Argonaute HITS-CLIP method;

(HITS-CLIP: HIgh-Throughput Sequencing of RNAs from *in vivo* Cross-Linking and Immuno-Precipitation)

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Day 1

General method for UV cross-linking of tissue/cell lines

For mouse tissue:

Harvest necortex from P13 mice and let tissue sit in ice cold HBSS until harvest is complete.

[In our case, half of P13 neocortex tissue suffices for high quality data using anti-Ago antibodies. If cell culture is used, HBSS can be used instead of PBS.]

Add 10 cell volumes of HBSS and triturate tissue first using a 5 or 10 ml pipette, and then again by adding a 100μl micropipette tip at the end of the pipette.

[Because UV light can penetrate a few cell layers, stringent trituration to the single-cell is not necessary.]

Irradiate suspension (using 10mls per 10 cm tissue culture plate) three times for 400mJ/cm^2 in Stratalinker (we use model 2400 from Stratagene). Mix suspension between each irradiation.

[The length of crosslinking should be optimized for each protein separately, as each RNA binding domain crosslinks with different efficiency, depending on the availability of aromatic amino acids. For a preliminary experiment, try 100, 200 and 400mJ/cm^2, and then use the shortest condition that gives >70% of the maximum signal.]

For cell culture:

Grow cells in a 100 or 150mm plates, rinse once with PBS, and place in Stratalinker with the cover off. Irradiate one time for 400mJ/cm^2 and additional 200 mJ/cm^2 in Stratalinker.

[Because cells grown in a single layer are all equally exposed to UV light, a single UV and additional half energy UV irradiations are sufficient.]

Collect suspension, pellet cells at 2500rpm for 5 min at 4°, re-suspend pellet in (~3x dry volume) of PBS and distribute 1ml of suspension to each eppie; quick spin at 4°, remove supernatant and freeze pellets at -80° until use (each tube is about 200 μl of cells).

To prepare **1x HBSS**:

50 ml 10x Hank's Balanced salt solution, Ca-Mg-free (Gibco, #14186-012)
5 ml 1M HEPES, pH 7.3
445 ml ddH_2O

Day 2

II. Immunoprecipitation

a. Solutions (to be made fresh)

1X PXL (Wash Buffer)
1X PBS (tissue culture grade; no Mg++, no Ca++)
0.1% SDS
0.5% deoxycholate
0.5% NP-40

5X PXL (High-salt Wash Buffer)
5X PBS (tissue culture grade; no Mg++, no Ca++)
0.1% SDS
0.5% deoxycholate
0.5% NP-40

1X PNK Buffer
50 mM Tris-Cl pH 7.4
10 mM $MgCl_2$
0.5% NP-40

1X PNK+EGTA Buffer
50 mM Tris-Cl pH 7.4
20 mM EGTA
0.5% NP-40

b. Bead preparation:

For each Eppie of crosslinked lysate use 400 μl of protein A Dynabeads (Dynal, 100.02).
Wash beads 3x with 0.1 M Na-phosphate, pH 8.0.

[We use high pH here because this increases antibody capture by Protein A. However, you may need to adapt all the immunoprecipitation buffers to the antibody that you use. For instance, we found that some antibodies don't work with the deoxycholate in the wash buffer.]

Resuspend beads in 350 μl 0.1 M Na-phosphate pH 8.0 and add 50 μl bridging Ab (2.4mg/ml; rabbit anti-mouse IgG; Jackson Immunoresearch: 315-005-008)

[Ago antibodies(2A8 or 7G1-1*) we use are mouse monoclonal. So we pre-incubate the beads with rabbit anti-mouse IgG to increase the avidity.]

Rotate tubes at room temperature for ~45 min.; wash 3x with 0.1 M Na-phosphate, pH 8.0.

Resuspend beads in 400 μl 0.1 M Na-phosphate pH 8.1 and add 3 μl of 2A8 or 12 μl of 7G1-1* ascite fluid (5mg/ml).

