

CLIP (*in vivo* Cross-Linking and ImmunoPrecipitation) method

I. general method for UV crosslinking of tissue/cell lines

For mice tissue:

Harvest forebrain / hindbrain from P8 mice (ICR strain); let tissue sit in ice cold HBSS until harvest is complete

(if you use more than 10 brains: Set up a 500 ml Stericup; cut out the cellulose filter and make a conical filter out of a sheet of 200 um nylon mesh, Pass tissue through the mesh using a flat-tip cell scraper; wash the tissue through the mesh with more PBS; the resulting cell suspension should be about 100 ml.)

If less than 10 brains are used, cells can be triturated using a 5ml pipette (a pipette tip can be added at the end).

Transfer the suspension to 10 or 50ml falcon tubes and spin at 2500 rpm 10' at 4°

Take off supernatant and resuspend cells in approximately 10 cell volume of HBSS

Irradiate suspension (using 10mls per 10 cm tissue culture plate) for 400mJ/cm² in Stratalinker

[For cell lines, grow cells in 150mm plates, rinse once with DMEM or PBS, and then add 10mls PBS/plate; then start method at the step above]

Collect suspension, and then add (3ml) HBSS to each irradiated plate to wash; collect this wash also

Pellet cells again at 2500rpm 10' at 4°; resuspend pellet in (~2x dry volume) of PBS

Distribute 1ml of suspension to each eppie; quick spin them at 4° and take off sup

Freeze pellets at -80° until use (each tube is about 250 µl of cells)

To prepare 1x HBSS add:

50 ml 10x Hank's Balanced salt solution, Ca-Mg-free, 10X Gibco, #14186-012

5 ml 1M HEPES, pH 7.3

445 ml ddH₂O

II. Immunoprecipitation

a. Solutions

1X PXL

1X PBS (tissue culture grade; no Mg⁺⁺, no Ca⁺⁺)

0.1% SDS

0.5% deoxycholate

0.5% NP-40

1X PNK+

50 mM Tris-Cl pH 7.4

10 mM MgCl₂

0.5% NP-40

b. Bead prep:

For each Eppie of crosslinked lysate prep use 300 μ l of protein A Dynabeads

Wash beads 3x with 1X PXL;

Resuspend beads in 240 μ l 1X PXL and add 60 μ l of rabbit anti-Nova.

Rotate tubes at room temp for 30-45 min.; wash each tube 3X with 1X PXL; if you are not yet ready to add crosslinked lysate, leave beads in last wash step

c. Crosslinked lysate workup

Resuspend each tube of crosslinked lysate using 400 μ l of 1X PXL; let sit on ice for 10 min.

Add 40 μ l RNAsin and 40 μ l of RQ1 DNase to each tube; incubate at 37° for 15 min, 1000 rpm.

Make a dilution of RNA T1 stock (1198 U/ μ l) at 1:500 in 1X PXL

Add 6 μ l of T1 dilution to each tube; incubate 37° for 10'

[Correct dilution of T1 is critical—you might need to do titrations of T1 to figure out what dilution will give you RNA CLIP tags of about 40-200 nt]

Spin lysates in pre-chilled micro-ultracentrifuge; 90K for 25' at 4°. (polycarbonate tubes in TLA 120.2 rotor)

[This step clears the lysate of all high-molecular weight material, like ribosomes, or very large RNPs.]

Carefully remove the supernatant and add supernatant to one prepared tube of beads; rotate the beads/lysate for 1 hour at 4°.

Wash with ice cold buffer:

2X w/ 1X PXL
3X w/ 1X PNK+

Resuspend beads in 80 µl of 1X PNK+ and add 10 µl of P³² γ-ATP and 10 µl of PNK enzyme

[This assures that all RNA has a 5' phosphate. Also, T4 PNK has a "resolving" activity that opens up the 2',3' cyclic phosphate at the 3' end of the RNA tags to give you some fraction of free 3' -OH. Cameron V, Uhlenbeck OC Biochemistry. 1977 Nov 15;16(23):5120-6. 3'-Phosphatase activity in T4 polynucleotide kinase.]

Incubate in themomixer at 37° and 1000 rpm for 20 min.
Finish reaction by adding 10 ul of 1 mM ATP. Let the reaction go another 5 min.

Wash 4X with 1X PNK+

Resuspend beads in 30 µl of 1X PNK+ and 30 µl of Novex loading buffer (DO NOT add any reducing agent) Incubate at 70° for 10 min at 1000 rpm. Isolate beads and take the sup for loading.

[The Novex 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 2 wells/tube of Novex NuPAGE 10% Bis-Tris gel

After gel run, transfer gel to S&S BA-85 nitrocellulose using the Novex wet transfer apparatus

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

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

Cut out the band corresponding to the MW of your protein, count in scintillation counter.

[Use a luminescent sticker so that you can align the filter back to the autorad. If strong radioactive band is seen after 1h exposure, you probably have enough RNA for the CLIP procedure. The protein will migrate ~5-10 kDa higher than normal due to the bound RNA tags. Because the digested RNase are of different sizes, ideally the resulting bands will be diffused within ~5kDa. A sharp radioactive band is a sign of overdigestion, meaning that the bound RNase are too small to be useful for CLIP assay.]

[The gel itself should be a lot less hot than the amount of radioactivity that you loaded. Most of the signal should be in the lower buffer. If there is still a lot of signal in the gel, it will likely be below a MW of about 20-15 kD. You can cut this off the gel before the transfer if your protein is greater than 30 kD.]



III. RNA isolation and purification

1X PK buffer
100 mM Tris-Cl pH 7.5
50mM 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 solution in 1X PK buffer; pre-incubate this stock at 37° for 20 min to kill any RNAses

[Use the film to allow you to cut out the corresponding piece of nitrocellulose using a scalpel blade. Cut this piece into as many smaller pieces as you can manage.]

Add 200 µl of protK solution to each tube of isolated NC pieces; incubate 20 at 37° at 1200 rpm

Add 200 µl protK/7M urea solution; incubate another 20 min at 37° at 1200 rpm

Add 400 µl “RNA phenol” and 130 µl of CHCl₃ to solution; 37° for 20' at **1400** rpm

[“RNA phenol” is pure phenol that has been equilibrated with 0.15M NaOAc pH 5.2, “CHCl₃” 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 and 1ml of 1:1 EtOH:isopropanol
(in case of small quantity of RNA, 0.5 µl of glycogen is necessary to precipitate it, but don't add more, otherwise the RNA ligase may be inhibited)

Ppt O/N at -20°

IV. RNA ligations

Spin down RNA. Check to see if you got decent precipitation of counts; if not, might have to add glycogen to tube.

Wash and dry pellet. Count RNA in scintillation counter. If possible save about 20% to run as an unligated control

RNA ligation:

1 μ l 10X T4 RNA ligase buffer (Fermentas)
1 μ l BSA (0.2 mg/ml)
1 μ l ATP (10 mM)
0.3 μ l T4 RNA ligase (3U, Fermentas)
1 μ l RL5 RNA linker @ 20 pmol/ μ l
5.7 μ l RNA resuspended in H₂O
10 μ l total

[It is important to add the RNA to the reaction last, after the L5 RNA linker. A vast excess of 5' RNA linker will ensure that the reaction is driven to a ligation between the 5' linker and the CLIP tag, instead of having the CLIP tag circularize. The linker itself cannot circularize (it is 5'-OH and 3'-OH), and the 5' linker-CLIP tag product cannot circularize.]

Incubate at 16° for 1 hour

Add 1 μ l RL3 RNA linker @ 40 pmol/ μ l, 0.5 μ l ATP (10 mM), 0.2 μ l T4 RNA ligase.

[As the RL3 linker is blocked at its 3' end with puromycin, it is only competent to ligate at its 5' end. The end result of the ligation will be RL5-CLIP tag-RL3 RNA and a prominent side reaction of RL5-RL3 linkers only. These will be removed by gel purification of the ligated products.]

Incubate 16° overnight

Add to reaction:

77 μ l H₂O
11 μ l 10X DNase I buffer
5 μ l RNasin
5 μ l RQ1 DNase
100 μ l total

Incubate 37° for 20 minutes

Add to reaction:

300 μ l H₂O
300 μ l "RNA phenol"
100 μ l CHCl₃

Vortex, spin and take aqueous layer

Precipitate by adding:
50 μ l 3M NaOAc pH 5.2
1 μ l glycoblue
1 ml 1:1 EtOH:isopropanol

Ppt. O/N @ -20°



V. Size purification of the ligated RNA

Spin, wash and dry RNA pellets; check recovery by counting in scintillation counter

Pour a 20% denaturing polyacrylamide gel (1:19 acrylamide)

Run entire RNA ligation on gel along with pre-ligation RNA; use hot phiX markers

[Autorad gel; the pre-ligation RNA should give a number for the modal size of the RNA tags—this number + 35 nt for the linkers should be the “sweet spot” for the ligated RNA. In general, I have found the modal size of the RNA tags to be about 50 nt. The smallest size I will take is about 60 nt, and I usually take a fraction of 60-100 nt and another of 100-200 nt. DO NOT use any material smaller than 60 nt- this fraction contains the 35 nt linker-linker dimer.]

Nucleic acid elution buffer

1 M NaOAc pH 5.2

1 mM EDTA

Cut out gel slices and place in Eppies; add 350 μ l of nucleic acid elution buffer and crush with a 1ml syringe plunger; incubate at 37° for 30 min at 1200 rpm

With a cut-off P1000 tip add gel slurry to a Costar SpinX column to which you have added a 1cm glass pre-filter.

