers).

9.8 Elute the ligation products from the gel slices overnight at 4°C shaking at 800 rpm in 350 µl 0.4 M NaCl. Add 1 µl of 100 µM 3' PCR primer as a carrier to facilitate the recovery of the ligation products.

9.9 Pipet the supernatant into a new siliconized tube and add 1 ml 100 % ethanol. Precipitate the RNA for 1 h on ice and spin at 20,000 g for 15 min at 4°C.

9.10 Remove the supernatant, air dry the pellets and dissolve in 5.6 µl H_2O.

Tip *Make sure that the loading of the gel is asymmetrical.*

Tip *You can recover unligated material by excising the gel region below the ligated 19 nt marker line since this represents 3’ ligated, 5’ unligated RNA fragments. Freeze these gel pieces. Should you later wish to perform another 5’ adapter ligation from the RNAs eluted from these gel pieces you have them stored as a backup.*

Step 10 cDNA library preparation / reverse transcription

(Day 6)

Overview The RNA ligated to both sequencing adapters is reverse transcribed and will be used for PCR in the subsequent step.

Duration *1.5 h*

10.1 Prepare the following reaction mix (multiplied by the number of samples plus one for the pipetting error):
1.5 µl 0.1 M DTT
3 µl 5x first-strand buffer
4.2 µl 10x dNTPs

10.2 Denature the RNA by incubating the tube for 30 s at 90°C and transfer the tube to a 50ºC thermomixer.

10.3 Add 8.7 µl of the reaction mix to each sample and incubate for 3 min at 50°C. Add 0.75 µl of Superscript III Reverse Transcriptase and incubate for 1 h at 42°C.

10.4 Prepare 150 mM KOH/ 20 mM Tris base and 150mM HCl and check on pH paper that a 1:1 mix results in a pH between 7.0 and 9.5. If not, change the ratios until the pH is within that range.

10.5 To hydrolyze the RNA, add 40 µl of 150 mM KOH/ 20 mM Tris base and incubate for 10 min at 90°C.

10.6 Neutralize the solution by adding 40 µl of 150 mM HCl (the exact volume depends on the ratio determined in step 10.4) and check the pH of the mixture by spotting 1 µl on pH paper. It should be between 7.0 and 9.5 so that the subsequent PCR is not inhibited. If necessary, readjust the pH by adding more base or acid.

Step 11 PCR amplification of cDNA library & sample preparation for sequencing
(Day 6)

Overview This step concludes the PAR-CLIP protocol. To minimize the distortion of the cDNA library composition by excessive PCR and to recognize possible failure during reverse transcription leading to false positive PCR results, we monitor the accumulation of the PCR product during a pilot PCR. To determine the minimal cycle number a small scale trial PCR is performed before the final large scale PCR. The PCR product is gel fractionated; the appropriately sized fraction is recovered from the gel and submitted to Solexa sequencing. *See figure 1D.*

Duration *8 - 9 h; you might wish to pause after the small scale PCR and continue with the large scale PCR on the following day.*

11.1 Prepare the following mix multiplied by the number of samples plus one for the negative control:
40 µl 10x PCR buffer,
40 µl 10x dNTPs,

2 µl of 100 µM 5’ PCR primer,
2 µl of 100 µM 3’ PCR primer,
272 µl H_2O
89 µl of the reaction mix will be used in the pilot PCR reaction to determine the minimal cycle number; the remainder will be needed for the large scale PCR (freeze the reaction mix if you do not plan to run the large scale PCR on the same day).
To 89 µl of the reaction mix add 10 µl cDNA and 1 µl *Taq* polymerase (5 U/µl). Remember to include a negative control (H_2O instead of cDNA).
Use the following cycle conditions: 45 s at 94°C, 85 s at 50°C, 60 s at 72°C.

11.2 To determine the necessary number of cycles for amplifying the cDNA library, remove 12 µl aliquots every other cycle starting with cycle number 12 up to cycle number 26. To remove aliquots from the PCR tube, temporarily pause the PCR cycler at the end of the 72ºC step. You can use a multichannel pipet to remove the aliquots.

