Chromatin Immunoprecipitation (ChIPs) Protocol
(Farnham Lab)

This protocol is based upon protocols from Mark Biggin, Dave Allis and Richard Treisman plus a fair amount of trial and error. We have successfully used this protocol with NIH 3T3, Friend, HeLa, Raji and CHO cells. However, you may have to optimize conditions for your specific cell type.

Day 1

1. Add formaledehyde directly to tissue culture media to a final concentration of 1%. We generally use 2 x 107 cells per antibody per timepoint. Fewer cells can be used but usually results in a lower signal to noise ratio. Incubate adherent cells on a shaking platform and suspension cells on a stir plate for 10 minutes at room temperature. Crosslinking for longer periods of time (>30 minutes) tends to cause cells to form into a giant crosslinked aggregate that can not be efficiently sonicated.

2. Stop the crosslinking reaction by adding glycine to a final concentration of 0.125 M. Continue to rock or spin at room temp for 5 minutes.

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

4. 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 370 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. Following addition of trypsin or PBS, scrape adherent cells from dishes. If you have used trypsin, inactivate the trypsin by adding a small amount of serum. Centrifuge scraped adherent or suspension cells and wash pellet once with 1X PBS plus PMSF (10 ul per ml).

6 Resuspend cell pellet in cell lysis buffer plus the protease inhibitors PMSF (10 ul per ml), aprotinin (1 ul per ml) and leupeptin (1 ul per ml). The final volume of cell lysis buffer should be sufficient so that there are no clumps of cells. Incubate on ice for 10 minutes. Cells can also be dounced on ice with a B dounce several times to aid in nuclei release.

7. Microfuge at 5,000 rpm for 5 minutes at 40 C to pellet the nuclei.

8. Resuspend nuclei in nuclei lysis buffer plus the same protease inhibitors as the cell lysis buffer. Incubate on ice for 10 minutes.

9. Sonicate chromatin to an average length of about 600 bp while keeping samples on ice (the time and number of pulses will vary depending on sonicator, cell type and extent of crosslinking). Microfuge at 14,000 rmp for 10 minutes at 40 C. At this point,

chromatin can be snap frozen in liquid nitrogen and stored at -700 C for up to several months.

10. Carefully remove the supernatant and transfer to a new tube. Preclear chromatin by adding blocked Staph A cells. Use 10-15 uls of preblocked Staph A cells for every 1 X 107 cells that you started with.

11. Incubate on a rotating platform at 40 C for 15 minutes, no longer. Microfuge at 14,000 rmp for 5 minutes.

12. Transfer supernatant to a clean tube and divide equally among your samples. Be sure to include a "no antibody" sample. We also include a "mock" samples which contains 1X dialysis buffer instead of chromatin (no antibody and mock are critical to control for nonspecific interactions and DNA contamination of Ip and wash solutions....the final output of this experiment is analyzed by PCR). Adjust the final volume of each sample with IP dilution buffer plus protease inhibitors if necessary. Samples volumes should be between 200 and 500 uls. Add 1 ug of antibody to each sample.

13. Incubate on the rotating platform at 40 C for at least 3 hours. Overnight is fine. If you are using monoclonal antibodies, add 1 ug of an appropriate secondary antibody and incubate for an additional 1 hour.

Day 2

14. Add 10 uls of blocked Staph A cells to each sample. Incubate on the rotating platform at room temp for 15 minutes, no longer.

15. Microfuge samples. Save the supernatant from the "no antibody" sample as "total input chromatin".

16. Wash pellets twice with 1.4 mls of 1X dialysis buffer (**if you are using a monoclonal antibody, omit the sarkosyl**) and four times with 1.4 mls of IP buffer (**pH 8.0 for monoclonal antibodies**). For each wash, dissolve the pellet in 200 uls of buffer and use an additional 200 uls of buffer to wash the pipet tip. Add an additional 1 ml of buffer. For each wash, incubate samples on a rotating platform for 3 minutes then microfuge at 14,000 rmp for 3 minutes at room temp. Try to remove as much buffer as possible after each wash without aspirating the Staph A cells. **Efficient washing is critical to reduce background.**

17. After the last wash, microfuge and remove the last traces of buffer. Elute antibody/protein/DNA complexes by adding 150 uls of IP elution buffer. Shake on vortexer for at least 15 minutes at setting "vortex 3". Microfuge at 14,000 rpm for 3 minutes. Transfer supernatants to clean tubes. Repeat and combine both elutions in the same tube.

