

Chromatin Immunoprecipitation

(submitted by Steve Innocente, Beggs Lab)

This protocol is adapted from *Current Protocols in Molecular Biology, Supplement 47, 1999*

Solutions/Buffers

- 37% Formaldehyde, • Glycine, • Protein-A or G Dynabeads,
- TE buffer pH 8 (1L = 10ml 1M Tris, 2ml 0.5M EDTA)

FA-1 lysis			Elution buffer			FA-2 lysis			ChIP Wash Buffer		
5ml	0.5M HEPES-KOH pH, 7.5	50mM		Tris-PH, 7.5	50mM	1.5ml	0.5M HEPES pH, 7.5	50mM		Tris pH 8.0	10mM
1.75ml	4M NaCl	140mM		EDTA	10mM	1875µl	4M NaCl	0.5M		LiCl	250mM
100µl	0.5M EDTA	1mM		SDS	1%	30µl	0.5M EDTA	1mM		EDTA	1mM
500µl	TritonX-100	1%				150µl	TritonX-100	1%		NP-40	0.5%
500µl	Sodium deoxycholate	0.1%				150µl	Sodium deoxycholate	0.1%		Sodium deoxycholate	0.5%
1 tablet	protease inhibitors (Mini)										
to 50ml with water. Make fresh			Store RT for 1 year			to 15ml with water. Make fresh			Store RT for 1 year		

Day1: Culture Growth and Crosslinking

1. Grow 200-400mls of yeast cells to an OD of 0.5-1.0 at 30°C (or suitable temperature). Take OD at time of harvest and make note of it, it will be needed later.
Use baffled flasks to increase oxygenation and rate of culture growth.
2. Add 5.4mls (for 200ml culture) or 10.8mls (for 400ml culture) of 37% formaldehyde (to a 1% final concentration) to culture in a drop-wise manner to crosslink DNA, RNA and proteins, shake gently at RT for 10 mins (or optimal time for crosslinking of selected protein).
Use formaldehyde that is fresh (<6months).
3. Add 30 (200ml culture) or 60 mls (400ml culture) of filter sterilised 2.5M Glycine. Shake at 23°C/RT for a further 5 mins to quench the crosslinking reaction.
Filter sterilisation of glycine will prevent fungal growth.
4. Transfer cells to a 500ml centrifuge pot and spin in JL 10.5 at 5000G for 10 mins at 4°C.
5. Resuspend pellet in 50 mls of ice-cold **PBS**, transfer to 50ml falcon tube,
6. Spin 3500rpm 5 min at RT (or 4°C, if desired).
7. Resuspend pellet in 20mls ice cold PBS by vortexing, spin 3500rpm 5min at RT (or 4°C).
8. Drain all PBS, invert and blot on tissue. Freeze pellet in -80°C till needed.
9. Add protease inhibitor tablet (e.g. one Complete tablet to 50ml FA lysis buffer) and PMSF (2mM) FA lysis buffer, place in -20°C freezer.

Day2/3: Sonication and Lysis

1. Defrost FA-lysis buffer with protease inhibitors on ice and keep cold.
2. Resuspend frozen cell pellet with 10mls FA-lysis buffer and spin 5 min.
3. Resuspend cell pellet in ice-cold FA-lysis culture to the following ratio: 250µl FA-lysis per 100 OD units of culture. Transfer to a 2ml sure-lock tube.
4. Add a 350µl volume of zirconia beads to cell solution. Screw down caps tightly (label tubes in a few places as sometime the labels can wear off).
The ratio of 350µl to 100 OD units of culture assumes lysis with zirconia beads in a vortexer. If using a bead-beater, add 1.0ml of lysis buffer and fill 1.5ml screw cap tube to top with zirconia beads.
5. Place in vortexer and vortex at full-speed for 45 min at 4°C.

Lysis with zirconia beads is quite efficient and should result in roughly 90% cell lysis in a vortexer. If using a bead beater, lysis is about 99%. Glass beads can also be used but they give about 15-25% lysis.

6. To separate the cell lysate from the zirconia beads, make a holder for the 1.5ml screw cap tube (place a 5ml syringe, with plunger removed, into a 15ml falcon tube).
7. Heat a 27G needle on Bunsen and puncture bottom of 1.5ml tube.
8. Place punctured tube in syringe holder in the falcon tube and pierce the lid also.
9. Spin samples in centrifuge at 3500rpm at 5 min at RT.
Important: If using a swinging-bucket centrifuge, check that the 1.5ml falcon-syringe holder with the 1.5ml screw cap tube will swing freely.
10. Ensure that all of the cell lysate has drained from the tube. If the hole is clogged, simply make another hole and spin again.
11. Transfer separated cell lysate to a fresh 1.5ml centrifuge tube.
12. Spin at 14000rpm 15mins 4°C and discard the supernatant.
This step may seem a bit odd, but the supernatant contains un-crosslinked proteins and non-chromatin associated proteins. The cell pellet contains the chromatin complexes. Quickly check the size of the cell pellet, a drastically smaller pellet in one sample can indicate incomplete lysis.
13. Add 1ml FA-lysis buffer and resuspend the pellet. Keep on ice.

Sonication

N.B. Sonication conditions differ for each sonicator, each probe and even for each strain. Conditions must be determined empirically. The sonication step is where most variability is introduced into the ChIP assay, be cautious to use exactly the same conditions each time to ensure reproducibility.

There are two schools of thought on sonication: One thinks that a few short high-power bursts are better. I prefer a gentler approach, numerous low-power sonication bursts. Both give similar sized DNA, but the low-power approach tends to give more usable protein and leads to better ChIP results. The protocol below details a low-power approach on a Branson Sonifier 250.

