Chromatin Immunoprecipitation from paraffin-embedded pathology tissue

1. Collect from one to four FFPE tissue sections per 1.5-ml tube.
2. Start the deparaffinization by applying 1 ml of xylene solution and incubate for 10 min at RT.
3. Centrifuge at 17,860g (15,000 r.p.m.) for 3 min at RT.
4. Carefully discard the supernatant and repeat Steps 2–4 four more times if necessary.
5. Resuspend the deparaffinized tissue in 1 ml of absolute (100%) ethanol and incubate for 10 min at RT.
6. Centrifuge at 17,860g for 5 min at 4 °C. Carefully discard the supernatant and resuspend the pellet in 1 ml of 95% (vol/vol) ethanol solution. Incubate for 10 min at RT.
7. Repeat Step 6 four more times, progressively increasing the percentage of water (by using 70%, 50% and 20% (vol/vol) ethanol solutions and, at the end, Milli-Q water).
8. Resuspend the rehydrated material in 500 µl of lysis buffer and incubate (in a rotating platform at 20 r.p.m.) for 30 min at RT.
9. Centrifuge at 17,860g for 3 min at 4 ^{o}C. Carefully aspirate and discard the supernatant and resuspend the pellet in 200 µl of digestion buffer.
10. Sonicate the sample three times for 30 s in an ice bath; cool the sample for 1 min between pulses.
11. Dilute 10 µl of the sonicated sample in 500 µl of distilled water and measure the absorbance spectrophotometrically at 260nm (A260). Quantify the amount of chromatin/DNA. The spectrophotometric measurement of the amount of chromatin/DNA at this step is an approximate evaluation of the material; it is used for the subsequent digestion with MNase (Step 12).
12. Add 1 U of MNase to every 10 µg of preheated chromatin sample (from Step 10, preheated for 5 min to 37 °C in a water bath) and incubate for 1 min at 37 °C. Stop the nuclease activity immediately by adding 25 µl of 0.5 M Na-EDTA (final concentration of 12.5 mM) and place the sample on ice.
13. Centrifuge at 17,860g for 3 min at 4 °C. Carefully aspirate the supernatant and resuspend sections in 200 µl of extraction buffer.
14. Sonicate the sample three times in an ice bath; cool the sample for 1 min between pulses.
15. Remove debris by centrifuging at 9,500g (10,000 r.p.m.) for 5 min at RT. Collect 170 µl of supernatant containing purified chromatin and store in a clean 1.5-ml tube at 4 °C.

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^{o}C with agitation.
12. Pellet beads by a brief centrifugation and collect supernatant fraction.

13. Save back 10% 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 elutions 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

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 µl 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.

Buffers:

Lysis buffer (fresh)

Mix 1 ml of TBS (10× stock solution), 250 µl of Tween-20 (20% (vol/vol) stock solution), 10 µl of RNAse A (stock solution 10 mg ml−1 in Milli-Q water), 10 µl of PMSF (stock solution, 100 mM in isopropanol) and fill with Milli-Q water to a final volume of 10 ml. Keep on ice until use. Final concentrations: 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% (vol/vol) Tween-20, 10 µg ml−1 RNAse A and 0.1 mM PMSF.

Critical: PMSF must be added just before use.

Digestion buffer (fresh)

Mix 1.1 g of sucrose, 500 µl of Tris-HCl (pH 7.4; 1 M stock), 40 µl of $MgCl_2$ (1 M stock), 10 µl of $CaCl_2$ (1 M stock) and 10 µl of PMSF (100 mM stock). Fill with Milli-Q water to a final volume of 10 ml. Keep on ice until use. Final concentrations: 50 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.32 M sucrose and 0.1 mM PMSF.

Critical: PMSF must be added just before use.

Extraction buffer (fresh)

Mix 5 ml of TBS (10× stock) and 500 µl of SDS (10% (wt/vol) stock); fill with Milli-Q water to a final volume of 50 ml. Final concentrations: 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% (wt/vol) SDS and 0.1 mM PMSF.

Critical: PMSF must be added just before use.