11.3 Analyze 6 µl of each sample on a 2.5 % agarose gel containing 0.4 µg/ml of ethidium bromide to check for consistency. Load a 25 bp ladder on each side and load all cycles from one sample next to each other in an ascending order.
The PCR products might appear as a double band with the higher band running at the expected length of about 95 - 110 nt and a lower band corresponding to the 3' adapter to 5' adapter ligation / template switch products running at about 65nt. Figure 1D illustrates a typical small scale PCR. The red arrows indicate the chosen number of cycles for the large scale experiment
Define the minimal cycle number for the cDNA amplification, which should be within the exponential amplification phase of the PCR, about 5 cycles away from reaching the saturation level of PCR amplification. For a typical PAR-CLIP experiment the minimal number of cycles is between 16 and 20 (**Pause point**: you can pause at any time before or after the large scale PCR).

11.4 Perform a 300 µl large scale PCR (100 µl per well) with the determined minimal number of cycles and combine all three PCR reactions. Again include a negative control.

11.5 Analyze 6 µl of the products next to the corresponding products from the pilot PCR on a 2.5 % agarose gel containing 0.4 µg/ml of ethidium bromide to check for consistency.

11.6 To the remaining 264 µl add 26.4 µl 3M NaCl and 1 ml 100 % EtOH. Precipitate for 1 h on ice and spin at 20,000 x g for 30 min at 4°C. Take off the supernatant, air dry the pellet and dissolve in 40 µl 1x DNA loading dye (5x DNA loading dye diluted in 1x TBE).

11.7 Divide the sample into two wells of a 2.5 % low melt agarose gel containing 0.4 µg/ml ethidiumbromide. Run the gel at 120 V for 2 - 3 h.
Do not overload the gel as this will compromise its separating capacity.

11.8 Visualize the DNA on a 365 nm transilluminator and excise the band corresponding to 95-110nt with a clean scalpel.

11.9 Purify the DNA using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer’s instructions. Include the isopropranol step as described for short fragments. Elute in 30 µl elution buffer.

11.10 Analyze 5 µl of the eluate on a 2.5 % agarose containing 0.4 µg/ml of ethidium bromide gel to ensure the removal of any unwanted amplified 5’adapter-3’adapter PCR products.

11.11 Submit 10 µl of the purified cDNA to Solexa sequencing.

Tip *If you have more than one PAR-CLIP sample prepare a 96-well plate with 3 µl 5x loading dye in the required number of wells so that you only have to pipet once per cycle with a multichannel pipet.*

Tip *Main goal of the preparative gel is to reduce non-informative sequence reads of unwanted 5'adapter-3'adapter PCR product. Do not overload the gel and run the gel as long as possible to achieve the best separation possible. Check intermittently that you do not run your samples into the buffer. Bromophenol blue runs roughly at the same length as the samples. Thus use either xylene cyanol as loading dye or do not add any loading dye to the PCR buffer but run an aliquot containing bromophenol blue next to your samples.*

Tip *Perform a second gel extraction if any 5'adapter-3'adapter products should still be visible after the first gel extraction.*

Addendum
Determination of incorporation levels of 4SU into total RNA

Overview To optimize crosslinking of protein to RNA it is useful to determine the fraction of substitution of uridine by 4SU. This is especially necessary when changing cell growth conditions or cell type. Total RNA is isolated and enzymatically degraded to monomeric ribonucleosides which are separated and quantified by HPLC analysis (Andrus and Kuimelis 2001).

Duration *Step 1: 15 min from cell harvest onwards, step 2: 2 h, step 3: 10 min and overnight incubation, step 4: as needed*

Equipment

Major equipment	CO_2 incubator for mammalian cell culture
	HPLC with a Supelco Discovery C18 (bonded phase silica 5 µM particle, 250 x 4.6 mm) reverse phase column (Bellefonte PA, USA)
	Thermomixer

Consumables	1.5 ml polypropylene tubes
	10-cm tissue culture dishes

Reagents & Chemicals	Acetonitrile
	Appropriate cell culture medium and selection antibiotics
	Bacterial Alkaline Phosphatase (Worthington Biochemical, LS006344)
	DMSO

	DTT
	Ethanol (100 %)
	Isopropanol
	$MgCl_2 \cdot 6H_2O$
	rA, rG, rC, rU and 4SU (Sigma, T4509)
	Snake Venom Phosphodiesterase (Worthington Biochemical, LS003926).
	TEAA (Acetic acid - triethylamine solution 1:1, Sigma, 09748)
	Tris base
	Tris-HCl
	TRIzol reagent (Invitrogen, 15596-026)

Reference oligoribonucleotides	CGUACGCGGAAUACUUCGA(**4SU)**U (e.g. from Thermo Scientific)
	CGUACGCGGAAUACUUCGAUU