[This protocol is optimized for Ago HITS-CLIP using half of neocortex from P13 mouse. If you are using less tissue, you can scale down the amount of bead and antibody used here.]

Rotate tubes at 4°C for ~4 hrs.; wash 3x with 1X PXL; if you are not yet ready to add crosslinked lysate, leave beads in last wash step.

c. 5' end labeling of RL3 (-P) linker (no phosphate)

[Prepare labeled 5'end linker before or during IP. We use pre-labeled 3'linker for AGO HITS-CLIP which gives more specific signal in autoradiogram than labeling isolated RNA after IP by PNK reaction]

2.4 μl RL3(-P) linker (50pmol/μl ; no phosphate in 5'end)
5 μl 10X PNK Buffer (NEB)
25 μl ^{32}P-γ-ATP
8 μl T4 PNK enzyme (NEB, M0201L)
3 μl RNAsin (Promega)
6.6 μl water
50 μl total

Incubate in Thermomixer R (Eppendorf) at 37° for 30 minutes
Add 0.2μl of 10mM ATP, and let the reaction go for an additional 5 minutes.

Resuspend the resin in the G-25 column (GE healthcare:27-5325-01) by vortexing.
Pre-spin the column for 1min at 735 x g, apply the sample to resin, and spin the column for 2min at 735 x g; if you are not yet ready to use, store it at -20°C.

[This protocol is for 10 samples; 5 µl for each sample. If you are using less than 10 samples, you can scale down the amount of materials used here.]

d. Partial RNA digestion and ultracentrifugation

Resuspend each tube of crosslinked lysate using ~700 μl of 1X PXL (with protease inhibitors; ~1 ml total volume); add 15 μl RNAsin (Promega) and let sit on ice for 10 min.

Add 30 μl of RQ1 DNAse (Promega, M6101) to each tube; incubate at 37° for 5 min, 1000 rpm.

[DNAse 1 is not supposed to work in the absence of Ca and Mg (ideally, it requires 25 mM $MgCl_2$ and 5 mM $CaCl_2$). However, at this step after cell lysis, the contaminating Ca++ and Mg++ from the cell lysate and high concentration of the DNAse are sufficient to digest the DNA.]

Make a dilution of RNAse A (USB 70194Y) at 1:100 in 1X PXL (high-RNAse).
Make a dilution of RNAse A (USB 70194Y) at 1:10,000 in 1X PXL (low-RNAse).

[Each experiment should be done in duplicate with two RNAse concentrations – the dilution depends also on the batch of RNAse, so in the first experiment, several dilutions should be tested. The over-digest experiment is used as a control to confirm that the size of the radioactive band on SDS-PAGE gel changes in response to different RNAse concentrations (which confirms that the band corresponds to a protein-RNA complex). In addition, this experiments helps determine the size of the immunoprecipitated RNA-binding proteins, because these proteins will be bound to short RNAs and will thus migrate as less diffuse bands only ~7kDa above the expected MW.]

Add 10μl of each RNAse dilution to the two duplicate tubes; incubate at 37° for 5 minutes.
Spin lysates in pre-chilled ultra-microcentrifuge (polycarbonate tubes in TLA 120.2 rotor), 30K for 20' at 4°C.

[You should determine the optimal speed for your protein. Higher speed (90K) is beneficial to clear the lysate of all high-molecular weight material, like ribosomes or very large RNPs, but in that case you need to make sure your protein stays in supernatant.]

Carefully remove the supernatant, and save 10μl for immunoblot analysis.

e. Immunoprecipitation

Add the rest of supernatant to one prepared tube of beads. Rotate beads/lysate mix for 2-4 hours at 4°.

Remove the supernatant and save 10μl for immunoblot analysis (in order to test the relative depletion of the antigen).