18. After the second elution, microfuge samples at 14,000 rpm for 5 minutes to remove

any traces of Staph A cells. Transfer supernatants to clean tubes. Add 1 ul of high concentration RNase A (10 mg per ml) and 5M NaCl to a final concentration of 0.3 M. Incubate samples in the 670 waterbath for 4-5 hours to reverse formaldehyde crosslinks. Add 2 and a half volumes of ethanol and precipitate at -200 C overnight.

Day 3

19. Microfuge samples at 14,000 rpm for 15-20 minutes at 40 C. Respin and remove residual ethanol. Allow pellets to air dry completely.

20. Dissolve each pellet in 100 uls of TE. Add 25 uls of 5X PK buffer and 1.5 uls of proteinase K to each sample. The "total" sample will be gunky and may have to be dissolved in a larger volume. Incubate in 450 waterbath for 1-2 hours.

21. Add 175 uls of TE to each sample. Extract once with 300 uls of phenol/chloroform/ isoamyl alcohol and once with 300 ul chloroform/isoamyl alcohol. Total input samples may need to be extracted twice.

22. Add 30 uls of 5M NaCl, 5 ugs of tRNA and 5 ugs of glycogen to each sample. Mix well then add 750 uls of ethanol. Precipitate in -200 C freezer overnight.

Day 4

23. Microfuge samples at 14,000 rpm for 20 minutes at 40 C. Allow pellets to air dry. Resuspend DNA in water or TE and analyze by PCR. We generally resuspend in Ip's in 30 uls and then dilute the "total" sample an additional 300 fold and use 2-3 uls for each PCR reaction.

Solutions

Cell Lysis buffer
5 mM PIPES pH 8.0
85 mM KCL
0.5% NP40
protease inhibitors

Nuclei Lysis buffer
50 mM Tris-Cl pH 8.1
10 mM EDTA
1% SDS
protease inhibitors

IP Dilution buffer

0.01% SDS
1.1% Trition X 100
1.2 mM EDTA
16.7 mM Tris-Cl pH 8.1
167 mM NaCl

1X Dialysis buffer
2 mM EDTA
50 mM Tris-Cl pH 8.0
0.2 % Sarkosyl (omit for monoclonal antibodies)

IP Wash buffer
100 mM Tris-Cl pH 9.0 (8.0 for monoclonal antibodies)
500 mM LiCl
1% NP40
1% deoxycholic acid

Elution buffer
50 mM NaHCO3
1% SDS

5X PK buffer
50 mM Tris-Cl pH 7.5
25 mM EDTA
1.25% SDS

Protease Inhibitors
100 mM PMSF in ethanol, use at 1:100
10 mg per ml aprotinin in 0.01 M HEPES pH 8.0, use at 1:1,000
10 mg per ml leupeptin in water, use at 1:1,000

Staph A Cells

Resuspend 1 gram of lyophilized Staph A cells (Boehringer Mannheim) in 10 mls of 1X dialysis buffer. Centifuge at 10,000 rpm for 5 minutes at 40 C. Repeat. Resuspend in 3 mls of 1X PBS plus 3% SDS and 10% BME. Boil for 30 minutes. Centifuge at 10,000 rpm for 5 minutes. Wash in 1X dialysis buffer and cetrifuge at 10,000 rpm for 5 minutes. Repeat. Resuspend in 4 mls of 1X dialysis buffer. Divide into 100 ul aliquots, snap freeze and store in liquid nitrogen.

To block Staph A cells

Thaw 1 tube (100 uls) of cells for approximately every 108 cells that you begin with. Add 10 of herring sperm DNA (10 mg/ml) and 10 uls of BSA (10 mg/ml) to each tube of Staph A cells. Incubate on the rotating platform at 40 C for at least 3 hours, overnight is fine. Before using, microfuge for 3 minutes. Remove supernatant and wash pellet twice in 1X dialysis buffer. Resuspend cells in a volume of 1X dialysis equal to the original starting volume.