ChIP Assay Protocol

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Formaldehyde cross-linking and chromatin immunoprecipitation assays of tissue culture cells are performed as described by Boyd and Farnham (MCB 29:8389-8399) and Y. Shang et. al. (Cell 103:843-852) with some modifications.

1. HC11 cells (2 x 10^7 in a 150 mm dish) are grown at confluence for three days and primed for 24 hr in Priming Media [0.5 M glutamine/5 μg/ml insulin/10% stripped donor horse serum/RPMI 1640 media] in the presence of 1 μg/ml hydrocortisone.

2. Following the addition of 1 μg/ml ovine prolactin for various times, cells are cross-linked by adding formaldehyde to a final concentration of 1% (0.68 ml of 37%/25 ml media) directly into the media and rocked for 10 min at room temperature.

3. Stop the cross-linking by adding glycine to a final concentration of 125 mM (3.75 ml of 1 M/25 ml media). Continue to rock at room temp for 5 min.

4. Wash the cell monolayers three times with ice-cold 1X PBS. Cells are then scraped into 1X PBS (1 ml) plus protease inhibitors and collected by centrifugation (700 x g for 4 min).

5. Cell pellets are resuspended in cell lysis buffer [5 mM Pipes (KOH), pH 8.0/85 mM KCl/0.5% NP-40] containing the following protease inhibitors 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM PMSF and incubated for 10 min on ice. The efficiency of cell lysis can be checked with trypan blue and if cells are not lysed, they can be dounced on ice with a type B homogenizer several times.

6. Nuclei are pelleted by centrifugation (5000 rpm for 5 min).

7. Resuspend nuclei in nuclear lysis buffer [50 mM Tris, pH 8.1/10 mM EDTA/1% SDS containing the same protease inhibitors as in cell lysis buffer]. Incubate on ice for 10 minutes.

8. Sonicate chromatin to an average length of about 600 bp while keeping samples on ice. For HC11 cells, I sonicate on power setting 5 using a Branson Sonifier 250 with a microtip in 20-sec bursts followed by 1 min of cooling on ice for a total sonication time of 3 min per sample. This procedure results in DNA fragment sizes of 0.3-1.5 kb.

9. Debris is cleared by centrifugation at maximum speed for 10 min at 4°C. At this point, the supernatant containing the chromatin can be snap frozen in liquid nitrogen and stored at -70°C for up to several months.

10. Transfer the supernatant to a new tube and dilute 5-fold in ChIP dilution buffer [0.01% SDS/1.1% Triton X-100/1.2 mM EDTA, 16.7 mM Tris, pH 8.1/167 mM NaCl plus protease inhibitors].

11. To reduce nonspecific background, pre-clear the sample with 80 μl of a salmon sperm DNA/protein A agarose slurry for 30 min at 4°C with agitation.

12. Pellet beads by a brief centrifugation and collect supernatant fraction.

13. Save back 20% of the total supernatant as total input control and process with the eluted Ips beginning with the cross-linking reversal step.

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

15. Collect immune complexes with 60 μl of the salmon sperm DNA/protein A agarose slurry for 1 hr at 4°C with rotation.

16. Beads were then washed consecutively for 3-5 minutes on a rotating platform with 1 ml of each solution:

a. low salt wash buffer [0.1% SDS/1% Triton X-100/2 mM EDTA, 20 mM Tris, pH 8.1/150 mM NaCl]
b. high salt wash buffer [0.1% SDS/1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl]
c. LiCl wash buffer [0.25 M LiCl/1% NP40/1% deoxycholate, 1 mM EDTA/10 mM Tris, pH 8.0]
d. twice in 1X TE buffer

17. Elute complexes by adding 250 μl of elution buffer [1% SDS/0.1 M $NaHCO_3$] to pelleted beads. Prepare buffer fresh each time. Vortex briefly to mix and 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. Reverse formaldehyde crosslinks by adding 1 μl 10 mg/ml RNase and 5 M NaCl to a final concentration of 0.3 M to the elutants and incubate in a 65°C water bath for 4-5 hours.
Add 2 1/2 volumes of 100% ethanol. Precipitate overnight at –20°C.

19. Pellet DNA and debris and resuspend in 100 μl of water. The input sample will be gunky. Add 2 μl of 0.5 M EDTA, 4 μl 1 M Tris, pH 6.5 and 1 μl of 20 mg/ml Proteinase K and incubate for 1-2 hours at 45°C.

20. Purify DNA using QiaQuick spin columns and elute in 50 μl/column 10 mM Tris, pH 8.0. Two μl of DNA is used in quantitative PCR reactions.

Supplemental Protocol: To make ssDNA/protein A agarose
(Courtesy of Upstate Biotechnology)

1. Make 100 ml of sterile TE (10 mM Tris, pH 8/1 mM EDTA, pH 8).

2. Combine: 50 mg BSA (the one used for diluting antibodies)
   0.5 ml Sodium Azide (from a 5% stock solution)
   Bring up to 47.5 ml with TE and filter sterize using a 0.2 μ filter)

3. Wash 20 ml of protein A beads (50% slurry catalog 16-125, Upstate Biotechnologies)
   twice using 15 ml of sterile TE.

4. Combine: 10 ml washed protein A packed beads
   4.0 mg salmon sperm DNA (sonicated)
   bring up to 20 ml using sterile TE/BSA/sodium azide solution
   rock 45 minutes at 4°C.

5. Aliquot and store at 4°C.