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iCLIP protocol

Procedure for investigating RNA-protein interaction using UV cross-linking.

UV Cross-Linking and Immunoprecipitation (CLIP)

Interest in RNA-protein interactions is booming as we begin to appreciate the role of RNA, not just in well-established processes such as transcription, splicing, and translation, but also in newer fields such as RNA interference and gene regulation by non-coding RNAs. CLIP is an antibody-based technique used to study RNA-protein interactions related to RNA immunoprecipitation (RIP), but differs from RIP in the use of UV radiation to cross-link RNA binding proteins to the RNA that they are bound to. This covalent bond is irreversible, allowing stringent purification conditions. Unlike RIP, CLIP provides information about the actual protein binding site on the RNA. Different types of CLIP exist, high-throughput sequencing-CLIP (HITS-CLIP), photoactivatable-ribonucleoside enhanced CLIP (PAR-CLIP), and individual nucleotide-resolution CLIP (iCLIP).

Here is a summary of the iCLIP protocol adapted from Konig et al. J. Vis. Exp. 2011. "iCLIP -Transcriptome-wide Mapping of Protein-RNA Interactions with Individual Nucleotide Resolution."

Further adapted from Huppertz et al. Methods. 2014. "iCLIP: Protein–RNA interactions at nucleotide resolution."

Protocol summary

1. UV irradiation to covalently cross-link *in vivo* protein-RNA complexes.

2. Cell lysis and partial RNA digestion.

3. Immunoprecipitation (IP) and dephosphorylation to purify the protein of interest together with the bound RNA.

4. Ligation of an RNA adapter to the 3' end of the RNA and radioactive labelling of the 5' end to allow for sequence specific priming of reverse transcription.

5. SDS-PAGE and membrane transfer to purify cross-linked protein-RNA complexes from free RNA.

6. RNA isolation from the membrane by proteinase K digestion of the protein to leave a polypeptide at the cross-link nucleotide.

7. Reverse transcription (RT) that truncates at the remaining polypeptide and introduces two cleavable adapter regions and barcode sequences.

8. Gel purification of cDNA by gel electrophoresis and size selection to remove free RT primer.

9. Ligation of primer to the 5' end of the cDNA: circularization and annealing of oligo-nucleotide to the cleavage site, then linearization to generate suitable templates for PCR amplification.

10. PCR amplification; high-throughput sequencing generates reads in which the barcode sequences are immediately followed by the last nucleotide of the cDNA. Since this nucleotide locates one position upstream of the cross-linked nucleotide, the binding site can be deduced with high resolution.

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iCLIP protocol

1. UV cross-linking of tissue culture cells

1.1. Remove media and add ice-cold PBS to cells (eg use cells grown in a 10 cm plate for three experiments and add 6 ml PBS).

1.2. Remove lid, place on ice and irradiate once with 150 mJ/cm^2 at 254 nm using a Stratalinker.

One or more negative controls should be maintained throughout the complete experiment. Knockout cells or tissue, as well as non-cross-linked cells, are good negative controls, while knockdown cells are not recommended.

Note. Optional 4-thiouridine pre-incubation and UV-A crosslinking could be used for certain proteins. 4-thiouridine enhances crosslinking of some proteins. (For this optional step follow the instructions below).

Optional – Thiouridine labeling and UV-A crosslinking (alternative to step 1.2)

• Add 50 µL of 4SU (stock concentration: 100 mM 4SU) to a 10 cm plate of cells cultured in 10 ml DMEM supplemented with FBS and pen strep to get a final concentration of 500 µM 4SU. Alternatively, add 10 µL of 4-thiouridine (stock concentration: 100 mM) to get a final concentration of 100 µM 4SU.

• Incubate cells with 4-thiouridine for 60 min at 500 µM or 8 h at 100 mM. Check cell viability.

• Aspirate the medium and add 6 mL of ice-cold PBS to cells growing in a 10 cm plate (enough for three immunoprecipitations). Remove the lid and place on ice.

• Irradiate once with 2 × 400 mJ/cm^2 in a Stratalinker 2400 with 365 nm bulbs, or an equivalent instrument.

1.3. Harvest cells with a cell scraper and transfer cell suspension to microtubes (eg 2 mL to each of three microtubes).

1.4. pellet cells (spin at top speed for 10 sec at 4°C), then remove supernatant.

1.5. Snap-freeze cell pellets on dry ice and store at -80°C until use.

The experiment could take up to a week. Avoid multiple cycles of freeze thaw.

1.6 Bead preparation

1.6.1 Add protein A or protein G beads (e.g. 100 µl magnetic beads per experiment) to a fresh microtube and wash beads 2x with lysis buffer.

1.6.2 Resuspend beads in lysis buffer (100 µL), add antibody (2-10 µg) and rotate tubes at room temperature for 30–60 min.

The amount of antibody required might need to be optimized. A no-antibody sample is a good negative control.

1.6.3. Wash beads 3x with lysis buffer (900 µL) and leave in the last wash until ready to proceed with the immunoprecipitation (step 3.1).

If an antibody is working in IP, this is a good indication that it will work in CLIP.

2. Cell lysis and partial RNA digestion

2.1. Resuspend cell pellet in lysis buffer with protease inhibitors (1 mL) and transfer to 1.5 mL microtubes.

Note. Here you can try an optional sonication step. Sonication shears the DNA, which releases proteins from the chromatin increasing recovery of protein–RNA complexes.

Optional – Sonication of samples

- Sonicate the sample on ice using a probe sonicator. The probe should not touch the tube sides to avoid foaming. Sonicate twice with 10 sec bursts at five decibels. Clean the probe by sonicating H_2O before and after sample treatment.

Or

- Use Bioruptor plus for five cycles with alternating 30 sec on/30 sec off at low intensity.

2.2. Add low RNase dilution (10 µL) and Turbo DNase (2 µL) to the cell lysate and incubate for exactly 3 min at 37°C, shaking at 1,100 rpm, then immediately transfer to ice.

2.3. Spin at 4°C at 22,000 g for 10 min and carefully collect the cleared supernatant (leave about 50 µL lysate with the pellet).

Each member of the laboratory should use their own set of buffers and reagents to easier identify potential sources of contamination. Ideal conditions for the RNase digestions may need to be optimized for every new batch of RNase.

3. Immunoprecipitation and dephosphorylation of RNA 3'ends

3.1. Remove lysis buffer from the beads (step 1.1.3) and add cell lysate (from step 2.3).

3.2. Rotate the samples for 2 h at 4°C.

3.3. Discard the supernatant, wash beads 2x with high-salt buffer (900 µL) and then 2x with wash buffer (900 µL). Rotate these washes for at least 1 minute at 4°C.

Optimization and stringent washing conditions are very important.

Note. In the case that standard iCLIP conditions are not stringent enough to specifically purify the protein of interest, a dual immunoprecipitation with urea denaturation can be used instead of the protocol described here. For more

information see Huppertz et al. Methods. 2014. "iCLIP: Protein–RNA interactions at nucleotide resolution."

3.4. Discard the supernatant, resuspend beads in PNK mix (20 µL) and incubate for 20 min at 37°C in a thermomixer.

3.5. Add wash buffer (500 µL), wash 1x with high-salt buffer and then 2x with wash buffer.

4. Linker ligation to RNA 3' ends and RNA 5' end labelling3. Immunoprecipitation and dephosphorylation of RNA 3'ends

4.1. Carefully remove the supernatant, resuspend beads in ligation mix A (20 µL) and incubate overnight at 16°C in a thermomixer.

4.2. Add wash buffer (500 µL), wash 2x with high-salt buffer (1 mL) and then 2x with wash buffer (1 mL). Carry out these washes with rotation at 4°C for 5 mins each.

4.3. Remove the supernatant, resuspend beads in hot PNK mix (4 µL) and incubate for 5 min at 37°C.

4.4. Remove the hot PNK mix and resuspend beads in 1x SDS-PAGE loading buffer (20 µL).

4.5. Incubate on a thermomixer at 70°C for 10 min.

4.6. Immediately place on a magnet to precipitate the empty beads and load the supernatant on the gel (see step 5).

5. SDS-PAGE and membrane transfer. Immunoprecipitation and dephosphorylation of RNA 3'ends

5.1. Load samples as well as a pre-stained protein size marker (5 µL) on a precast 4-12% Bis-Tris gel, and run the gel for 50 min at 180 V in 1x MOPS running buffer (according to manufacturer's instructions).

Gels with constant pH 7 are recommended.

5.2. Remove the gel front and discard as solid waste (contains free radioactive ATP).

5.3. Transfer the protein-RNA complexes from the gel to a nitrocellulose membrane using a wet transfer apparatus (transfer 1 h at 30 V depending on manufacturer's instructions).

