EB ChIP Protocol

This protocol is a combination of Odom and colleagues (Odom et al., 2004) with a Maniatis lab protocol (Daniel Smith and Diana Saville update March, 2004) and my own modifications. These are designed to reduce the number of input cells to approximately 2x10^6 cells per precipitation. I don’t ever bother to spin down at 4º, but this may be necessary for some proteins. However, I do chill BSA/PBS, Lysis Buffer and Buffer 3 on ice before using. Some protocols say it is OK to freeze chromatin preps, but I always find a reduction in quality of ChIPs – sometimes they don’t work at all. So I go straight through the entire protocol.

Embryoid Body Preparation
Thaw ES cells (Gli160-6 act uses 17.5% FBS – NO NEO) and expand to 3 x 10cm dishes per sample. This will be enough for ~5 chips and (and input controls).

When the 3x10cm dishes are ready to split after two days, trypsinize, spin down and resuspend in ES medium and pre-plate on gelatinized T-160 flasks to reduce the number of feeders. Use 2 flasks for 3 10cm dishes. Let site for 45minutes- 1 hour. Take supernatant and spin down. Resuspend in 5-10ml of DFNB medium (50%DMEM, 25%F12, 50%Neurobasal medium, 1XB-27 supplement, Glutamine, Penicillin/Streptomycin and 0.1mM 2-mercaptoethanol).

Count cells and adjust to 1x10^6 per ml of DFNB. Plate out the equivalent of 6x10cm dishes using 6 well plates (=18x35mm or 3entire plates; Corning COSTAR Ultra Low Cluster #3471) 1x10^6 cells per well in a total 5ml of media. So you will need 1.8x10^7 ES cells to plate these out.

After two days, change media to DFNB containing 500nM retinoic acid and 1µM of the Hh agonist Ag1.5 ((Frank-Kamenetsky et al., 2002)Ag1.5 provided by Curis, Inc.). EBs were grown for an additional 3 days to induce neural progenitor stages at which point cultures were harvested for ChIP.

Day 1
Preblock and bind antibody to magnetic beads (exactly as per Odom et al, 2004).
- Wash 100ul Dynal Magnetic Beads (Sheep anti-mouse #M-280) in 1ml fresh BSA/PBS.
- Collect beads by spinning at 3000rpm for 1 minute.
- Wash beads in 1.5ml BSA/PBS 2 times, collecting beads with magnet.
- Add 9µg of Anti-FLAG®M2 to 250ml BSA/PBS and use this to resuspend washed beads (Sigma F-3165) (Pre-aliquot antibody into 3µl aliquots and store all at -20º).
- Incubate overnight on a rotating platform at 4º, making sure beads are agitating so they won’t settle. I use 2ml screw cap tubes so the liquid doesn’t accumulate at the bottom of the tube.
- The next day (Day 2), immediately before use, rinse 3 times with 1.5ml BSA/PBS. Resuspend in 150µl of BSA/PBS. Use 15µl per ChIP (0.1X beads).
**Day 2**

Pool all plates of EBs to a 50ml conical vial. Let the EBs settle by gravity, aspirating most of the supernatant. Transfer the balance to a 15ml conical vial, rinsing with PBS to remove residual EBs. Pulse spin and aspirate liquid. Add 3ml 2X TE (10ml TE + 25mg fresh trypsin (Sigma#T-4665). Incubate 5 minutes at 37º. Immediately add 7ml of cell culture media (I use MEF media containing 10% serum – the idea is to quickly inactivate the trypsin). Add 1ml crosslinking buffer (make up a fresh solution each time).

-Crosslink for exactly 30 minutes at room temperature using mild agitation on a platform shaker. Stop by immediately adding 550µl of 2.5M Glycine and incubate 5 minutes on ice.

-Spin down 5 minutes at 2000 rpm on Heraeus centrifuge.

-Aspirate liquid – be very careful not to suck up pellet, removing the last bit manually with pipetman. Resuspend in 5ml of Lysis Buffer and incubate 10 minutes at 4º.

-Spin down 8’ at 2500 rpm.

-Remove liquid and resuspend pellet in 6ml buffer 2 (all solutions from this point on must have fresh protease inhibitor. Incubate 10 minutes at room temperature.

-Spin down 8’ at 2500 rpm.

-Resuspend in 3.1ml buffer 3 in a 15ml conical vial (chill empty vial on ice). The pellet will not go into solution at all, consisting of insoluble white clumps. Chromatin is not soluble under these conditions until it is sheared. Keep everything on ice in protease inhibitor-containing solutions.

-Using a Branson 450 sonifier (Maniatis lab), 12 times - I use setting 5 for the first eight times, then I switch the setting to 6 (constant power, duty 100%). These are 30 second bursts, allowing suspension to cool on ice for about 1 minute between pulses. Be careful to “wipe” off residual liquid from tip using the 15ml conical vial so you don’t lose to much volume. Sometimes it is necessary to reduce the power on the final four sonications to 4 (instead of 6) if there is lots of foaming.

-Measure remaining volume (should be about 2.8ml). Take a 16µl sample and run out on a 0.8% agarose gel – you should see a smear with a maximal peak around 2kb. Then add the appropriate amount of a fresh, 20% Sarkosyl solution (powder Sigma#L9150-50G made up in buffer 3) to get a final concentration of 0.5%. Rock for 10 minutes at room temperature.

