ChIP Protocol Boss Laboratory

General Notes – Prepare reagents in dishes freshly rinsed in ddH_2O. Use fresh ddH_2O to make reagents. Use separate chemicals for ChIP only to make solutions and pour or use sterile stripettes. Do <u>not</u> autoclave 1.7 mL tubes, tips, or reagents. Wear gloves at all times and keep ALL ChIP reagents marked and separate from other reagents.

Prepare Chromatin

1. To ensure ample cells, use 40 x 10^6 cells. Add formaldehyde directly to tissue culture media to a final concentration of 1%. Stock formaldehyde is usually 37%, so dilute 1:37. For adherent cells, tilt dish and add to pooled media. Crosslink for 10 min at room temp. with mild shaking.
   25 mL – 675 uL 20 mL – 540 uL 10 mL – 270 uL

2. Add protease inhibitors to 2 mL Cell Lysis buffer per sample and 0.5 mL Nuclei Lysis buffer per sample. Keep Cell Lysis cold and Nuclei Lysis room temp..

3. For adherent cells, pour off media and wash with cold PBS. For suspension cells, pellet at 3000g for 5 min and resuspend in cold PBS, then go to step 5. **All steps from here on must be on ice unless otherwise noted.**

4. Scrape adherent cells in a small volume of PBS and then wash plate out with PBS. Pellet at 3000g for 5 min. **Use barrier tips from this point on.**

5. Resuspend in 1 mL PBS and transfer to 1.7 mL tube. Pellet (3000g for 5 min) and resuspend in 1 mL Cell Lysis buffer. Ensure there are no clumps, then let sit 5 min on ice. Pellet (3000g for 5 min) and repeat.

6. Pellet cells (3000g for 5 min) and resuspend in 0.5 mL Nuclei Lysis. Ensure no clumps. You can freeze here at –70°C overnight.

7. Sonicate chromatin to an average size of 600 bp with a microtip on power setting #3. We use a program of 4 sec. on, 4 sec. off, for 10 reps. Repeat that program 4 times for each sample, alternating samples in between so they can cool. Take care not to froth the sample, which occurs due to changes in tip depth. Clean the tip with ddH_2O and then EtOH using a Kimwipe between samples.

8. Spin samples at 14000 rpm at 4°C to remove precipitates. Take care to distinguish between cell debris and precipitating SDS due to cold. Warm the sample between your fingers if SDS precipitates. Snap freeze at –70°C if not used immediately. Degree of sonication can be checked here by combining 10 uL of sample and 2 uL of 5M NaCl at 65°C overnight. Phenol chloroform, precipitate, dry, and resuspend. Run 1/4 on 2-3% agarose with standard.

Immunoprecipitate

1. Prepare 140 uL protein A slurry per sample. Per mL, add 24 uL of 10 mg/mL salmon sperm, 24 uL of10 mg/mL tRNA, and 100 ul 10 mg/mL BSA. Rotate for 15min.

2. Add protease inhibitors to 1 mL IP Dilution buffer per sample. To preclear the chromatin of background binding, combine 950 uL IP Dilution with 80 uL protein A slurry and 50 uL chromatin sample.

3. Rotate at 4°C for 30 minutes to 1 hour. Pellet protein A (3000g for 5 min) and transfer supernatant to fresh tube.

4. Add antibodies to samples and rotate at 4°C overnight. Remember to include an irrelevant antibody control. Use 5 to 10 μg antibody per sample or titrate specifically. Rotate extra supplemented protein A overnight. A titration may also be done with homemade antibodies to determine the correct concentration needed.

5. Spin tubes (3000g for 1 min) to remove fluid from cap. Add 60 uL protein A and rotate 1 hr.

Wash and Elute

1. Aliquot 2 mL each of IP Dilution, TSE-500, LiCL/Detergent, and 1xTE buffers per sample. Chill on ice.

2. Pellet protein A (3000g for 5 min). ****SAVE** 400 uL supernatant from irrelevant antibody IP. Leave at room temp. and process these samples with the other elutions.** Aspirate supernatant from samples and discard. It is more important to leave the beads than to get 100% of the liquid.

3. Wash with 1 mL of IP Dilution and rotate for 10 min at 4°C. Pellet (3000g for 5 min) and aspirate supernatant. Repeat this wash step once more.

