

11. Solutions

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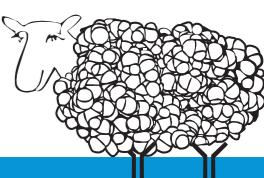
UV Cross-Linking and Immunoprecipitation (CLIP)

CLIP is an antibody-based technique used to study RNA-protein interactions.



RNA binding proteins are cross-linked to the RNA they are bound to with UV radiation, and CLIP provides information about the actual protein binding site on the RNA.

This leaflet contains an iCLIP protocol summary adapted from Konig et al. J. Vis. Exp. 2011



Lysis buffer:
50 mM Tris-HCl, pH 7.4
100 mM NaCl
1% Igepal CA-630
0.1% SDS
0.5% sodium deoxycholate
Protease inhibitors (add fresh each time)

High-salt buffer:
50 mM Tris-HCl, pH 7.4
1 M NaCl
1 mM EDTA
1% Igepal CA-630
0.1% SDS
0.5% sodium deoxycholate

Low RNase dilution:
1/500 RNase I dilutions for library preparation

High RNase dilution:
1/50 RNase I dilutions to control for antibody specificity

Wash buffer:
20 mM Tris-HCl, pH 7.4
10 mM MgCl₂
0.2% Tween-20

PNK mix:
15 µl water
4 µl 5x PNK pH 6.5 buffer [350 mM Tris-HCl, pH 6.5; 50 mM MgCl₂; 25 mM dithiothreitol];
0.5 µl PNK enzyme
0.5 µl RNasin

Ligation mix A:
9 µl water
4 µl 4x ligation buffer [200 mM Tris-HCl;
40 mM MgCl₂; 40 mM dithiothreitol]
1 µl RNA ligase
0.5 µl RNasin
1.5 µl pre-adenylated linker L3 [20 µM]
4 µl PEG400

Hot PNK mix:
0.4 µl PNK
0.8 µl ³²P-γ-ATP
0.8 µl 10x PNK buffer
6 µl water

PK buffer:
100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA

PKurea buffer:
100 mM Tris-HCl pH 7.4
50 mM NaCl
10 mM EDTA
7 M urea

RNA/primer mix:
6.25 µl water
0.5 µl Rclip primer [0.5 pmol/µl]
0.5 µl dNTP mix [10 mM]

RT mix:
2 µl 5x RT buffer
0.5 µl 0.1M DTT
0.25 µl Superscript III reverse transcriptase

Ligation mix B:
6.5 µl water
0.8 µl 10x CircLigase Buffer II
0.4 µl 50 mM MnCl₂
0.3 µl Circligase II

Oligo annealing mix:
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

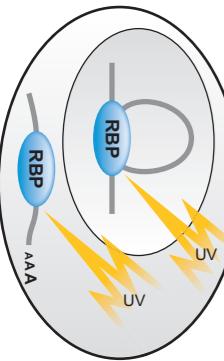
For a detailed protocol, please check out www.abcam/protocols, further information on the iCLIP protocol can be found at Konig, J., Zarnack, K., Rot, G., Curk, T., Kayikci, M., Zupan, B., Turner, D. J., Luscombe, N. M., Ule, J. "iCLIP - Transcriptome-wide Mapping of Protein-RNA Interactions with Individual Nucleotide Resolution." *J. Vis. Exp.* (50), e2638, DOI: 10.3791/2638 (2011).

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iCLIP Protocol

Bench guide summary protocol with all the important steps from cell lysis to sequencing...

UV crosslinking *in vivo*



1. UV cross-linking of tissue culture cells

1. Remove media, add ice-cold PBS to cells (e.g. for three experiments use cells from 10 cm plate, add 6 ml PBS).
2. Remove lid, place on ice, irradiate once with 150 mJ/cm² at 254 nm using a Stratalinker®.
3. Harvest cells with a cell scraper, transfer cell suspension to microtubes (3x 2 ml).
4. Pellet cells (spin at top speed, 10 sec, 4°C), then remove supernatant.
5. Snap-freeze cell pellets on dry ice, store at -80°C until use.

Tip: Knockout cells or tissue as well as non-cross-linked cells are good negative controls.

1.1. Bead preparation for later immunoprecipitation (IP)

- 1.1.1. Add protein A (or protein G) beads (e.g. 100 µl magnetic beads per experiment) to a fresh microtube, wash beads 2x with lysis buffer.
- 1.1.2. Resuspend beads in lysis buffer (100 µl), add antibody (2-10 µg), rotate tubes (room temperature, 30-60 min).
- 1.1.3. Wash beads 3x with lysis buffer (900 µl), leave in last wash until IP (step 3.1).

Tip: A no-antibody sample is a good negative control.

2. Cell lysis and partial RNA digestion

- 2.1. Resuspend cell pellet in lysis buffer (1 ml), transfer to 1.5 ml microtubes.
- 2.2. Add low RNase dilution (10 µl) and Turbo DNase (2 µl) to cell lysate, incubate (exactly 3 min, 37°C, 1,100 rpm shaking), then immediately transfer to ice.
- 2.3. Spin (4°C, 22,000 g, 20 min), carefully collect only cleared supernatant.

3. IP and dephosphorylation of RNA 3'ends

- 3.1. Remove lysis buffer from beads (step 1.1.3), add cell lysate (from step 2.3).
- 3.2. Rotate samples (2 h, 4°C).
- 3.3. Discard supernatant, wash beads 2x with high-salt buffer (900 µl), then 2x with wash buffer (900 µl).
- 3.4. Discard supernatant, resuspend beads in PNK mix (20 µl), incubate (20 min, 37°C).
- 3.5. Add wash buffer (500 µl), wash 1x with high-salt buffer, then 2x with wash buffer.