Wash beads with ice-cold buffer:
2x 1X PXL (Wash Buffer)
2x 5X PXL (High-salt Wash Buffer)
2x 1X PNK Buffer

[The first time CLIP is done for a particular protein, you can skip steps III-IV, and go straight to step V. Continue until the exposure of membrane to X-ray film and check the following:
1. Do I get radioactive band ~7kDa above the MW of my protein in high RNAse experiment?
2. Does my band disappear in the control experiments? Possible controls are no UV crosslink, irrelevant antibody pulldown, knockout organism, RNAi knockdown, or no transfection of a tagged construct.
3. Does this band move up and become more diffuse in low-RNAse experiment? If yes, you are dealing with an RNA-binding protein, and you can in your following experiments proceed to step III.
[Continue to step III with the sample that contained low RNAse (1:300,000). Skip steps III and IV with the over-digest sample – leave it at 4° until step V.]

III. CIP Treatment (On-Bead)

8 μl 10x dephosphorylation buffer (Roche, 712023)
3 μl alkaline phosphatase (Roche, 712023)
2 μl RNAsin (Promega)
67 μl water
80 μl total (per 400 ⌈l of beads)

Incubate in Thermomixer R (Eppendorf) at 37° for 20 min (1000 rpm every 2 minutes for 15 seconds).

Wash 1X with 1X PNK Buffer
Wash 1X with 1X PNK+EGTA Buffer
Wash 2X with 1X PNK Buffer

IV. 3' RNA Linker Ligation (On-Bead)

8 μl 10X T4 RNA ligase buffer (Fermentas)
8 μl BSA (0.2 μg/μl)
8 μl ATP (10 mM)
2 μl T4 RNA ligase (Fermentas)
2 μl RNAsin (Promega)
5 μl Labeled hot RL3 (12pmol, Prepared in IIc)
47 μl water
80 μl total

Add 80 μl of ligase mix to each tube of beads.

Incubate at 16°C for 1hr in Thermomixer R (Eppendorf) (1000 rpm every 2 minutes for 15 seconds).

Add 4μl of RL3(20pmol/μl; with Phosphate on 5'end) , and let the reaction go for overnight.

Day 3

Wash 1x with 1X PXL buffer
Wash 1x with 5X PXL buffer
Wash 3x with 1X PNK buffer

V. PNK Treatment (On-Bead)

8 μl	10X PNK Buffer (NEB)
1 μl	cold ATP (10mM)
4μl	T4 PNK enzyme (NEB, M0201L)
2 μl	RNAsin (Promega)
65 μl	water
80 μl	total

Add 80 μl of PNK mix to each sample and incubate in Thermomixer R (Eppendorf) at 37° for 20 minutes (1000 rpm every 4 minutes for 15 seconds).

Wash 1x with 1X PXL buffer
Wash 1x with 5X PXL buffer
Wash 3x with 1X PNK buffer

VI. SDS-PAGE & nitrocellulose transfer

[Do western blot for input and post-IP; load equal volumes.]

Re-suspend the beads in 30 μl of 1X PNK+ and 30 μl of Novex loading buffer (without reducing agent).
[Antibody bands may interfere in the way your protein runs on the gel; if the MW of your protein is less than 50kDa, DO NOT add any reducing agent.]

[The Novex NuPage gels are critical. A pour-your-own SDS-PAGE gel (Laemmli) has a pH during the run that can get to ~9.5 and can lead to alkaline hydrolysis of the RNA. The Novex NuPAGE buffer system is close to pH 7.]

Load 1 tube per 2 wells of a 10 well Novex NuPAGE 10% Bis-Tris gel. Run the gel at 175V in the cold room.

[Some pre-stained molecular weight markers may run differently on Novex NuPage gels. We use rainbow marker (Amersham, RPN800), which runs at the expected molecular weights.]

[The gel itself will be a lot less hot than the amount of radioactivity that you loaded. Most of the signal will be in the lower buffer, which results from free ATP and small free RNAs that run out of the gel. Free RNA of up to 100 nucleotide length will migrate on the gel below 30 kDa.]