Solutions & buffers

Step 1

4-thiouridine containing growth medium

Component	**Stock**	**Final conc.**	**Amount/liter medium**
4-thiouridine (in DMSO)	1 M	100 µM	100 µl

Step 2

1M DTT

Component	**Stock**	**Final conc.**	**Amount/ 10 ml**
DTT	n/a	n/a	1.54 g
H_2O to 10 ml	n/a	n/a	

Step 4

HPLC buffer A

Component	**Stock**	**Final conc.**	**Amount/liter**
Acetonitrile	100 %	3 %	30 ml
TEAA	2 M	0.1 M	50 ml
H_2O to 1 liter	n/a	n/a	

HPLC buffer B

Component	**Stock**	**Final conc.**	**Amount/liter**
Acetonitrile	100 %	90 %	900 ml
H_2O to 1 liter	n/a	n/a	100 ml

Protocol

1. Cell culture
   1.1 Grow HEK293 cells on a 10-cm plate in regular medium supplemented with 100 µM 4SU for 16 h prior to harvest. As a control, also grow cells without adding 4SU.
   1.2 Decant the growth medium.

2. Extraction of total RNA
   2.1 Add 1 ml of TRIzol reagent directly onto the plate and isolate total RNA according to the manufacturer's instructions.
   2.2 Add 0.1 mM DTT (prepare a 1M stock which can be frozen for storage) to isopropanol and ethanol wash steps as well as to the subsequent reactions to prevent oxidization of the thiocarbonyl group, yielding disulfides or uridine.
   2.3 Dissolve the RNA pellet in 30 µl H_2O.
   2.4 Determine the concentration of the obtained RNA. Expect to obtain about 50 - 100 µg total RNA per 10-cm plate.

3. Dephosphorylation and enzymatic hydrolysis of total RNA to ribonucleosides
   3.1 Set up the following reaction:

40 µg total RNA	x µl
1 M $MgCl_2$	0.4 µl
0.5 Tris-HCl (pH 7.5 at RT)	2 µl
Bacterial Alkaline Phosphatase	4 µl (equals 0.4 U)
Snake Venom Phosphodiesterase	2.4 µl (equals 0.09 U)
H_2O	to 28.8 µl

   Digest for 16 h at 37°C.
   As an additional control also digest and analyze synthetic RNAs with and without 4SU.

4. HPLC
   4.1 Separate ribonucleosides on a Supelco Discovery C18 reverse phase column (bonded phase silica 5 µM particles, 250 x 4.6 mm, Bellefonte PA, USA).
   4.2 Use an isocratic gradient of 0% B for 15 min, 0 to 10 % B for 20 min, 10 to 100% B for 30 min with a 5 min 100 % B wash between runs (see figure 2).
   4.3 Calculate the absorption ratios from the known sequence first which are then used to estimate the incorporation rate for 4SU (in our experiments between 1.4 - 2.4% of U is substituted by 4SU).
   4.4 Confirm U and 4SU retention times by co-injection with standards.
   4.5 Calculate the substitution ratio of 4SU by dividing the area under the curve by the extinction coefficients of rU versus 4SU at 260 nm versus 330 nm.

Nucleoside	Extinction coefficient at 260 nm (pH 7.0)	Extinction coefficient at 330 nm
rA	12340	0
rC	7020	0

rG	10240	0
rU	9720	0
4SU	9720	17000

9. References

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10. TOPICS

Topic Class	Keyword
Methods List the methods used to carry out this protocol (i.e., for each step).	1 RNA-protein crosslinking
	2 UV crosslinking
	3 Immunoprecipitation
	4 Small RNA cDNA library preparation
	5 Solexa sequencing
Process List the biological processes addressed in this protocol.	1 Posttranscriptional gene regulation
	2 RNA processing
	3 mRNA stability
	4 mRNA transport
	5 mRNA splicing
Organisms List the primary organism used in this protocol. List any other applicable organisms.	1 Mammalian cells
	2
	3
	4
	5
Pathways List any signaling, regulatory, or metabolic pathways addressed in this protocol.	1
	2
	3
	4
	5
Molecule role/function List any cellular or molecular functions or activities addressed in this protocol.	1 RNA recognition by RBPs via specific RNA-binding motifs
	2
	3
	4
	5
Phenotype List any developmental or functional phenotypes addressed in this protocol (organismal or cellular level).	1 n/a
	2
	3
	4
	5
Anatomy List any gross anatomical structures, cellular structures, organelles, or	1 n/a
	2
	3
	4

macromolecular complexes pertinent to this protocol.	5
Diseases List any diseases or disease processes addressed in this protocol.	1 n/a
	2
	3
	4
	5
Other List any other miscellaneous keywords that describe this protocol.	1 Defining RNA-binding motifs
	2
	3
	4
	5

11. VIDEO

Please refer to this link (http://www.jove.com/index/Details.stp?ID=2034) for a video illustrating the first day of experiments.

