ChIPping for Dummies

Compiled from S.B.

Cells of interest: Figure on $1x10^7$ per IP. They should of course be healthy and not overly confluent.

The Day Before….

Blocking Staph A cells

1. Thaw 1 tube (100mLs each) of Staph A cells (1 tube/$5x10^8$ cells).
2. Add 5uL of herring sperm DNA (10mg/mL) and 5 uLs BSA (10mg/mL) for each tube (combine tubes if you perchance) and mix by pipetting.
3. Incubate on rocking/rotating platform at 4C overnight.
4. The next day, spin for 3mins and remove supernatant. Wash pellet twice with 1mL dialysis buffer without sarkosyl and repellet. After final wash resuspend in a volume of 1x dialysis buffer WITH PMSF (10uL/mL – so 1UL/100UL BUFFER) equal to the original starting volume (100uL x number of tubes). Staph A's are now good stored at 4C for two weeks.

Crosslinking cells

1. Crosslink cells by adding 37% stock formaldehyde directly to tissue culture media to a final concentration of 1%. I like to place formaldehyde into 50mL conical tubes, and add media directly to this.
2. Rock cells for 10mins at room temp. Crosslinking for long periods (>30mins) may cause cells to aggregate and not sonicate efficiently.
3. Stop the crosslinking by adding 10X glycine solution (1.25M) – final concentration of 0.125M. Continue to rock at room temp for 5 mins.
4. Spin cells down in conical tubes, wash twice in PBS and repellet. Use plastic for all transfers and washes! Calculate cell numbers before final spin down. Snap freeze pellets and store at -80C.

Day 1

Chromatin preparation from cell pellets

1. Keep samples on ice. Use 2mL screwcap tubes for entire procedure. Be paranoid about contamination…use barrier tips etc.
2. Add protease inhibitors to Cell Lysis Solution: PMSF (10uL/mL), aprotinin (1uL/mL in the -20, use aliquots and do not refreeze), leupeptin (1uL/mL, 4C)
3. Add CLS to cell pellets, try 1~2 mLs.
4. Incubate on ice for 10 mins.
5. Dounce cells with B dounce, 20 strokes. After douncing transfer sample back to 2mL tubes, wash dounce with alconex or SDS, H_2O, 95% EtOH, and lastly H_2O

6. Centrifuge at 5,000rpm for 5minutes at 4C to pellet nuclei. Before centrifuging, may want to take an aliquot and view nuclei (Trypan Blue stained, etc)
7. Resuspend nuclei in 1mL Nuclei Lysis Buffer (plus the 3 protease inhibitors)
8. Incubate on ice for 10mins, can go longer here as you prep Staph A cells (see above)

Sonication

1. Rinse sonicator tip with detergent, H_20, ETOH before use and after
2. Sonicate (Sonicator setting, number of pulses, +pulse duration all determined experimentally)
3. 1 minute wait between pulses, cells on ice
4. Centrifuge at 14K for 10min at 4C

Blocking

1. Remove supernatant to new tube. Preclear by adding 50uL Staph cells/1x10^8 cells
2. Incubate at 4C for 15mins, no longer.
3. Centrifuge at 14K for 5min, 4C

Immunoprecipitation

1. Transfer supernatent to clean tube and measure volume. Divide equally for IP (10^7 cells/IP).

Total Volume/ N + 0.2 where N is the number of IP reactions

2. Save the 0.2 (20%) as Total Imput sample, put this away in the 4C at this point

3. Adjust the final volume by adding 2x volume IP dilution buffer and protease inhibitors. (just to IP samples)

4. Include a mock sample containing equal volume nuclei/IP buffers instead of chromatin
5. Include a No antibody sample with chromatin alone
6. Add 1 ug of antibody to each sample (or whatever is optimal)
   Anti-EBNA1 (IH4) rat monoclonal 10ug/1x10^7 cells = 100uL
   Anti-E2F4 (C-20) rabbit polyclonal (C0504) 10ug/1x10^7 cells = 5uL
   [this is a positive control for the whole ChIP experiment. It should pull down DHFR promoter]
   Anti-Rat Rabbit IgG 20ug/1x10^7 cells = 10uL
   [this is a nonspecific antibody to see background pulldowns. It also bridges anti-EBNA1 to the Staph-A cells]
7. Incubate on rotating platform at 4C overnight