-Spin at 3500 rpm for 10 minutes.
-Remove supernatant – you should have enough for 5 ChIPs – put 500µl into each of three 2ml screw cap tubes. Make sure to reserve 2.5 ul of Chromatin as Input control (store at -20º). **IF YOU DON’T DO THIS, YOU WILL BE UNABLE TO TEST ChIPs LATER ON!!!**

-Add 150µl of ChIP Cocktail Mix and 10ul of freshly rinsed beads (see Day1 above) per chip and incubate overnight on a rotating platform at 4º with brisk shaking (so beads don’t settle).

**Day 3**
The following can be performed in the cold room, although I just work quickly with all solutions kept on ice at room temp. I use an aspirator for all these steps – a glass Pasteur pipet with a fresh yellow tip for each tube. As long as you aspirate while the tube is in the bead precipitator, you can get rid of all liquid without losing any beads at all.
-Use magnetic stand to precipitate beads.
-Rinse five times with 1ml RIPA buffer
-Rinse once with 1ml TE-plus-50mM NaCL
-Spin 3k for 1-2 min. and aspirate residual TE buffer
-Add 100µl Elution buffer (this strips the protein::DNA complex from the antibody).
-Elute at 65º for 15 minutes, briefly vortexing every 2 minutes (I don’t have any evidence, but I believe this to be an important step. So I time everything with a stop-watch, and incubate the tubes in a 65º water bath).
-Spin down beads for 1minute at 14K.
-Remove all 100µl of supernatant (so after spinning, transfer to magnetic stand so you can get out all the liquid). Remove input tube from -20º, add 97.5ul of elution buffer (1 input is more than enough for all three ChIP samples).
-Reverse crosslinks by incubating overnight at 65º.

**Day 4**
This is the first breathing room you have. If necessary, you can remove samples from 65º and store at -20º until convenient for you. These steps are virtually identical to Odom and colleagues (Odom et al., 2004).
-Add 100µl TE to IP and Input fractions.
-Add RNaseA to a final concentration of 0.2µg/µl (4µl of a 10mg/ml stock) and incubate at 37º for 1 hour.
-Add ProteinaseK to a final concentration of 0.2µg/µl (4µl of a 10mg/ml stock) and incubate at 55º for 2 hours.
-Extract once with Phenol::Chloroform (I am very careful not to get residual phenol – I don’t use phase lock tubes, but these would probably work well).
-Add 20µg of Glycogen (Ambion #9510).
-Add 20µl of 5M Ammonium Acetate and 550µl of Absolute EtOH(prechilled) and incubate at -20º for 15 minutes or more.
-Spin 15 min. at max. speed and wash with 500µl of 75% EtOH
-Resuspend pellets in 60µl of 10mM Tris, pH 8.0. This is your ChIPed product. I always test enrichment by qPCR at this stage. After that, it can be amplified using the LMPCR protocol.

**Solutions:**
N.B. For all solutions where protease inhibitor is used, make a 50X stock, using 1tablet of Complete Mini, EDTA Free (Roche#11836170001). This makes a “slurry” – you have to use a pestal or forcep to crunch up the pellet first. Then vortex right before aliquoting the appropriate amount. Therefore, most of these solutions below will last as “stocks” since you only need a few ml at a time.

**BSA/PBS Solution** (always use cold)
250mg Albumin, Frac. 5 (Sigma #A3059-10G)
50ml PBS (I use standard PBS containing Ca and Mg).
Remake fresh each week, store at 4º.

**Crosslinking Buffer** (keeps at room temp.)
1.25 ml 4M NaCl
100µl 0.5M EDTA
50µl 0.5M EGTA
2.5ml 1M Hepes NaOH 8.0
31.25ml H2O
*Immediately before use, add 298ul of 37% Formaldehyde to 702ul of the above pre-mix.

**Lysis Buffer** (keep on ice)
2ml 1M Hepes KOH, pH7.5
1.4ml 4M NaCl
80µl 0.5M EDTA
4ml Glycerol
2ml 10% NP40
1ml 10% Triton-X 100
28.72 ml H2O
40ml. Add protease inhibitor immediately before use

**Buffer 2** (keep at room temperature)
1.6ml 4M NaCl
64µl 0.5M EDTA
32µl 0.5M EGTA
160µl 2M Tris 8.0
29.5ml H2O
Add protease inhibitor immediately before use

**Buffer 3** (keep on ice)
40µl 0.5M EDTA
20\(\mu l\) 0.5M EGTA
100\(\mu l\) 2M Tris 8.0
19.64 ml H2O
Add protease inhibitor immediately before use

**ChIP Cocktail Mix** (make up fresh each time before use)- scale up as necessary
16.25 4M NaCl
6.5\(\mu l\) 10% Sodium Deoxycholate
65\(\mu l\) 10% Triton-X100
62.5\(\mu l\) H2O
150\(\mu l\)

**RIPA Buffer** *(Young Lab)* – no need to filter sterilize; good for several months
200ml:
155.6 ml ddH2O
20ml 10% NP40
14ml 10% Sodium Deoxycholate *(fresh)*
400\(\mu l\) 0.5M EDTA
10ml 1M Hapes-KOH pH7.5
4.24g Lithium Chloride
200ml

**Elution Buffer** *(fresh each time)*
Per 1ml:
25\(\mu l\) 2M Tris, pH 8.0
20\(\mu l\) 0.5M EDTA
100\(\mu l\) 10% SDS
1ml

**References**