2005.6
Xiaobing
Gozani Lab

Chromatin Immunoprecipitation for in *vivo* histone binding Protocol (shortcutting methods from Rosen Lab, Farnham Lab and Abcam)

Cross-Linking

1. Cells (2×10^7) are grown at confluence for three days. Add formaledehyde directly to tissue culture media to a final concentration of 1% (271 µL of 37% formaldehyde (Sigma, catalogue # F-8775) into 10 ml medium, 680 µl per 25 ml medium). Incubate adherent cells on a shaking platform and suspension cells on a stir plate for 10 minutes at room temperature.

2. Stop the crosslinking reaction by adding glycine to a final concentration of 0.125 M (1 mL of 1.25 M stock in 10 mL medium). Continue to rock at room temp for 5 minutes.

3. For adherent cells, aspirate the media and rinse plates twice with cold 1X PBS. For suspension cells, centrifuge and wash cell pellet twice with cold 1X PBS.

4. **Optional:** *Add an appropriate volume (we use 5 mls per 500 cm2 dish) of 1X PBS* ***or*** *a 20% 1X trypsin/EDTA (tissue culture grade) solution in 1X PBS. Incubate at 37^0 C for 10 minutes if using trypsin (we have found this step useful for cells which are difficult to swell. Thus, for cell types that are easily swelled, this step may not be necessary).*

5. Scrape adherent cells from dishes into 1 ml PBS plus PI and PMSF (10 ul per ml). Centrifuge scraped adherent or suspension cells and wash pellet once with 1X PBS. Pellet cells by centrifuging at 2000 rpm for 5 min at 4°C and carefully remove supernatant.

   5.1 At this point cell pellets may be snap frozen in liquid nitrogen and stored at -80°C for subsequent sonication, IP reaction setup.

6 Resuspend cell pellet in 1ml cell lysis buffer plus the protease inhibitors. Incubate on ice for 10 minutes.

7. Microfuge at 5,000 rpm for 5 minutes at 4^0 C to pellet the nuclei.

Sonication/Chromatin shearing

8. Resuspend nuclei in **200 µl** nuclei lysis buffer plus protease Inhibitors. Incubate on ice for 10 minutes.

9. Sonicate chromatin to an average length of about 600 bp while keeping samples on ice.

   9.1 the time and number of pulses will vary depending on sonicator, cell type and extent of crosslinking.
   9.2 Setting: 10% output, 20-sec burst (0.5 sec pulse and 1 sec break) followed by 1-2 min cooling on ice, repeat 6-9 times to total sonication time of 2-3 min per sample. Brason Sonifier 250.

10. Microfuge at 13,000 rpm for 10-20 minutes at 4^0 C. Take the supernatant.

    10.1 At this point, chromatin can be snap frozen in liquid nitrogen and stored at -80^0 C for up to several months.

11. Transfer the supernatant into a new tube, dilute 5-fold in ChIP dilution buffer (to 1 ml).

Prescearing/Immunoprecipitation

12 [**Optional**] Preclear the sample is to add 80 µl of samon sperm DNA/protein A agarose slurry for 30 min at 4°C with agitation. Spin down (4000 rpm, 2 min) the beads and take the supernatant.

12.1 An alternative method to preclear chromatin by adding blocked Staph A cells. Use 10-15 uls of preblocked Staph A cells for every 1 X 10^7 cells that you started with. Incubate on a rotating platform at 4^0 C for 15 minutes, no longer. Microfuge at 14,000 rmp for 5 minutes.

13. Save back 100 µl of the total supernatant as 11% **total input control**.

14. The rest of the supernatant is divided into two fractions: one for a no antibody control and the second is incubated with 1-5 µg of antibody overnight at 4°C with rotation.

Wash and reverse crosslink

15 Add 30 µl of the salmon sperm DNA/protein A agarose 50% slurry, rotate for 1 hr at 4°C (or 60 µl FLAG-agarose M2 25% slurry).

16. Beads were then washed consecutively for 3-5 minutes on a rotating platform with 1 ml of each solution: 2x Low salt wash buffer---2x high salt wash buffer---1x LiCl wash buffer---2x TE buffer. For each wash, incubate samples on a rotating platform for 3 minutes then microfuge 4000 rpm 2 min.

17. After the last wash, microfuge and remove the last traces of buffer. Add proper

amount (180 µl) SDS-PAGE sample buffer, boiled at 96^{o}C for >30 min. Spin and save supernatant. Load 10-20 µl for PAGE.

Solutions

ChIP Cell Lysis buffer	**Stock**	**Vol for 1 L**
20 mM TrisHCl pH 8.0	1M	20 ml
85 mM KCl	3 M	28 ml
0.5% NP40	10%	50 ml
ddH2O		883 ml
Nuclei Lysis buffer		
50 mM Tris-Cl pH 8.0	1 M	50 ml
10 mM EDTA	0.5 M	20 ml
1% SDS	10%	100 ml
ddH2O		830 ml
ChIP Dilution buffer		
0.01% SDS	10%	1 ml
1.1% Trition X 100	10%	110 ml
1.1 mM EDTA	0.5 M	2.2 ml
20 mM Tris-Cl pH 8.0	1 M	20 ml
167 mM NaCl	4 M	41.8 ml
ddH2O		825 ml
Low salt wash buffer		
0.1% SDS	10%	10 ml
1% Trition X 100	10%	100 ml
2 mM EDTA	0.5 M	4 ml
20 mM Tris-Cl pH 8.0	1 M	20 ml
150 mM NaCl	4 M	37.5 ml
ddH2O		828.5 ml
High salt wash buffer		
0.1% SDS	10%	10 ml
1% Triton X-100,	10%	100 ml
2 mM EDTA,	0.5 M	4 ml
20 mM Tris, pH 8.0,	1 M	20 ml
500 mM NaCl	4 M	125 ml
ddH2O		741 ml
LiCl wash buffer		
0.25 M/0.5M LiCl	1 M	250 ml
1% NP40	10%	100 ml
1% deoxycholate,		10g
1 mM EDTA	0.5 M	2 ml
20 mM Tris, pH 8.0	1 M	20 ml
ddH2O		~600 ml

TE buffer		
10 mM TrisHCl 8.0	1 M	10 ml
1 mM EDTA	0.5 M	2 ml

To make ssDNA/protein A agarose

(Courtesy of Upstate Biotechnology)

1. Make 1 ml of sterile TE (10 mM Tris, pH 8/1 mM EDTA, pH 8).
2. Combine: 0.5 mg BSA (the one used for diluting antibodies)
   5 ul Sodium Azide (from a 5% stock solution)
3. Wash 200 ul of protein A beads (50% slurry catalog 16-125, Upstate Biotechnologies) twice using 15 ml of sterile TE.
4. Combine: 100 ul washed protein A packed beads
   40 ug salmon sperm DNA (sonicated)
   bring up to 200 ul using sterile TE/BSA/sodium azide solution
5. Rock 45 minutes at 4$_{o}$C.
6. Aliquot and store at 4$_{o}$C