ChIP Protocol – April 2013
He Lab

Make the following solutions before starting:

Day 1:

Cell Lysis Buffer(CLB) (4 degree)
5 mM PIPES-NaOH pH 8.0
85 mM KCl
0.5% NP40

Nuclei Lysis Buffer (NLB) (RT)
50 mM Tris pH 8.1
10 mM EDTA
1% SDS

RIPA buffer (4 degree)
10 mM Tris pH 7.6
1 mM EDTA
0.1% SDS
0.1% Sodium deoxycholate
1% Triton X-100

Day 2:

Low Salt Wash Buffer (4 degree)
20 mM Tris pH 8.1
2 mM EDTA
150 mM NaCl
0.1% SDS
1% Triton X-100

High Salt Wash Buffer (4 degree)
20 mM Tris pH 8.1
2 mM EDTA
500 mM NaCl
0.1% SDS
1% Triton X-100

LiCl Wash Buffer (4 degree)
0.25 M LiCl
10 mM Tris pH 8.1
1 mM EDTA
1% NP40
1% Sodium deoxycholate

TE (4 degree)
10 mM TRIS-HCl pH8.0
1 mM EDTA

Elution Buffer (RT)
50 mM Tris pH 8
10 mM EDTA
1% SDS

Day 1:

Harvesting Cells and Crosslinking

1. Transfer cells and media from the tissue culture flasks into either 50-ml conical tubes or centrifuge bottles.
   Depending on the target protein, the number of cells required can vary. For histone marks, we used around 10 million cells.
2. Add 1/10 volume of 11% formaldehyde to reach a final concentration of 1%.
3. Incubate at room temperature with shaking for 10 minutes.
4. Stop the cross-linking reaction by adding 1/20 volume of 2.5M glycine to reach a final concentration of 0.125 M.
5. Incubate at room temperature with shaking for 5-10 minutes
6. Pellet cells by centrifuging at 2,000 rpm for 5 minutes at 4 degrees.
7. Place cells on ice and remove the supernatant. Add an equal volume of cold PBS and gently resuspend the cells, then centrifuge again.
8. Carefully remove supernatant and either proceed to sonication or snap-freeze in liquid nitrogen for storage at -80°C or in liquid nitrogen.
   Frozen cross-linked cells appear to be stable indefinitely. Therefore, this is a convenient step in the protocol to collect many samples for testing multiple antibodies, biological replicates, controls, etc.

Sonication

All steps should be done on ice unless otherwise stated.

1. Dissolve 1 tablet of protease inhibitors (Roche) in 10 mL of CLB.
2. If starting from frozen chromatin, thaw the frozen chromatin on ice.
3. Use a wide opening tip to resuspend cell pellets gently in 1 mL cold CLB containing protease inhibitors.
4. Transfer the suspensions to low adhesion microcentrifuge tubes.
5. Rotate on an end-to-end rotator for 10 minutes at 4 degrees.
6. Centrifuge at 800 x g for 10 minutes at 4 degrees.
7. Carefully aspirate the supernatant without disturbing the nuclei pellet.
8. Again, gently resuspend the pellet in 1 mL cold CLB containing protease inhibitors.
9. Rotate on an end-to-end rotator for 10 minutes at 4 degrees.
10. Centrifuge at 800 x g for 10 minutes at 4 degrees.
11. Dissolve 1 tablet of protease inhibitors in 10 mL of NLS at RT.
    The amount of NLS required depends on the sonication method. For example, a probe sonicator may require 2-3 mL per sample, while a Covaris disruptor requires 600 uL per sample.
12. Carefully aspirate the supernatant without disturbing the pellet.
13. Resuspend in the appropriate volume of NLB containing protease inhibitors to lyse the nuclei.
14. Transfer to sonication tubes and place on ice.
15. Sonicate using a probe sonicator or Covaris acoustic disruptor.

Sonication conditions for the desired fragment size should be determined prior to performing ChIP. For ChIP-qPCR, make the major fraction between 300-1000 nt; for ChIP-seq, make 200-500 nt.

16. Transfer the sonicated lysates to low adhesion microcentrifuge tubes.
17. If lysates are cloudy, warm on bench top briefly to dissolve SDS precipitate.
18. Centrifuge at max speed 15 min at 4 degrees.
19. Transfer the supernatant containing fragmented chromatin to new low adhesion microcentrifuge tubes.
    Sonicated chromatin may be snap frozen and stored at -80 degrees.
20. Spec the chromatin to determine the DNA concentration, using NLB without SDS as a blank.
21. Transfer 10 uL of the supernatant to new tubes and purify the DNA by phenol chloroform extraction and ethanol precipitation. Run on a gel to verify the correct fragment size.

Preparing DynaBeads

To save time, prepare either before/during sonication or during dialysis

1. Prepare a solution of PBS ph 7.4 + 5 mg/ml BSA (Sigma A9647). Make enough for 4 mL per IP reaction.
2. Aliquot 50 uL of DynaBeads to a low adhesion microcentrifuge tube for each IP reaction (remember to include IgG control).
   You may use Protein A, Protein G, or Protein A + G conjugated DynaBeads, depending on the antibody isotype.
3. Add 1 mL of PBS + BSA to each aliquot of DynaBeads.
4. Mix well by inverting several times, load onto a magnetic stand to pellet the beads, then aspirate the PBS + BSA.
5. Repeat the wash twice more.
6. In the remaining PBS + BSA, dissolve 1 tablet of protease inhibitors per 10 mL of PBS + BSA.
7. After the last wash, resuspend the beads in 1 mL of PBS + BSA + protease inhibitor and add the desired amount of antibody. *For histone marks, we use 3 ug of antibody* per IP reaction.
8. Rotate at 4 degrees for at least 2 hours.

Dialysis of lysate

This is an optional step to remove excess SDS from the lysate. Do this if the antibody doesn't work well.

1. Transfer the sonicated chromatin to 15 mL conicals.
   Normalize the amount of chromatin across samples based on DNA concentration.
2. Add cold RIPA buffer to make a total volume of 3 mL.
3. Transfer the lysates by syringe to Slide-A-Lyzer dialysis cassettes (Pierce, 3-12 mL capacity, 3500 MWCO).
4. Submerge the cassettes in 300 mL of cold RIPA buffer.
5. Incubate with stirring at 4 degrees for 2 hours.
6. Transfer the lysates to 15-ml tubes.
7. Determine the protein concentration by BCA assay.

8. Add enough RIPA buffer + protease inhibitors to bring the sample with the lower concentration to 4 mL. Then dilute the sample with the higher concentration in RIPA + PI such that the concentrations are equivalent.
9. Transfer 100 uL of each sample to a new microcentrifuge tube as the input. Store at -80.

Immunoprecipitation

1. Pellet the antibody-incubated beads using a magnetic stand. Aspirate the supernatant.
2. Wash the beads twice with 1 mL PBS.
3. Split the lysate equally into each tube containing antibody-incubated beads.
4. Incubate overnight while rotating at 4 degrees.

Day 2

1. Perform the following washes at 4 degrees. For each wash, pellet the beads on the magnetic stand, aspirate the supernatant, add 1 ml of wash buffer, invert several times to disperse the beads, and rotate for 3 minutes.
    2 times with low salt buffer
    3 times with high salt buffer
    4 times with LiCl buffer
    1 time with TE
2. Resuspend the beads in 200 uL of elution buffer.
3. Heat at 65 degrees for 2 hours to elute.
4. Centrifuge at max speed for 3 minutes to pellet the beads.
5. Transfer the supernatant to new microcentrifuge tubes.
6. Thaw inputs and add 100 uL of elution buffer to make a total volume of 200 uL.
7. Transfer IP samples and inputs to PCR tubes.
8. Heat overnight at 65 degrees in a thermocycler to reverse cross-linking.

Day 3

1. Purify the DNA using the Qiagen PCR purification kit.
2. Elute in 100 uL EB (from the kit).
3. Proceed to qPCR or high-throughput sequencing library preparation.