Binding of cellular nuclear proteins to GST-LANA fusion protein:

1. Prepare 1mg each of GST-LANA and GST-2TK proteins (see detail protocol at end)
2. Prepare 6mg nuclear extract from BC3 cells (use 2 billion cells) (see detail protocol at end)
3. Take 200ul **GST beads** only and 6mg of **nuclear extract** from BC-3 (making sure that glycerol concentration does not go above 10%, consider the glycerol present in the nuclear extract also)
4. Mix GST beads and nuclear extract in binding buffer and incubate at 4^0C for 2 hours with continuous rotation.
5. Centrifuge the content (GST beads + Nuclear extract) at 2000g/5min/4^0C and take the supernatant in a fresh tube (save the beads too)
6. Wash beads with same binding buffer 4 times by centrifugation 2000g/5min/4^0C and store as beads control.
7. Preclear with **GST-2TK beads** (400ug) for 4 hours at 4^0C with continuous rotation.
8. Centrifuge the content (GST-2TK beads + Nuclear extract) at 2000g/5min/4^0C and take the supernatant in a fresh tube (save the beads too)
9. Wash beads with same binding buffer 4 times by centrifugation 2000g/5min/4^0C and store as GST-2TK control-1.
10. Divide the supernatant from previous step into two and incubate half with **GST-LANA fusion protein** beads (800ug) and half with **GST-2TK beads** (800ug) overnight with continuous rotation.
11. Centrifuge thecontents at 2000g/5min/4^0C and take the beads.
12. Wash beads with same binding buffer 4 times by centrifugation 2000g/5min/4^0C.
13. Collect the beads and boil at 95^0C. Load in SDS-PAGE with proper controls.
14. Stain the gel with coomassie blue and excise the bands unique to GST-LANA lane.
15. Sequencing.

Binding Buffer

10mM Tris pH 7.5
2mM EDTA pH 8.0
150mM NaCl
Glycerol 10% final conc. desired
DTT 5mM
PMSF 1mM
NP-40 0.5%
Protease Inhibitor:
- Apotinin 5 ug/ml
- Pepstatin 5ug/ml
- Leucopeptin 5ug/ml

Preparing Nuclear Extracts*

*see buffer section for reagents

1) Collect between 20 – 100 million cells (Depends on specific experiment) and centrifuge at 1800-2000 rpm for 10 mins at 4C.
2) Aspirate media and wash once in cold PBS. **If pellet is larger than 250ul divide sample into multiple tubes.
3) Spin at 1800-2000 rpm for 10 mins at 4C.
4) Aspirate PBS and resuspend in 4 times the volume of the pellet in NE Buffer A. (500ul – 1ml).
5) Incubate on ice for 1 hour. Put one douncer for every sample on ice.
6) Transfer to douncer and homogenize with 25 strokes.
7) Transfer homogenized sample to 1.7ml eppendorf tubes and centrifuge at 2000rpm for 5mins at 4C.
8) Aspirate supernatant and resuspend the pellet in 1ml of NE Buffer A. Centrifuge at 2000rpm for 5mins at 4C.
9) Aspirate supernatant and resuspend the pellet in 2 times it volume of NE Buffer B.
10) Incubate on ice for 30mins. Centrifuge at 13000rpm for 20mins at 4C.
11) Transfer supernatant to fresh 1.7ml eppendorf tube and add an equal volume of NE Buffer C.
12) Aliquot into small volumes (100-200ul).
13) Save 1 tube of each sample for Western Blot and Bradford Assay. Snap freeze all other samples in a dry ice/ isopropanol bath. Store at -80C.

Bradford Assay

Mix:

795ul H2O
5ul Protein (NE)
200ul Bradford Reagent

Measure OD 595 or 600

Need to do standard curve with BSA to determine concentration of protein

NE Buffer A (add Protease Inh.)

10mM HEPES pH7.9 – 100ul 1M
10mM KCl – 100ul 1M
1.5mM MgCl2 – 15ul 1M
ddH2O – to 10 mls

NE Buffer B (add Protease Inh.)

20mM HEPES pH 7.9 – 200ul 1M
10% glycerol – 1 ml 100%
420mM NaCl – 840ul 5M
1.5mM MgCl2 – 15ul 1M

0.2 mM EDTA – 4ul 0.5M
ddH2O – to 10 mls

NE Buffer C (add Protease Inh.)

20mM HEPES pH 7.9 – 200ul 1M
30% glycerol – 3 ml 100%
1.5mM MgCl2 – 15ul 1M
0.2 mM EDTA – 4ul 0.5M
ddH2O – to 10 mls

Protease Inhibitors for 20ml Buffer

Aprotinin	40ul
Pepstatin A	20ul
Leupeptin	4ul
PMSF	200ul

GST-LANA protein preparation:--

1. Inoculate 3 ml LB+Amp [100ug/ml] with a single GST colony from a freshly streaked plate. Incubate overnight at 37^{o}C, 250 RPM.
2. The following morning, use 2.5ml of the overnight culture to inoculate 250 ml of LB + Amp [100ug/ml] (1:100 dilution). Incubate at 37^{o}C, 250 RMP until O.D.$_{600}$ = 0.6
3. Induce the translation of the GST protein by adding 0.5-1.0 mM IPTG (this quantity may vary depending on the fusion protein). Incubate at 37^{o}C, 250 RPM for 4 hours.
4. Pellet cells with a 5000 RPM spin for 10 minutes at 4^{o}C. Discard supernatant.
5. Place cell pellet on ice. It is important to keep cells cold from here on! Resuspend cells in 5ml STE buffer. Transfer cells to cold 30ml centrifuge tubes. Pellet cells again at 5000 RPM for 10 minutes at 4^{o}C. Discard supernatant.
6. Resuspend cells in 1.5ml NETN + PMSF+Aprotinin+Pepstatin. Set on ice for 15 minutes.
7. Add 75ul 1M DTT and 900ul 10% Sarkosyl in STE. Sonicate cells for one minute at setting #3. Sonicate another minute at setting #3. Keep cells on ice during sonication.
8. Pellet cell debris with 10,000 RPM spin for 10 minutes at 4^{o}C. Transfer supernatant to 6ml culture tube. Add 1.5ml 10% Triton-X 100 in STE and 100ul Glutathione Sepharose beads. Rotate at 4^{o}C for 2 hours.
9. Transfer sample to a 2ml microcentrifuge tube. Pellet beads with 3000 RPM spin for 3 minutes at 4^{o}C.
10. Wash beads 5 times in NETN + PMSF+Aprotinin+Pepstatin, aspirating the supernatant between washes. (Be careful not to aspirate out the beads!)
11. Resuspend beads in 250ul of NETN + PMSF+Aprotinin+Pepstatin.

12. Remove a sample of the beads (5ul) and place with Lysis loading buffer (10ul). Heat samples at 95oC for 10 minutes. Run a mini-SDS-PAGE with the appropriate percent acrylamide, use BSA controls to determine amount of protein.

Recipes

STE, pH 7.5	For 100ml:
100mM NaCl →	2ml 5M NaCl
10 mM Tris →	1ml 1M Tris, pH 7.5
1 mM EDTA→ 0.5M EDTA	0.2ml 0.5M EDTA

NETN, pH 8.0	For 100ml:
0.5% NP40→	0.5ml NP40
20mM Tris→	2ml Tris, pH 8.0
1 mM EDTA→	200 ul 0.5M EDTA
100 mM NaCl	2 ml 5 M NaCl

Add protease Inhibitors to NETN:
For every 100 ml, add:
1 ml 100mM PMSF
50ul Pepstatin
50ul Aprotinin