12. IMAGES

Figure 1

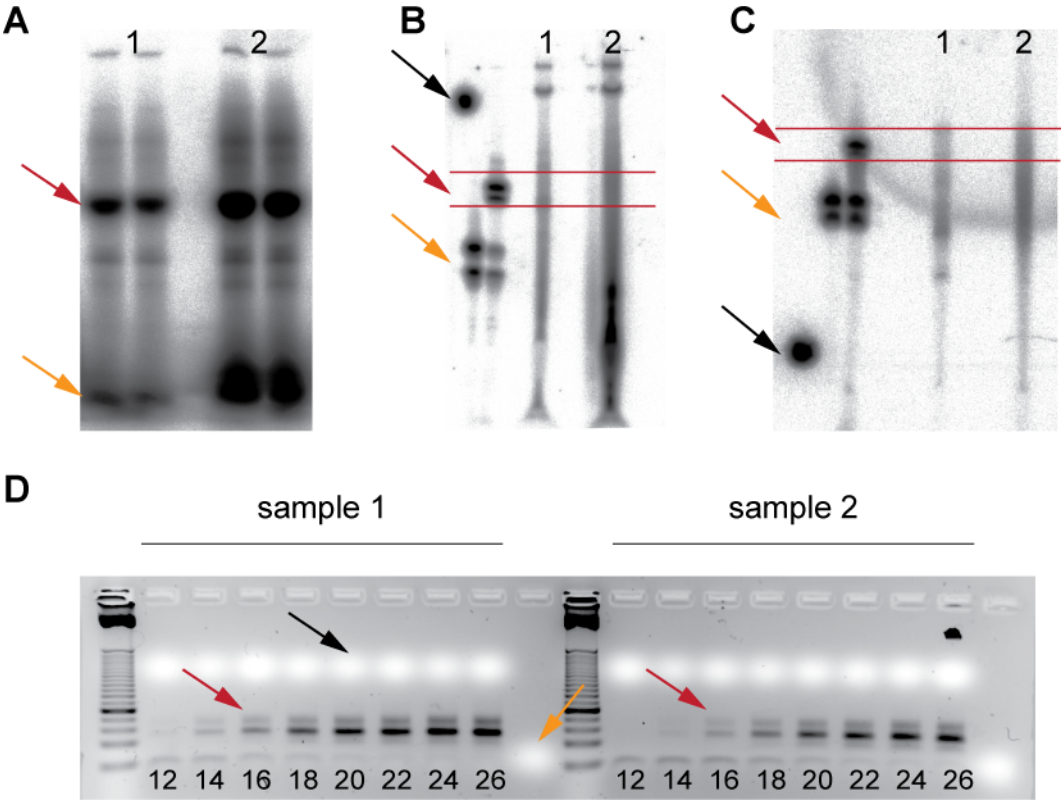

Figure 1: Selected PAR-CLIP experimental steps. A) SDS-PAGE gel of crosslinked and 5'radiolabelled RNA-protein complex immunoprecipitates. The red arrow points to the radioactive bands corresponding to the expected size of the RNA-binding protein (FUS, running at 75 kDa), the yellow to the radioactive running front. B) 8 M urea polyacrylamide gel after 3'adapter ligation. The black arrow indicates one of the inserted little radioactive gel pieces to facilitate alignment of gel to printout, the red to the 3'ligated size markers and the area which was cut from the gel and further processed; the yellow arrows shows the unligated 3'size markers. C) 8 M urea polyacrylamide gel after 5'adapter ligation. The black arrow indicates one of the inserted little radioactive gel pieces to facilitate alignment of gel to printout, the red to the 5'ligated size markers and the area which was cut from the gel and further processed; the yellow arrows shows the unligated 5'size markers. D) Agarose gel after small scale trial PCR. The black arrow points to the position of migration of the xylene cyanol loading dye, the yellow to the bromophenol blue loading dye running close to the gel front. Bands of about 75 and 100 bp mobility are detectable, representing insert-less 5'adapter-3'adapter PCR side product and expected insert-containing PCR product, respectively. The red arrows indicate the number of cycles chosen for the large scale experiment. A 25 bp ladder is loaded to the left of each set of experiments; the fourth band from the bottom corresponds to 100 bp. The negative control was performed but is not shown.