After gel run, transfer gel to S&S BA-85 nitrocellulose using the Novex wet transfer apparatus.
Transfer 1 h at 30V in NuPAGE Transfer Buffer with 10% methanol.

[This pure nitrocellulose is a little fragile, but it works better for the RNA/protein extraction step.]

After transfer, rinse the nitrocellulose filter in 1X PBS, and gently blot on Kimwipes; wrap membrane in plastic wrap and expose to autoradiogram.

[Most free RNA will pass through the membrane, so due to the loss of free RNA in the transfer step, the membrane will be less hot than the gel.]

[Use a luminescent sticker, so that you can later align the filter back to the autorad.]

Day 4

[In case of Ago, we usually see a signal on the autoradiogram after ~2 hours exposure if half of neocortex from p13 brain tissue was used as starting material. If much less material is used, an overnight exposure will sometimes be necessary to see a decent signal.]

We don't clone RNAs from the over-digested sample, but use it to determine the specificity of the RNA-protein complexes by the following steps:

1. ***Look at the over-digested sample, and see if you have a band ~7kDa above the expected MW of your protein (~100kDa for Ago).***
2. ***Calculate the distance from the closest contaminating band, and estimate the relative signal strength of your protein relative to other bands.***
3. ***You should have no problem purifying RNA specific for your protein if your protein band migrates more than 10kDa away from any other protein band.***

The RNA-protein complexes that were digested by low RNAse will appear as a diffuse radioactivity with a modal size of ~15-20kDa above the expected MW of your protein.

[In case of Ago, we usually see two different modal sizes. One is ~110kDa from Ago-miRNAs complex and the other is ~130kDa from Ago-mRNA complex]

4. ***Average MW of 50 nucleotides long RNA is ~16 kDa. As the tags contain 20 nucleotides long linker (L3), the position of protein-RNA complex that will generate CLIP tags longer than 50 nucleotides is 20 kDa above the expected MW of the protein.***
5. ***Because the RNAse digestion is random, the tag sizes will vary from ~50-150 nucleotides, and thus the complex will migrate more diffuse than the low RNAse complex (where the tag sizes are ~20-60 nucleotides).***
6. ***In order to be able to later separate CLIP tags specific to different proteins, you need to cut a band as thin as possible (~3kDa wide band) approximately 20 kDa above the expected MW of your protein.***
7. ***In addition, cut also two bands below and above this band, with ~15 kDa distance. The lower band should not contain any RNA longer than 15 nucleotides specific to your protein (but it will show RNA bound to any smaller proteins).***

Cut out thin bands (~4-8kDa wide) using a clean scalpel blade, and put the nitrocellulose pieces into separate microtubes. The smaller the pieces, the better. Count radioactivity in a scintillation counter.

VII. RNA Isolation and Purification

1X PK Buffer:
100 mM Tris-Cl pH 7.5
50 mM NaCl
10 mM EDTA

1X PK Buffer/7M urea (this buffer must be fresh):
100 mM Tris-Cl pH 7.5
50mM NaCl
10 mM EDTA
7 M urea

Make a 4mg/ml proteinase K (Roche, 1373196) solution in 1X PK Buffer; pre-incubate this stock at 37° for 20 min to kill any RNAses.

Add 200 μl of proteinase K solution to each tube of isolated nitrocellulose pieces; incubate 20 min at 37° at 1000 rpm.

Add 200 μl 1x PK/7M urea solution; incubate another 20 min at 37° at 1000 rpm.

Add 400 μl RNA phenol (Ambion, 9710) and 130 μl of $CHCl_3$ to solution; 37° for 20' at 1000 rpm.

[RNA phenol can also be prepared by equilibrating pure phenol with 0.15 M NaOAc pH 5.2; $CHCl_3$ is chloroform 49:1 with isoamyl alcohol.]

Spin tubes at full speed in microcentrifuge; take aqueous phase Add 50 μl 3M NaOAc pH 5.2, 0.75 μl of glycogen (Ambion, 9510) and 1ml of 1:1 EtOH:isopropanol.

