CLIP (*in vivo* Cross-Linking and Immuno-Precipitation) method

Day1

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

For mouse tissue:

Harvest tissue and let tissue sit in ice cold PBS until harvest is complete.

[In our case, 20mg of tissue suffices for high quality data using anti-Nova antibodies. If neuronal tissue is use, HBSS can be used instead of PBS.]

Add 10 cell volumes of PBS (or 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 100-400mJ/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 100-400mJ/cm^2 in Stratalinker.

[Cell culture can be used to express tagged proteins when immunoprecipitation from tissue is not possible due to lack of specific antibodies.]

[Because cells grown in a single layer are all equally exposed to UV light, a single crosslinking is sufficient.]

Collect suspension, pellet cells at 2500rpm for 3 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

II. Immunoprecipitation

Day2

a. Solutions

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

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 MgCl2
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 8.1.

[We use high pH here because this increases antibody capture by Protein A. However, you may need to adopt 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. If your antibody is mouse IgG1, you can pre-incubate the beads with rabbit antimouse IgG.]

Resuspend beads in 320 µl 0.1 M Na-phosphate pH 8.1 and add 80 µl of rabbit anti-Nova.

Rotate tubes at room temperature for 30-45 min.; wash 3x with wash buffer; if you are not yet ready to add crosslinked lysate, leave beads in last wash step.

c. Partial RNA digestion and ultracentrifugation

Resuspend each tube of crosslinked lysate using ~600 µl of wash buffer (~1ml total volume); let sit on ice for 10 min (if tissue is resistant to lysis, for example adult brain tissue, you may want to sonicate it at this step).

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 MgCl2 and 5 mM CaCl2). 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 wash buffer (high-RNAse).

Make a dilution of RNAse A (USB 70194Y) at 1:5000 in wash buffer (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. If you have only one tube of a lysate, divide it into two and continue with half the amounts. 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.]

[We use RNAse A, but other RNAses can also be used, such as RNAse T1 – in that case, about 50 fold more RNAse should be used.]

Add 10µl of each RNAse dilution to the two duplicate tubes; incubate at 37° for 10 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. Save also 20µl for TOTAL control ***[this can be used to clone the control RNA tags bound to the total pool of RBPs]***.

d. Immunoprecipitation

Add the rest of supernatant to one prepared tube of beads. Rotate beads/lysate mix for 1 hour 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 wash buffer
2x high-salt wash buffer ***(if this step decreases the efficiency of IP, you can omit it)***
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 (1:300000). Skip steps III and IV with the over-digest sample – leave it at 4° until step V.]

III. CIP Treatment (On-Bead)

CIP mix:

8 µl	10x dephosphorylation buffer (Roche, 712023)
3 µl	alkaline phosphatase (Roche, 712023)
69 µl	water
80 µl	total

Incubate in Thermomixer R (Eppendorf) at 37° for 10 min (1000 rpm every 3 minutes for 15 seconds)

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

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

[If your protein binds DNA, then

Linker mix:
8 μl 3' RNA linker @ 20 pmol/μl
32 µl water
40 μl total

Add 40 µl of linker mix to each tube of beads

Ligase mix:
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)
14 µl water
40 μl total

Add 40 µl of ligase mix to each tube of beads (final linker concentration is 2 pmol/μl)

Incubate at 16°C overnight in Thermomixer R (Eppendorf) (1000 rpm every 5 minutes for 15 seconds)

Day3
Wash 3X with 1X PNK buffer

V. PNK Treatment (On-Bead)

PNK mix:
8 µl 10X PNK Buffer (NEB)
2 µl P^{32}-γ-ATP
4 µl T4 PNK enzyme (NEB, M0201L)
66 µl water
80 µl total

Add 80 µl of PNK mix to each sample and incubate in Thermomixer R (Eppendorf) at 37° for 10 minutes (1000 rpm every 4 minutes for 15 seconds).

