**iChIP / enChIP-SILAC**

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0. Cell culture

(1) Culture cells to be analyzed and negative control cells in Heavy and Light condition medium (SILAC Protein Quantitation Kit, Thermo Fisher Scientific; 13C6 15N4 L-Arginine-HCl, Thermo Fisher Scientific (if necessary)), respectively. 5 x 107 cells each (e.g. Ba/F3, DT40) are prepared.

At least 5 cell passages are necessary to label proteins in Heavy condition medium.

1. Formaldehyde crosslinking of cells

(1) Mix labeled (Heavy) and non-labeled (Light) cells. Total 1 x 108 cells.

(2) Add 37% formaldehyde for 1% final concentration into the culture medium containing the cells. Incubate at 37 °C for 5-10 min (usually 5 min).

(3) Stop crosslinking by adding 1.25 M Glycine solution for 127 mM final concentration. Incubate at room temperature for 10 min.

(4) Collect cells by centrifugation (1,300 rpm, 4 °C for 5 min).

(5) PBS wash twice. Collect the pellet (cells). Note that the cells can be stored at -80 °C.

2. Preparation of chromatin

(1) Suspend the fixed cells in 50 ml of CLB. Incubate on ice for 10 min.

(2) Centrifuge at 2,000 rpm, 4 °C for 8 min. Discard carefully the supernatant.

(3) Suspend the pellet in 50 ml of NLB. Incubate on ice for 10 min. Vortex every 2-3 min.

(4) Centrifuge at 2,000 rpm, 4 °C for 8 min. Discard carefully the supernatant.

(5) Suspend the pellet in 50 ml of PBS. Centrifuge at 2,000 rpm, 4 °C for 10 min. Collect the pellet as the chromatin fraction. Note that the chromatin fraction can be stored at -80 °C after immediate freezing in liquid nitrogen.

3. Sonication of chromatin

(1) Suspend the collected chromatin fraction in 4 ml of MLB3. Transfer the suspension into five 1.5 ml microtubes (800 μl x 5).

(2) Sonication of the chromatin using Ultrasonic disruptor UD-201 (TOMY SEIKO). Condition is as follows:

Output: 3

Duty: 100%

10 - 18 cycles of sonication for 10 sec and cooling on ice for 20 sec

Keep the position of the tip of the sonication bar approximately 0.5 cm away from the tube bottom.

(3) Centrifuge at 13,000 rpm, 4 °C for 10 min. Collect the supernatant in a 15 ml tube (Total 4 ml). Note that the supernatant can be stored at -80 °C after immediate freezing in liquid nitrogen.

4. Reverse crosslinking (Evaluation of sonication of chromatin)

(1) Suspend 10 μl of the fragmented chromatin in 85 ul of distilled water.

(2) Add 4 μl of 5M NaCl. Incubate 65 °C overnight.

(3) Add 1 μl of 10 mg/ml RNase A. Incubate 37 °C for 45 min.

(4) Add 2 μl of 0.5M EDTA (pH 8.0), 4 μl of 1M Tris (pH 6.8), and 1 μl of 20 mg/ml Proteinase K. Incubate 45 °C for 1.5 h.

(5) Pick up 10 μl for electrophoresis in 1% agarose gel without ethidium bromide. 100 V for 30 min.

(6) Gel staining with ethidium bromide or other substitutes for 0.5-1 h.

5. Preparation of Dynabeads conjugated with antibody

(1) Transfer 1500 μl Dynabeads-protein G (Invitrogen) in six new 2 ml tubes (250 μl x 6; 3 is for anti-FLAG, 3 is for control mouse IgG).

(2) Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.

(3) Add 1.5 ml PBS with 0.01% Tween-20, each tube. Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.

(4) Repeat the step (3).

(5) Add 1.5 ml PBS with 0.01% Tween-20 and 0.1% BSA, each tube.

(6) Add 25 μg antibody (anti-FLAG antibody or control mouse IgG) per tube (total 25 μg x 3, respectively). Rotate 4 °C overnight.

(7) Centrifuge briefly. Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.

(8) Add 1.5 ml PBS with 0.01% Tween-20, each tube. Invert several times and centrifuge briefly. Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant by pipetting.

(9) Repeat the step (8), twice. The Dynabeads are ready for the next step.

6. Chromatin immunoprecipitation

(1) Add 500 μl of 10% Triton X-100 (in MLB3) and 500 μl of MLB3 into 4 ml fragmented chromatin.

(2) Transfer the chromatin solution into three tubes, in which the Dynabeads conjugated with control IgG were prepared at the step 5-(9) (1670 μl x 3 tubes). Rotate 4 °C for 1h.

(3) Put the tubes on a magnet stand and wait for 3 min.

(4) Transfer the supernatant into the three tubes, in which the Dynabeads conjugated with anti-FLAG antibody were prepared at the step 5-(9). Rotate 4 °C overnight.

(5) Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant using a pipetting.

(7) Wash 1: Add 1.5 ml of LSB. Rotate 4 °C for 5 min. Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant using a pipetting. Repeat wash, again.

(8) Wash 2: Repeat the step (7) with HSB instead of LSB.

(9) Wash 3: Repeat the step (7) with LiCl buffer instead of LSB.

(10) Wash 4: Add 1.5 ml of TBS-CA630. Rotate 4 °C for 5 min. Put the tubes on a magnet stand and wait for 3 min. Discard the supernatant using a pipetting.

(11) Elution: Add 133 μl of Elution Buffer per tube. Incubate at 37 °C for 20 min. Put the tubes on a magnet stand and wait for 3 min.

(12) Transfer the supernatant (total: 133 µl x 3 = ca. 400 μl) into a 1.5 ml microtube, in which 1 ml of 2-propanol, 50 μl of 3M NaAc (pH 5.2), and 5 μl of 20 mg/ml glycogen were added. Precipitate proteins at -20 °C overnight.

(13) Centrifuge at 15,000 rpm for 30 min at 4 °C. Discard the supernatant.

(14) Add 1 ml of 70% ethanol. Centrifuge at 15,000 rpm for 10 min at 4 °C. Discard the supernatant completely using a pipetting.

(15) Suspend pellets in the three tubes in 40 μl of 2 x Sample buffer. Vortex 5 min to completely dissolve the precipitant. Incubate 100 °C for 30 min (protein denaturing and reverse-crosslinking).

6. SDS-PAGE

(1) Set up standard SDS-PAGE with the sample prepared in 5-(16).

(2) Electrophoresis until proteins are loaded 1 cm in gel.

(3) After Coumassie Brilliant Blue staining or Silver staining, the stained parts (1 cm) are cut into 5 slices and collect individually for Mass analysis.

**REAGENTS**

**Cell Lysis Buffer (CLB):** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5% IGEPAL CA-630, protease inhibitor cocktails

**Nuclear Lysis Buffer (NLB):** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% **lauroylsarcosine**, protease inhibitor cocktails

**Modified Lysis Buffer 3 (MLB3):** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktails

**Low Salt Buffer (LSB):** 20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktails

**High Salt Buffer (HSB):** 20 mM Tris, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitor cocktails

**LiCl Buffer:** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% IGEPAL CA-630, 0.5% sodium deoxycholate, protease inhibitor cocktails

**TBS-CA630:** 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% IGEPAL CA-630, protease inhibitor cocktails

**Elution Buffer:** TBS-CA630, 500 μg/ml 3X FLAG peptide (Sigma, F4799)

**2 x Sample buffer:** 125 mM Tris pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue