150405 **iChIP/enChIP-RNA-Seq**

1. Formaldehyde crosslinking of cells

(1) Culture target cells. Use 2 x 107 cells (e.g. Ba/F3, DT40) for chromatin preparation.

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

 Cell volume 30 ml

 37% formaldehyde 810 µl

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

 Cell volume 30 ml

 1.25 M Glycine 3.05 ml

 1.25 M Glycine Glycine MW: 75.07

 Glycine 18.8 g / 200 ml

(4) Collect cells by centrifugation (300 × g, 4 °C for 5 min).

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

2. Preparation of chromatin (/ 2 x 107 cells)

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

**Cell Lysis Buffer (CLB)** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5% IGEPAL CA-630, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *40 ml*

 1 M Tris (pH 8.0) 400 µl

 0.5 M EDTA 80 µl

 IGEPAL CA-630 200 µl

 Complete-Mini 4 tablets

 RNasin plus RNase inhibitor 5 µl

DDW 39.32 ml

(2) Centrifuge at 830 × g, 4 °C for 8 min. Discard carefully the supernatant.

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

**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**, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *40 ml*

 1 M Tris (pH 8.0) 400 µl

 0.5 M EDTA 80 µl

 5 M NaCl 4 ml

 Triton X-100 400 µl

 10% sodium deoxycholate 2 ml

 30% lauroylsarcosine 666 µl

 RNasin plus RNase inhibitor 5 µl

Complete-Mini 4 tablets

 DDW 32.46 ml

 10% sodium deoxycholate

 sodium deoxycholate 1 g / 10 ml

(4) Centrifuge at 830 × g, 4 °C for 8 min. Discard carefully the supernatant.

(5) Suspend the pellet in 10 ml of PBS. Centrifuge at 830 × g, 4 °C for 10 min. Collect the pellet as the chromatin fraction. The chromatin fraction can be stored at -80 °C after immediate freezing in liquid nitrogen.

3. Sonication of chromatin (/ 2 x 107 cells)

(1) Suspend the collected chromatin fraction in 800 µl of **MLB3**. Transfer the suspension into a 1.5 ml microtube.

**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, 1 x protease inhibitors, 40 U/ml RNase inhibitor

 *10 ml*

 1 M Tris (pH 8.0) 100 µl

 0.5 M EDTA 20 µl

 0.1 M EGTA 50 µl

 5 M NaCl 300 µl

 10% sodium deoxycholate 100 µl

 10% SDS 100 µl

 Complete-Mini 1 tablet

 RNasin Plus RNase Inhibitor 10 µl

DDW 9.33 ml

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

 Output: 3

 Duty: 100% (continuous)

 Time: Free

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

2 min on ice after 5 - 6 cycles

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

(3) Centrifuge at 16,000 × g, 4 °C for 10 min. Collect the supernatant (800 µl). The supernatant can be stored at -80 °C after immediate freezing in liquid nitrogen.

4. Reverse crosslinking (Evaluation of fragmentation of chromatin)

(1) Suspend 10 µl of the fragmented chromatin in 85 µl 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) Prepare 1% agarose gel w/o staining dye.

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

(6) Use 10 µl for electrophoresis in 1% agarose gel w/o staining dye. 100 V for 30 min.

(7) Stain the gel with staining dye for 0.5-1 h.

5. Preparation of Dynabeads conjugated with antibody

(1) Prepare two 1.5 ml tubes, one for pre-clearing using normal mouse IgG and the other for

incubation with the anti-FLAG Ab. Add 150 μl of Dynabeads-Protein G to each tube.

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

(3) Add 1 ml PBS with 0.01% Tween-20. Put the tube on a magnet stand and wait for 2 min. Discard the supernatant by pipetting.

 PBS-0.01% Tween-20

 PBS 10 ml

 10% Tween-20 10 µl

(4) Repeat the step (3).

(5) Add 1 ml PBS with 0.01% Tween-20 and 0.1% BSA.

 PBS-0.01% Tween-20

 PBS 10 ml

 10% Tween-20 10 µl

 7.5% BSA 133 µl

(6) Add 15 µg antibody (anti-FLAG antibody Sigma F1804 or control IgG for pre-clear). Rotate 4 °C **overnight**.

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

(8) Add 1 ml PBS with 0.01% Tween-20. Invert several times and centrifuge briefly. Put the tube 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) Transfer 0.8 ml of the fragmented chromatin, which corresponds to chromatin extracted from 2 x 107 cells, into a new 1.5 ml tube.

(2) Add 200 µl of 5% Triton X-100 (in MLB3) (final 1%).