Add 10µl of 1mM ATP, and let the reaction go for an additional 5 minutes

Wash 3X with **1X PNK+**

VI. SDS-PAGE & nitrocellulose transfer

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.]

Mix 20 µl of TOTAL control (supernatant from IP) and 10 µl of Novex loading buffer

Incubate at 70° for 10 min at 1000 rpm and take the supernatant for loading.

[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.

[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.

[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.]

Day4

[In case of Nova, we usually see a signal on the autoradiogram after 30 minutes exposure if ~100mg tissue was used as starting material. If much less material is used, an overnight exposure will sometimes be necessary to see a decent signal. That's OK – with the current protocol, ~20mg of starting material, which needs an overnight exposure to detect the protein-RNA complex, still gives high quality data (only mouse sequences cloned).]

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.***
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.

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 (~3kDa wide) using a clean scalpel blade, and put the nitrocellulose pieces into separate microtubes. Count radioactivity in a scintillation counter.

From the TOTAL control lane, cut out a very wide band from 30kDa up to almost the top of the gel. Cut the band into small pieces and try to fit it into a single microtube.

8. ***This control should contain RNA tags bound to a wide spectrum of the cellular RNA-binding proteins. It should not contain free RNA with size less than 100kDa, because such RNAs migrate lower than 30kDa. This control tags can later be compared with the tags that were isolated as bound to your protein.***

VII. 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 (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 CHCl3 to solution; 37° for 20' at 1000 rpm

[RNA phenol can also be prepared by equilibrating pure phenol 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, 0.5 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°.

[Continue with protein specific tags to step IX (skip steps VIIIa-VIIId). TOTAL control sample is optional – it will give RNA tags bound to a large pool of RNA-binding proteins in your sample – these tags can later be used as a control for the tags specific for your protein. With the TOTAL control sample, include steps VIIIa-VIIId and then continue to step IX.]

VIII(a). Dephosphorylation (only for TOTAL control)

Spin down RNA (10' at max speed in microcentrifuge). Check to see if you got decent precipitation of counts; if not, might have to add glycogen to tube. Wash and dry pellet.

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

Count RNA in scintillation counter. Resuspend in 26.5 µl H_20.

dephosphorylation:
0.5 µl of alkaline phosphatase (20U/µl)
3 µl of NEB Buffer 3
26.5 µl of RNA

incubate 30' at 37°

Add to reaction:
300 µl H2O
300 µl "RNA phenol"
100 µl CHCl3

Vortex, spin and take aqueous layer

Precipitate by adding:
50 µl 3M NaOAc pH 5.2
0.5 µl glycogen
1 ml 1:1 EtOH:isopropanol

Ppt. 1h or overnight @ -20°

VIIIb. 3' RNA linker ligation (only for TOTAL control)

Spin down RNA. Wash and dry pellet. Resuspend in 8 µl of H_20. Save 1 µl for unligated control.

RNA ligation:
1 µl 10X T4 RNA ligase buffer (Fermentas)
1 µl BSA (0.2 mg/ml)
1 µl ATP (10 mM)
0.1 µl T4 RNA ligase (3U, Fermentas)
0.1 µl RL3 RNA linker @ 20 pmol/µl
6.8 µl RNA resuspended in H_20
10 µl total

[As the L3 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 tag-L3 RNA.]

Incubate at 16° for 1 hour or overnight (in a PCR tube, in PCR thermoblock)

Add to reaction:
200 µl H2O
300 µl "RNA phenol"
100 µl CHCl3

Vortex, spin and take aqueous layer

Precipitate by adding:
50 µl 3M NaOAc pH 5.2
0.5 µl glycogen
1 ml 1:1 EtOH:isopropanol

Ppt. 1h or overnight @ -20°

VIIIc. RNA 5' end phosphorylation (only for TOTAL control)

Spin down RNA. Wash and dry pellet. Resuspend in 7 µl of H_20

RNA phosphorylation:
1 µl 10X PNK buffer (NEB)
1 µl PNK enzyme
1 µl P^{32}-γ-ATP ATP
7 µl RNA
10 µl total