[0.5 µl of glycogen is necessary to precipitate small quantity of RNA, but don't add more, otherwise the RNA ligase may be inhibited.]

Precipitate overnight at -20°.

Day 5

IX. 5' RNA Linker Ligation

Spin down RNA (10' at max speed in microcentrifuge). Check to see if you got decent precipitation of counts. Wash and dry pellet.

[It is important to wash well, as residual salt can decrease ligation efficiency. We recommend two washes with 150 µl cold 75% ethanol. One can vortex the second wash and spin down the RNA again for 10' at max speed in microcentrifuge. Don't over-dry – 2' in speedvac is usually enough.]

Count RNA in scintillation counter. Resuspend in 5.9 μl H_2O.

RNA ligation:
1 μl 10X T4 RNA ligase buffer (Fermentas) 1 μl BSA (0.2 μg/μl)
1 μl ATP (10 mM)
0.1 μl T4 RNA ligase (3U, Fermentas)
1 μl RL5 or RL5D RNA linker @ 20 pmol/μl
4.1 μl

Add 5.9 μl RNA resuspended in H_2O
10 μl total

[The linker itself cannot circularize (it has 5'-OH and 3'-OH), and the CLIP tag-3' linker product cannot circularize (It has 5'-P and 3'-Pmn).]

Incubate at 16° for 1-5 hours (or overnight).

Add to the reaction:
79 μl H2O
11 μl 10X DNAse I Buffer
5 μl RNAsin
5 μl RQ1 DNAse

Incubate 37° for 20 minutes.

Add:
300 μl H_2O
300 μl "RNA phenol"
100 μl $CHCl_3$

Vortex, spin and take aqueous layer.

Precipitate by adding: 50 μl 3M NaOAc pH 5.2
0.5μl of glycogen (Ambion, 9510)
1 ml 1:1 EtOH:isopropanol

Precipitate overnight (or 1h) @ -20°.

Day 6

X. RT-PCR.

Spin down the RNA. Wash and dry the pellet. Count RNA in scintillation counter to see if you got decent precipitation of counts. Re-suspend in 8 μl of H_2O.

RT reaction:
Mix 8 μl of the ligated RNA and 2 μl of DP3 (5 pmol/μl – i.e. 1 pmol/μl final concentration) and 3 µl 3 mM dNTPs.

Heat 65° for 5 min; chill and quick spin.

Add:
1 μl 0.1 M DTT
4 μl 5X SuperScript RT Buffer
1μl RNAsin
1 μl SuperScript III (Invitrogen, 18080-044)
20 μl total

Incubate at 50° for 45 min., 55° for 15min., 90° for 5 min., leave at 4°.

PCR reaction:
27 μl Accuprime Pfx Supermix (Invitrogen, 12344-040)
0.75 μl DP5 primer, 20 pmol/μl
0.75 μl DP3 primer, 20 pmol/μl
2 μl of the RT reaction
30.5 μl total

Settings: 95° 2' Cycle 25-35x (depending on how much RNA you started with): 95° 20'' / 58° 30'' / 68° 20'' 68° 5'.

[Do each tube in duplicate. Run one sample and keep the second one as backup.]

Day 7

Pour a 10% denaturing poly-acrylamide gel. We use Owl vertical electrophoresis system (P9DS-2).
In this case, we prepare 20ml of the following for each gel:
8.4 g urea (Fisher, U15-3)
5 ml 40% 19:1 Acrylamide:Bis-acrylamide (Fisher)
4 ml 5x TBE
water to 20 ml total

immediately before pouring the gel, add:
200 μl 10% APS
7.5 μl TEMED

We use the following 2x loading buffer for polyacrylamide gels:
95% formamide, deionized (Sigma F-9037)
5% 100mM EDTA, pH 8
Bromophenol blue + Xylene cyanol (Sigma B-3269)

Run the entire PCR reaction; use low molecular weight markers (we use 3 μl of Amplisize Molecular Ruler, Biorad – don't heat it), visualize DNA by immersing the gel for 10 minutes in 10.000-fold dilution of SYBR Gold (Molecular Probes) in TBE.