 MLB3 5 ml

 Triton X-100 250 µl

(3) Transfer all (1 ml) of the chromatin solution into the tube, in which the Dynabeads conjugated with control IgG were prepared at the step 5-(9). Rotate 4 °C for 1h.

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

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

(6) Put the tube on a magnet stand and wait for 3 min. Discard the supernatant using a pipet.

(7) Add 1 ml of **LSB**. Rotate 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant using a pipet. Repeat again (total 2 times).

**Low Salt Buffer (LSB)** 20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *10 ml*

 1 M Tris (pH 8.0) 200 µl

 0.5 M EDTA 40 µl

 5 M NaCl 300 µl

 Triton X-100 100 µl

 10% SDS 100 µl

 Complete-Mini 1 tablet

 RNasin plus RNase inhibitor 1.3 µl

DDW 9.26 ml

(8) Wash 3 and 4: Repeat the step (7) with **HSB** x 2.

**High Salt Buffer (HSB)** 20 mM Tris, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *10 ml*

 1 M Tris (pH 8.0) 200 µl

 0.5 M EDTA 40 µl

 5 M NaCl 1 ml

 Triton X-100 100 µl

 10% SDS 100 µl

 Complete-Mini 1 tablet

 RNasin plus RNase inhibitor 1.3 µl

DDW 8.56 ml

(9) Wash 5 and 6: Repeat the step (7) with **LiCl buffer** x 2.

**LiCl Buffer** 10 mM Tris, pH 8.0, 1 mM EDTA, 0.25 M LiCl, 0.5% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *20 ml*

 1 M Tris (pH 8.0) 200 µl

 0.5 M EDTA 40 µl

 8 M LiCl 625 µl

 IGEPAL CA-630 100 µl

 10% sodium deoxycholate 1 ml

 Complete-Mini 2 tablets

 RNasin plus RNase inhibitor 2.5 µl

DDW 18.035 ml

(10) Add 1 ml of TBS with 0.1% IGEPAL CA-630. Rotate 4 °C for 5 min. Put the tube on a magnet stand and wait for 3 min. Discard the supernatant using a pipet.

**TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) with 0.1% IGEPAL CA-630**, 1 x protease inhibitors, 5 U/ml RNase inhibitor

 *10 ml*

 1M Tris-HCl (pH 7.5) 500 µl

 5 M NaCl 300 µl

 IGEPAL CA-630 10 µl

 Complete-Mini 1 tablet

 RNasin plus RNase inhibitor 1.3 µl

DDW 9.19 ml

(11) Elution: Add 200 µl of 500 µg/ml 3xFLAG peptide (Sigma, F4799) in TBS with 0.1% IGEPAL CA-630 (40 U/ml RNase inhibitor). Incubate at 37 °C for 20 min. Put the tube on a magnet stand and wait for 3 min. Transfer the supernatant (200 µl) into the 1.5 ml microtube.

 3xFLAG peptide (5 mg/ml) 50 µl

 TBS w/ 0.1% IGEPAL CA-630 450 µl

(12) Repeat the elution step.

(13) Mix the supernatant (200 µl + 200 µl = 400 µl) with 16 μl of 5 M NaCl and incubate at 65 °C for 2 h.

(14) Add 1 ml of Isogen II reagent to the sample. Vortex for 15 sec and then incubate at room temperature for 5–15 min. Centrifuge the sample at for 15 min at 12,000 × g and room temperature.

(15) Transfer the supernatant to a new 1.5 ml tube and add 5 μl of p-bromoanisole. Vortex for 15 sec and then incubate at room temperature for 3–5 min. Centrifuge the sample for 10 min at 12,000 × g and room temperature.

(16) Transfer the supernatant (approximately 1 ml) into a new 2 ml tube and add 1 ml of 2-propanol. Invert the tube and incubate at room temperature for 10 min. Load the mixture onto a Zymo-Spin IIC column from the Direct-zol RNA Miniprep kit. Centrifuge the column for 1 min at 12,000 × g and room temperature.

(17) Wash the column with 400 μl of RNA Wash Buffer (included with the Direct-zol RNA

Miniprep kit). 10.7) Add 80 μl of DNase I cocktail (mixture of 5 μl of DNase I (1 U/μl), 8 μl of 10× DNase I Reaction Buffer, 3 μl of DNase/RNase-free water, and 64 μl of RNA Wash Buffer) to the column. Incubate the sample at 37 °C for 15 min and then centrifuge at for 30 sec at 12,000 × g and room temperature.

(18) Wash the column with 400 μl of RNA PreWash Buffer (included with the Direct-zol RNA

Miniprep kit) twice.

(19) Elute the RNA with 50 μl of DNase/RNase-free water. The eluted RNA can be used for RNA sequencing.