Incubate 20 min at 37° (in a PCR tube, in PCR thermoblock)

Add 1 µl cold ATP (10 mM) and incubate 10 more min at 37°

Add to reaction:
300 µl H2O
300 µl "RNA phenol"
100 µl CHCl3

Vortex, spin and take aqueous layer

Precipitate by adding:
50 µl 3M NaOAc pH 5.2
0.5 µl glycogen
1 ml 1:1 EtOH:isopropanol

Ppt. 1h @ -20°

VIIId. Size purification of the ligated RNA (only for TOTAL control)

Pour a 10% denaturing polyacrylamide gel (1:19 acrylamide)

Run entire ligated RNA on gel along with pre-ligation RNA; use hot phiX markers (Promega, E3511)

[Run the gel so that 24nt marker is at the bottom]

Autorad gel at –80° by supporting it on GelBond PAG Film, wrapping in Saranwrap, and using Stratagene luminescent markers for orientation

Nucleic acid elution buffer
1 M NaOAc pH 5.2
1 mM EDTA

Cut out a band from the gel corresponding to RNA size of 70-80 nucleotides (corresponding to 50-60 nucleotide insert size) and place in a microtube; add 350 µl 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 colum (Corning Incorporated, 8161) to which you have added a 1cm glass pre-filter (Whatman, 1823010).

Spin full speed in microcentrifuge and take sup.

Add:
0.5 µl glycogen
1 ml 1:1 EtOH:isopropanol

Ppt. O/N

IX. 5' RNA Linker Ligation

(Day5)

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 6µl H_20.

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 L5 RNA linker @ 20 pmol/µl
4.1 µl

Add 5.9 µl RNA resuspended in H_20
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 hour (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 H2O
300 µl "RNA phenol"
100 µl CHCl3

Vortex, spin and take aqueous layer

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

Precipitate overnight (or 1h) @ -20°.

X. RT-PCR.

Day6

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_20.

RT reaction:
Mix 8 µl of the ligated RNA and 2 µl of P3 (5 pmol/µl – i.e. 1 pmol/µl final concentration)

Heat 65° for 5 min; chill and quick spin

Add:
3 µl 3 mM dNTPs
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 30 min; 90° for 5 min., leave at 4°.

PCR reaction:
27 µl Accuprime Pfx Supermix (Invitrogen, 12344-040).
0.5 µl P5 primer, 30 pmol/µl
0.5 µl P3 primer, 30 pmol/µl
2 µl of the RT reaction
30 µl total

Settings:
95° 5'
Cycle 25-35x (depending on how much RNA you started with): 95° 20'' / 61° 30'' / 68° 20''
68° 5'

Day7
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)]

[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. For instance, we see a complex corresponding to a 35kDa protein bound to RNA - RNA that was bound to this protein is amplified by RT-PCR, but there is no overlap between the RNA bands that were bound to Nova or p35 protein.]

Cut out the DNA of 80-90nt, extract DNA with QIAEX II kit (follow protocol for polyacrylamide gel), and re-suspend in 20 µl of water.

Week2

XI. Concatemerization

PCR
95μl Accuprime Supermix pfx
1 μl P5BanIa (100 μM)
1 μl P3BanIa (100 μM)
3 μl Qiaex II purified RT-PCR product
100 μl total

Settings:
95° 5'
Cycle 15-20x (depending on how much DNA you started with): 95° 15'' / 60° 30'' / 68° 30''
68° 5'

Save 5 µl of the reaction to analyze on 2% agarose gel as "undigested". Bring volume up to 200 µl with water.

Extract and purify the DNA
Add 200µl Phenol/Chloroform (Sigma, P3803), vortex, spin at room temperature 5 min, collect the aqueous fraction, add 200µl CHCl3 (chloroform 49:1 with isoamyl alcohol), vortex, spin 5 min, collect the aqueous fraction.