5.4. Following the transfer, rinse the membrane in PBS buffer, then wrap it in clingfilm and expose it to a film at -80°C for 30 min, 1h and then overnight.

A fluorescent sticker next to the membrane later allows to align the film and the membrane. The success of the experiment can be monitored at the autoradiograph of the protein-RNA complex after membrane transfer.

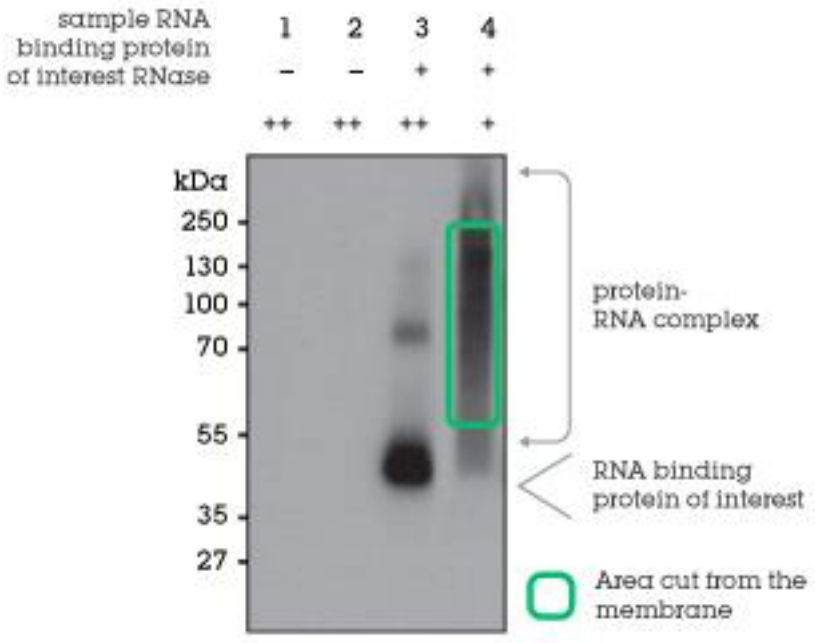

Figure 1: Schematic representation of a typical autoradiograph

Control experiments should give no signal on autoradiograph. In the autoradiograph of the low-RNase samples, diffuse radioactivity should be seen above the molecular weight of the protein. For high-RNase samples, this radioactivity is focused closer to the molecular weight of the protein.

6. RNA isolation

6.1. Isolate the protein-RNA complexes from the membrane using the autoradiograph from step 5.4 as a mask. Cut this piece of membrane into several small slices and place them into a 1.5 mL microtube.

6.2. Add PK buffer (200 µL) and proteinase K (10 µL) to the membrane pieces, and incubate shaking at 1,100 rpm for 20 min at 37°C.

6.3. Add PK urea buffer (200 µL) and incubate for 20 min at 37°C.

6.4. Collect the solution, add it together with RNA phenol/chloroform (400 µL) to a 2 ml Phase Lock Gel Heavy tube and incubate shaking at 1,100 rpm for 5 min at 30°C.

6.5. Separate the phases by spinning for 5 min at 13,000 rpm at room temperature.

6.6. Carefully transfer just the aqueous layer into a new tube.

6.7. Add glycoblue (0.75µL) and 3 M sodium acetate pH 5.5 (40 µL) and mix. Then add 100% ethanol (1 mL), mix again and precipitate over night at -20°C.

7. Reverse transcription

7.1. Spin for 20 min at 15,000 rpm at 4°C, then remove the supernatant and wash the pellet with 80% ethanol (0.5 ml).

7.2. Resuspend the pellet in RNA/primer mix (7.25 µL). For each experiment or replicate, use a different Rclip primer containing individual barcode sequences (see 11).

7.3. Incubate for 5 min at 70°C before cooling to 25°C.

7.4. Add RT mix (2.75 µL) and incubate 5 min at 25°C, 20 min at 42°C, 40 min at 50°C and 5 min at 80°C before cooling to 4°C.

7.5. Add TE buffer (90 µL), glycoblue (0.5 µL) and sodium acetate pH 5.5 (10 µL) and mix; then add 100% ethanol (250 µL), mix again and precipitate over night at -20°C.