4. Linker ligation to RNA 3' ends and RNA 5' end labelling

- 4.1. Remove supernatant, resuspend beads in ligation mix A (20 µl), incubate (over night, 16°C).
- 4.2. Add wash buffer (500 µl), wash 2x with high-salt buffer (1 ml), then 2x with wash buffer (1 ml).
- 4.3. Remove supernatant, resuspend beads in hot PNK mix (8 µl), incubate (5 min, 37°C).
- 4.4. Remove hot PNK mix, resuspend beads in 1x SDS-PAGE loading buffer (20 µl).
- 4.5. Incubate (on thermomixer, 70°C, 10 min).
- 4.6. Immediately precipitate empty beads, load supernatant on gel (step 5).

5. SDS-PAGE and membrane transfer to remove free RNA

- 5.1. Load samples and pre-stained protein size marker (5 µl) on precast 4-12% Bis-Tris gel, run gel (50 min, 180 V, 1x MOPS running buffer).
- 5.2. Remove gel front, discard as solid waste (contains free radioactive ATP).
- 5.3. Transfer protein-RNA complexes from gel to a nitrocellulose membrane using a wet transfer apparatus (transfer 1 h, 30 V, depending on manufacturer's instructions).
- 5.4. Following transfer, rinse membrane in PBS buffer, then wrap it in clingfilm and expose it to a film at -80°C for 30 min, 1h and then over night.

6. RNA isolation

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

Tip: Control experiments should give no signal on autoradiograph.

- 6.2. Add PK buffer (200 µl) and proteinase K (10 µl) to membrane pieces, incubate (20 min, 37°C, 1,100 rpm shaking).
- 6.3. Add PKurea buffer (200 µl), incubate (20 min, 37°C).
- 6.4. Collect solution, add it together with RNA phenol/chloroform (400 µl) to a 2 ml Phase Lock Gel Heavy tube, incubate (5 min, 30°C, 1,100 rpm shaking).
- 6.5. Separate phases by spinning (5 min, 13,000 rpm, room temperature).
- 6.6. Carefully transfer just the aqueous layer into a new tube.
- 6.7. Add glycoblu (0.5 µl), 3 M sodium acetate pH 5.5 (40 µl), mix. Then add 100% ethanol (1 ml), mix again, precipitate (over night, -20°C).

7. Reverse transcription (RT)

- 7.1. Spin (20 min, 15,000 rpm, 4°C), then remove supernatant, wash pellet with 80% ethanol (0.5 ml).
- 7.2. Resuspend pellet in RNA/primer mix (7.25 µl). For each experiment or replicate, use different Rclip primer containing individual barcode sequences (see online protocol).
- 7.3. Incubate (5 min, 70°C), then cool to 25°C.
- 7.4. Add RT mix (2.75 µl), incubate (5 min, 25°C, then 20 min, 42°C, then 40 min, 50°C and 5 min, 80°C), then cool to 4°C.
- 7.5. Add TE buffer (90 µl), glycoblu (0.5 µl), sodium acetate pH 5.5 (10 µl), mix; then add 100% ethanol (250 µl), mix again, precipitate (over night, -20°C).

8. Gel purification of cDNA

- 8.1. Spin down, wash samples (see 7.1), then resuspend pellets in water (6 µl).
- 8.2. Add 2x TBE-urea loading buffer (6 µl), heat samples (80°C, 3 min) directly before loading on a precast 6% TBE-urea gel. Also load a low molecular weight marker.
- 8.3. Run gel (40 min, 180 V, depending on manufacturer's instructions).
- 8.4. Use a razor blade to cut three bands of cDNA fractions at 120-200 nt, 85-120 nt and 70-85 nt (see online protocol for details). Marker lane can be stained and imaged to control sizes after cutting.
- 8.5. Add TE (400 µl), crush gel slice with a 1 ml syringe plunger, incubate (2 h, 37°C, 1,100 rpm shaking).
- 8.6. Place two 1 cm glass pre-filters into a Costar SpinX column, transfer liquid portion of sample to column, spin (1 min, 13,000 rpm) into a 1.5 ml tube.
- 8.7. Add glycoblu (0.5 µl), sodium acetate pH 5.5 (40 µl), then mix sample. Add 100% ethanol (1 ml), mix again, precipitate (over night, -20°C).

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

- 9.1. Spin down, wash samples (see 7.1), then resuspend pellets in ligation mix B (8 µl), incubate (1 h, 60°C).
- 9.2. Add oligo annealing mix (30 µl), incubate (1 min, 95°C), then decrease temperature every 20 sec by 1°C until 25°C are reached.
- 9.3. Add BamHI (2 µl), incubate (30 min, 37°C).
- 9.4. Add TE (50 µl), glycoblu (0.5 µl), mix. Add sodium acetate pH 5.5 (10 µl), mix, then add 100% ethanol (250 µl), mix again, then precipitate (over night, -20°C).

10. PCR amplification

- 10.1. Spin down, wash samples (see 7.1), then resuspend pellet in water (19 µl).
- 10.2. Prepare PCR mix, run PCR programme: 94°C for 2 min, [94°C for 15 sec, 65°C for 30 sec, 68°C for 30 sec]_{25-35 cycles}, 68°C for 3 min, 4°C for ever.
- 10.3. Mix PCR product (8 µl) with 5x TBE loading buffer (2 µl), load on a precast 6% TBE gel. Stain gel with Sybrgreen I, analyse with a gel imager.
- 10.4. The barcode in Rclip primers allows to multiplex different samples before submitting for high throughput sequencing. Submit 15 µl of library for sequencing, store rest.

Tip: Control experiments should give no products after PCR amplification, and yield no unique sequences after high-throughput sequencing.