Precipitate DNA (2 hours or overnight at -20°C) by adding 14 μl 5M NaCl (0.1M final) and 500 μl ethanol (2.5 volumes).

BanI Digest:
Spin down at max speed for 15' to pellet DNA, wash pellet 2x with 75% EtOH, dry pellet.

Re-suspend pellet in 80 μl of 1X NEBuffer 4. Add 4 µl of BanI Enzyme (20U/µl) and incubate at 37°C for 3 hours.

Bring volume up to 200 μl with water. Extract and purify the DNA. (Add 200µl Phenol/Chloroform (Sigma, P3803), vortex, spin at room temperature 5 min, collect the aqueous fraction, add 200µl CHCl3 (chloroform 49:1 with isoamyl alcohol), vortex, spin 5 min, collect the aqueous fraction. Precipitate DNA (2 hours or overnight at -20°C) by adding 14 μl 5M NaCl (0.1M final) and 500 μl ethanol.)

Ligation:
Spin down at max speed for 15' to pellet DNA, wash pellet 2x with 75% EtOH, dry.

Resuspend pellet in 67 μl H_2O, save 1.5 μl as "unligated"

65.6 μl BanI digest resuspended in H_2O
8 μl 10X T4 DNA Ligase Buffer (NEB)
1.2 μl P5BanIa (100 μM)
1.2 μl P3BanIa (100 μM)

Before adding the ligase, incubate the sample at PCR: 65°C for 10', 4 deg, keep on ice (in order to quench the short cleaved ends and prevent re-ligation to tags)

4 μl T4 DNA Ligase (2000U/μl) (NEB)
80 μl total

Incubate overnight at 16°C.

Next morning, add 1 µl of T4 DNA Ligase to tube and keep at 16°C for an additional hour.

Desalt on G-25 column (Amersham) and run on a 2% low melting (NuSieve) agarose gel. Separate well, cut out and purify the desired ligation concatemer product using Qiaex II.

XII. TOPO Cloning and Sequencing

Generate A-overhang:
3.9 μl DNA (Qiaex II extracted)
0.5 μl 10X Taq Buffer
0.5 μl 10mM dATP
0.1 μl Taq Polymerase 5U/μl (0.5U)
5 µl total

20' at 72° (in PCR cycler)

immediately proceed to cloning:
4 μl DNA
1 μl salt solution
1 μl pCR2.1-TOPO vector (Invitrogen, K450001)
6 µl total

mix gently and incubate 5-10' at room temperature (store 3 μl that you don't use in first day cloning at -20°C for potential subsequent transformation).

Transformations:
add 2 µl of reaction into a vial of Top10 competent cells
incubate 10' on ice, 30'' at 42°, 2' ice

add 250 μl SOC medium, 1h shaking at 37°
spread 10-50 μl on amp plates (before that, add 40μl of X-gal per plate)
(to make x-gal stock: 400mg X-Gal in 10ml dimethylformamide, store in dark vials at –20°C)

The next day pick the white colonies and isolate the DNA following the Quiagen miniprep protocol.

Sequence using M13F primer (GTAAAACGACGGCCAG)

XIII. Linker and primer sequences

[The RNA linkers need to be gel purified. Run 50µl 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):

L5: 5'-OH AGG GAG GAC GAU GCG G 3'-OH
L3: 5'-P GUG UCA GUC ACU UCC AGC GG 3'-puromycin

DNA primers (from Operon):

P5: 5'-AGGGAGGACGATGCGG-3'
P3: 5'-CCGCTGGAAGTGACTGACAC-3'

Concatemerization Primers (from Operon):

P5Ban1a: 5'-CAGCCAACAGGCACCAGGGAGGACGATGCGG-3'
P3BanIa: 5'-GACTAGCTTGGTGCCGCTGGAAGTGACTGACAC-3'
*Red: BanI Site (GGYRCC)