1. Prepare a wet-ice ethanol bath in a smallish square or rectangle chamber (10x15cm by 8cm high, I use a glass slide chamber). Place tubes into a floaty-thingy and place into ice-ethanol bath. Try to wedge the floaty-thingy so it doesn't move around and remains level.
2. Use micro-tip at 20% power, pulsed 10.0sec ON and 10.0sec for a total of 1:30min of sonication time (eg. total time that sample is actively sonicated).
3. Place chamber in sonicator booth and raise platform so the microtip is 2-3mm from the bottom of the 1.5ml tube. Ensure the probe is directly centred and not contacting the sides of the tube.
This should avoid foaming and maximise the force delivered by the sonicator.
4. Following sonication keep samples on ice and centrifuge 14000rpm, 4°C for 30 min.
5. Transfer supernatant to new 1.5 ml falcon tubes and pool the sonicated lysate from like samples.

6. Remove 20µl of lysate to check sonication fragment size (place in 500µl PCR tube). Add 460µl of Elution buffer and 20µl of Proteinase K (50mg/ml) and incubate overnight in a PCR block at 42°C for 2 hours followed by a hold at 65°C to reverse the protein-DNA crosslinks.
 - a. The next day add 5µl of RNase cocktail and incubate 1hr at 37°C.
 - b. Extract with 500µl of phenol/chloroform/isoamyl alcohol.
 - c. Precipitate with 500µl of isopropanol and 50µl 3M Sodium acetate. Spin and resuspend DNA in 20µl of water.
 - d. Run on a 1.5-2.0% agarose gel. Fragment sizes should be between 100-1000bp with the bulk being between 200-600bp.
7. Optional: Add 100µl of 50% protein-A and/or G sepharose/FA-1 lysis, rotate 4°C for 1hr to pre-clear sample. Spin samples in cold centrifuge at 1000G at 4°C, 5 min.
8. Determine the protein concentration of cell lysate using your method of choice.
9. Remove supernate and store at -80°C. This is the whole cell extract for use in IPs (WCE/pre-cleared)

Day 4: Immunoprecipitation

I have tried various types of Protein A and G to immunoprecipitate and have found that the Invitrogen Dynabeads gives the lowest signal to noise ratio (the cleanest IPs). I tend to use 15µl of a polyclonal antibody or an antibody directed to a protein tag. For histone antibodies (eg. H3diMeK4 from Upstate) I use 2.5µl per IP and for the RNA PolII (8WG16) antibody I use 7µg. 15µl of either Protein A or G Dynabeads is sufficient.

I. Pre-binding and Pre-blocking of Antibodies and Beads:

1. Remove enough beads for all IPs and place in magnetic rack.
2. Remove supernatant and wash beads with 1ml of FA-lysis buffer. Repeat twice more.
3. Place in magnetic rack again and remove supernatant. Resuspend beads in original volume with FA-lysis buffer.
4. Dilute antibody in 185µl of FA-lysis buffer into a 1.5ml tube. Add 15µl of Dynabeads giving 200µl total of beads/antibody.
5. Incubate antibody-bead complexes on a rotating wheel for 1hr at 4°C.
6. Place beads in magnetic rack, remove supernate and wash once with 500µl of FA-lysis buffer, resuspend beads in 150µl of FA-lysis buffer.
7. Add 150µl of blocking buffer to beads and incubate on a rotating wheel for a further 1hr at 4°C.
8. Place beads in magnetic rack, remove supernate and wash once with 500µl of FA-lysis buffer.
9. Resuspend beads in 200µl pre-blocking buffer (1ml FA-1 with 10µl glycogen (20mg/ml), 10µl BSA (20mg/ml) and 10µl tRNA (20mg/ml))
10. Pre-block beads on rotating wheel for 30-45min at RT.
11. Place beads in magnetic rack, remove supernate and wash once with 500µl of FA-1 lysis buffer.
12. Resuspend beads/antibody in 50µl of FA-lysis buffer.

II. Immunoprecipitation, Washes and Crosslink Reversal.

1. Add pre-bound and pre-blocked beads to 50µg of ChIP lysate diluted to 150µl with FA-lysis buffer.
2. Incubate on a rotating wheel at RT for 60-90min.
3. Centrifuge beads at 3000rpm RT, place in magnetic rack. Discard supernatant.
4. Resuspend beads in 1ml of FA-lysis buffer.
5. Wash the beads as follows, place in magnetic rack then allow beads to 'stick' to the magnetic side of the tube. To wash I just rotate the tube 180° in the rack allowing the beads to flow through the tube and stick to the other side. Rotate again and remove supernate. Wash as follows:
 - a. Twice with 1ml of FA-lysis buffer
 - b. Twice with 1ml High-salt FA-lysis buffer
 - c. Twice with 1ml LiCl wash buffer
 - d. Twice with 1ml TE
6. Resuspend beads in 495µl ChIP Elution buffer, add 5µl of Proteinase K (50mg/ml, Roche PCR grade) and transfer to a 500µl PCR tube.
7. For input sample add 50µg of ChIP extract to 495µl of elution buffer in a 500µl PCR tube. Add 5µl of Proteinase K (50mg/ml).
8. Reverse crosslinks by placing in PCR machine overnight at 42°C for 2hrs and 12hrs at, 65°C.
9. Purify DNA using Promega Wizard PCR cleanup kit following the manufacturer's directions.

The Promega Wizard kits are superior to Qiagen as they can bind 4 times the DNA and the membrane binding buffer is a 5X concentrate and allows for a single loading of the 500µl ChIP sample.
10. Elute DNA with 200µl of dH₂O containing 5µl of RNase A/T1 cocktail (Ambion).
11. Store samples at -20°C until needed.

- ChIP samples are now ready for end-point or quantitative PCR.