8. Gel purification of cDNA

8.1. Spin down and wash the samples (see 7.1), then resuspend the pellets in water (6 µL).

8.2. Add 2x TBE-urea loading buffer (6 µL) and heat samples to 80°C for 3 min directly before loading on a precast 6% TBE-urea gel. Also load a low molecular weight marker for subsequent cutting (see below).

8.3. Run the gel for 40 min at 180 V depending on manufacturer's instructions. This leads to a reproducible migration pattern of cDNAs and dyes (light and dark blue) in the gel.

Note. You could also try a further phenol/chloroform extraction step here as carryover of urea and polyacrylamide can decrease CircLigase II efficiency. CircLigase II is present in ligation mix B (step 9.1).

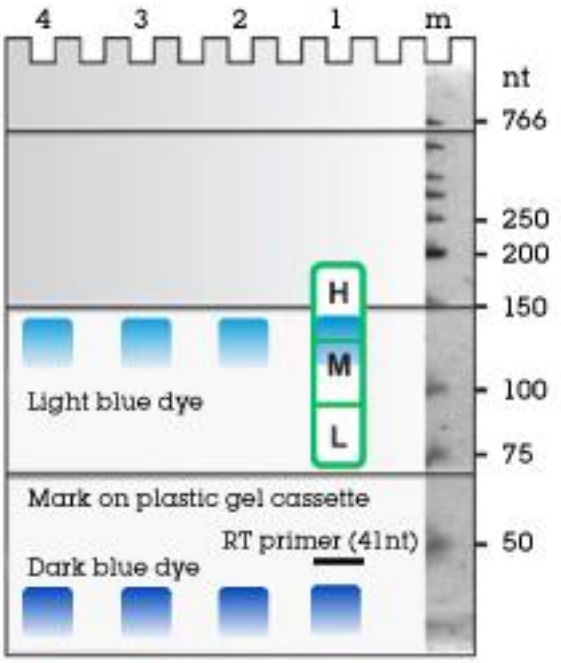

Figure 2: Schematic representation of migration pattern of cDNAs.

8.4. Use a razor blade to cut (red line) three bands of cDNA fractions at 120-200 nt (high (H)), 85-120 nt (medium (M)) and 70-85 nt (low (L)). Start by cutting in the middle of the light blue dye, divide the medium and low fractions and trim the high and low fractions. Use vertical cuts guided by the pockets and the dye to separate the different lanes (in this example 1-4). The marker lane (m) can be stained and imaged to control sizes after the cutting. Fragment sizes are indicated on the right.

8.5. Add TE (400 µL) and crush the gel slice into small pieces using a 1 mL syringe plunger. Incubate with shaking at 1,100 rpm for 2 h at 37°C.

8.6. Place two 1 cm glass pre-filters into a Costar SpinX column and transfer the liquid portion of the sample to the column. Spin for 1 min at 13,000 rpm into a 1.5 mL tube.

8.7. Add glycoblue (0.5 µL) and sodium acetate pH 5.5 (40 µL), then mix the sample. Add 100% ethanol (1 mL), mix again and precipitate over night at -20°C.

9. Ligation of primer to the 5'end of the cDNA

9.1. Spin down and wash the samples (see 7.1), then resuspend pellets in ligation mix B (8 µL) and incubate for 1 h at 60°C.

9.2. Add oligo annealing mix (30 µL) and incubate for 1 min at 95°C. Then decrease the temperature every 20 sec by 1°C until 25°C is reached.

9.3. Add BamHI (2 µL) and incubate for 30 min at 37°C.

9.4. Add TE (50 µL) and glycoblue (0.5 µL) and mix. Add sodium acetate pH 5.5 (10 µl) and mix, then add 100% ethanol (250 µL) and mix again, then precipitate over night at -20°C.

Avoid contamination with PCR products from previous experiments by spatially separating pre- and post-PCR steps. Ideally, analysis of PCR products and all subsequent steps should be performed in a separate room.

10. PCR amplification

10.1. Spin down and wash the samples (see 7.1), then resuspend the pellet in water (19 µL).

10.2. Prepare the PCR mix and run PCR programme:

94°C for 2 min,

[94°C for 15 sec, 65°C for 30 sec, 68°C for 30 sec] x25–35 cycles,

68°C for 3 min,

4°C forever.

The primer sequences used are for solexa sequencing, other systems may require adjustment of the primers.

10.3. Mix PCR product (8 µL) with 5x TBE loading buffer (2 µL) and load on a precast 6% TBE gel. Stain the gel with Sybrgreen I and analyze the PCR products with a gel imager; this allows monitoring of the success of the experiment prior to sequencing of the iCLIP library.

The gel image of the PCR products should show a size range that corresponds to the cDNA fraction (high, medium or low) purified in step 8.4. Note that the PCR primers P3Solexa and P5Solexa introduce an additional 76 nt to the size of the cDNA. Primer dimer product can appear at about 140 nt.

10.4. The barcode in the Rclip primers allow to multiplex different samples before submitting for high throughput solexa sequencing.

10.5. Submit 15 µL of the library for sequencing and store the rest.

Tip: Control experiments should give no products after PCR amplification, and high-throughput sequencing of control libraries should return very few unique sequences.

11. Linker and primer sequences

11.1. Pre-adenylated 3' linker DNA (aliquots of 20 µM of the DNA adapter): L3 /5rApp/AGATCGGAAGAGCGGTTCAG/3ddC/

11.2. Rclip reverse transcriptase primers with different barcodes (desalted and not gel-purified):

Rclip1 X33NNAACCNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip2 X33NNACAANNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip3 X33NNATTGNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip4 X33NNAGGTNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip5 X33NNCGCCNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip6 X33NNCCGGNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip7 X33NNCTAANNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip8 X33NNCATTNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip9 X33NNGCCANNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip10 X33NNGACCNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip11 X33NNGGTTNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip12 X33NNGTGGNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip13 X33NNTCCGNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip14 X33NNTGCCNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip15 X33NNTATTNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

Rclip16 X33NNTTAANNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC

11.3. X33 = 5' phosphate

11.4. Cut oligo: GTTCAGGATCCACGACGCTCTTCaaaa

11.5. P5Solexa:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

11.6. P3Solexa:
CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCC GATCT

12. Reagents

Lysis buffer

50 mM Tris-HCl, pH 7.4

100 mM NaCl

1% NP-40

0.1% SDS

0.5% sodium deoxycholate

Protease inhibitors (add fresh each time)

High-salt Wash

50 mM Tris-HCl, pH 7.4

1 M NaCl

1 mM EDTA

1% NP-40

0.1% SDS

0.5% sodium deoxycholate

Low RNase dilution High RNase dilution

1/500 RNase I dilutions for library preparation 1/50 RNase I dilutions to control for antibody

specificity

Wash buffer PNK mix

20 mM Tris-HCl, pH 7.4

10 mM $MgCl_2$

0.2% Tween-20

15 µL water

4 µL 5x PNK pH 6.5 buffer [350 mM Tris-HCl, pH 6.5; 50 mM $MgCl_2$; 5 mM dithiothreitol];

0.5 µL PNK enzyme

0.5 µL RNasin

Ligation mix A Hot PNK mix

9 µL water

4 µL 4x ligation buffer [200 mM Tris-HCl; 40 mM $MgCl_2$; 4 mM dithiothreitol]

1 µL RNA ligase

0.5 µL RNasin

1.5 µL pre-adenylated linker L3 [20 µM]

4 µL PEG400

0.4 µL PNK

0.8 µL 32P-γ-ATP

0.8 µL 10x PNK buffer

6 µL water

PK buffer PKurea buffer

100 mM Tris-HCl pH 7.4

50 mM NaCl

10 mM EDTA

100 mM Tris-HCl pH 7.4

50 mM NaCl

10 mM EDTA

7 M urea

RNA/primer mix RT mix

6.25 µL water

0.5 µL Rclip primer [0.5 pmol/µL]

0.5 µL dNTP mix [10 mM]

2 µL 5x RT buffer

0.5 µL 0.1M DTT

0.25 µL Superscript III reverse transcriptase

Ligation mix B Oligo annealing mix

6.5 µL water

0.8 µL 10x CircLigase Buffer II

0.4 µL 50 mM MnCl2

0.3 µL Circligase II

26 µL water

3 µL FastDigest Buffer

1 µL cut oligo [10 µM]

PCR mix

19 µL cDNA

1 µL primer mix P5/P3 solexa

10 µM each

20 µl Accuprime Supermix 1 enzyme

Useful references:

Konig et al. J. Vis. Exp. 2011. "iCLIP -Transcriptome-wide Mapping of Protein-RNA Interactions with Individual Nucleotide Resolution."

Huppertz et al. Methods. 2014. "iCLIP: Protein–RNA interactions at nucleotide resolution."