Chromatin immunoprecipitation under 3D-mimetic conditions Entered by Lixin Wang
Janes Lab Protocols 8/10/11

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1. Plate MCF10A-5E cells at 12,500 cells/cm^2 in 15-cm dishes under normal morphogenesis conditions (assay medium + 2% matrigel + 5 ng/ml EGF). Change medium every four days as with 3D culture.
   - Cells are seeded at high density because horizontal proliferation on the plate is minimal under these conditions; cells will proliferate as "mounds" on the tissue culture surface
   - Cultures can be maintained up to 10 days and roughly mimic the major stress programs of 3D acini; beyond this time, the cultures begin to diverge from 3D conditions
   - Each 15-cm plate is sufficient for one chromatin immunoprecipitation and a matched IgG control
2. On the day of interest, add methanol-stabilized 37% formaldehyde (Fisher Scientific #F79-500) directly into the culture medium to a final concentration of 1% and fix the cells at room temperature for 5–10 min.
   - *Beyond 10 minutes, the chromatin will be overfixed and methylene crosslinks will be difficult to reverse at the later stages*
3. Stop the fixation by adding 1/20 volume of 2.5 M glycine for 5 min at room temperature.
4. Wash the plates twice with cold PBS. Scrape the cells into the tube with 1 ml cold PBS
5. Centrifuge at 140 rcf for 3 min at 4°C.
6. Resuspend the cell pellet with 300 µl of lysis buffer and incubate on ice for 10 min.
7. Sonicate for six 8-min cycles of 25-sec pulses with 35-sec intervals at 4°C.
   - *Tubes must be kept on ice throughout the sonication to avoid overheating the chromatin*
   - *This sonication profile on a Bioruptor creates genomic fragments 750–800 bp in length*
8. Centrifuge for 20 min at 14,000 rcf at 4°C and collect the supernatant.
   - *After centriguation, set aside 20 µl of the supernatant as the input chromatin fraction*
9. Dilute the remaining soluble chromatin tenfold in dilution buffer.
   - *The purpose of the dilution buffer is to reduce the concentration of SDS and improve the chances of a successful immunoprecipitation*
10. Perform immunoclearing by incubating 1 ml soluble chromatin with 50 µl Protein A Plus Ultralink Resin (Thermo Scientific, #53142) for 2 hrs at 4°C.
11. Spin at max speed on a benchtop centrifuge for 1 min and collect supernatant.
12. Split the precleared chromatin into two tubes.
13. Add specific antibody of interest to one tube and an equal amount of normal IgG to the other tube as a matched control
14. Incubate overnight at 4°C on the nutator.
15. Add 50 µl Protein A Plus Ultralink Resin and incubate for 2–4 hrs at 4°C on the nutator.
16. Centrifuge at 300 rcf for 2 min at 4°C and aspirate the supernatant.
17. Wash beads sequentially for 10 min on ice with:
    a. 1 ml RIPA (once)
    b. 1 ml RIPA supplemented with 500 mM NaCl (three times)
    c. 1 ml LiCl buffer (two times)
    d. 1 ml TE buffer (two times)
18. Add 500 µl elution buffer to the beads and incubate at 65°C overnight,
    - *Remember to reverse the crosslinks from the 20ul Input fraction collected in Step #8 with 480ul elution buffer*
19. Centrifuge at 300 rcf for 3 min and save the supernatant.
20. Add RNase (Sigma #R5503) to a final concentration of 100 µg/ml and incubate at 37°C for 30 min.
21. Add proteinase K (Sigma #P2308) to a final concentration of 200 µg/ml and incubate at 56°C for 90 min.
22. Add 500 µl phenol-chloroform in a fume hood. Vortex thoroughly and spin at max speed on a benchtop centrifuge for 1 min.
23. Transfer about 450 µl of the aqueous (top) fraction to a new tube, add 50 µl of 3 M NaOAc (pH 5.2), and 1 µl 20 mg/ml glycogen (Invitrogen #10814-010). Vortex.

24. Add 1 ml ice-cold 100% EtOH, vortex, and incubate at –20°C for at least 30 min.
25. Spin for 20 min at max speed on a benchtop centrifuge.
26. Carefully aspirate supernatant and wash pellet with 500 µl 70% EtOH at room temperature.
27. Spin for 1 min at max speed on a benchtop centrifuge.
28. Carefully aspirate supernatant and remove residual EtOH by hand with a pipette tip.
29. Air dry pellets for 5–10 min at room temperature.
30. Dissolve pellet in 40-200 µl water.
31. Quantify genomic loci by quantitative PCR (see Janes_RTqPCR.pdf).

Buffer recipes

- **Lysis buffer**
  50 mM Tris.HCl (pH 8.0)
  1% SDS
  5 mM EDTA
  10 μg/ml aprotinin (from 10 mg/ml stock in water; stored at –20°C)
  10 μg/ml leupeptin (from 10 mg/ml stock in water; stored at –20°C)
  1 μg/ml pepstatin (from 1 mg/ml stock in MeOH; stored at –20°C)
  1 mM PMSF (from 100 mM stock in isopropanol; stored at –20°C)

- **Dilution buffer**
  20 mM Tris.HCl (pH 8.0)
  1% Triton X-100
  2 mM EDTA
  150 mM NaCl
  10 μg/ml aprotinin (from 10 mg/ml stock in water; stored at –20°C)
  10 μg/ml leupeptin (from 10 mg/ml stock in water; stored at –20°C)
  1 μg/ml pepstatin (from 1 mg/ml stock in MeOH; stored at –20°C)
  1 mM PMSF (from 100 mM stock in isopropanol; stored at –20°C)

- **RIPA buffer**
  50 mM Tris-HCl (pH 7.5)
  150 mM NaCl
  1% Triton X-100
  0.5% sodium deoxycholate
  0.1% SDS
  5 mM EDTA
  10 μg/ml aprotinin (from 10 mg/ml stock in water; stored at –20°C)
  10 μg/ml leupeptin (from 10 mg/ml stock in water; stored at –20°C)
  1 μg/ml pepstatin (from 1 mg/ml stock in MeOH; stored at –20°C)
  1 mM PMSF (from 100 mM stock in isopropanol; stored at –20°C)

- **LiCl buffer**
  10mM Tris-Cl (pH 8.0)
  1% NP-40
  1% sodium deoxycholate
  1 mM EDTA
  0.25 M LiCl

- **Elution buffer**
  10mM Tris-Cl (pH 8.0)
  0.5% SDS
  1 mM EDTA
  200 mM NaCl