**iChIP / enChIP to purify genomic DNA**

Originally developed by Toshitsugu Fujita on September 18, 2012

Modified by Hodaka Fujii on December 29, 2013

1. 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 (1,300 rpm, 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

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

**Cell Lysis Buffer (CLB)** 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% IGEPAL CA-630, 1 x protease inhibitors

 *40 ml*

 1 M Tris-HCl (pH 8.0) 400 µl

 0.5 M EDTA 80 µl

 IGEPAL CA-630 200 µl

 Complete-Mini 4 tablets

 DDW 39.32 ml

(2) 2,000 rpm, 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-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% **lauroylsarcosine sodium salt**, 1 x protease inhibitors

 *40 ml*

 1 M Tris-HCl (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 sodium salt 666 µl

 Complete-Mini 4 tablets

 DDW 32.46 ml

 10% sodium deoxycholate

 sodium deoxycholate 1 g / 10 ml

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

(5) Suspend the pellet in 10 ml of PBS. 2,000 rpm, 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

(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-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1 x protease inhibitors

 *10 ml*

 1 M Tris-HCl (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

 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) 13,000 rpm, 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 at 65°C overnight.

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

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

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

(6) Gel staining with staining dye for 0.5-1 h.

5. Preparation of Dynabeads conjugated with antibody

(1) Transfer 30 µl Dynabeads-Protein G (Invitrogen) in a new 1.5 ml tube.

(2) Put the tube on a magnet stand and wait for 2 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 10 ml

 10% Tween-20 10 µl

(4) Repeat the step (3).

(5) Add 300 µl PBS with 0.01% Tween-20 and 0.1% BSA.

 PBS 10 ml

 10% Tween-20 10 µl

 7.5% BSA 133 µl

(6) Add 3 µg antibody (e.g. anti-FLAG antibody Sigma F1804, control IgG). Rotate at 4°C overnight.

(7) Spin down briefly. Put the tube on a magnet stand and wait for 2 min. Discard the supernatant by pipetting.

(8) Add 300 µl PBS with 0.01% Tween-20. Invert several times and spin down briefly. Put the tube on a magnet stand and wait for 2 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 160 µl of the fragmented chromatin, which corresponds to chromatin extracted from 4 x 106 cells, into a new 1.5 ml tube.

(2) Add 340 µl of MLB3 1.47% Triton X-100 (final 1%).

 MLB3 1 ml

 Triton X-100 14.7 µl

(3) Transfer all (500 µl) 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 2 min.

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

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

(7) Wash 1: Add 1 ml of LSB. Rotate 4 °C for 10 min. Put the tube on a magnet stand and wait for 2 min. Discard the supernatant by pipetting. Repeat wash with LSB.

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

 *20 ml*

 1 M Tris-HCl (pH 8.0) 400 µl

 0.5 M EDTA 80 µl

 5 M NaCl 600 µl

 Triton X-100 200 µl

 10% SDS 200 µl

 DDW 18.52 ml

(8) Wash 2: Repeat the step (7) with HSB x 2.

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

 *20 ml*

 1 M Tris-HCl (pH 8.0) 400 µl

 0.5 M EDTA 80 µl

 5 M NaCl 2 ml

 Triton X-100 200 µl

 10% SDS 200 µl

 DDW 17.12 ml

(9) Wash 3: Repeat the step (7) with LiCl buffer x 2.

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

 *20 ml*

 1 M Tris-HCl (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

 DDW 18.035 ml

(10) Wash 7 and 8: Repeat the step (7) with **TBS with 0.1% IGEPAL-CA-630** x 2.

**TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) with 0.1% IGEPAL CA-630**

(11) Elution: Add 150 µl of 500 µg/ml 3xFLAG peptide (Sigma, F4799) in TBS with 0.1% IGEPAL CA-630. Incubate at 37 °C for 20 min. Put the tube on a magnet stand and wait for 3 min.

 3xFLAG peptide (5 mg/ml) 50 µl **66 µl**

 TBS w/ 0.1% IGEPAL CA-630 450 µl **594 µl**

(12) Repeat the elution step. Total 300 µl.

(13) Add

 5 M NaCl 12 µl final 200 mM

 0.5 M EDTA 0.6 µl 1 mM

(14) 65°C, overnight.

(15) Add **3 µl** of 10 mg/ml RNase A. Incubate 37°C for 1 hr.

(16) Add

 10% SDS **16 µl**

 Proteinase K **10 µl**

(17) Incubate at 45°C for 2 hr.

(18) Purify DNA using ChIP DNA Clean & Concentrator kit (Zymo Research, D5205). Add 1.5 ml of ChIP DNA Binding Buffer.

(19) Transfer mixture into a Zymo-Spin Column in a Collection Tube.

(20) 15,000 rpm, 30 sec.

(21) Discard flow-through.

(22) Wash with 200 µl of Wash Buffer x 2.

(23) Elute DNA with 50 µl of Elution Buffer.