4. Wash twice each as above (in step 3) with TSE-500, LiCL/Detergent, and 1xTE buffers in order. The wash process takes about 3 hours. Alternate samples (half rotating, half spinning and aspirating) if you have more than 15.

5. Elute from the protein A beads with 250 uL of IP Elution. Vortex hard and rotate 15 minutes at room temp.

6. Pellet prot A (3000g for 5 min) and **Save Supernatant**. Repeat elution once more and pool with the first eluate.

7. Add 20 uL 5M NaCl to each sample (including irrelevant antibody supernatant) and incubate at 65°C 4 hours to overnight. This reverses the formaldehyde crosslinks.

8. Mix 10 uL 0.5M EDTA, 20 uL 1M Tris pH 6.5, and 2 uL 10 mg/mL proteinase K (in 1XTE) per sample. Add 32 uL per sample and incubate for 1 hour at 55-65°C.

9. Extract once with 300 uL of room temp. PCIA (25:25:1). Centrifuge (14,000 for 5 min) at room temp. and **save top aqueous layer**. Add to a new tube containing 15 uL 5M NaCl and 5 uL 10 mg/mL tRNA.

10. Mix and add 1 mL of 100% ethanol (reagent alcohol). Precipitate at –20°C for at least an hour.

11. Centrifuge (14,000 for 20 min) at 4°C and **carefully** aspirate alcohol. Wash once with 500 uL 70% ethanol, centrifuge (14,000 for 5 min), and aspirate. Dry in speed vacuum 3 minutes or on bench top.

12. Resuspend in 30 ul purchased ddH_2O. Vortex hard. After resuspension, dilute irrelevant antibody supernatant 1:30. Use 3 ul per PCR reaction, preferably RealTime.

Solutions

20% SDS
    20g into 100 H_2O

100 mM PIPES pH 8.0 (FW 302.4)
    3g into 100 mL final volume. pH with 1.75 mL 10N NaOH, then dropwise with 2N NaOH. Will not dissolve before 8.

1M Tris 8.1 (FW 121.1)
    60.5g into 500 mL final volume. pH with 17 mL Conc. HCl, then dropwise.

1M Tris 6.5 (FW 121.1)
    12.1g into 100 mL final volume. pH with 7 mL Conc. HCl, then dropwise.

0.5 M EDTA (FW 372.4)
    18.6g into 100 mL final volume. Heat to 65°C, then pH with 10N NaOH. Will not dissolve before 8.

5 M NaCl
    29.2g NaCl into 100 mL final volume. Heat to dissolve.

Cell Lysis Buffer	for 500 mL
5 mM PIPES pH 8.0	25 mL 100mM PIPES pH 8.0
85 mM KCl	3.17g KCl
0.5% NP40	2.5 mL NP40 (IGEPAL)

(PMSF, leupeptin, pepstatin A, and aprotinin fresh)

Nuclei Lysis Buffer	for 100 mL
50 mM Tris pH 8.1	5 mL Tris pH 8.1
10 mM EDTA	2 mL 0.5 M EDTA
1% SDS	5 mL 20% SDS

(PMSF, leupeptin, pepstatin A, and aprotinin fresh)

IP Dilution Buffer	for 500 mL
0.01% SDS	0.25 mL 20% SDS
1.1%Triton X-100	5.5 mL Triton X-100
1.2 mM EDTA	1.2 mL 0.5 M EDTA
16.7 mM Tris pH 8.1	8.35 mL 1 M Tris 8.1
167 mM NaCl	4.9g NaCl

TSE-500 Wash	for 500 mL
0.1% SDS	2.5mL 20% SDS
1% Triton X-100	5 mL Triton X-100
2 mM EDTA	2 mL 0.5 M EDTA
20 mM Tris pH 8.1	10 mL Tris pH 8.1
500 mM NaCl	14.6g NaCl

LiCl/Detergent Wash	for 500 mL
100 mM Tris pH 8.1	50 mL 1 M Tris pH 8.1
500 mM LiCl	10.6g LiCl
1% NP40	5 mL NP40 (IGEPAL)
1% deoxycholic acid	5g deoxycholic acid

Elution Buffer	
50 mM $NaHCO_3$	0.42g $NaHCO_3$
1% SDS	5 mL 20% SDS