**I. Chromatin Preparation**

**Buffers and solutions**

Protease Inhibitor, 50x stock

Completely dissolve 1 Roche complete protease inhibitor cocktail (PIC) tablet into 1 ml H2O.

0.2M PMSF

dissolve in ethanol.

Homogenizer buffer

1x PBS

0.1% Triton X-100

add DTT (final:1mM), PMSF(0.1mM), 1x PIC (from 25x stock), before use.

Nuclei Lysis Buffer (RIPA buffer) 50 ml

Tris pH 7.5 20 mM 1M 1 ml

EDTA 1 mM 0.5M 0.1 ml

NaCl 150 mM 5M 1.5 ml

NP-40 1% 10% 5 ml

Na-deoxycholate 0.5% 5% 5 ml

SDS 0.1% 20% 0.25 ml

H2O 37.15 ml

Add PMSF and PIC before use

***Procedure***

• Resuspend embryos in 5 – 10 ml homogenization buffer/g embryo, homogenize with a A (loose) pestle with 7-10 strokes. (for small amount of embryos directly use B pestle)

• Further dounce 20 -40 strokes with a B (tight) pestle.

• check embryo disruption under microscope, the lyses may not be efficient with certain dounces. Dounce more times if needed.

• Transfer to a 15 ml falcon tube or microcentrifuge tube if volume < 1.5ml. Add NP40 to 0.5%, mix well.

• Spin at 1250xg, at 4 °C, for 10 min. Remove supernatant

• Resuspend the nuclei gently in 5 ml nuclear lysis buffer/g starting material, by gently pipetting up and down or if necessary dounce with A pestle. Transfter to microcentrifuge tube.

# more stringent wash can be done at this step with SDS at 1% and sarkosyl at 2% (without NP-40 and Na-deoxycholate), which is followed by another nuclei lysis buffer wash.

• Spin at full speed for 5 min. remove supernatant.

• Resuspend pellet in 0.5 ml nuclei lysis buffer. Homogenized with B pestle to aid resuspension if necessary.

Note: the sample volume should be between 0.5 – 2 ml if sonication is carried out in 15 ml falcon tube

• Fragment chromatin in a 15 ml (polyethylene, or polystyrene. The later, clear plastic, is better) conical tube, or microcentrifuge tube if sample volume is small, in a bioruptor. Set power at H, and carry out 6 rounds of 10 min cycles at setting of 15s on : 45s off until the average fragment size is about 300 bp (4 – 10 rounds will be needed). Note: if more than one sample is processed, to achieve similar fragmentation between samples, the sample volumes should be adjusted to about the same, and the number of tubes that are processed the same time also has effect.

• Transfer chromatin to microcentrifuge tube(s). Spin at full speed in a microcentrifuge for 10 min. Transfer the chromatin solution to new tube. Remove 10 ul, and carry out step 12. For the rest, flash freeze and store it at -80oC.

• Check chromatin recovery, concentration, and size range:

• To the chromatin sample and the sample from step 6, add 140 ul of 10mM Tris-HCl pH8, 0.15 M NaCl, 1mM EDTA pH8, 0.5%SDS, + 1 ug RNase A. incubate at 37oC for 1 hr.

• incubate at 65oC, 4 hr to overnight.

• Add 10 ug PK, 55oC, 2 hrs

• purify DNA with Qiagen MinElute kit

• nanodrop to measure DNA recovered

• Bioanalyzer

**II. Chromatin Immunoprecipitation**

**Material, Buffers, and Soultions**

chromatin in RIPA buffer

50x PIC

protein A-dynabeads: use 1-4 ug Ab/50 ul beads

or anti-rabbit IgG – dynabeads: use 1-10 ug Ab/50 ul beads

note: protein A-dynabeads can bind 8 ug IgG/mg beads; at 30mgbeads/ml, 50 ul beads has 12 ug IgG capacity. In contrast, for 2 mg/ml protein – sephacryl beads, the capacity is as high as 20 ug/ul beads

0.2 M PMSF

RIPA buffer 50 ml

~ 10 ml is needed for each sample

Tris pH 7.5 20 mM 1M 1 ml

EDTA 1 mM 0.5M 0.1 ml

NaCl 150 mM 5M 1.5 ml

NP-40 1% 10% 5 ml

Na-deoxycholate 0.5% 5% 5 ml

SDS 0.1% 20% 0.25 ml

H2O 37.15 ml

Add PMSF and PIC before use

0.5M RIPA

To the RIPA buffer, adjust NaCl concentration to 0.5 M.