[If 3kDa wide band was cut from the membrane with the SDS-PAGE gel separated complexes, the resulting DNA tags will vary about 10-20nt in size. In case of the band that was cut ~20kDa above the expected MW of your protein, the DNA amplified from the RNA tags specific for your protein will appear as a band migrating between ~80-100 nucleotides (average RNA insert size ~50 nucleotides + 36 nucleotides for the linkers)]

[In case of Ago, we usually see two different bands here. One is ~56 nucleotides (20+36) and the other is ~80-100 nucleotides (50+36). Based on the sequence results, we confirmed that majority of ~56 nucleotides are from miRNAs and ~80-100 nucleotides are from target RNAs. In case of band that was cut ~110kD, we usually see ~56 nucleotides but from ~130kD, we usually see both ~56 and ~80-100 nucleotides bands.]

[When analyzing the PCR gel, you need to go back and look at the SDS-PAGE gel and find out how many bands migrated faster than your protein-RNA complex.]

Cut out the DNA of 80-100nt, extract DNA with QIAquick Gel Extraction Kit (follow "user-developed" protocol for extracting DNA fragments from polyacrylamide gels).

XI. Re-PCR with Solexa Fusion Primers

PCR reaction:
27 µl Accuprime Pfx Supermix (Invitrogen, 12344-040)
0.5 µl DSFP5 primer, 20 pmol/µl
0.5 µl DSFP3 primer, 20 pmol/µl
3µl of the RT reaction
31 µl total

Settings: 95° 2'
Cycle 6-14x (depending on how much DNA you started with): 95° 20'' / 58° 30'' / 68° 40''
68° 5'

Pour a 2% agarose (Nusieve or MetaPhor) / Ethidium Bromide gel.

Run entire PCR product; use low molecular weight markers (we use 5 µl of Amplisize Molecular Ruler). Visualize DNA on UV box. Cut out DNA of 150-170 nt, extract DNA with QIAquick Gel Extraction Kit following protocol for extracting DNA fragments from agarose gels).

XII. Quantitation of DNA

We use the Quant-it DNA Assay Kit, High Sensitivity (Invitrogen, Q-33120) to determine DNA concentration (follow manufacturer's instructions).
Make 10-30 µl of 10 nM DNA per sample; Submit for Solexa Sequencing.

XIV. Linker and primer sequences

[The RNA linkers need to be gel purified. Run 50 µl of 500 µM stock of deprotected RNA on 20% polyacrylamide gel, visualize the RNA by UV shading, cut out the band and purify as described above.]

RNA linkers (from Dharmacon):

RL5: 5′-OH AGG GAG GAC GAU GCG G 3′-OH
RL5D: 5′-OH AGG GAG GAC GAU GCG Gr(N)r(N) r(N)r(N)G 3′-OH
RL3: 5′-P GUG UCA GUC ACU UCC AGC GG 3′-puromycin
RL3(-P): 5′-OH GUG UCA GUC ACU UCC AGC GG 3′-puromycin

DNA primers (from Operon):

DP5: 5′-AGG GAG GAC GAT GCG G-3′
DP3: 5′-CCG CTG GAA GTG ACT GAC AC-3′

Solexa Fusion Primers (from Operon):

DSFP5: 5'-AATGATACGGCGACCACCGACTATGGATACTTAGTCAGGGAGGACGATGCGG- 3'
DSFP3: 5'-CAAGCAGAAGACGGCATACGACCGCTGGAAGTGACTGACAC- 3'

Solexa Sequencing Primer (from Operon):

SSP1: 5'-CTA TGG ATA CTT AGT CAG GGA GGA CGA TGC GG-3'