Figure 2

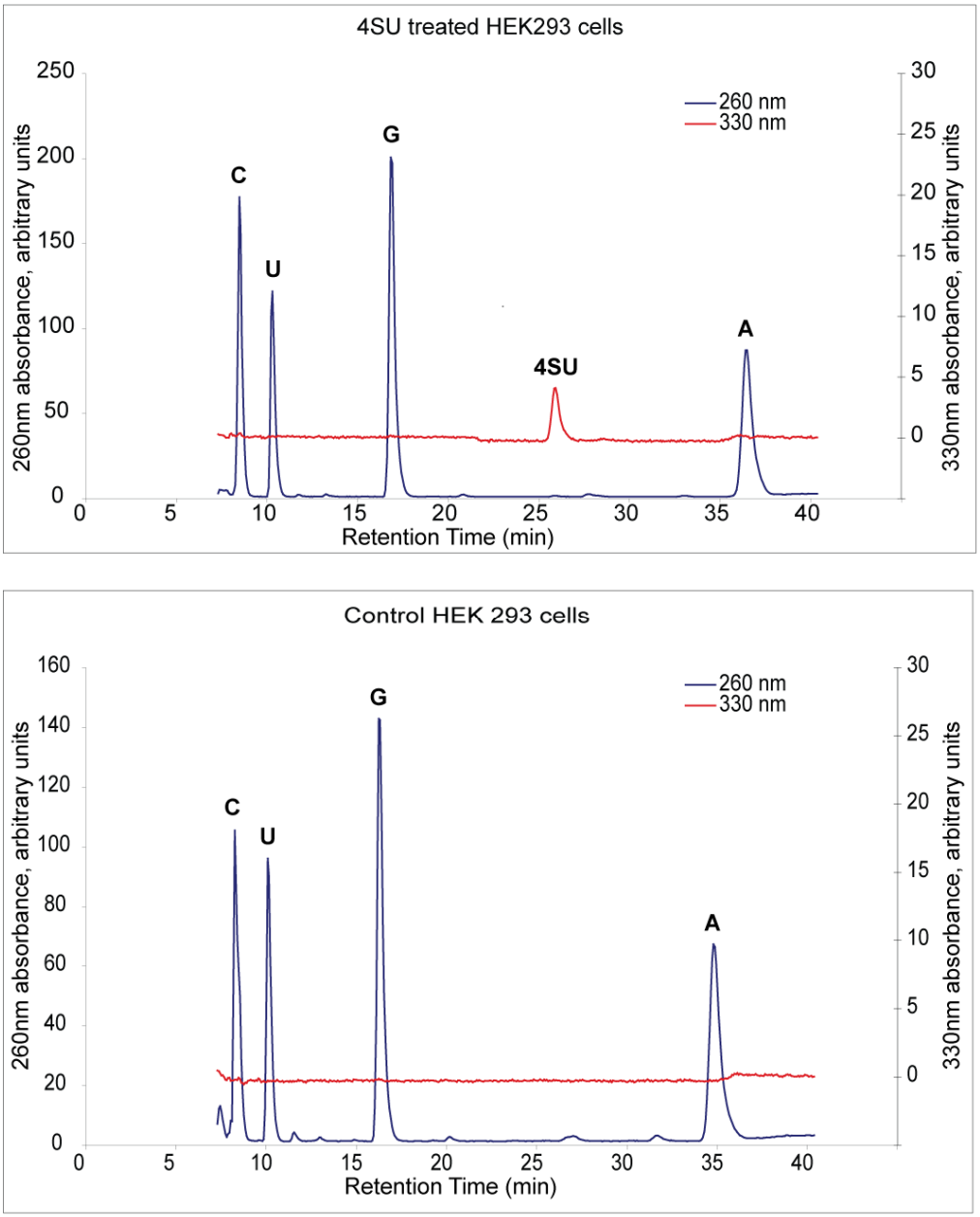

Figure 2: HPLC trace of extracted total RNA to estimate 4SU incorporation into HEK293 cells. Please refer to the main text for a detailed description.