LiCl buffer 15 ml

Tris-Cl pH 8.0, 10 mM 1 M 0.15 ml

EDTA, 1 mM 0.5M 30 ul

LiCl, 500 mM 8 M 0.94 ml

NP40 1% 10% 1.5 ml

sodium deoxylcholate 1% 5% 3 ml

H2O 9.4 ml

Add PMSF to 0.1 mM

TE 20 ml

10 mM Tris-Cl pH 8.0, 1 M 0.2 ml

1 mM EDTA, 0.5M 40 ul

H2O 19.8 ml

Direct Elution Buffer (10mM Tris-HCl pH8, 0.3M NaCl, 5mM EDTA pH8, 0.5%SDS): Dynal beads: Make fresh each use

• 1.0M Tris-HCl pH8 0.1 ml

• 5M NaCl 0.6 ml

• 0.5M EDTA pH8 0.1 ml

• 20% SDS 0.25 ml

• ddH20 8.95 ml

• Total Vol 10 ml

**Procedure**

Day 1

**block an aliquot of dynabeads**: In a low-retention tube, add 50µl of a thoroughly resuspended bead slurry (use a widebore pipette tip). Place the tube in the magnetic separation stand, wait ~1minute, remove the storage solution, and replace with 1ml PBS/ 0.1% Triton X-100/ ~~3%~~ 2% BSA. Incubate on a nutator in the cold room overnight.

**Prepare IP samples:** in a low retention 1.5ml microcentrifuge tube, transfer the per-IP volume of sheared chromatin and add fresh RIPA buffer to make a final volume of 600µl. Add an appropriate amount of IP antibody to each tube. Incubate IP samples overnight on a nutator at 4°C.

Day 2

• Spin the overnight-incubated antibody/chromatin mixtures, full speed 4°C for 5’.

• In the meantime, collect the tubes with blocked Protein A Dynabeads and load them in the magnetic separation stand. This can be at room temperature. remove the blocking solution.

• Once the spin is complete, transfer the supernatants to the tubes with the magnetic beads (label appropriately).

• Incubate at 4°C for 1h on a nutator

• Perform the following wash steps with 0.8ml of **COLD** buffer. Flick tubes to resuspend beads and incubate each wash for 5min on rocker at 4°C. Place tube in magnetic stand. Invert several times. Allow beads to clump and discard supernatant.

• 1 times with RIPA-150

• 2 times with RIPA-500

• 2 times with LiCl buffer. Aspirate suds after second wash.

• 2 times with 1xTE Buffer, pH8.0. Don’t need to incubate on rocker at this step. Aspirate suds after final wash.

• Resuspend beads in 200ul freshly made Direct Elution Buffer.

• Incubate for 4hrs or O/N at 65C to reverse crosslink (briefly vortex sample every ½ hour for the first few hours).

• Quick spin sample. Place in magnetic stand. Allow beads to clump and transfer supernatant to a new low-bind tube.

• Add 1 ul RNase A (20 mg/ml) and incubate at 37C for 30 min.

• Add 3ul of Proteinase K (10 mg/ml) and incubate for 1-2hrs at 55C.

**DNA Clean Up, PCR**:

There are several ways to do DNA clean up. Most quickly, samples can be processed directly through a Qiagen PCR Purification kit following the standard protocol with some exceptions: I use 900µl of Buffer PB instead of 500µl for the initial dilution, I wash twice with 750µl Buffer PE, and I elute in 80µl (for ChIP-PCR) or 40µl (for ChIP-seq) of Buffer EB after incubating at room temperature for 3 minutes .

For PCR, I first make two 10-fold dilutions of the Input sample (to yield a set of 2%, 0.2%, and 0.02%). Samples are then analyzed in duplicate by QPCR using SYBR Green detection. I design primers using PrimerBLAST (NCBI) using the default temperature parameters, and limiting product size to be between 50 and 150 base pairs. Each 20µl QPCR reaction contains 2µl of the ChIP product. Primers to single-copy loci typically amplify 2% input between 22 and 26 cycles, the negative control comes up around 32 cycles and the positive results are somewhere in between (or sometimes greater than 2%, in which case: congratulations).