Day 2

Washing and crosslink reversal

1. If you are using antibodies that are not rabbit, add 1ug (or predetermined amount) of secondary antibody, incubate for 1hr at 4C. Next add 10uL blocked/washed Staph A cells to each sample. Incubate at ROOM TEMP 15mins rotating platform, no longer

2. Spin at 14K 4min at 4C

3. Wash pellets twice with 1.4mLs 1x dialysis buffer (add PMSF 10ul/mL). Be sure to use appropriate monoclonal or polyclonal dialysis and wash buffers. Resuspend pellet with 700uls buffer, then add the final 700uL. Incubate samples each time at room temp (rocking) for 3 minutes then spin at 14K 4 mins at 4C.

4. Wash four times with 1.4mLs IP wash buffer (and PMSF). Follow steps as above

5. After last wash, dump buffer, spin pellets again (ORIENT TUBES CORRECTLY). Aspirate last traces of buffer.

6. Elute by adding 150uLs IP elution buffer (no inhibitors). Shake on vortexer 15mins setting 3, room temp. Spin at 14K 3 mins at 4C. Transfer supernatent to clean tubes. Repeat and combine both eluates

7. Spin samples at 14K for 5mins to remove all Staph A cells, transfer to new tube. Add 12uL 5M NaCl (0.2M final concentration). Prepare 20% totals by bringing volume up to 300uL with elution buffer, adding 12uL 5M NaCl. Incubate all samples in 67C waterbath at least 4 hours to reverse crosslinks

8. Clean up samples using Quiagen PCR clean up kit.

Sugden Lab.

ChIP Recipies

Cell Lysis Buffer (for 100mLs)

5 mLs 0.1M PIPES pH 8.0	5mM PIPES pH 8.0
8.52 mLs 1M KCl	85mM KCl
500 uLs IGEPAL (CA-630)	
ddH_2O to volume	

Nuclei Lysis Buffer (for 100mLs)

5 mLs 1M Tris pH 8.0	50mM Tris-Cl pH 8
2 mLs 0.5M EDTA	10mM EDTA
5 mLs 20% SDS	1% SDS
88 mLs ddH_2O	protease inhibitors, see below

IP Dilution Buffer (for 200mLs)

100 uLs 20% SDS	0.01% SDS
2.2 mLs Triton-X 100	1.1% Triton-X 100
480 uLs 0.5M EDTA	1.2 mM EDTA
3.34 mLs 1M Tris pH 8	16.7mM Tris-Cl pH 8
6.68 mLs 5M NaCl	167mM NaCl
ddH_2O to volume	

1X Dialysis Buffer (for 1L)

4 mLs 0.5 EDTA	2mM EDTA
50 mLs 1M Tris	50mM Tris-Cl pH 8
ddH_20 to volume	
0.2% Sarkosyl added for polyclonal antibodies	

IP Wash Buffer (for 1L)

100 mLs Tris pH **(8 for mono, 9 for poly abs)**	100mM Tris
21.2 g LiCl	500mM LiCl
10 mLs NP40	1% NP40
10 g deoxycholic acid	1% deoxycholic acid

Elution Buffer (for 100 mLs)

0.42 g $NaHCO_3$	50mM $NaHCO_3$
5 mLs 20% SDS	1% SDS
90 mLs ddH_2O	

Protease Inhibitors

0.0871 g PMSF in 5 mLs isopropanol (aliquot to 1mL samples, keep at –4C, two weeks) use at 1:100 (stock is 100mM)
10mg/mL aprotinin in 0.01M HEPES pH 8.0, use at 1:1000
10mg/mL leupeptin in water, use at 1:1000

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