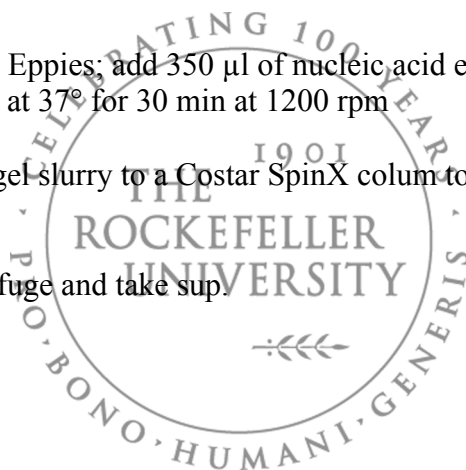
Spin full speed in microcentrifuge and take sup.

Add:

2 μ l glycoblue

1 ml 1:1 EtOH:isopropanol

Ppt. O/N



VI. cDNA and PCR

Spin, wash and dry RNA; count RNA again in scintillation counter

Resuspend RNA in 9 μ l H₂O and add 2 μ l of DP3@ 5 pmol/ μ l

Heat 65° for 5 min; chill and quick spin

Add:

2 μ l 10mM dNTPs

2 μ l 0.1 M DTT

4 μ l 5X SuperScript RT buffer

0.5 μ l RNAsin

0.5 μ l SuperScript II

Incubate at 42° for 30 min; then 90° for 5 min.

Chill

PCR reaction:

4 μ l 10X Pfu buffer

4 μ l DP5 primer, 5 pmol/ μ l

4 μ l DP3 primer, 5 pmol/ μ l

4 μ l radiolabelled DP5 primer

4 μ l 2.5 mM dNTPs

1 μ l Pfu

16 μ l water

3 μ l of the RT reaction

Cycle 30x:

94° 30''

61° 30''

72° 30''

Pour a 10% denaturing polyacrylamide gel, run entire PCR reaction; use hot phiX markers, autorad gel.

[There should be a sharp band at 35nt, and less sharp from 39-48 nt—these are all PCR products of the linker-linker ligation. The good bands should run in the same place as where you cut them out, 60-100 nt and 100-200 nt. It is possible that the larger fraction (the 100-200 nt fraction) will show products below 100 nt - this is okay.]

Cut out the major bands of ~60-200nt; purify as RNA (described in step V).
Resuspend the purified DNA in 10 μ l of water.



VII. Re-PCR:

PCR reaction:

10 μ l Accuprime Taq supermix

2 μ l DP5 primer, 5 pmol/ μ l

2 μ l DP3 primer, 5 pmol/ μ l

6 μ l gel-purified DNA

Cycle 15x:

94° 30''

61° 30''

72° 30''

Pour a 4% metaphor agarose gel, run entire PCR reaction, cut out the appropriate bands and extract DNA with QIAEX kit (Qiagen).



VIII. TOPO Cloning and sequencing

optional step to ensure that all DNAs have an A overhang:

Generate 3' A end:

3.5 μ l DNA

0.5 μ l 10x Taq buffer

0.5 μ l 10 mM dATP

0.5 μ l Taq polymerase (5U) incubate at 72° for 20'

place on ice and use immediately in the TOPO cloning reaction

TOPO clone:

2-4 μ l DNA

H₂O to 4 μ l

1 μ l salt solution

1 μ l pCR4-TOPO vector

mix gently and incubate min 5' at RT (store 3 μ l that you don't use in first day cloning at -20° for potential subsequent transformation)

For transformations, add 2 μ l of reaction into a vial of Top10 competent cells

10' on ice

30'' at 42°

2' ice

add 250 μ l SOC medium, 1h shaking at 37°

spread 10-50 μ l on amp plates (with IPTG & X-gal)

(make x-gal stock: 400mg X-Gal in 10ml dimethylformamide, store in dark vials at -20

make IPTG stock: 238 IPTG/10ml ddH₂O, filter sterilize, store at 4°

add 40 μ l of each X-gal and IPTG per plate)

next day pick the white colonies, miniprep the DNA

Sequence using M13 forward primer

Dilute primer to 5 μ M

Submit:

16 μ l miniprep DNA

2 μ l primer (10 pmole final)

VIII. Linker and primer sequences

[IT IS CRITICAL TO RIGOROUSLY GEL PURIFY THE RNA OLIGOS (AND THE DNA OLIGOS FOR THE FIRST PCR STEP)!!!!]

RNA linkers (from Dharmacon)

RL5: 5' -OH AGG GAG GAC GAU GCG G 3' -OH

RL3: 5' -P CGA GAU GGC GGC UUC CUG C 3' -puromycin

DNA primers (from Operon)

DP5: AGG GAG GAC GAT GCG G

DP3: GCA GGA AGC CGC CAT CTC G

